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Résumé

Des particules ultrafines y compris nanométriques, enrichies en métaux (notées PM) sont émises dans l'atmosphère en zones industrielles et urbaines par diverses activités anthropiques : recyclage des métaux, incinération des déchets, trafic routier... À l'échelle mondiale, ces PM sont transférées vers les écosystèmes terrestres et aquatiques, avec des conséquences sur la qualité des plantes et la santé humaine. En ce qui concerne l'utilisation, la biodisponibilité et l'(éco)toxicité des produits chimiques, la réglementation et les pressions de l'espace public se sont récemment renforcées en Europe (réglementation REACH, 2007) et plus largement dans le monde (Système Global Harmonisé en Chine, 2014). Dans ce contexte socio-scientifique mondial, des études d'impacts environnement-santé sur tout le cycle de vie des PM sont indispensables. La thèse visait tout d'abord à étudier le devenir et l'impact des métaux des PM: leur cinétique de transfert et les mécanismes de phytodisponibilité, phytotoxicité, et les risques pour la santé humaine lors de l'ingestion. Ensuite, à travers le cas de potagers urbains en Chine, une étude socio-scientifique a été réalisée afin de proposer des moyens de gestion durable des risques environnement-santé. Plusieurs expériences ont été effectuées en conditions contrôlées et *in situ* pour des légumes contrastés et couramment consommés (laitues, choux, épinards, etc.), selon différentes voies de transfert (foliaire et/ou racinaire) et différentes PM riches en métaux (Pb, Cd, Cu...). La cinétique d'absorption, la translocation, et l'écotoxicité des métaux ont été étudiés en relation avec la morphologie des plantes, la spéciation des polluants et leur localisation dans les feuilles. Les interactions particules-phyllosphère ont été investiguées par des techniques de microscopie et de spectroscopie complémentaires et des tests de phytotoxicité. Enfin, la bioaccessibilité humaine (protocole BARGE *in vitro*) a été mesurée pour évaluer l'exposition aux métaux induite par les PM.

Les légumes peuvent accumuler des quantités importantes de métaux par absorption foliaire lorsque des particules fines pénétrent par les stomates. Ce phénomène a été observée à la fois dans des expériences contrôlées et sur le terrain, même après un lavage efficace. Des PM ultrafines de PbO (<10 microns) et de nano-CuO (<50 nm) induisent une forte phytotoxicité (réduction de la biomasse et des échanges gazeux, nécroses). La phytotoxicité n'est pas simplement régie par la concentration totale en métaux car des bio-transformations se produisent et modifient les formes chimiques des métaux. La phyto-toxicité des PM peut également être associée à leur stress oxydant (Espèces Réactives de l'Oxygène). Des observations en microscopie et spectroscopie (SEM-EDX, RMS et EPR) ont permis de renseigner l'interaction particules-phyllosphere: 1) Des métaux (CdO, Sb_2O_3 et nano-CuO) piégés dans des stomates ont été observés, favorisant leur absorption foliaire; 2) L'analyse par résonance paramagnétique électronique (EPR) a permis la mise en évidence d'un changement de spéciation du cuivre par biotransformation (transformation de CuO en Cu (II)-complexe) dans les tissus des feuilles; 3) Des nécroses ont été observées à la surface des feuilles après l'exposition à CuO et certains stomates déformés ont été mis en évidence dans ces zones nécrosées. Nous avons observé une influence significative de la nature du métal (Cd, Sb, Zn et Pb), de l'espèce végétale et du type d'exposition (foliaire/racinaire) sur la bioaccessibilité gastro-intestinale des éléments. La bioaccessibilité gastrique des métaux dans les légumes suit l'ordre : Cd ≈Zn> Pb> Cr ≈Cu> Sb. En outre, la bioaccessibilité gastrique du métal était significativement plus élevée dans les légumes que dans les sources de PM, certainement en raison de changements de spéciation chimique. A proximité d'un incinérateur de déchets ou d'une autoroute, l'absorption foliaire des PM induit des concentrations élevée en métaux dans les plantes, en plus du transfert sol-plante. 60-79% des métaux présents dans les légumes peuvent être biodisponibles par ingestion humaine, avec une valeur maximale obtenue pour le Cd. Une bioaccessibilité humaine relativement élevée a été mesurée, suggérant un risque potentiel pour la santé en cas de consommation régulière. Les quotients de danger (HQ) du Cd et Pb sont plus élevés que ceux des autres métaux : leur contribution au risque sanitaire est donc élevée. Les jardins potagers étudiés présentent un risque sanitaire faible (cas de l'incinérateur) ou modéré (cas de l'autoroute) à l'égard de la consommation humaine des légumes étudiés. Mais, une exposition supplémentaire à différents polluants est souvent possible. L'exposition humaine peut-être réduite en choisissant certaines espèces de plantes et en améliorant la procédure de lavage. Ces résultats sont utiles pour concilier objectifs économiques et santé publique, dans le cadre de la transition écologique actuellement initialisée en Chine. Enfin, cette thèse souligne l'importance de prendre en compte l'influence de l'atmosphère en plus de la qualité du sol pour estimer la qualité des plantes consommées cultivées en zone anthropisée (fermes et jardins de cuisine), pour la gestion durable des agricultures urbaines.

Mots clés: Agriculture urbaine durable; Gestion des risques environnement et santé; Métaux et métalloides; Ultrafines PM, Cinétique de transfert ; Phytotoxicité; Absorption foliaire et/ou racinaire; (Eco) toxicité; Bioaccessibilité.

Abstract

Ultrafine particles including nanosized enriched with metal(loid)s (PM) are emitted into the atmosphere of industrial or urban areas by various anthropogenic activities: recycling factories, waste incinerator, road traffic, etc. At the global scale, these PM can transfer into the atmosphere, soil and water ecosystems and have consequences on plant quality and human health. Concerning the uses, bioavailability and (eco)toxicity of chemicals, regulation and public space pressures recently increased in Europe (Reach regulation, 2007) and more widely in the world (Global Harmonized System in China, 2014). In that global socio-scientific context, studies of environmental and health impacts throughout the life cycle of PM are of crucial sanitary concern. The PhD aims first to study metal(loid)s present in the PM: their transfer kinetic and mechanism of phytoavailability, phytoxicity, human health risks-ingestion bioaccessibility. Then, through the case of vegetable gardens near an incinerator and a highway in China, a socio-scientific study was performed in order to give suggestions for sustainable environmental and health risk management for these sites. Several controlled and field experiments were therefore performed for contrasted and currently consumed vegetables (lettuce, cabbage, spinach, etc.), foliar and/or root exposed to various metal(loid)s enriched PM (Pb, Cd, Cu...). Kinetic of metal(loid)s uptake, translocation, and ecotoxicity were studied in relation with plant morphology, pollutant speciation and localization in leaves. PM-phyllosphere interactions were highlighted by complementary microscopy and spectroscopy techniques and phytotoxicity tests. Finally, human bioaccessibility (using *in vitro* ingestion BARGE protocol) were performed to investigate the human health consequences of PM pollution and, a socio-scientific study was realized in collective gardens near incinerator and highway in China.

Vegetables can significantly accumulate metal(loid)s by foliar uptake when metal(loid)s enriched atmosphere fine particles directly enter into leaves through stomata apertures. Foliar uptake of different metal(loid)s was observed both in control and field experiments, even after meticulous washing. Ultrafine PbO (<10µm) and nano-CuO (<50nm) particles caused serious phytotoxicity (reduced biomass and gaseous exchange, and necrosis) after interaction with leaf surface, and leaf to root metal transfer was observed. Phytotoxicity of metal(loid)s is not simply governed by their total concentration, but also depended on their potential bio-transformation. Metal effects and behavior depend on metal and plant nature, metal concentration, exposure mode and duration. Toxicity of PM may be associated with their oxidant stress to target cells (Reactive Oxygen Species formation). Microscopy and spectroscopy (SEM-EDX, RMS, and EPR) observations provide evidence for particle-phyllosphere interactions: 1) Metals (CdO, Sb_2O_3 and nano-CuO) trapped within stomata were observed, which permits their uptake by vegetable leaves; 2) Electron paramagnetic resonance (EPR) analysis clearly evidenced Cu speciation biotransformation (from CuO (source) to Cu(II)-

complex) in leaf tissues; 3) Necrosis was observed in leaf surface after CuO exposure and some deformed stomata were evidenced in many necrosis areas. We observed a significant influence of the nature of metal (Cd, Sb, Zn and Pb), plant species and the way of metal transfer (foliar/root) on bioaccessibility measures. The bioaccessibility of metals in vegetables was in the order of Cd ≈Zn >Pb >Cr ≈Cu >Sb, both in controlled and field exposure conditions. Moreover, the gastric bioaccessibility of metal(loid)s was significantly higher in vegetables than in PM sources, certainly due to chemical speciation changes. For a social-scientific study near waste incinerator and highway, we found that atmosphere PM fallouts can induce significant metal foliar uptake in addition to soil-plant transfer. Bioaccessibility results showed that 60-79% of metals in vegetables can be bioavailable by human ingestion, with a maximum value for Cd. The relatively high metal human bioaccessibility was measured suggesting a potential health risk in the case of regular consumption. Hazard quotient of Cd and Pb are higher than that for other metals suggesting their higher contribution to health risk. Vegetable gardens present a low (waste incinerator) or moderate (highway) health risk with respect to human consumption quality of the investigated vegetables, but the additive exposure to different pollutants is often possible. Since local residences must live in those urban areas, sustainable management strategies should be developed such as reduce human exposure by selecting plant species and improve washing procedure. These results come within the framework of the ecological transition currently initialized in China to reconcile economic goal and public health. Finally, our studies highlight the importance to take into account atmosphere and soil quality on consumed cultivated plants in urban area (farms and kitchen gardens), for sustainable management of urban agricultures and quality of vegetables cultivated in megacities at the global scale.

Key words: Sustainable urban agriculture; Human risk management; Metal(loid)s; Ultrafine PM; Transfer kinetic; Phytotoxocity; Foliar and/or root uptake; (Eco)toxicity; Bioaccessibility.

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Glossary

PM: Particulate Matters

REACH: Registration, Evaluation and Authorisation of CHemicals

SVHC: Substance of Very High Concern

ICPE: Installation Classified for Environmental Protection

ICP-MS: Induced Coupled Plasma-Mass Spectrometry

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

STCM: Société de Traitements Chimiques des Métaux. The Society of Chemical Treatments of Metals.

SEM-EDX: Scanning Electron Microscopy coupled with Energy Dispersive X-Ray microanalysis

RMS: Raman Microspectrometry

EPR: Electron Paramagnetic Resonance

ppm: part per million

µXRF: Micro-X-ray Fluorescence

ToF-SIMS: Time of Flight Secondary Ion Mass Spectrometry

ROS: Reactive Oxygen Species. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides.

UBM: Unified BARGE Method

BARGE: Bioaccessibility Research Group of Europe

TF: Translocation Factor

GEF: Global Enrichment Factor

MTE: Metal Ttrace Elements

DTT assay: Dithiothreitol assay

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Chapter 1 Introduction:Contexte scientifique global et objectifs

► Depuis des siècles, les substances chimiques ont de nombreuses utilisations dans la vie quotidienne des hommes. Cependant, certaines de ces substances chimiques peuvent entraîner des effets (éco)toxiques qui questionnent alors leur utilisation. C'est le cas de certains métaux ou métalloïdes qui sont soumis à autorisation dans le règlement européen REACH qui vise en particulier, lorsque possible : (i) la réduction du nombre de substances chimiques commercialisées, (ii) une meilleure information des citoyens sur les risques environnement-santé liés à l'utilisation de certaines substances chimiques et les moyens de les réduire, (iii) le remplacement des tests sur animaux par des tests in vitro.

► Ces dernières décennies, la proportion de particules ultrafines incluant des nanoparticules riches en métaux et métalloïdes a augmenté dans la troposphère. Les raisons de cette augmentation de PM sont multiples et parfois surprenantes comme :

-l'utilisation de filtres plus efficaces par les industries qui a entrainé une réduction des quantités de particules émises dans l'environnement (en masse ou volume) mais une proportion de fines PM plus grande;

-ou le développement des activités réduction des déchets et de recyclage (dans une optique d'économie circulaire) qui produisent des PM via l'utilisation d'incinérateurs par exemple.

Les activités de récupération et recyclage des métaux contribuent au développement durable mais produisent des PM (Batonneau et al, 2004; Ettler et al., 2005; Uzu et al, 2010). L'incinération des déchets est considérée comme une pratique efficace pour réduire le volume des déchets et produire de l'énergie (électricité et chauffage urbain), mais les incinérateurs ont généré un vif débat dans les pays occidentaux en raison des émissions de polluants parfois détectés dans l'atmosphère (Buonanno et Morawska, 2014). En France, les installations d'incinération ou de co-incinération de déchets non dangereux sont des installations classées pour la protection de l'environnement (ICPE), soumises à autorisation. Elles sont classées dans la rubrique 2771 de la nomenclature ICPE intitulée « Installation de traitement thermique de déchets non dangereux ». Le plan national santé-environnement (PNSE) 2015-2019, met l'accent sur la gestion de l'exposition humaine aux polluants via l'alimentation en particulier dans le cas des nanoparticules.

► En Chine, la pollution a fortement augmenté durant les trois dernières décennies avec l'industrialisation du pays. Un grand nombre de métropoles chinoises comptent parmi les plus polluées de la planète (Lucotte, 2009). Une montée en puissance dans l'espace public des discussions relatives à la prise en compte des liens environnement-santé est donc actuellement observée. En 2013, le ministère chinois de l'Environnement a d'ailleurs reconnu l'existence de "villages du cancer" dans certaines régions particulièrement polluées du pays (Ralston AFP, 22.02.2013) : air très chargé en particules fines ou déchets industriels. Le gouvernement reconnaît que «des produits chimiques toxiques et nocifs» sont parfois utilisés en Chine et «mettent potentiellement en danger la santé humaine et l'environnement sur le long terme».

►Les particules en suspension dans l'air présentent une large gamme de tailles allant des PM_{10} (diamètre aérodynamique de 10 µm ou moins) à la taille nanométrique (diamètre < 100 nm) et elles peuvent être transportées sur de longues distances. Les PM_{10} sont prises en compte par l'Organisation Mondiale de la Santé (OMS, 1987) et le règlement de la Commission européenne (CE) n ° 221/2002 concernant l'évaluation de la qualité de l'air (Commission européenne, 2002) en raison de leurs effets néfastes sur l'environnement et la santé humaine. Si les $PM_{2,5}$, PM_1, et nanoparticules contribuent relativement peu à la quantité totale (en masse) de particules émises dans l'environnement, elles sont plus critiques en termes d'impacts environnementaux et sanitaires (Uzu et al, 2009, 2011b; Fernández Espinosa et al ., 2002) : solubilité élevée et fort potentiel de pénétration pulmonaire après inhalation. En outre, ces PM peuvent impacter les écosystèmes terrestres (Donisa et al., 2000; Van Hees et al, 2008; Foucault et al, 2012), ce qui conduit à la contamination des sols (Schreck et al, 2011; Pelfrene et al ., 2013) et des plantes consommées (Uzu et al, 2010; Austruy et al, 2014).

►Parmi les polluants mesurés dans ces PM, les métaux et métalloïdes sont souvent observés (Faiz et al., 2009). Certains comme Cu, Fe, Zn, Mn peuvent jouer un rôle essentiel pour les plantes et/ou les animaux, d'autres tels que Cd, As, Pb et Hg sont non essentiels et peuvent entraîner des effets néfastes pour la santé, même en quantités infimes (Martorell et al, 2011 ; Ferré-Huguet et al, 2008;. Martí-Cid et al, 2008a). Certains comme Pb, Cd ou As ont des impacts (éco)toxiques (US EPA, 2000). Or, en raison de multiples activités (traitement des déchets, application d'engrais, certains épandages, etc.), une augmentation des concentrations en métaux et métalloïdes est observée dans l'atmosphère et les sols. En conséquence, les plantes cultivées dans ces environnements pollués peuvent absorber des polluants et présenter aussi des signes de phyto-toxicité (Verma et al., 2007). Plusieurs chercheurs ont étudié en conséquence l'accumulation des métaux dans les cultures par transfert sol-plante, les facteurs qui en sont responsables, et les effets engendrés sur la santé humaine et animale (Tubek et al., 2008). Par exemple, une réduction de la croissance des plantes a été observée en raison de la présence de niveaux élevés de métaux (Cu, Pb, et Hg) par Merry et al. (1986).

►Le plomb est naturellement présent dans les sols (fond pédogéochimique) et des activités anthropiques telles que l'exploitation minière, les traitements des métaux, le recyclage des batteries peuvent accroitre les concentrations et modifier les formes chimiques de ce métal dans l'environnement. L'exposition au plomb peut entraîner des effets délétères en particulier sur le développement intellectuel des enfants (Jarup, 2003). La nourriture est la principale source d'exposition au plomb des populations, en particulier lorsque des légumes comestibles ont été pollués via leurs systèmes racinaires ou par absorption foliaire (Finster et al., 2004). Le cadmium est connu pour sa biodisponibilité relativement élevée en comparaison avec les autres métaux. L'exposition au Cd peut poser des effets néfastes sur la santé : attaque rénale, fragilité osseuse (OMS, 1992; Jarup, 2003). Les denrées alimentaires sont la principale source de Cd pour l'homme (Autorité européenne de sécurité des aliments, 2009 ; Alloway, et al., 1990). Le cuivre peut être utilisé comme un supplément nutritionnel pour les animaux, et c'est un élément essentiel pour la croissance des plantes qui est donc apporté aux sols comme engrais et aussi fongicide en agriculture biologique. Mais à long terme, des usages répétés de « bouillie bordelaise » peuvent entrainer des pollutions de la couche cultivée du sol et des eaux souterraines. Par exemple, son utilisation prolongée conduit à 200-500 mg kg^{-1} Cu dans les sols viticoles en France (Brun et al., 1998). Le Zn est également un élément essentiel dans la nutrition des plantes, des animaux et des humains, mais à des concentrations élevées, le Zn a des effets néfastes sur les écosystèmes (Eisler, 1993 ; Miretzky et al., 2004). L'écotoxicité du Zn est observée en particulier lorsque des apports anthropiques sont à l'origine de concentrations élevées (sols agricoles traités avec des boues d'épuration, déchets de fonderie, sols urbains et péri-urbains) et que le pH du sol est acide (Chaney, 1993). Le Zn en excès peut s'accumuler dans les tissus végétaux, les concentrations phytotoxiques sont supérieure à 300 µg g^{-1} (Foy et al., 1978).

►Les parties comestibles des légumes couramment cultivés en zones urbaines (radis, choux, tomates, carottes, haricots verts, etc.) présentent parfois des concentrations en métaux (Cd, Cr, Pb) plus élevées par rapport aux végétaux « témoins ». Certaines cultures comme les choux, les épinards et les haricots verts, accumulent une grande partie des métaux (Pb et Cd) dans leurs racines. Selon Pichtel et Bradway (2008), la laitue peut accumuler des métaux lourds dans ses tissus. Des études ont également montré que la végétation peut efficacement adsorber et réduire les quantités de particules dans l'air en les capturant au niveau de leurs parties aériennes (Freer-Smith et al, 1997; Prusty et al., 2005). L'efficacité de l'interaction entre les PM et les végétaux dépend de la morphologie des feuilles, des conditions météorologiques (Freer-Smith et al., 1997; Zhao et al. , 2002), etc. Des études récentes ont démontré que les feuilles des plantes peuvent accumuler des métaux lourds issus de PM atmosphériques (Tomasevic et al., 2005;. Uzu et al, 2010). La biosurveillance des métaux en trace dans l'atmosphère a aussi été très étudiée (de Temmerman et Hoenig, 2004; Schintu et al, 2005; Espinosa et Oliva, 2006; Rossini Oliva et Mingorance, 2006; Rodriguez et al, 2008; Bermudez et al, 2009; Gonzalez-Miqueo et al, 2010).

► Lorsque les aérosols atmosphériques ont des concentrations élevées en métaux, par exemple aux environs des entreprises de traitement de déchets ou recyclage des métaux, il est indispensable d'étudier la qualité des végétaux (Hutchinson et Whitby, 1974; Lùbersli et Steinnes, 1988; Lyanguzova Chertov et 1990; Koricheva et Haukioja, 1995; Kozlov et al., 1995) qui peut être influencée à la fois par des transferts racinaires (Lin et Xing, 2007, 2008; Stampoulis et al, 2009; Ma et al, 2010;. Yin et al, 2011; Lombi et al., 2011) et/ou foliaires (Tomasevic et al., 2005; honneur et al, 2009;. Uzu et al., 2010). Les concentrations en métaux des parties aériennes des végétaux sont fréquemment utilisées comme des biotests, indices de pollution (Doncheva, 1978; Kryuchkov, 1993; Koricheva et Haukioja, 1995; Kozlov et al., 1996; RuohomaÈki et al, 1996;. Zvereva et al., 1997 ; Caggiano et al., 2005; Rossini Oliva et Mingorance, 2006;. Rodriguez et al, 2008; Bermudez et al, 2009; Gonzalez-Miqueo et al, 2010). Cependant, la plupart des études sur l'absorption foliaire des métaux sont anciennes et n'informent pas sur les mécanismes impliqués (Little, 1978; Ward et Savage, 1994). Peu de données sont actuellement disponibles sur le devenir des PM au niveau de la phyllosphère en relation avec la forme et la taille des PM et la spéciation des métaux. Le transfert foliaire, ses interactions avec le transfert racinaire, la phytotoxicité induite et la cinétique restent encore peu connus. Pourtant, la voie de transfert pourrait influencer la compartimentation et la spéciation et enfin la bioaccessibilité gastro-intestinale des métaux présents dans les plantes.

►L'apport alimentaire est la principale voie d'exposition aux métaux lourds pour les populations dans le monde sauf dans le cas de l'inhalation de particules ultrafines enrichies en métaux (Tripathi et al., 1997). Les légumes constituent une partie importante de l'alimentation humaine, car ils contiennent des glucides, protéines, vitamines, minéraux et fibres nécessaires pour la santé humaine (Suruchi et Khanna, 2011). Ainsi l'information relative aux concentrations en métaux lourds dans les produits alimentaires et les quantités de produits consommés sont indispensables pour évaluer les risques sanitaires (Zhuang et al., 2009). La plupart des travaux de recherche ont porté sur l'évaluation des risques potentiels pour la santé des populations habitant dans le voisinage de sites dangereux, comme les mines et les fonderies : exposition aux métaux lourds par le biais de la consommation des cultures agricoles polluées (Zhuang et al, 2009; Sipter et al, 2008; Cui et al., 2004; Zheng et al, 2007 ; Sridhara Chary et al, 2008;. Wang et al., 2005; Khan et al., 2008). Les mesures de bioaccessibilité des métaux présents dans des matrices ingérées (par exemple des légumes pollués) permettent d'évaluer la fraction des polluants biodisponible pour l'homme. La détermination des métaux bioaccessibles constitue une étape importante pour l'évaluation des risques sanitaires. Le groupe BARGE (BioAcessibility Research Group of Europe) a mis au point des tests *in vitro* afin de répondre à la réglementation REACH (réduction des tests sur des animaux) (European Commission, 2010). La bioaccessibilité gastro-intestinale des polluants suite à l'ingestion de PM enrichies en métaux, de sols ou de légumes pollués peut donc être mesurée *in vitro* et venir donner une bonne évaluation de la biodisponibilité *in vivo*.

Cette thèse s'intègre dans une dynamique de recherche relative aux transferts sol-plante-atmosphère des métaux présents dans l'environnement sous forme particulaire: thèse de G. Uzu et projet DIMENSION. **L'objectif plus précis de cette thèse était** d'étudier la bioaccessibilité des métaux présents dans les végétaux en relation avec les conditions d'exposition et ceci à la fois en conditions contrôlées et sur le terrain.

Le travail de thèse est axé sur :

1) l'évaluation de l'impact des éléments traces métalliques (MTE) des PM sur les légumes consommés, l'évolution de la biodisponibilité et de la phytotoxicité.

2) l'étude des mécanismes de transfert foliaire des métaux par les plantes : cinétique de transfert, quantités d'exposition et facteurs de translocation (feuilles vers racines).

3) l'étude des interactions particules-phyllosphère : le devenir des métaux dans/sur les feuilles des plantes est influencé par des transformations bio-physico-chimiques. En outre, ce travail a également pour objectif d'étudier la localisation et la spéciation des métaux présents à l'intérieur des feuilles, par microscopie électronique à balayage couplée à la microanalyse à dispersion d'énergie des rayons X (SEM-EDX) et la résonance paramagnétique électronique (RPE) d'analyse. Le travail a été réalisé en collaboration avec le LAboratoire de Spectrochimie Infrarouge et Raman (LASIR) (Thèse Vincent Dappe, Université de Lille 1, 2015).

4) l'étude de la biodisponibilité humaine des MTE dans diverses plantes contaminées en fonction des paramètres physico-chimiques, la nature des PM et des conditions d'exposition. Cette étape permet d'évaluer le risque pour la santé via des quotients de risque calculés avec des concentrations totales et bioaccessibles des métaux.

Cette étude est utile pour la surveillance des industries réglementées et des activités agricoles, favorisant l'agriculture durable (gestion des pollutions) dans les zones urbaines, ainsi que l'amélioration de la réglementation afin de réduire l'exposition humaine aux métaux. Ce projet est important pour la gestion des zones d'agricultures urbaines en Chine, France et d'autres pays industrialisés, et sera utile pour les urbanistes et les gestionnaires de risques environnementaux qui cherchent à concilier objectifs économiques et santé publique.

Ce travail de thèse participe à éclairer les mécanismes de transfert des métaux dans le système air-sol-plante et à clarifier la relation santé-environnement. Les détails de l'étude sont présentés dans le chapitre 2 (Matériels et Méthodes) et les chapitres 3, 4, 5 (Résultats et Discussion).

Chapter 1 Introduction : Global scientific context and objectives

► At the global scale, atmosphere pollution by ultrafine particles including nano enriched with metal(loid)s (PM) represents a high sanitary risk for population living in urban or industrial areas and megalopolis. Actually, these PM can originate from both natural and anthropogenic activities, however, anthropogenic emissions is significant higher than natural emissions (Pb, Cd, Cu, Sb…) (Catinon et al., 2011). Therefore the total amounts of metal(loid)s released into the environment by industries are now strictly controlled in Europe (Glorennec et al., 2007), particularly some metals (Cd, Pb, Ni, As, Hg) are classified as "Substance of Very High Concern" by the European REACH regulation (EC 1907/2006), that require imposed monitoring throughout their life cycle (from manufacturing to disposal).

► The **reasons** for the increased metal(loid)s enriched PM are various: 1) manifold PM sources; 2) loose regulation in some countries; and sometimes surprising as 3) the use of more efficient filters by industries (that has led to a reduction in the quantities of particles emitted into the environment (by weight or volume) but a greater proportion of PM) and 4) high temprature during the metal-recycling activities or waste incineration (with a view of circular economy) etc.

Recently, for industrialized countries, emissions of PM from industrial activities have largely declined notably due to regulatory changes and the closure of some sites (Schreck et al. 2013). Different trapping devices were used to limit the unwanted emissions from factories. However, this treatment systems are not always effective especially for fine and ultrafine particles that can escape these sensing devices (Biswas and Wu, 2005) and ultimately pollute the environment (Shi et al., 2012; Waheed et al., 2011). Actually, significant amounts of metal enriched airborne fine and ultrafine particles (PM – rich in As, Cd, Cr, Cu, Ni, Pb, Zn...) are observed in industrial and urban areas with concentrations exceeding sometimes the French and European regulations thresholds (Bu-Olayan and Thomas 2009; Harrison and Yin 2010; Moreno et al. 2010; Shahid et al. 2013; Zhang et al. 2005). For some countries (Eastern Mediterranean and South-East Asia), which produce and export worldwide large quantities of items, regulation on air quality is increasing gradually, but currently allowed levels of PM emitted into the environment are relatively high (WHO, 2014a,b).

Metal-recycling activities contributed to sustainable development. Respect to the management policy of governments, this kind of factories help to handle the large amount of waste, convert waste into resources and recycle metals (Schreck et al., 2012b; Zhang et al., 2014). In France, they are installation classified for environmental protection (ICPE). Nevertheless, they also emit metal enriched PM (Nair et al., 2010; Schreck et al., 2012b; Uzu et al., 2010). Indeed, recyclers of lead-acid batteries are currently the main source of particulate air emissions rich in lead and other metal(loids) (i.e. metal trace elements – MTE – as As, Sb, Cu, Zn, Sb, etc.) (Dumat et al., 2006).

In the waste management, incineration is considered a good practice for reducing the waste volume and recovering its energy to produce electricity and district heating. In France, the incineration or co-incineration of non-hazardous waste facilities are classified as ICPE entitled "Heat treatment plant for non-hazardous waste". However, incinerators have generated a strong debate in western countries about their emissions of ultrafine particles (Buonanno and Morawska, 2014). The French National Health and Environment Plan (NESP) 2015-2019, focuse on the management of human exposure to pollutants through food and particularly in the case of nanoparticles. In China, the government is planning to close certain waste incinerators due to public space pressures: such as manifestation in favor of the environment (5000 inhabitants of Hangzhou (west of Shanghai) protested for three days against a new incinerator project). Therefore, due to increasing regulation, the managers of these companies or factories are responsible for implementing management strategies to minimize impacts on the environment and human health.

► Airborne particles present a large range of **size** from PM_{10} (aerodynamic diameter of 10 µm or less) to nano PM (diameter less than 100 nm). PM_{10} is a target pollutant for the World Health Organization (WHO, 1987) and the European Commission Regulation (EC) No. 221/2002 on ambient air quality assessment (European Commission, 2002) due to its adverse effects on the environment and human health. PM2.5, PM1, and nanoparticles (fine and ultrafine metallic particles, defined by the U.S. Environmental Protection Agency (US EPA) as <2.5-mm and <0.1-mm diameter, respectively) are less contribute to the total amount of particle emitted in terms of mass, but they are more critical in terms of their environmental and health impacts (Uzu et al., 2011a, 2009). Due to their high inhalation potential, PM could precipitate in the nose or throat region, and ultrafine particles are even lung penetrating. PM can also dry or wet deposited on terrestrial ecosystems (Donisa et al., 2000; Foucault et al., 2012; van Hees et al., 2008), leading to contamination of soils (Pelfrêne et al., 2013b;

Schreck et al., 2011), consumed plants (Austruy et al., 2014; Uzu et al., 2010). These pollutants induce health risk especially when contaminated plants are consumed by human.

► **Metal(loid)s** are concentrated in the finest particle size fractions (Harrison and Yin, 2010) even nanometric (Midander et al., 2012; Pöschl, 2010; Taiwo et al., 2014). The main threats to plants and human health from metal(loid)s are associated with exposure to lead (Pb), cadmium (Cd), copper (Cu), zinc (Zn), chromium (Cr), antimony (Sb), etc. Pb and Cd are nonessential and can cause adverse health effects even in trace quantities, at the reverse side Cu and Zn play essential role in plants and animals (Khillare et al., 2012; Martorell et al., 2011). However, with antropogenic activities (waste treatment, fertilizer application, atmospheric deposition, etc.), increased concentrations of metal(loid)s is observed in the atmosphere and soil. Consequently, the cultures grown in these polluted environments can absorb pollutants and result in phyto-toxicity (Verma et al., 2007). Several researchers have studied therefore metal accumulation in crops by soil-plant transfer, the factors involved in it, and its effect on human and animal health (Tubek and Tubek, 2008). A reduction in plant growth was observed due to the presence of high levels of metals (Cu, Pb, and Hg) by Merry et al. (1986).

Pb in particular, is an environmental contaminant that occurs naturally and, to a greater extent, from anthropogenic activities such as mining and smelting and battery manufacturing. Pb is considered to be the most phytotoxic heavy metal, and is accumulated in varying plant organs, though its accumulation is more detectable with plant roots, compared to shoots. Lead exposure in human body can cause a wide spectrum of health problems, ranging from small effects on metabolism and intelligence to convulsions, coma, renal failure and death (Papanikolaou et al., 2005). Long term exposure to Pb may lead to memory deterioration, prolonged reaction times and reduced ability to understand. Children may be affected by behavioral disturbances and learning and concentration difficulties (Jarup, 2003). Food is the major source of exposure to Pb and a possible risk for the population can be caused by the bioaccumulation of Pb in the edible vegetables because maybe taken up in edible plants from the soil via the root system, by direct foliar uptake and translocation within the plant, and by surface deposition of particulate matter (Finster et al., 2004). According to International Agency for Research on Cancer (IARC) evaluation, inorganic Pb compounds are probably carcinogenic to humans (IARC, 2006).

Cd has serious health consequences and ecosystem impacts. Cd exposure may pose adverse health effects, including kidney damage and possibly also bone effects and fractures (Jarup, 2003). In particular foodstuffs are the main source of human intake of Cd (European Food Safety Authority, 2009) that accumulates in the leaves of plants and, therefore, leafy vegetables grown in contaminated soils are more relevant compared to seed or root crops (Alloway et al., 1990). Pb and Cd are two main heavy metals regulated by European commission for contaminants in foodstuffs (European Commission, 2001).

Cu and Zn have non-carcinogenic hazardous effect to human health when exposures exceed the tolerable reference dose (US EPA, 2000). Actually, copper can be used as a nutritional supplement for animals (e.g. feed supplements), and is an essential element for plant growth, therefor used as fungicides and fertilizers in agriculture, however long term of copper application can lead to pollution on soil layer and groundwater, for example, its prolonged use lead to 200–500 mg kg^{-1} Cu in the vineyard soils in France (Brun et al., 1998). Zn is also an essential element in the nutrition of plants, animals and human. Zn toxicity in crops is far less widespread than Zn deficiency. However, Zn toxicity occurs in agricultural soils treated with sewage sludge, mine and smelting wastes, and in urban and peri-urban soils enriched by anthropogenic inputs of Zn, especially in low-pH soils (Chaney, 1993). Zn accumulates in excess in the plant tissues, reaching toxic concentrations (>300 μg g^{-1}) for plants (Beyer et al., 2013).

Cr is an important element especially in metallurgical/steel or pigment industry (Waseem et al., 2014). The toxic form of Cr occurs in +6 oxidation state (Cr(VI)), Cr(VI) in the forms of chromate (CrO_4^{2-}), dichromate (CrO_4^{2-}), and CrO_3 is considered the most toxic forms of chromium, as it presents high oxidizing potential, high solubility, and mobility across the membranes in living organisms and in the environment. Cr(III) in the forms of oxides, hydroxides, and sulphates is less toxic as it is relatively insoluble in water, presents lower mobility, and is mainly bound to organic matter in soil and aquatic environments (Oliveira, 2012). **In plants**, particularly crops, Cr at low concentrations (0.05–1 mg L^{-1}) was found to promote growth and increase yield, but it is not considered essential to plants (Paiva et al., 2009). Cr uptake, translocation, and accumulation depend on its speciation, which also conditions its toxicity to plants. Symptoms of Cr toxicity in plants are diverse and include decrease of seed germination, reduction of growth, decrease of yield, inhibition of enzymatic activities, impairment of photosynthesis, nutrient and oxidative imbalances, and mutagenesis (Oliveira, 2012). Few serious adverse effects have been associated with excess intake of Cr from food (Hellwig et al., 2006).

Sb is potencially toxic at very low concentrations and has no known biological functions (Smichowski, 2008). The majority of Sb pollution appears to originate from mining and industrial emission sources (Wilson et al., 2010). Shotyk et al. (2005) presented a comprehensive review covering different aspects of the anthropogenic impacts on the biogeochemistry and cycling of Sb. Special attention was paid to atmospheric emissions of Sb to the environment and its occurrence in the atmosphere, soils, sediments, plants and waters. Agricultural soils may become polluted with Sb through wet and dry deposition, from mining, manufacturing and municipal discharges and through the addition of soil amendments such as chemical fertilizers, sewage sludge and fly-ash (Crecelius et al., 1974; He, 2007). Although Sb is not an essential element, this metalloid can easily be taken up by plant roots from soils (Ainsworth et al., 1991). Many countries are currently concerned about Sb pollution (He et al., 2012).

► In this global context, it's necessary to study:

1) the interactions between particles and plants as well as the behaviour and response of the plants to these contaminants (the mechanisms involved in transfer),

2) the plant quality in case of PM transfer (phytotoxicity),

3) human exposure to fine and untrafine particles after plant and soil ingestion.

► For the sustainable development of agriculture, a major concern is the potential transfer of metal(oid)s contaminants within vegetables both *via* the plant **root exposure** to contaminated soils (Ge et al., 2000) and **foliar transfer**, when PM deposites can be entrapped by vegetable leaves (Larue et al., 2014; Schreck et al., 2012b).

Root transfer has been largely studied (Lin and Xing, 2007; Ma et al., 2010; Stampoulis et al., 2009) due to increased heavy metal concentration in the soil that polluted by various factors including the disposal of municipal and industrial wastes, application of fertilizers, atmospheric deposition and discharge of wastewater on land (Verma et al., 2007). It has been commonly observed that crops and vegetables grown in heavy metal-contaminated soils tend to have a greater accumulation of heavy metals in their edible or non-edible parts than those grown in uncontaminated soils (Sharma et al., 2007).

Although many studies attributed the contamination of plants via soil-plant transfer, it has recently been demonstrated that 25 to 40% of the total plant metal content can be derived from **leaf transfer** coming from industrial particles (Nowack and Bucheli, 2007; Schreck et al., 2012b). Due to increase in atmospheric pollution, the foliar uptake of pollutants is nowadays seriously considered for vegetable quality concern. Since few years, several studies have therefore been conducted on foliar metal transfer (Xiong et al., 2014a), actually, plant leaves can accumulate heavy metals from the atmospheric aerosols (Tomasević et al., 2005; Uzu et al., 2010; Xiong et al., 2014a) and reduce PM amount in the atmosphere; i.e. foliar dust(Freer-Smith et al., 1997; Prusty et al., 2005). The dust-retention abilities of vegetation depend on several factors, such as the different types of tree canopy, leaf and branch density and leaf morphology, as well as prevailing meteorological conditions (Freer-Smith et al., 1997; Zhao et al., 2002). The biomonitoring of trace metals in the atmosphere has been well studied (De Temmerman and Hoenig, 2004; Lee et al., 2005; Oliva and Mingorance, 2006; Rodríguez et al., 2008).

Therefore, in addition to knowing soil-plant transfer of metal, atmosphere-plant transfer of metal is equally important for risk assessment studies. However, most studies on foliar uptake of metals are not recent and do not provide information on the mechanisms involved (Little, 1978; Ward and Savage, 1994). Few data are currently available on the fate of PM at the phyllosphere in relation to the form and size of the PM and metal speciation. Foliar transfer and its interactions with the root transfer, phytotoxicity and kinetics are little known. Since the transfer channel could influence compartmentalization and speciation and finally metal bioaccessibility (loid) s in plants, it is necessary to well understand the foliar transfer mechanisms.

►**Vegetable quantity** is a serious problem with the increased PM in atmosphere and soil pollution, which were used for the studies of metal transfer in numerious previous studies (Ali and Al-Qahtani, 2012; Cao et al., 2010a; De Leon et al., 2010; Monteiro et al., 2009; Schreck et al., 2013). Foliar metals (Pb, Cu and Cd) uptake can induce high phytotoxicity(Salim et al., 1993). In order to better understanding the **phytotoxicity** of PM, several sensitive parameters are required. Recently, reports have shown that trace metals cause damages on plant leaves, stomata and leaf proteins (Pourrut et al., 2013; Xiong et al., 2014a). At cellular level, Pb toxicity results in overproduction of reactive oxygen species (ROS) (Shahid et al., 2014b, 2014c). Morphological and growth parameters showed a decrease in root and shoot growth (Schreck et al., 2011) and alterations in root branching pattern in *Lactuca sativa L.* after Pb treatment (Capelo et al., 2012). A reduction in plant growth was observed due to the presence of elevated levels of heavy metals like copper, lead and mercury (Merry et al., 1986). Physiological processes, such as photosynthesis and water status, are sensitive to trace metals as reported by Monni et al.(2001) and Austruy et al.(2013) working on different plant species. Photosynthesis has been found to be one of the most sensitive plant processes and the effect of the metal is multiracial(Mateos-Naranjo et al., 2012; Shahid et al., 2014a). Moreover, Le Guédard et al. (2012) reported that leaf fatty acid composition is a considerable biomarker of early effect of trace metals. Therefore, plant biomasses, gaseous exchange and fatty acis composition were chosed for phytotoxicity study.

►**Human can be exposed to fine and untrafine PM by plant ingestion. Dietary intake** is the main route of human exposure to heavy metals except inhalation (Tripathi et al., 1997). Vegetables constitute an important part of the human diet since they contain carbohydrates, proteins, vitamins, minerals and fibers required for human health (Khanna, 2011). Thus information about heavy metal concentrations in food products and their dietary intake is very important for assessing their risk to human health (Zhuang et al., 2009). Many research works have focused on the assessment of potential health risks by bioaccessibility for inhabitants in the vicinity of hazardous sites, such as mines and smelters, because of their exposure to environmental heavy metals via consumption of farm crops (Hu et al., 2013, 2012; Pelfrêne et al., 2012; Uzu et al., 2014). **Metal bioaccessibility** linked to PM ingestion from vegetables could be evaluated to determine potential risks for humans. Determination of metals bioaccessibility from ingested polluted matrices (soils and vegetables) constitutes an important step for health risk assessment. Indeed, a bioaccessibility adjustment factor is necessary to accurately assess the potential health risks to avoid overestimation of the risk (Huang et al., 2014). The BARGE (BioAccessibility Research Group of Europe) research group developed *in vitro* tests (Figure 1) in order to respond to REACH regulation (decrease tests performed on animals). The gastro-intestinal bioaccessibility of pollutants by ingestion of metal-rich PM, polluted soils or vegetables are therefore can be measured *in vitro* and then provide a good assessment of bioavailability *in vivo*.

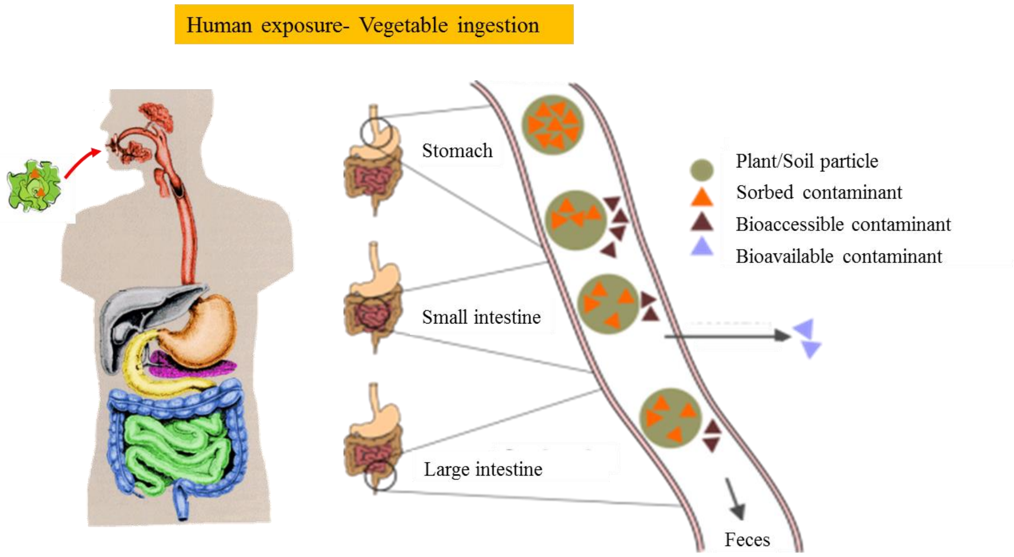

Figure 1 Bioavailability of metals, risk management for human health by vegetable ingestion.

This thesis is part of a dynamic research on soil-plant-atmosphere transfers of metal(loid)s in the environment in particulate form: Thesis G. Uzu and DIMENSION project. **The precise objective of this thesis was** to study the bioavailability of metal(loid)s in plants in relation to the exposure conditions and this both in controlled conditions and field.

<u>The PhD work focused on:</u>

1) Assessing the impact of metal(loid)s from PM on consumed vegetables, based on the determination of bioavailability as well as phyto-toxicity on such plants.

2) Studying metal foliar transfer mechanism by plants, transfer kinetic with exposure duration and quantities, and translocation factor (leaf to root).

3) Studying particles-phyllosphere interactions, the fate of metals in/on plant leaves influenced by possible bio-physicochemical transformations and their mechanism.

Additionally, this study also aimes to investigate the localization and speciation of metals present on and inside leaves by scanning electron microscopy coupled with energy dispersive X ray microanalysis (SEM-EDX) and electron paramagnetic resonance (EPR). This work was done in collaboration with the LASIR (Thesis of Vincent Dappe, University of Lille 1, 2015).

4) Studying the human bioavailability for metal(loid)s in various contaminated plants in relation to physicochemical parameters, PM type and exposure condition. This step permits to evaluate the health risk via hazard quotient calculated with total and bioaccessible concentrations.

This study is useful for monitoring regulated industries and agriculture activities, favoring sustainable agriculture (managing pollutions) in urban areas, as well as improving procedure to reduce human exposure to metal(loid)s. This project is important for management of urban agriculture area in China, France and other industrialized countries and will be helpful to urban planners and environmental risk managers who seek to reconcile economic goal and public health. The following scheme presents the global scientific context with the various PM in the ecosystem and relevant sanitary risk, and the different questions discussed in this work (Figure 2). The work of the thesis participates in enlightening the mechanisms in the air-plant-soil system and clarifying the relationship between human and environment. The details of the work are presented in the chapter 2 (Material and Methods) and the chapters 3, 4, 5 (Results and Discussion).

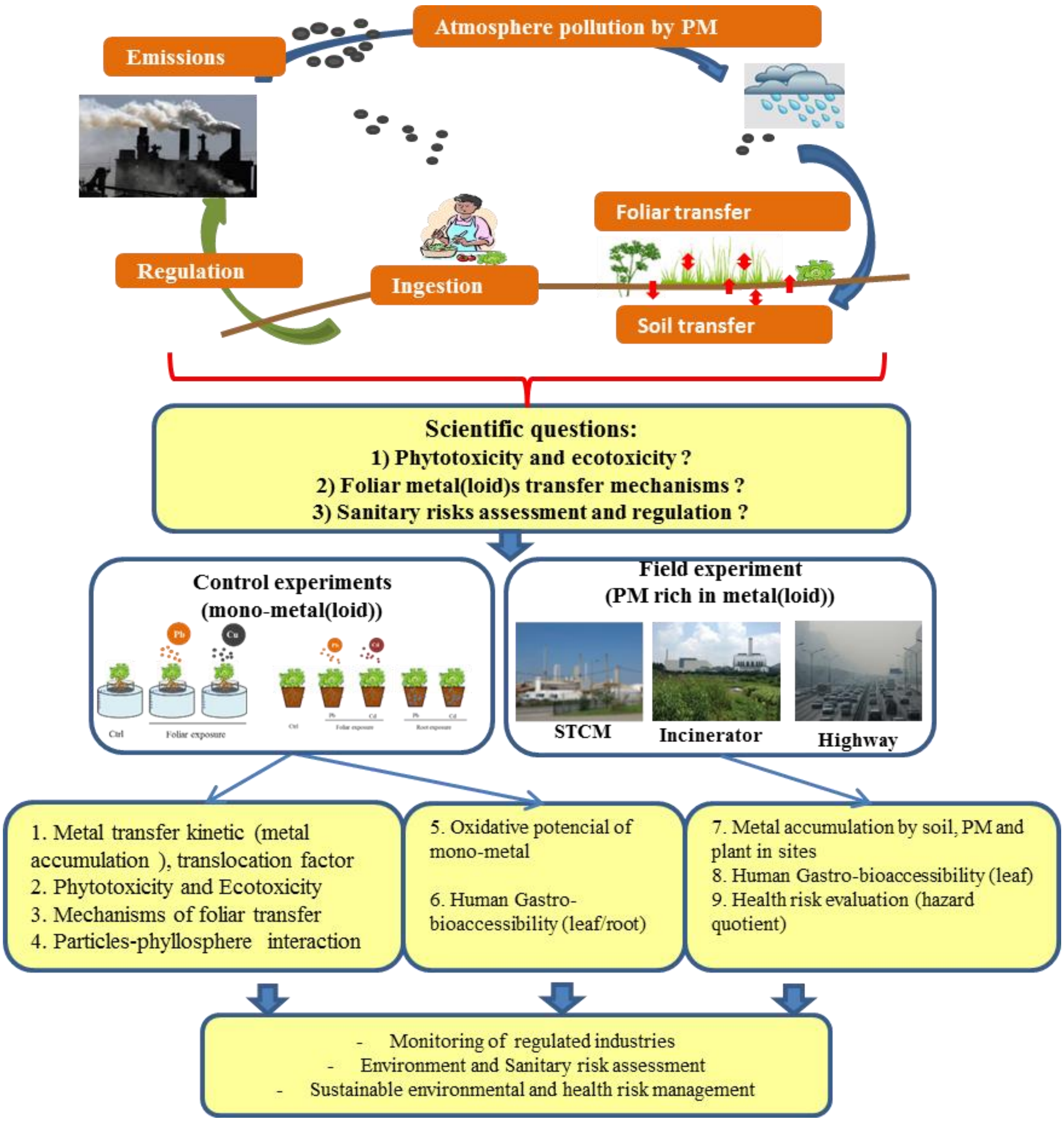

Figure 2 The general scientific context of the thesis.

The manuscript is divided into 6 chapters detailed below:

Chapter 1 (introduction both in French and English): The first chapter describes the context of the work and a literature review of problems induced by fine and ultrafine particles, vegetable quality, the bioaccessibilibty of metal particles and human health.

Chapter 2: The second chapter describes the study sites and the materials and methods used for the control and field experiments.

Chapter 3: In the third chapter we present the results on the control conditions by exposed vegetables with lead (sub-microparticles) and copper (nano particles). This part is presented in a form of two submitted articles «Kinetic study of phytotoxicity induced by foliar lead uptake for vegetables exposed to fine particles and implications for sustainable urban agriculture» and « Foliar uptake and phytotoxicity of copper oxide nanoparticles and consequences for sustainable urban agricultures». This chapter studies metal transfer kinetic with exposure duration and exposure quantities, metal translocation factor (leaf to root), assessed the impact of MTE on the vegetables consumed by humans (phytotoxicity by foliar transfer). Moreover, the metal foliar transfer mechanism by plants, particles-phyllosphere interactions, the fate of metals in/on plant leaves influenced by possible bio-physicochemical transformations chemicals (mechanisms) were also studied.

Chapter 4: The fourth chapter presents the result of health risk of PM enriched with the various metal(loid)s observed in the atmosphere of urban areas (lead recycling factory) and manufactured mono-metallic oxide particles. This chapter is presented in a form of two accepted articles published in 2014: «Foliar uptake and metal(loid) bioaccessibility in vegetables exposed to particulate matter» and «Lead and Cadmium Phytoavailability and Human Bioaccessibility for Vegetables Exposed to Soil or Atmospheric Pollution by Process Ultrafine Particles». This chapter compares the foliar uptake of several metals (Process-PM, CdO, Sb_2O_3 and ZnO), different vegetables and their bioaccessibility, and the influence of exposure conditions (foliar exposure and root exposure) on the metal bioaccessibility.

Chapter 5: The fifth chapter presents the field study of soil-plant-atmosphere metals transfers in the case of waste incinerator and highway; consequences for pollutants human bioaccessibility; human exposure assessment incorporate bioaccessibility measurements. We measured the metal accumulation by soil, PM and plants, discussed the regulation values of metal pollutant for soil and plant. Non-carcinogenic risk assessment was calculated both with total and bioaccessible metals. This study permits to provide more accurate information on metal uptake by plants in real scenarios, highlight the importance of bioaccessible metal concentration on human health risk assessment. The improved method and tests permit a better risk evaluation.

Chapter 6: The conclusions and perspectives are present in the sixth chapter.

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Chapter 2 Materials and Methods

► In order to respond to different objectives of the thesis, experiments of PM characterization, phytotoxicity tests, atmosphere-plant transfer mechanisms, as well as risk assessment were conducted. This chapter describes all the techniques, experimental set-up and protocols used in this study. Further details are provided in the various targeted chapters.

In the present study, edible parts of vegetables were used as biomarkers to assess the toxicity of PM enriched in metal(loid)s. The parameters determined in these experiments include:

1) Metal(loid)s accumulation by roots and leaves using ICP-OES and ICP-MS.

2) Phytotoxicity tests based on lettuce and cabbage with shoot growth, gaseous exchange and fatty acid parameters.

3) Metal localization and chemical speciation studied by microscopy (SEM-EDX), Raman microspectrometry (RMS) and electron paramagnetic resonance (EPR) analysis.

4) Gastro-bioaccessibility of PM and edible parts of vegetables.

2.1 Study sites

To investigate the metal(loid) transfer through vegetables exposed to fine and ultrafine PM and the associated health risks, two industrial areas that can emit PM enriched with metal(loid)s were chosen for the present study: a lead recycling plant (Société de Traitements Chimiques des Métaux : STCM, Toulouse, France) and a waste incinerator (Guangzhou, China). Moreover an urban site near a highway (Guangzhou, China) was also studied.

2.1.1 Société de Traitements Chimiques des Métaux (STCM)

2.1.1.1 Presentation of the company

STCM is a secondary lead smelter which currently recycles batteries (Schreck et al., 2011) and other lead materials (scraps, waste, oxides, etc.). It is an installation classified for environmental protection (ICPE).

STCM produces soft lead and lead alloys (calcium - antimony - tin). With 65,000 tons of lead per year, STCM is the leading producer of recycled lead in France. The main markets are the batteries, ballistics, ionizing protections building. The company produces 25% of the world recycled lead (also called secondary lead).

Established in 1952, STCM began operations in Toulouse, France. In 1967, a second factory was established in Bazoches-Les-Gallerandes (Loiret, France). Initially, family-oriented company, STCM was bought by the German group Metallgesellschaft in 1993 then by the American group Quexco in 1996, through its UK subsidiary ECO-BAT Technologies. The company is particularly voluntary on environment and health issues. Indeed, the STCM is certified ISO 9001 and ISO / TS 16949 (at the request of cars customers) since August 2003 for its management system of quality (SMQ, *Système de Management de la Qualité*). The ISO 14001 certification was obtained in September 1999 and recertified together with the OHSAS 18001 certification since December 2002 for its environmental, health and safety management systems (respectively SME, *Système de Management de l'Environnement*, and SMS, *Système de Management de la Santé et de la Sécurité au travail*). All these certifications are grouped in an integrated management system (*SMI, Système de Management Intégré*).

2.1.1.2 STCM smelter in Toulouse

The study site is located in 30-32 Avenue de Foundeyre, 31200 Toulouse (STCM-Toulouse). It is in urban area of Toulouse and southwest of France (43°38’12’’ N, 01°25’34’’ E). At its creation in 1952, the plant in Toulouse was far away from residential area. Over time, the area has gradually developed. The north of Toulouse became the industrial center of the city. The STCM-Toulouse with an area of 23 650 m² is now surrounded by a fuel depots (Site Seveso II), a dairy, and subdivisions. In this context, and for strategic and economic reasons, STCM had opted of expropriation infrastructure furnaces and therefore stop his melting and refining activity in 2011. Regarding to the legislation on activities of ICPE, and in respect to the polluter pays principle, the company will rehabilitate its contaminated soil by Pb and other metals and metalloids (Cd, Cu, Zn, Sb, etc.) depending on the future use of the soil. This environment is described in the Figure 1 below.

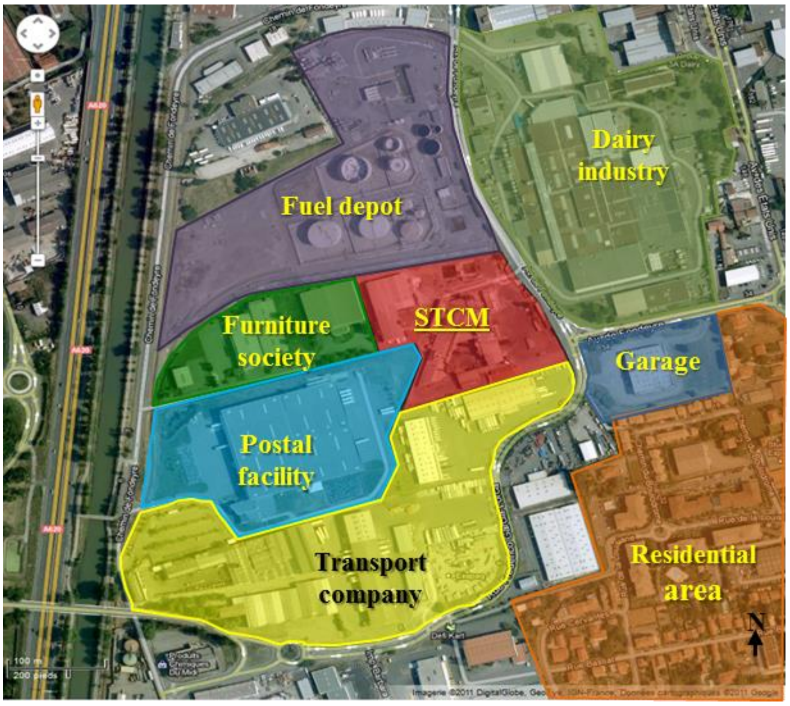

Figure 1 Aerial map of STCM Toulouse and its nearby infrastructures (Source: Foucault et al., 2012, from Google Map adaptation).

2.1.2 Area near waste incinerator and highway

The second study is performed in collective gardens located near a waste incinerator and a highway in Guangzhou, China. Incineration is often proposed as the treatment of processing municipal solid and/or hazardous wastes (Schuhmacher et al., 1997), however substantial PM can be emitted from waste incinerator and contains significant Cd and Pb (Cr, Ni, Cu...) (Li et al., 2015). Road traffic is also an important source of PM due to tires, wear of brakes, other vehicle components, exhaust emissions and re-suspended road dust, and contains variable quantities of Cu, Zn, Ni, and Pb (Hu et al. 2003;Johansson et al. 2009; Piatak et al. 2004).

The studied incinerator is running since 2006, with an area of 101778 m^2 and daily waste processing capacity of 1040 t, representing about 1/7 of local household waste. And, it is the only waste incinerator in Guangzhou. The sample site is located less than 0.5 km away from the incinerator (Figure 2a). The vegetable garden near highway (<0.2 km) is located in Guangzhou Higher Education Mega Center (urban area, more than 30 km away from the incinerator). A bus station is just outside the garden (Figure 2b).

Figure 2 Photography of the sampling area near a waste incinerator(a) and highway(b).

2.2 Experimental set-up in controlled or field conditions of plant culture and exposure

2.2.1 Vegetable sampling.

Several kinds of vegetables were chosed, and then grown and collected in the sampling sites to study foliar and root transfer of various metals (Pb, Cd and Cu, Zn, etc.) (Table 1): (i) Lettuce (*Lactuca sativa* L.), a leafy vegetable that has been used to test soil–plant transfer of metals (Alexander et al., 2006; Waisberg et al., 2004), and it is a very good bioaccumulator of heavy metals and nutrients (Pichtel and Bradway, 2008); (ii) Cabbage (*Brassica oleracea*), a leafy green biennial, grown as an annual vegetable for its densely-leaved heads; (iii) Radish (*Raphanus sativus* L.), an edible root vegetable of the Brassicaceae family that was domesticated in Europe (Lewis-Jones et al., 1982) in pre-Roman times, which shown to accumulate metals in roots to a higher extent than others members of *Brassicaceae and* was chosen for root pollutant uptake; (iv) Spinach (*Spinacia oleracea* L.), an edible flowering plant in the family of Amaranthaceae, native to central and southwestern Asia; (v) Water spinach (*Ipomoea aquatica* Forsk.), an important leafy vegetable in Southeast Asia, India, and southern China, but easily polluted by Hg, Cd, and Pb (Göthberg et al., 2002) and (Xin et al., 2010); (vi) Parsley, an herb used to give fragrance to different food products (Doğru and Erat, 2012); (vii) Leaf lettuce (*Lactuca sativa* (L.) *var. longifoliaf*), Leaf mustard (*Brassica juncea* (L.) Czern Coss), Purslane (*Portulaca oleracea* L.), Welsh onion (*Allium fistulosum* L.), Bitter lettuce (*Cichorium endivia* L.) and Amaranth (*Amaranthusmangostanus* L.) were collected from Guangzhou because they are representative vegetable species and currently eaten by local residences. Leaf mustard can hyper accumulate Cd and many other soil trace elements. Amaranth was found to accumulate higher Cd than other vegetables in the study of Alam et al. (2003).

Therefore lettuce and cabbage were chosen for foliar pollutant (Pb and Cu) uptake in our control experiments. Spinach and lettuce were used in order to study foliar uptake and bioaccessibility of metal(loid)s in vegetables exposed to PM (enriched in Pb) and mono-metallic oxide particles (CdO, Sb_2O_3 and ZnO). Lettuce, radish and parsley were chosen to study Pb and Cd phytoavailability and human bioaccessibility after soil or atmospheric pollution exposure. Leaf lettuce, water spinach, leaf mustard, welsh onion, purslane, bitter lettuce and amaranth are used to study metal pollution near a waste incinerator and highway in Guangzhou, China (Table 1).

Table 1 Plant species studied in controlled conditions and field experiments.

Plant species	Botanical name	Objectives
Lettuce	*Lactuca sativa* L.	Foliar/root transfer and bioaccessibility
Cabbage	*Brassica oleracea*	Foliar transfer
Radish	*Raphanus sativus* L.	Foliar/root transfer and bioaccessibility
Spinach	*Spinacia oleracea* L.	Foliar transfer and bioaccessibility
Parsley	*Petroselinum crispum*	Foliar/root transfer and bioaccessibility
Water spinach	*Ipomoea aquatica* Forssk.	Pollution from incinerator and highway
Leaf lettuce	*Lactuca sativa* (L.) var. *longifolia*	Pollution from incinerator
Leaf mustard	*Brassica juncea* (L.) Czern. & Coss.	Pollution from incinerator
Purslane	*Portulaca oleracea* L.	Pollution from incinerator
Welsh onion	*Allium fistulosum* L.	Pollution from incinerator and highway
Bitter lettuce	*Cichorium endivia* L.	Pollution from incinerator
Amaranth	*Amaranthusmangostanus* L.	Pollution from highway

2.2.2 Soils for experiments

Soils for microculture experiments were sampled in the peri-urban zone of Toulouse 43°35′57.24″N, 1°23′41.15″E. Acidic digestion and ICP-OES analyses were performed to confirm the absence of trace metallic element contamination. The soil exhibiting the following physico-chemical characteristics: rich in organic matters (OM): organic matter 44.7 g kg^{-1}, organic carbon 26 g kg^{-1}; optimum pH (6.5); average cationic exchange capacity (CEC): 12.3 cmol(+) kg^{-1}; granulometry (%): clay 13.3, thin silt 24.8, rough silt 23.5, thin sand 22.5, rough sand 15.9 (Schreck et al., 2011). This soil was sieved through a 2-mm mesh size before use.

Soil for long-term experiments:

1) The uncontaminated control soil used in this study was purchased from a market garden (Support de culture NFU 44 551, Batanic-Serre Salève, Saint Julien en Genevois). The soil contained primarily peat moss, clay, crushed expanded clay and vegetable

composted (organic matter, 36% [w/w] of gross product, 66% [w/w] of dry weight; NPK 8-4-8 in 1 kg m^{-3}: N, 1000 mg kg^{-1}; P, 500 mg kg^{-1}; and K, 1000 mg kg^{-1}).

2) The polluted soil was collected from the STCM site. For several decades, atmospheric fallouts of metal-enriched particles have induced high concentrations of Pb and other metals such as Cd and Cu in nearby top-soils (Schreck et al., 2011; Uzu et al., 2009). Thus, the sampled soil is contaminated with a complex mixture of metals: Pb, Cu and Cd at the concentrations of 2546, 23 and 3 mg kg^{-1} of dry weight, respectively. This historically polluted soil was sampled from the 0-25 cm top-soil layer, air-dried at room temperature for a week, disaggregated and, finally, sieved to retain aggregates smaller than 2 mm. This soil (pH=6, organic matter 5.2%) is mainly composed of 12% clay, 47% silt and 41% sand (Lévêque et al., 2013).

Rhizosphere Soil

Rhizosphere soil is defined as the soil adhering to roots and directly influenced by root exudates (Hinsinger, 2001). Rhizosphere soils were sampled after separation from bulk soil by carefully shaking the root system. Rhizosphere soil was preferred than bulk soil in order to better assess the soil-root pathways of metal(loid)s (Uzu et al., 2014). In this study, rhizosphere soil in the vegetable gardens near waste incinerator and highway were investigated to acquire pollutant information in those sites.

2.2.3 Metal-PM characterization and reactivity

The metals studied in this work refer to: Pb, Cd, Cu, Zn, Sb, Cr, etc. High purity monometallic and monospeciation fine (<1 µm) and ultrafine (<0.1 µm) particles were purchased from Aldrich® (CdO, CuO, PbO, $PbSO_4$, Sb_2O_3, and ZnO) and Acros Organics® ($CdCl_2$). Metal(loid) enriched PM were collected in the different sites from secondary lead smelter (STCM), waste incinerator and highway. PM in the smelter courtyard were sampled with 9.2 cm diameter high density polyethylene (HDPE) Nalgene funnels connected to 2 L HDPE Nalgene bottles fixed on posts 2m above ground (Schreck et al., 2012b). The funnels and bottles were acid washed before initial use. Sample bottles filled with high purity water were used as references (Schreck et al., 2012b). Atmospheric fallouts were measured using plastic Owen gauges that enable wet and dry atmospheric depositions to be recorded (Schreck et al., 2012a). According to Uzu et al. (2011a, 2011b, 2009), the process (crushing, fusion, reduction and refining) PM from the secondary lead smelter contains particles with the following diameter distributions: 9, 50, 20 and 21 % in the $PM_{>10}$, PM_{10}, $PM_{2.5}$ and PM_1 fractions, respectively. Lead is the major element (33 % of total metals content), and its speciation was found mainly to be PbS, $PbSO_4$, $PbO.PbSO_4$ and PbO. Some minor elements were also measured: Cd (0.2 %), Sb (0.55 %) and Zn (0.2 %).

The PM from vegetable gardens near waste incinerator and highway were sampled by collecting dust from the cement surface of house roof/window at each sampling site with a vacuum cleaner. According to Zeng et al. (2014), air particles settled on cement surfaces located in the vegetable gardens were collected in order to represent air particles deposited on the vegetable leaves.

The physico-chemical properties of mono-metallic particles are listed in Table 2. Their specific areas ranged from 4.3 to 25 m^2 g^{-1}. The water solubility of these metal(loid) oxides is low due to the particulate status of the metal(loid)s, which leads to a very low ionic (dissolved) fraction. Our previous study (Goix et al., 2014), proposes global threat scores to prioritize the harmfulness of anthropogenic fine and ultrafine metallic particles emitted into the atmosphere at the global scale. The metal(loid) oxides currently observed in the atmosphere (CdO, CuO, PbO, $PbSO_4$, Sb_2O_3, and ZnO) were assessed by performing complementary *in vitro* tests: ecotoxicity, human bioaccessibility, cytotoxicity, and oxidative potential. Based on the combination of (eco)toxicity and physicochemical results, hazard classification of the particles is proposed as follow: $CdCl_2$>CdO>CuO>PbO>ZnO>$PbSO_4$>Sb_2O_3 (Goix et al., 2014).

The particle aggregates observations performed by SEM-EDX are present in Figure 3 from Goix et al. (2014). Cd compounds exhibited the highest threat score due to their high cytotoxicity and bioaccessible dose, whatever their solubility and speciation, suggesting that cadmium toxicity is due to its chemical form rather than its physical form. CdO was in a monteponite mineralogical form, and was not soluble whatever the media. By contrast, the Sb_2O_3 threat score was the lowest due to particles with low specific area and solubility, with no effects except a slight oxidative stress. The fine and ultrafine metallic particles physicochemical properties reveal differences in specific area, crystallization systems, dissolution process, and speciation, various mechanisms may influence their biological impact. CuO was present in two main mineralogical forms, one of them being fibrous. The specific area presented a medium value and numerous aggregates of nanometric particles were observed. It exhibited a high threat score with high cytotoxicity (despite the essential character of Cu) and high oxidative potential (high dithiothreitol (DTT) activity) (Figure 4). ZnO was identified as zincite. Nanometric particles strongly aggregated, explaining its medium specific surface area. Its threat score was medium, characterized by high bioaccessible dose and ecotoxicity after long-term exposure (Goix et al., 2014). Sb_2O_3 was present in two mineralogical forms: senarmontite and valentinite. Individual particles were mainly cubic, but some fibrous shapes were observed. The specific area was low and the particles almost insoluble. Its threat scorewas the lowest calculated with no effects except a slight oxidative stress (Figure 4). PbO was identified as a mix of massicot and litharge with high specific surface area. Strong aggregation of particles was hypothesized to cause heterogeneity of suspensions during the tests. No reproducible results were available for PbO cytotoxicity and ecotoxicity assays (Goix et al., 2014).

Table 2 Physico-chemical properties of mono-metallic particles.

	Supplier	**CAS**	**Particle size (µm)**	**Specific area (m^2 g^{-1})**	**Water Solubility**	**Purity (%)**	**MW(g mol^{-1})**
CdO	SIGMA-ALDRICH	1306-19-0	~1µm (PM_1)	4.3	0.227 mg L^{-1} (20°C)[2]	99.5 %	128.41
Sb_2O_3	SIGMA-ALDRICH	1309-64-4	< 0.25 µm (PM_1)	15.6	0.017 mg L^{-1} (20°C)[2]	≥ 99.9 %	291.52
ZnO	SIGMA-ALDRICH	1314-13-2	< 0.1 µm $PM_{0.1}$	15-25	1.6 mg L^{-1} (20°C)[2]	≥ 99.0 %	81.39
$CdCl_2$	ACROS organics Product of:ES	10108-64-2	For analysis ACS,anhydrous	2.31[1]	Very hygroscopic , 1400 g L^{-1} (20 ºC)		183.31
$PbSO_4$	SIGMA-ALDRICH	7446-14-2		0.06[1]	0.404 g L^{-1} (25 ºC); 0.032 g L^{-1} (15 ºC)	99.995%	303.25
PbO	SIGMA-ALDRICH	1317-36-8	<10µm	29.2[1]	Insoluble in water, soluble in acids and alkalies	≥ 99.9 %	223.19
CuO	SIGMA-ALDRICH	1317-38-0	<0.050 µm (TEM)	12.55[1]	Insoluble		79.55

[1] Measured specific area by Goix et al.(2014).
[2] Measured water solubility by Xiong et al.(2014a).

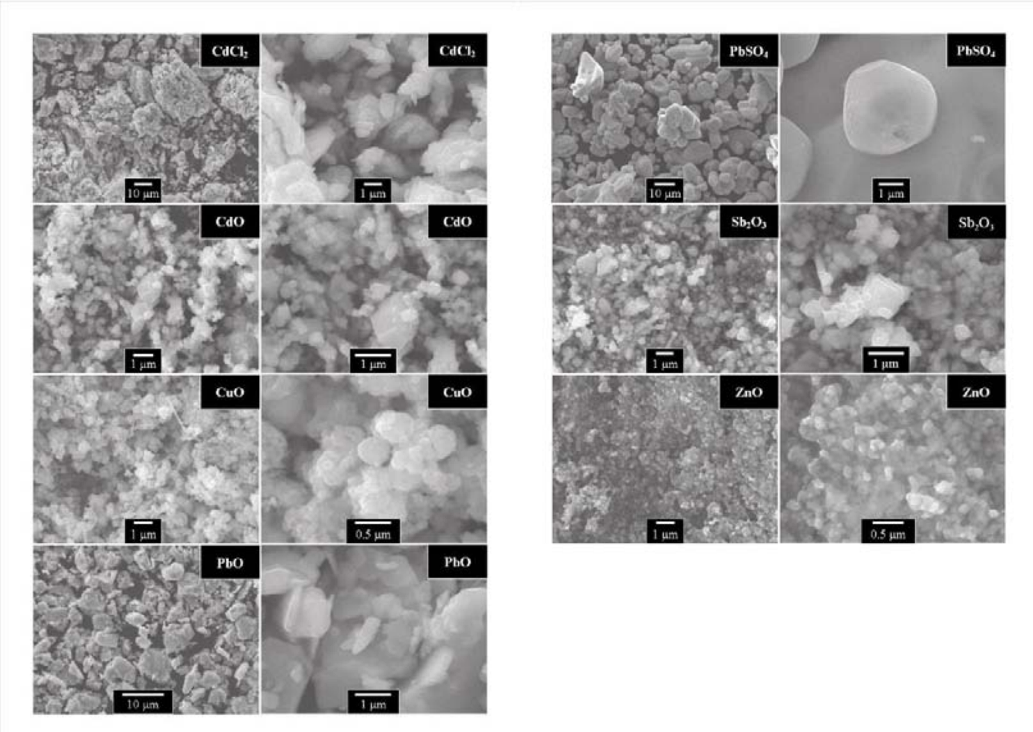

Figure 3 Particle aggregates observations performed by SEM-EDX (Goix et al., 2014).

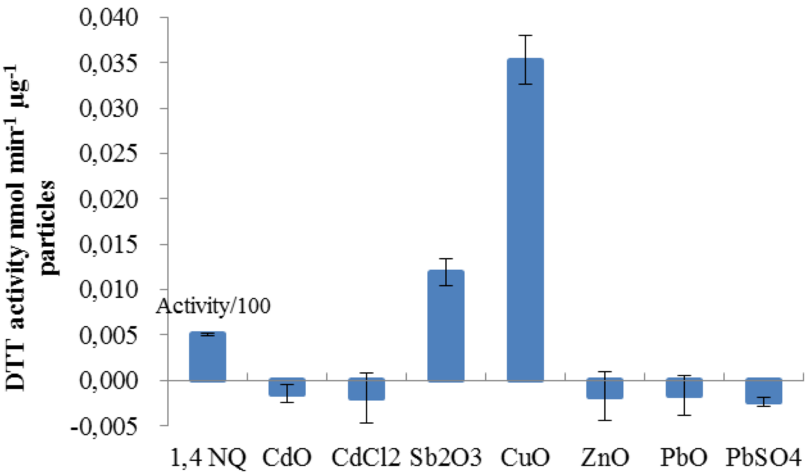

Figure 4 DTT activity as a function of different PM.

2.2.4 Foliar transfers studied by micro-culture experiments

2.2.4.1 Micro-culture device (RHIZOtest®)

Micro-culture was performed with RHIZOtest® device (ISO/CD 16198), as described by (Uzu, 2009). The device used hydroponic solutions for pre-culture (Figure 5 ①). During this period, root and foliar growth were fast without contamination. In test culture period (Figure 5 ②), the device allowed soil–plant contact indirectly, roots separated from the soil by a membrane, can absorb nutrients from soil (Bravin et al., 2010). Thereafter, the different compartments of plant can be analyzed separately. The experiments were carried out in controlled conditions with a day/night temperature regime of 25±2 °C (16 h) / 20±2 °C (8 h) and a light intensity of 425 ± 50 photons µmol m^{-2} s^{-1}. The relative humidity was adjusted to 65±5%.

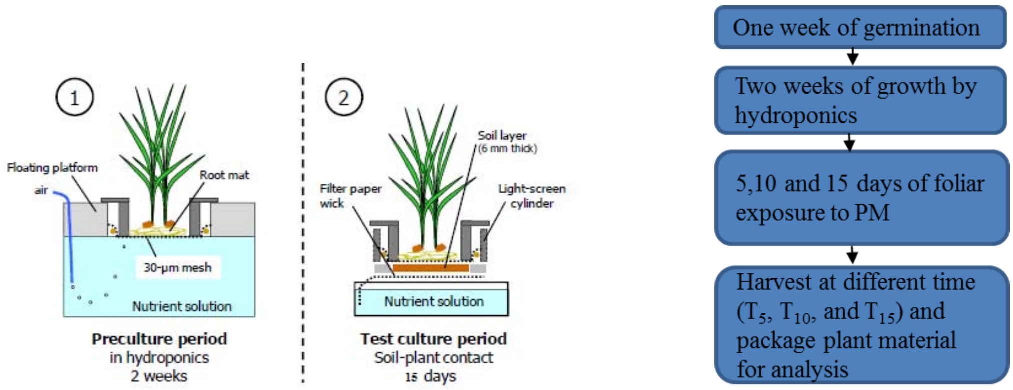

Figure 5 Steps for Micro-culture device (ISO, 2011).

2.2.4.2 Experimental set-up: particle deposition on vegetable leaves

Seed germination was carried out with commercial lettuce, cabbage and spinach seeds (from Botanic®, France). Seeds are firstly immersed in a 6% (v/v) solution of hydrogen peroxide (H_2O_2) for 10 min to ensure surface sterility followed by rinsing thoroughly with distilled water, and then germinated on moistened filter paper in a germination chamber under optimal conditions (in darkness at 22°C of temprature and 100% humidity by germination solution (Table 3)). After one week of seed germination, when the primary roots were about 2-3 cm in length, the healthy and uniform seedings were transplanted to PVC tanks (Figure 5 ①) containing growth solution (Table 3). After pre-culturing of 15 days, plants were transferred in individual pots containing 9.1g of uncontaminated soil layer (Figure 5 ②), each pot was filled with 400ml incubation solution (Table 3).

Table 3 Nutrient solution according to the phases of RHIZOtest® (ISO/CD 16198).

	Chemicals	Concentrations
Germination solution (Pre-culture period)	$CaCl_2 \cdot 2H_2O$	0.6mM
	H_3BO_3	2 µM
Hydroponic growth solution (Pre-culture period)	KH_2PO_4	0.5 mM
	KNO_3	2 mM
	$Ca(NO_3)_2 \cdot 4H_2O$	2 mM
	$MgSO_4 \cdot 7H_2O$	1 mM
	$CuCl_2 \cdot 2H_2O$	0.2 µM
	H_3BO_3	9.98 µM
	$MnCl_2 \cdot 4H_2O$	2 µM
	$ZnSO_4 \cdot 7H_2O$	1µM
	$Na_2MoO_4 \cdot 2H_2O$	0.05 µM
	NaFe(III) (EDTA)	0.1 mM
Incubation solution (Test culture period)	KH_2PO_4	0.05 mM
	KNO_3	2 mM
	$Ca(NO_3)_2 \cdot 4H_2O$	2 mM
	$MgSO_4 \cdot 7H_2O$	1 mM

Three-weeks-old plants were then exposed to monometallic (Pb, Cu, Cd, Sb, and Zn) particles or process PM with an applicator brush onto the entire leaf surface according to the method described by (Xiong et al., 2014a). Surfaces of leaves were moistened with a hand spray first, and the brush was only used to deposit the PM onto the leaf surfaces without spreading. The homogeneity of the solution distribution on the leaves and the reproducibility of the technique were confirmed in previous tests (Xiong et al., 2014a). Indeed, we used this method because the deposition of dry PM with an applicator brush without pre-wetting can mechanically bring the particles into the leaf and favor coarse particle uptake, whereas fine particles are mainly present in a wet deposit (Xiong et al., 2014a). To avoid artifacts during our experiments, no surfactants were used (whereas these products are sometimes used to favor particle dispersion). In order to precisely determine the quantity of pollutants deposited on the leaf surface, the applicator brush was rinsed with 20 mL of distilled water after PM deposit (Xiong et al., 2014a). The rinse water was then centrifuged, and after removing the supernatant, the particles were mineralized with aqua regia, and the metal(loid) concentrations were measured. This procedure therefore allows knowing the exact amount of PM deposited on the leaves. Then, the interaction between PM and leaves could be simulated in quite a realistic way. Furthermore, a geotextile membrane was placed between aerial parts and roots to protect them from the deposition of PM and then to avoid root transfer (Hurtevent et al., 2013; Xiong et al., 2014a). The characteristics of the studied metal(loid) particles were listed in Table 2.

The design of exposure duration and quantity, and the choice of the different metals and plant species were performed according to several scientific goals.

Pb and Cu foliar exposure was performed during 5, 10, 15 days to allow the PM foliar uptake by plant (lettuce and cabbage). Three treatments were defined in function of the metal quantities deposited: a control condition without any metal input (0 mg), 10 mg and 250 mg of PM inputs. Totally, the experimental design consisted of 36 plants for each plant species, including three exposure durations, three exposure quantities and four replicates (Figure 6). This experiment permits to study the metal transfer kinetic and the involved transfer mechanisms and phytotoxicity.

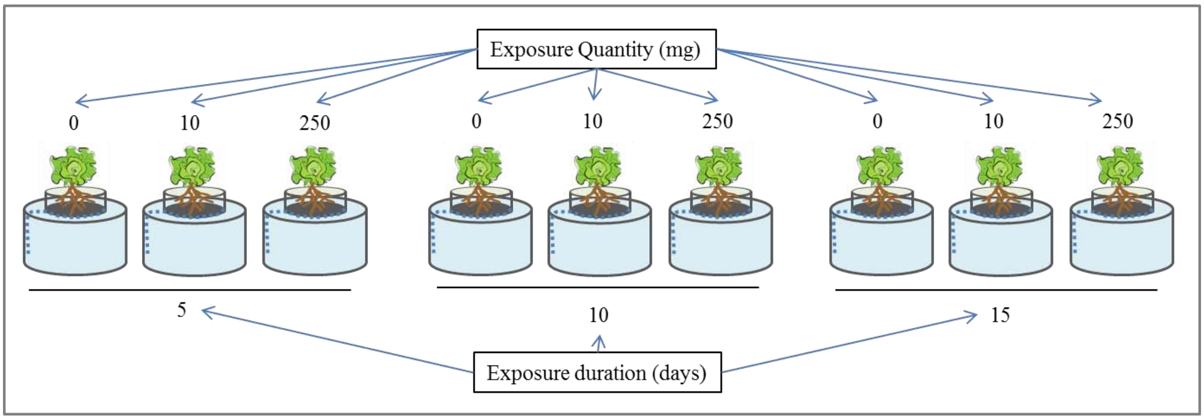

Figure 6 Experimental design of foliar exposure of lettuce and cabbage with Pb and Cu particles.

Exposure experiment with process PM (mainly enriched with lead) and mono-metallic commercial species as oxides (CdO, Sb_2O_3 and ZnO) were performed with plants (spinach and cabbage) during 3 weeks with five replicates per treatment. Vegetable leaves were exposed to a total quantity of 300 mg of PM. This experiment permits to study metal gastric bioaccessibility associated with the consumption of vegetables in the case of foliar metal(loid) uptake in order to highlight the influence of metal(loid) nature on bioaccessibility and health risk.

After exposure, plants were harvest for analyzing metal concentration, translocation, phytotoxicity, bioaccessibility, compartmentalization and metal speciation.

2.2.5 Field experiment: gastric bioaccessibility of uptaken lead and cadmium after foliar and root transfers

The field experiment was performed in pots (Figure 7) in the courtyard of the STCM factory, as described before. Foliar or root metal plant uptake experiments were performed using three different vegetable species: (i) lettuce *(Lactuca sativa*), (ii) radish (*Raphanus sativus*), and (iii) parsley (*Petroselinum crispum*).

For the foliar transfer experiments, 45 pots, containing one plant each and 4 kg of unpolluted soil, were placed for 6 weeks in the courtyard of the secondary Pb smelter. The annual emission of PM from the smelter is 328 kg (Schreck et al., 2012b). These emitted PM have been previously characterized by Uzu et al. (2011a, 2011b, 2009). The size distribution of PM is mainly comprised in the 1-100 µm size range (89% in volume fraction). Lead is the major element in these atmospheric fallouts with 33% of the total metal content and the second major metal is Cd at 2.7%. Lead speciation in PM was found to be mainly PbS, $PbSO_4$, $PbO.PbSO_4$ and PbO (Uzu et al., 2011b).

During the vegetable exposure experiments, Pb concentration in industrial atmospheric fallouts was controlled in the smelter courtyard by collecting bulk depositions in Owen gauges (Taylor and Witherspoon, 1972), following the procedure described by Schreck et al. (2012b) and Gandois et al. (2010). Metal concentrations of Pb and Cd in atmospheric fallouts were respectively 450 ± 7 and 0.8 ± 0.1 mg $m^{-2}.month^{-1}$.

Here, the plants were only exposed to atmospheric fallouts by placing a geotextile membrane on the top of the unpolluted soil (25.5 ± 1.6 mgPb and 0.45 ± 0.05 mgCd kg^{-1} of dried soil) to avoid soil contamination and metal transfer via root uptake (Uzu et al., 2010; Schreck et al., 2012a, 2012b).

For the root transfer experiments, twenty-five plants of each species were individually cultivated for 6 weeks under an unpolluted atmosphere in 5 L pots containing each 4 kg of polluted soil (2000 mgPb and 1.2 mgCd kg^{-1} of dried soil) collected from the smelter's site. The soil was therefore contaminated by the same atmospheric PM fallouts as previously described for foliar metal transfer. The physico-chemical properties of the soil were: pH_{water} = 8.5, CEC = 6.9 cmol(+) kg^{-1}, amounts of soil organic matter and carbonates ($CaCO_3$) were respectively: 6 and 4 g kg^{-1}. Light, temperature, humidity and pluviometry were controlled during all the experiment as reported by Schreck et al. (2012b).

Plants were harvested after 6 weeks and used for further analysis-metal concentration, translocation, phytotoxicity, bioaccessibility, compartmentalization and speciation.

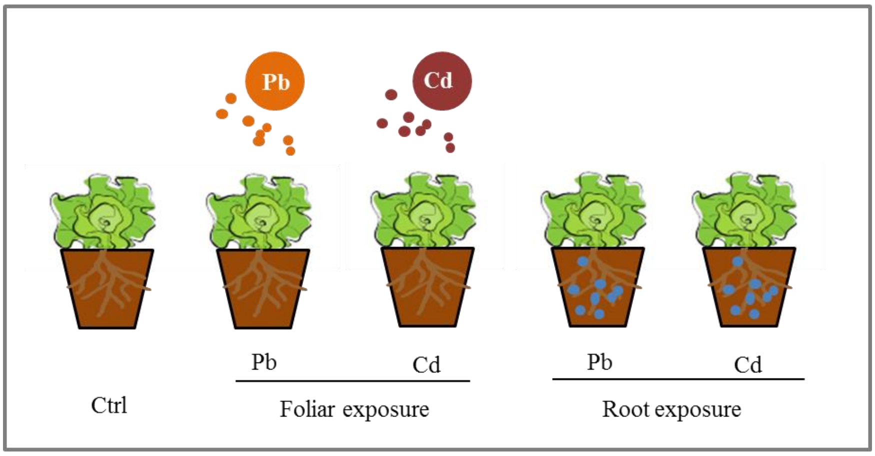

Figure 7 Foliar and root exposure with Pb and Cd particles from STCM (example for lettuce).

2.3 Sample preparation and analysis

2.3.1 Trace metal element analysis

The procedures for trace metal element analysis in vegetable and soil samples are presented in Figure 8.

The Protocol of acid mineralization of plant samples is performed as follows: Vegetables were harvested at different endpoint and stored in sealed polyethylene/paper bags. Afterward, vegetables were separated to root and shoot parts, and fresh weighted. A two-step washing method with deionized water was performed for vegetable leaves (Birbaum et al., 2010; Uzu et al., 2010) in order to reproduce the scenario of consumption by humans. The PM desorption procedure consisted in a global washing of plant leaves, first in running tap water for 30 s and then in two baths of deionized water for 1 min. Vegetable leaves were oven-dried at 40 °C for 72 h, weighted and then grinded and sieved to < 250 µm particle size. This particle size is believed to be ingested or directly available for ingestion by children (Gron and Andersen, 2003). Vegetables samples (0.125 g per sample) were then acid mineralized in a 1:1 mixture of HNO_3 (65%) and H_2O_2 at 80˚C for 4h (Uzu et al., 2014) with a Digiprep® instrument from SCP Science producer, which is a block digestion system allowing fast and uniform plant samples digestion. This Digiprep is equipped with a temperature sensor, thus, making it possible to work at desired temprature.

The Protocol of acid mineralization of soil samples is performed as follows: Soil samples were collected in the same time of vegetables. Soil samples were air dried at room temperature, grounded and homogenized. All prepared samples were sealed in clean polythene bags in the refrigerator (5˚C) before analysis. Mineralization of soil samples (0.5g per sample) was performed in aqua-regia (1/4 HNO_3 and 3/4 HCl) at 80˚C for 4h by Digiprep® instrument (Lévêque et al., 2014).

Finally the digested samples were filtered through the 0.45µm fine filters, appropriate diluted and stored at 4˚C for metal concentration analysis (Radojevic and Bashkin, 1999). Metal(loid)s concentrations were measured by inductively coupled plasma-optical emission spectrometry ICP-OES (IRIS Intrepid II XXDL) or inductively coupled plasma-mass spectrometry (ICP-MS, X Series II, Thermo Electron). Method control was performed with blank and reference samples submitted to the same treatment (mineralization and assay). The accuracy of measurements was checked using reference materials: (i) Virginia tobacco leaves for plant tissues, CTA-VTL-2, from the National Water Research Institute, Canada. (ii) Swiss loess soil for soil samples, RTH 912, from the Wageningen Evaluating Programs for Analytical Laboratories, Netherlands. The detected limits for Cr, Cu, Zn, Cd, Sb and Pb were respectively 0.1, 1.3, 2.2, 0.2, 0.2 and 0.3 µg L^{-1}.

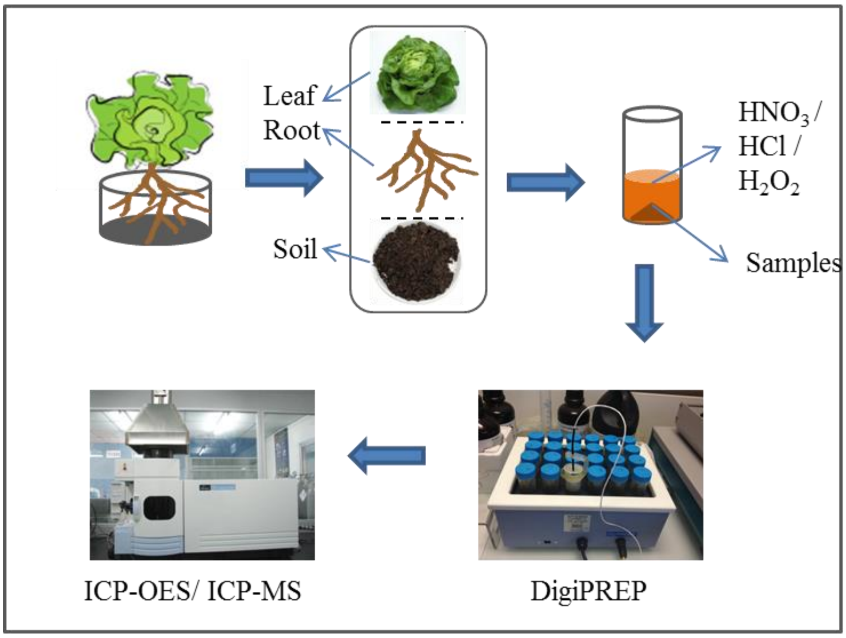

Figure 8 Trace metal element analysis in vegetable and soil samples.

Trace metal translocation factor (TF) in vegetables were determined by the equation $TF=C_{root}/C_{leaf}$ in case of foliar exposure, where C_{root} and C_{leaf} are metal concentrations (mg kg^{-1}) in roots and aerial parts, respectively (Serbula et al., 2012; Xiong et al., 2014b). TF>1 indicates that the vegetable translocates metals effectively from shoot to root (Marchiol et al., 2004a). Plant global enrichment factor (GEF) was calculated as $GEF=C_{plant}/C_{control}$, where C_{plant} and $C_{control}$ are metal concentrations (mg kg^{-1}) in plant parts (leaves and roots) from the polluted samples and the control samples, respectively. According to Mingorance et al.(2007), values of GEF>2 in the samples are considered to be enriched.

2.3.2 Phytotoxicity

Metal(loid)s phytotoxicity was assessed through plant biomasses, gas exchange and fatty acids composition determination of plants.

2.3.2.1 Plant biomass.

Plant Biomasses were measured including the shoot length, number of leaves, fresh and dry weight of leafs and roots, water content and tolerance index (TI) of shoot growth.

Shoot length was measured from the bottom of the shoot to the endpoint of the highest leaves (Figure 9). Shoot growth was expressed as the increased shoot length during exposure compare to the first exposure day.

The plants were fresh weighted (FW) and oven-dried to determine the dry weight (DW), and water content (water %) was calculated according the fresh and dry weight of plants using following formula:

$$Water\ \% = \frac{FW - DW}{FW} 100\%, \text{ Eq. 1}$$

TI evaluates the plant tolerance against or susceptibility to the composite effects of metals. According to Mahmood et al.(2007), data of individual growth parameter (G_x) was normalized (G_i) relative to the maximum value (G_{max}) of that parameter in the data set, G_i = (G_x G_{max}^{-1}). Summing all the G_i's and then divided by the total number of G_i's calculated the TI = $\Sigma(G_x\ G_{max}^{-1})\ n^{-1}$. The TI of shoot growth ranged from 0 to 1, with highly tolerant when it approaches to 1 and having extremely susceptible to the composite effects of heavy metal when it approaches to 0.

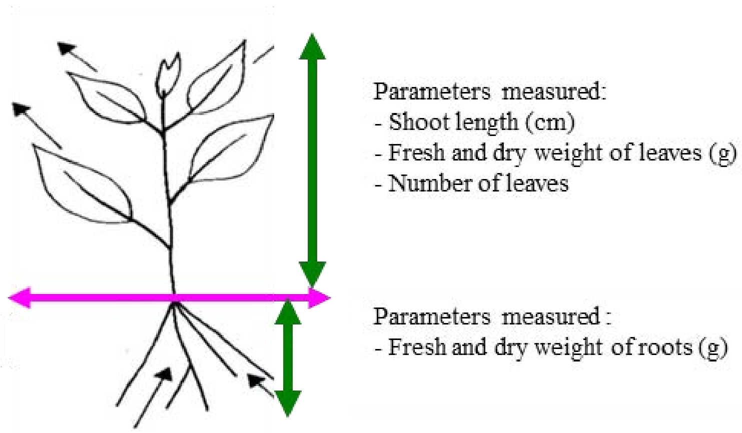

Figure 9 Schematic diagram of plant biomass measurement.

2.3.2.2 Gas exchange

Leaf gas exchange measurements were performed at greenhouse in four plants per treatment. Measurements were performed on two well exposed and fully expanded leaves. Gas exchange parameters such as net photosynthesis (Pn), stomatal conductance (gs), transpiration rate (Tr) and intercellular CO_2 concentration (Ci) were recorded simultaneously using a portable infrared gas analyzer (LI-COR 6400 XT) for estimating the influence of metal stresses on photosynthesis. The infrared gas analyzer system (IRGAs) was equipped with a clamp-on leaf chamber that possesses 6 cm^2 of leaf area (Figure 10). According to Majer and Hideg (2012), IRGAs measure the reduction in transmission of infrared wavebands caused by the presence of CO_2 between the radiation source and a detector. The reduction in transmission is a function of the concentration of CO_2. Thus the measurements of gas exchange are based on the differences in CO_2 and H_2O in an air stream that is flowing into the leaf cuvette (reference cell) compared to the air stream flowing out of it (sample cell). The rate of CO_2 uptake ($\mu mol\ m^{-2}\ s^{-1}$) is used to assess the rate of photosynthetic carbon assimilation, while the rate of water loss ($mol\ H_2O\ m^{-2}\ s^{-1}$) is used to assess the rate of transpiration and stomatal conductance.

Gas exchanges measurements were performed under irradiance at 425 $\mu mol\ m^{-2}\ s^{-1}$ and at the temperature of 23±2 °C. During the measurement, humidity was fixed at 65%, CO_2 concentration was maintained at a constant level of 380 $\mu mol\ mol^{-1}$ using a LI-6400-01 CO_2 injector with a high pressure liquid CO_2 cartridge source.

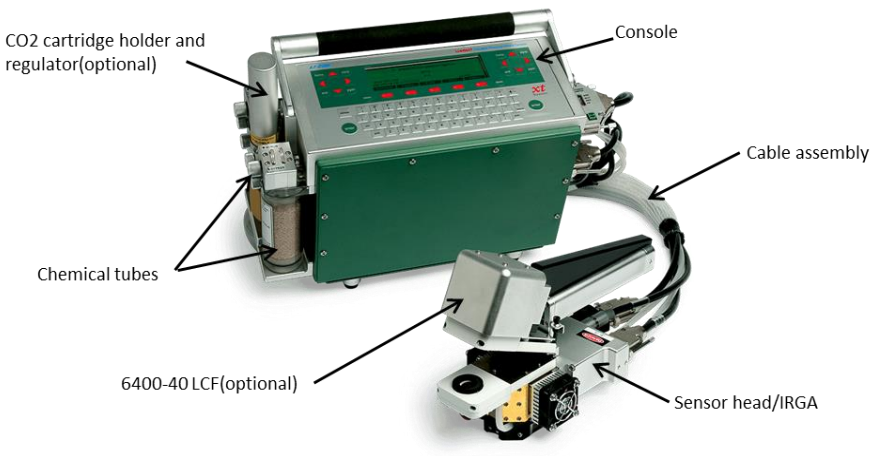

Figure 10 Portative photosysthesis measure analysis.

2.3.2.3 Fatty acid composition, analysis and identification

The fatty acid composition of lettuce leaves is modified after exposure to metals. A standardized foliar fatty acid ratio (C18:3/(C18:2 + C18:1 + C18:0)) is nowadays available to diagnose soil contamination by metals *ex situ* (AFNOR, 2012) and it was also successfully used in field (Le Guédard et al., 2012a, 2012b).

For fatty acid composition analysis, one cm^2 of fresh young leaf (secondary developed leaf) was collected for every sample and then immediately placed in screw-capped tubes containing 500 µL methanol acidified with 2.5% H_2SO_4 solution. Samples were stored at 4 °C before analysis. Fatty acid analysis and identification were performed according to Le Guédard et al. (2012a, 2009) and Schreck et al. (2013): leaf samples stored in acidified methanol were heated to 80 °C for 1 h and then 1.5 mL of H_2O and 0.75 mL of hexane were added after cooling. Fatty acid methyl esters (FAMEs) were extracted into hexane by vigorous shaking and a two-phase system was established by centrifugation (1500 g, 5 min). Separation of FAMEs in the hexane phase was performed by gas chromatography (Hewlett Packard 5890 series II) on a 15 m × 0.53 mm Carbowax column (Alltech) with flame ionization detection. The temperature ramp was performed (Le Guedard et al., 2008): the initial temperature of 160 °C was held for 1 min, followed by a 20 °C min^{-1} ramp to 190 °C and a second ramp of 5 °C min^{-1} to 210 °C, and maintained for 6 min. FAMEs were identified by comparing their retention times with standards (Sigma Chemical, St. Louis, MO, USA).

In general, the lipid composition of leaf consisted of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and alpha linolenic acid (C18:3). Lipid composition was dominated by polyunsaturated fatty acids mainly represented by linolenic acid (C18: 3) which accounted for 39.6-49.0% and 37.9-51.8% of the total fatty acid content, respectively in lettuce and cabbage in our study.

The work done by Le Guédard et al.(2012a, 2009) showed that the fatty acid ratio (Eq.2) in plant leaves has the indication characteristics of metabolic effect after the plant exposure to metals and has been used to diagnose the impact of foliar metal uptake by Schreck et al.(2013). Moreover, the index Z, defined by the Eq.3, is also a parameter allowing to evidence metal contamination (can present a descending trend for contaminated plants when exposure duration is more than two weeks). It is the product of three fatty acid concentration ratios (C16:1/C16:0, C18:3/C18:0 and C18:1/C18:2, Eq.3) with different rates (1, 0.57, and 0.23 respectively), which can be used when exploring the relationships between uptake and phytotoxicity (Schreck et al., 2013). Thus, change in the ratio of lipids in leaves can be used as a biomarker of stress when plants are exposed to PM by foliar way.

$$Fatty\,acid\,ratio = \frac{C18:3}{C18:0+C18:1+C18:2} \times 100\% \qquad \text{Eq.2}$$

$$Z = \left(\frac{C16:1}{C16:0}\right) \times \left(\frac{C18:3}{C18:0}\right)^{0.57} \times \left(\frac{C18:1}{C18:2}\right)^{0.23} \qquad \text{Eq. 3}$$

2.3.3 Transfer to human: risk assessment after plant ingestion-Gastric bioaccessibility tests.

Bioaccessibility tests were performed according to the Unified BARGE (BioAccessibility Research Group of Europe) Method (UBM) (Cave et al. 2006; Foucault et al. 2013; Uzu et al. 2011). This *in vitro* method is used for simulating the human digestive procedure by synthetic digestive fluids. The concepts of gastric bioaccessibility and oral bioavailability are fundamentally important for quantifying the risks that are associated with oral exposure to environmental contaminants(Guerin et al., 2015; Wragg et al., 2011).

The UBM measure is based on a successive extractions simulating saliva, gastric and intestinal steps. The fluids (Table 4) used in the procedure are composed of organic and inorganic products with compositions comparative to human digestive fluids. Digestive fluids were prepared the day before the test and allowed to be stirred overnight with a magnetic stirrer. The digestive fluids are prepared each time a test is scheduled in vitro, and maybe stored for 48h at 4˚C. On the test day, the fluids are allowed to warm to 37˚C at least 2 hours before use, the temperature is then maintained at 37 ° C during the extraction procedure. The pH of each solution should be checked before use and must be in the range specified in Table 4. Othervise, the pH should be adjusted with hydrochloric acid (37% HCl) and /or sodium hydroxide (1M NaOH).

Table 4 Composition of digestive solutions for testing in vitro United BARGE Method (UBM, Denys et al., 2009).

	Saliva solution (pH = 6.5 ± 0.5)	**Gastric solution (pH = 1.0 ± 0.2)**	**Duodenal solution (pH = 7.4 ± 0.2)**	**Bile solution (pH = 8.0 ± 0.2)**
Inorganic solution *	448 mg KCl 444 mg NaH_2PO_4 100 mg KSCN 285 mg Na_2SO_4 149 mg NaCl 0.9 ml 1M NaOH	1376 mg NaCl 133 mg Na_2PO_4 412 mg KCl 200 mg $CaCl_2$ 153 mg NH_4Cl 4.15 ml HCl (37% w/w)	3506 mg NaCl 2803 mg $NaHCO_3$ 40 mg KH_2PO_4 282 mg KCl 25 mg $MgCl_2$ 90 µl HCl (37% w/w)	2630 mg NaCl 2893 mg $NaHCO_3$ 188 mg KCl 90 µl HCl (37% w/w)
Organic solution*	100 mg of urea	325 mg of glucose 10 mg Glucuronic acid 42.5 mg of urea 165 mg of glucosamine hydrochloride	50 mg of urea	125 mg of urea
Reagents added to the inorganic and organic solutions	72.5 mg of alpha-amylase 25 mg mucin 7.5 mg Uric acid	500 mg of BSA* 1.500 mg mucin 500 mg of pepsin	100 mg $CaCl_2$ 500 mg BSA 1.500 mg of pancreatin 250 mg of lipase	111 mg $CaCl_2$ 900 mg BSA 3000 mg of pig bile

* BSA = Bovine Serum Albumin

* masses indicated for 250 ml of solution

For each vegetable or soil sample (dried and ground <250µm), 0.6 g is weighed and introduced into centrifuge Nalgene ® polycarbonate tubes. For each sample, tests are carried out in triplicates. The protocol simulates the human gastro-intestinal tract through 3 different compartments: mouth (5 minutes), stomach (1 hour) and small intestine (4 hours). The procedure of the in vitro test is presented in Figure 11. For gastro phase, samples is firstly mixed with 9 ml of saliva solution (pH 6.5) and shaken for 5 min. Then 13.5 mL of gastric solution (pH 1.0) is added to the suspension. The pH of the solution is adjusted to 1.2 using HCl (37%) or NaOH (1M) if necessary. The suspension is mixed using an end-over-end rotation agitator at 37 °C for 1 h. At the end of the gastric phase, the pH of the suspension is checked again to be in the 1.2–1.6 range otherwise sampled were re-prepared. This step is very important for making sure not to underestimate the values of metal (precipitate with increasing pH) bioaccessibility. If the pH is within the desired range, the gastric phase was extracted by centrifuging the suspension at 3000 g for 5 min.

Corresponding to the intestinal phase, samples firstly followed the gastric phase procedure without extraction by centrifuging, then duodenal solutions (27 ml, pH = 7.4) and bile solution (9ml, pH = 8.0) are added. The pH of the solution is checked and must be in the range 6.3 ± 0.5. Otherwise, it should be adjusted with HCl (37%) or NaOH (1M). The suspension is then placed in an an end-over-end shaker for 4 h at 37 °C. The pH is measured again and adjusted to 6.3 ± 0.5 if necessary. Finally, after centrifugation at 3,000 g for 5 min, the supernatant is collected, acidified (1 mL HNO_3 65%) and stored at 4 °C. All the samples obtained at the extraction steps of the procedure: gastric and duodenal will be analysed by ICP/MS or ICP/OES according to the searched metal.

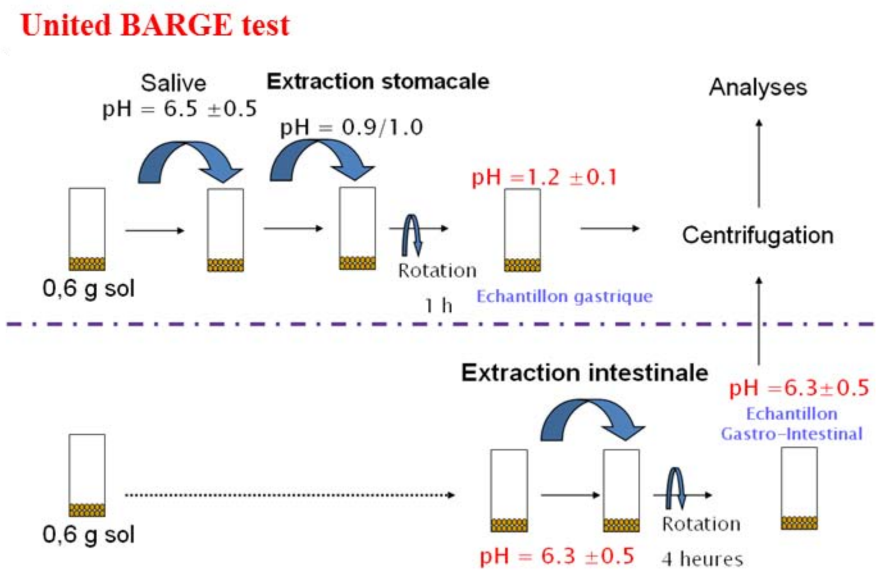

*Figure 11 Schema of in vitro United BARGE test (Caboche, 2009)***.**

The results are expressed in mg of bioaccessible contaminant per kg of solid matrix (vegetable or soil). They could then be expressed as percentage of bioaccessible contaminant by comparison to total metal content in the solid sample (Eq.4).

$$\%\ bioaccessible = \frac{Bioaccessible\ metal\ concentration\ (mg\ kg^{-1})}{Total\ metal\ concentration\ (mg\ kg^{-1})} \times 100 \qquad \text{Eq.4}$$

This test performed on vegetables and polluted soils in a large range of concentrations could then give an overall picture of human ingestion risks linked to exposed vegetable (and/or soil) consumption.

2.3.4 Compartmentalization of metal(loid)s in the plants in relation with exposure conditions

Microscopic and spectroscopic observations. Leaf observations on a microscopic scale were performed on vegetables by using complementary techniques. The localization and speciation of metals present on and inside leaves were investigate by scanning electron microscopy coupled with energy dispersive X ray microanalysis (SEM-EDX), Raman microspectrometry (RMS) and electron paramagnetic resonance (EPR) analysis. The work was done in collaboration with the LASIR (Vincent Dappe Thesis, University of Lille 1, 2015). These methods were chosen because they have a low spatial resolution to get elemental distribution and speciation of metals with few micrometer depth resolutions. SEM-EDX provide elemental distribution on leaf surface, the morphology and elemental composition of PM deposits were determined at a higher resolution by SEM-EDX, which has a low sensitivity (about 1000 mg kg^{-1}) but a better lateral resolution than micro-X-ray fluorescence (µXRF) (Schreck et al., 2012b). RMS provided the molecular composition of metal-rich areas. In most cases, leave areas (or particles) were analyzed by both SEM-EDX and RMS after a careful relocalization; thus, electronic and optical images as well as elemental and molecular compositions could be compared (Uzu et al., 2010). EPR is the best method allowing identification of copper (II) complexes. Actually, copper (II) cations exhibit many valuable features for the application of EPR spectroscopy. With its d9-configuration (S= ½, I = 3/2), it shows a strong deviation from a regular electron-density distribution. Thus, one can get information about the type of complex formed by an adsorbed species and its geometry. Moreover, paramagnetic species such as radicals can be successfully detected. Microscopy and spectroscopy are complementary tools to determine metal distribution and speciation in leaves.

A) Scanning electronic microscopy coupled with EDX (SEM-EDX). The adaxial leaves surfaces were observed using an optical microscope and SEM-EDX. The optical images was recorded with an Olympus BX 41 microscope equipped with a ×100 (N.A 0.9) objective. SEM-EDX measurements were carried out using a Quanta 200 FEI instrument equipped with a Quantax EDX detector to characterize the morphology of the leaf surfaces, elemental distribution in metal-enriched areas and the particles-phyllosphere interactions. Portions of leaves were dried, fixed on a carbon substrate and covered with carbon before analysis (Schreck et al., 2012b). The apparatus was operated in low-vacuum mode (~133 Pa) at 20 kV and with a pressure of 0.98 Torr. A working distance of 10 mm between the probe and the sample was set for optimal analysis. The recovering rate of the leaves by the particles was estimated by image analysis of back scattering emission images (BSE). ImageJ software was used for image analysis to estimate the distribution of the particles on the leaf surfaces. Fixed parameters were kept similar for image analysis of all BSE images. Binary images creation (black and white image) from BSE images gives the total number of particles on the defined area as well as the corresponding surface area of the particles. The recovering rate was estimated by calculating the ratio between the total area covered by particles and the total surface of the field defining in the image, i.e the total leaf surface. In order to be representative, 5 images of about 230×210 μm^2 by leaf were systematically recorded, given a total surface of 48.3 mm^2 for each leaf. Finally, the recovering rates of stomata were also estimated through the similar procedure. The rate calculation was performed on a selected area of the BSE image including stomata, solely.

B) Raman Microspectrometry (RMS)

The particle composition was characterized using RMS. The measurements were performed using a Raman HR 800 UV (HORIBA), equipped with a ×80 Mitutoyo (N.A. 0.55) objective and a solid MDB 266 system laser beam (Coherent Laser Group) emitted at 266 nm. The excitation using a UV laser (266nm) was necessary to probe the surface of leave, since many biologically important molecules such as chlorophyll have an intense fluorescence emission when excited in the visible range (400 nm $< \lambda <$ 800 nm) (Uzu et al., 2010). The Raman spectra were analyzed on the basis of the shifts in Raman peak values, changes in full width at half-maximum (FWHM) ratios of Raman bands, and normalized intensity variations.

C) Electron Paramagnetic Resonance (EPR) analysis

Metal speciation in lettuce leaves contaminated by fine and ultrafine particles was studied using EPR in continuous waves (CW). EPR spectroscopy experiments were performed at a fixed microwave frequency, the X-band (9.6 Ghz) using a Brüker ELEXSYS E500 spectrometer in CW. The microwave power and amplitude modulation were set to 100 mW and 4G, respectively. The spectra were collected at room temperature on the contaminated leave directly inserted in 8 mm quartz tube. The g-factor, which is a constant of proportionality, was determined in order to have information on the properties of metal in a certain environment. When there is a hyperfine structure, it is possible to determine the hyperfine constant A which corresponds to the distance between two consecutive stripes. The accuracy of the determination of EPR parameters was ±0.005 and ±0.0005 for g values of metal ions and radicals, respectively. The accuracy of the estimation of the hyperfine splitting constant was $\pm 10^{-4}$ cm^{-1}. For pure powder CuO nanoparticles, typical EPR spectrum showed three equally spaced peaks related to the hyperfine coupling of Cu^{2+} electron spin (S= 1/2) with its nuclear spin (I=3/2). The parallel components g// and A// were determined from the spectrum, the values were 2.337 and 179 10^{-4} cm^{-1}, respectively. The perpendicular region cannot be precisely determined from experimental spectrum. Speciation of Cu was determined through the comparison of parallel component values deduced from our experimental spectra and literature values.

Besides the three methods mentioned before, other observation and analysis methods for particles-phyllosphere interaction are also listed in the Table 5.

Table 5 Observation and analysis methods for particles-phyllosphere interactions.

Observation and analysis methods	**FUNCTION**
Micro-X-ray fluorescence (μXRF)	Map elemental distributions of centimeter regions of leaves
Time-of-flight secondary ion mass spectrometry and imaging (ToF-SIMS)	Timely identification typical clusters of the molecule; Spatial distribution of clusters; Depth profiling
X-ray diffraction(DRX)	Identifies the majority speciation but does not detect minor mineralogical phases (<1% by weight), or amorphous phases.
Extended X-ray Absorption Fine Structure (EXAFS)	Average atomic speciation of the sample for a particular target element

2.4 Statistical analysis

Statistical analysis. Total and bioaccessible metal(loid) concentrations are subjected to statistical analysis. Metal concentrations in plants are subjected to analysis of variance (ANOVA), using software software SPSS 18.0, Statistica, Edition '98 (StatSoft Inc., Tulsa,OK), and R software (R Development Core Team, 2014) with the ade4 package. Data were tested for normal distribution and homogeneity of variance preceding the ANOVA. Mean and range values (mean±standard deviation) were used to assess contamination levels of metals in soil, PM, and plants. Significant differences (p-value < 0.05) were determined using the LSD Fisher test, Duncan's test, and Student's t-test. The associations between parameters were investigated using partial correlation analysis, linear regression, and principal component analysis (PCA). Association between pairs of variables was investigated using Pearson correlation coefficient and t-test. The coefficient of determination (R^2) was used to indicate how well observed outcomes fit a statistical model, as the proportion of total variation of outcomes explained by the model.

In total, Figure 12 describes all consumed vegetables, measured metals, protocols, and techniques used in this study.

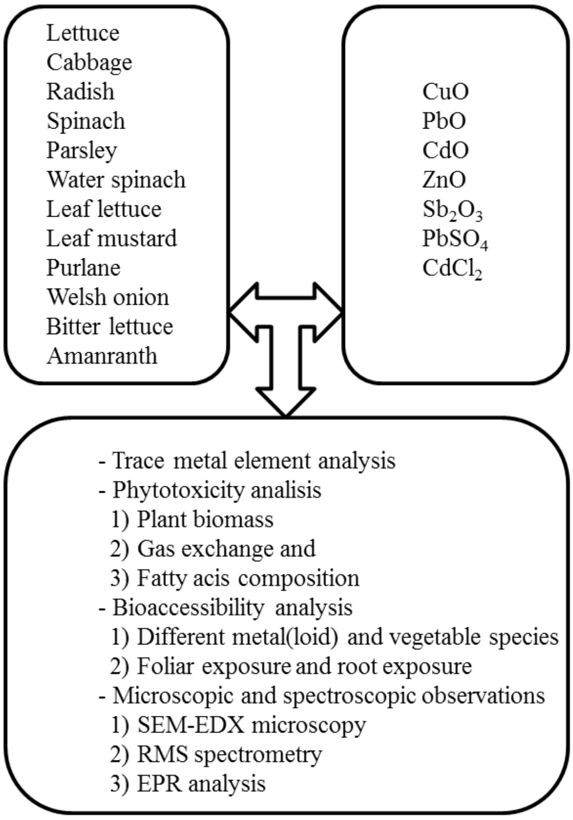

Figure 12 Studied vegetables, measured metals and various analysis methods.

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RESULTS & DISCUSSION

► Using the above-mentioned experimental techniques, several short or long term experiments were carried out to investigate metal foliar uptake, enrironmental and health risk of metal(loid)s, sustainable development and management of urban agriculture near industries or urban areas. The following chapters describe the Results and Discussion section of this work. They are presented as scientific publications published or submitted.

In the first part of this section (Chapter-3), several control experiments were performed to study the effects of foliar exposure by non essencial or essencial metals (Pb and Cu). The transfer mechanisms, phytotoxicity, transfer kinetics and translocation factor were studied.

Secondly, studies were performed with process PM and mono-metallic oxide particles in greenhouse, or directly near the lead recycling factory, to study the metal foliar transfer and oral bioaccessibility. The oral bioaccessibility of metal(loid)s from PM and vegetables after ingestion were studied under foliar and root exposure (Chapter-4).

Then, in order to get more accurate information on the uptake of metals by plants in real scenarios, we invested soils, vegetables and particulate matters near a waste incinerator and a highway (Chapter-5). Therefore, this chapter is focused on metal(loid) effects on the agriculture in industrial and urban areas, and consequently their health risk.

Finally, the health risks for humans were studied both in control and field conditions. A global scheme of phytotoxity, ecotoxicity on vegetables and health risk assessment is proposed for metal(loid)s.

Then, our results highlight substancial development and propose solutions to better manage a sustainable agriculture in urban and peri-urban areas.

Chapter 3 Lead and copper phytotoxicity for lettuce and cabbage in case of foliar exposure - vegetable quality

Forwords

Nowadays, metal rich airborne fine and ultrafine particles (PM_{10}, $PM_{2.5}$ and PM_1 – rich in As, Cd, Cr, Cu, Ni, Pb, Zn...) is a significant phenomenon with atmosphere pollution. Some metals (Cd, Pb, Ni, As, Hg) are classified as "Substance of Very High Concern" (REACH Regulation) and their presence in fine particles makes them highly toxic (associated with the increased reactivity and surface area). The emission of metal-rich PM from industries can cause contamination of soil (Han et al. 2009; Lévêque et al. 2014; Li et al. 2014) and plants (Fernández Espinosa and Rossini Oliva, 2006; Uzu et al., 2014; Zhou et al., 2014).

Since in the previous studies, most of the work of vegetable pollution were focused on root transfer, the effect of polluted soils on vegetables have been well documented, while the pollution based on foliar transfers were significantly less studied.

In this chapter, we investigated therefore, the impacts of metal on vegetables in case of foliar transfer. This chapter presents the results of experiment in controlled conditions with Pb and Cu in function of particle size. This study aims to improve the understanding the particles-phylosphere interaction with foliar pathway:

A) Metal accumulation, translocation; Phytotoxicity; Transfer kinetics;

B) Compartmentalization and speciation; Transfer mechnisms, Bio-transformation.

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3.1 Kinetic study of phytotoxicity induced by foliar lead uptake for vegetables exposed to fine particles and implications for sustainable urban agriculture

Forewords

Lead (Pb) is an nonessential element for plants, and it's one of the main hazardous pollutant observed in the environment that originates from numerous sources (Shu et al., 2012). Owing to its strong (eco)toxicity and high persistence, Pb is considered by various regulations (European Reach regulation and European regulation on consumed plants) as the most important pollutant to study.

Actually, fine PbO particles currently observed in the environment are highly reactive particles due to their low size (<10μm) and high specific surface areas (29.2 m^2 g^{-1}) (Goix et al., 2014). Since the pollutants in the atmosphere could be removed by both dry and wet depositions (dry particulate deposition could contribute up to 60% of the total fluxes) (Galarneau et al., 2000), and it has recently been demonstrated that 25 to 40% of the total plant metal content can be derived from leaf transfer coming from industrial particles (Nowack and Bucheli 2007; Schreck et al. 2012), therefore atmosphere-plant transfer of metal is important for vegetable quality and risk assessment studies in addition to previous knowledge on soil-plant metal transfer.

Therefore, the objective of this preliminary study was to invest the foliar transfer of metal-rich fine and ultrafine particles: kinetic study of phytoavailability and phytotoxicity. Different leafy vegetables cultivated in RHIZOtest® devices were exposed in greenhouse for 5, 10 and 15 days to various PbO PM doses. First, the kinetic of transfer was evaluated in relation with lead concentration and exposure duration, phytotoxicity was then assessed (biomass, gas exchanges, water content and leaf fatty acid composition).

►This chapter is presented in a form of an accepted article by the Journal of Environmental Science in 2015.

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Kinetic study of phytotoxicity induced by foliar lead uptake for vegetables exposed to fine particles and implications for sustainable urban agriculture

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Abstract

At the global scale, foliar metal transfer occurs for consumed vegetables cultivated in numerous urban or industrial areas with polluted atmosphere. But, the kinetic of metal uptake, translocation and involved phytotoxicity were never jointly studied when vegetables are exposed to micronic and sub-micronic particles (PM). Different leafy vegetables (lettuces and cabbages) cultivated in RHIZOtest® devices were therefore exposed in greenhouse for 5, 10 and 15 days to various PbO PM doses. Kinetic of transfer and phytotoxicity were assessed in relation with lead concentration and exposure duration.

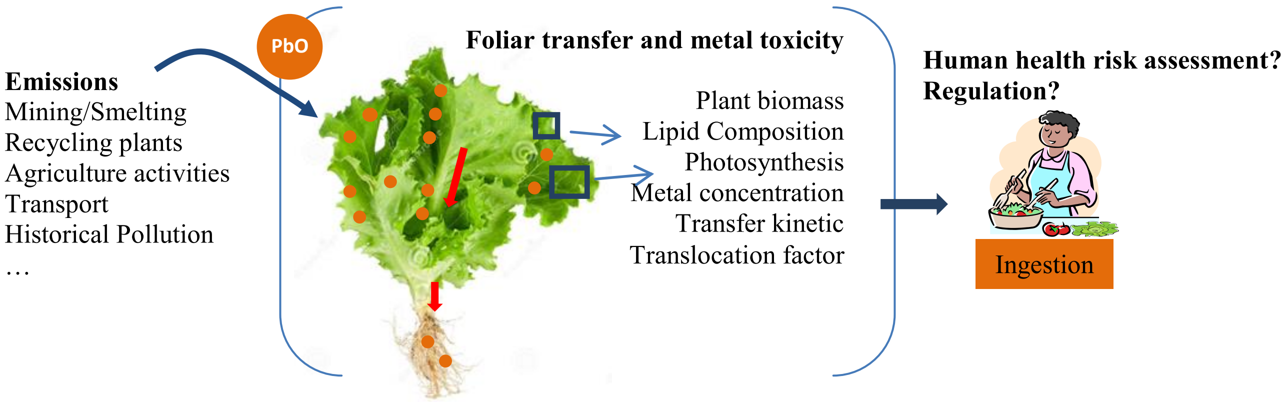
A significant Pb accumulation in leaves (up to 7392 mg·kg-1 DW in lettuce) with translocation to roots was observed. Lead foliar exposure resulted in a significant phytotoxicity: lipid composition change, decrease of plant shoot growth (up to 68.2 % in lettuce) and net photosynthesis (up to 58 % in lettuce). The phytotoxicity results indicated a plant adaptation to Pb and a higher sensitivity of lettuce in comparison with cabbage.

Air quality needs therefore to be considered for health and quality of vegetables grown in polluted areas such as certain megacities (in China, Pakistan, Europe...) and furthermore to assess the health risks associated with their consumption.

Key words: metallic particles, vegetables, foliar uptake, transfer kinetics and phytotoxicity.

Graphical abstract

The scientific context, main content and perspectives of the study.



3.1.1 Introduction

Urban agriculture is progressively developed in order to respond to sustainable aims of cities. However, environmental pollution needs to be assessed and reduced (Mombo et al., 2015; Pierart et al., 2015). Particularly, the proportion of metal(loid) micronic and sub-micronic particulate matters (PM) including nanoparticles has increased in the atmosphere with the expanding urban areas and development of industries (Luo et al., 2011; Zhao et al., 2012; Austruy et al., 2014; Uzu et al., 2014). According to Seinfeld (1986), natural sources of PM enriched with metals are generally coarse particles, while the metals in fine and ultrafine PM are related to anthropogenic sources. Fine and ultrafine PM are often highly reactive due to their large surface-area-to-volume ratio (Barrie, 1992; Gillespie et al., 2013). These PM can interact with terrestrial ecosystems (Schreck et al., 2012a, 2014), waters (Diop et al., 2014; Gupta et al., 2014), soils (Stampoulis et al., 2009; Schreck et al., 2011; Shahid et al., 2011, 2013a) and plants (Uzu et al., 2010; Hu et al., 2011; Xiong et al., 2014a). Thus, metal(loid)s carried by PM can induce a sanitary risk linked to polluted plants (Polichetti et al., 2009; Perrone et al., 2010; Xiong et al., 2014b).

Owing to increased use in numerous anthropogenic activities, lead is widely observed in all ecosystems at the global scale (Pourrut et al., 2011). Due to its strong (eco)toxicity and persistence, Pb is therefore considered by various European regulations such as REACH law. Fine PbO particles currently observed in the environment are highly reactive due to their low size (<10μm) and high specific surface areas (29.2 m^2 g^{-1}) (Goix et al., 2014). According to Zia et al. (2011), food is the major source of human exposure to Pb due to possible Pb bioaccumulation in the edible parts of vegetables. Lead pollution of leafy vegetables can be caused both by root transfer from polluted soils (Ma et al., 2010; Yin et al., 2011; Lombi et al., 2011) and by direct foliar uptake (Honour et al., 2009; Uzu et al., 2010; Schreck et al., 2012a, b). As atmospheric pollution increased since decades, the foliar way is nowadays currently taken into account (Xiong et al., 2014a). But, most of the studies focus on metal accumulation or shoot/leaf elongation (Little, 1978; Ward and Savage, 1994; Abbas and Akladious, 2012).

The PM-retention abilities of vegetables depend on several factors, such as leaf surface area, leaf longevity and cuticular structure (Freer-Smith et al., 1997; Barber, 2004; Rico et al., 2011; Schreck et al., 2012a).

Metals cause damages on plant leaves, stomata and leaf proteins (Pourrut et al., 2013; Xiong et al., 2014a). At cellular level, Pb toxicity results in overproduction of reactive oxygen species (ROS) (Shahid et al., 2014a, b). Morphological and growth parameters showed a decrease in root and shoot growth (Schreck et al., 2011) and alterations in root branching pattern in Lactuca sativa L. after Pb treatment (Capelo et al., 2012). Physiological processes, such as photosynthesis and water status, are particularly sensitive to metals (Monni et al., 2001; Austruy et al., 2013; Mateos-Naranjo et al., 2012; Shahid et al., 2014c). Moreover, Le Guédard et al. (2012a) reported that leaf fatty acid composition is a considerable biomarker of early effect of metals.

In that context, the kinetic of foliar transfer and Pb phytotoxicity was studied in controlled conditions with lettuce (Lactuca sativa L.) and cabbage (Brassica oleracea L var. capitata cv. Snowball), leafy vegetables which are currently cultivated and consumed at the global scale. The vegetables were exposed to PbO fine particles, thus, metal accumulation, biomass (leaf elongation, aerial mass), gaseous exchanges (Pn, gs) and leaf fatty acid composition were measured in function of exposure time.

3.1.2 Materials and Methods

3.1.2.1 Experimental conditions and set up

Micro-culture was performed with RHIZOtest® device (ISO 16198) in this study (Bravin et al., 2010). The device used hydroponic solutions for pre-culture (Fig. 1①). During this period, root and foliar growth were fast without contamination. In test culture period (Fig. 1 ②), the device allowed soil–plant contact indirectly, roots separated from the soil by a membrane, can absorb nutrients from soil. Thereafter, the different compartments of plant can be analyzed separately. The nutrient solutions according to the phases of RHIZOtest® are present in Table 4 (supporting information (SI)). The experiments were carried out in a controlled chamber with a day/night temperature regime of 25±2 °C (16 h) / 20±2 °C (8 h) and a light intensity of 425 ± 50 photons µmol m^{-2} s^{-1}. The relative humidity was adjusted to 65±5%.

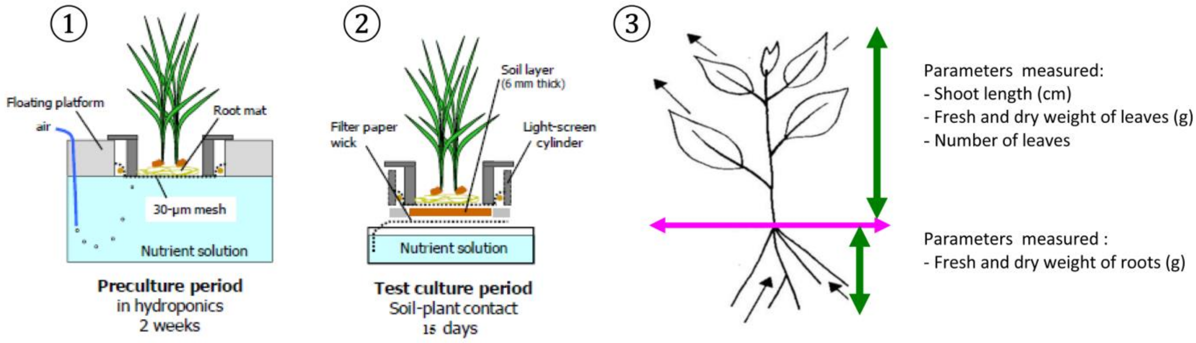


Fig. 1 Steps for Micro-culture by RHIZOtest® devices ①②, and parameters used for biomass measurement ③.

Leafy vegetables (lettuces and cabbages) are widely cultivated for human consumption and regularly grown in farms in China, Europe, and other countries (Khan et al., 2008; Uzu et al., 2009; Waisberg et al., 2004; Schreck et al., 2012a). They have short life-cycle and large surface interception, which are useful to investigate the atmospheric transfer of metals and therefore been the subject of several studies of metal transfer (Monteiro et al., 2009; Cao et al., 2010; De Leon et al., 2010; Schreck et al., 2013). The experimental design consisted of 36 plants for each plant species, including three durations, three exposure quantities and four replicates.

After pre-culture, plants were grown two weeks on a control unpolluted soil, which exhibited the following physico-chemical characteristics as described by Schreck et al. (2011): high contents of organic matter (44.7 g kg^{-1}), an optimum pH (6.5) and an average cationic exchange capacity (12.3 $cmol^{+}$ kg^{-1}). The Pb concentration of unpolluted soil was 25.5±1.6 mg kg^{-1} DW, as described by Uzu et al. (2010) and Schreck et al. (2012a). Because this study focused on the foliar transfer of metals, a geotextile membrane was placed between root and shoot parts to protect the roots from PM fallouts and then to avoid metal transfer via root uptake (Xiong et al., 2014a; Hurtevent et al., 2013).

Vegetable leaves were then exposed to PbO particles with an applicator brush onto the entire leaf surface (Xiong et al., 2014a). Note that the leaf surfaces were moistened with a hand spray first, and the brush was only used to deposit the PM onto the leaf surfaces without spreading. The homogeneity of the solution distribution on the leaves and the reproducibility of the technique were confirmed in previous tests (Xiong et al., 2014a). We used this method because the deposit of dry PM with an applicator brush without pre-wetting can mechanically bring the particles into the leaf and favor PM uptake (Xiong et al., 2014a). The PbO characteristics were as following: CAS number 1317-36-8, PM size < 10 µm, specific area 29.2 m^2g^{-1}, purity > 99.99%, and molecular weight of 223.19 g mol^{-1}. The metal foliar exposure was performed during 5, 10 and 15 days to allow PbO uptake by plant leaves. Three treatments were defined in function of metal quantities deposited: a control condition without any metal input (0 mg), 10 mg and 250 mg PbO inputs. These concentrations correspond to the Pb concentrations reported in atmospheric fallouts near a secondary Pb smelter which was 450±7 mg m^{-2} $month^{-1}$ (Xiong et al., 2014b), and concentrations occurring in previous field experiment conducted by Schreck et al. (2013) in industrial area (PM fallouts of 100 µg cm^{-2} $week^{-1}$). We define lettuces and cabbages exposed to 0 mg, 10 mg and 250 mg PbO respectively as L_0, L_{10}, L_{250} and C_0, C_{10}, C_{250}.

Furthermore, in order to precisely determine the quantity of pollutants deposited on the leaf surface, the applicator brush was rinsed with 20 mL of distilled water after PM deposit (Xiong et al., 2014a). The metal concentration of the rinse water was measured and the final PM amounts really deposited on leaf surfaces were 0, 5.61 and 218.78 mg respectively for 0, 10 and 250 mg of PbO deposited with the brush.

3.1.2.2 Metal concentrations in plant leaves and roots

After harvest, root and shoot biomasses were separated and properly washed with deionized water based on human ingestion scenario so as to remove particles deposited on the leaf surface (Birbaum et al., 2010; Uzu et al., 2010; Xiong et al., 2014a). The samples were first rinsed with deionized water, and then immersed for 10 min in deionized water and rinsed another time with deionized water (Hong et al., 2014). The root and leave samples were oven-dried at 40 °C to constant weight (about 72 h), grinded and sieved to < 250 µm particle size. Mineralization of plant samples (0.125 g per sample) was performed in aqua-regia ($^1/_4$ HNO_3 and $^3/_4$ HCl) at 80°C for 4h by Digiprep® instrument (Xiong et al., 2014a). The digested samples were then filtered through 0.45 µm filters and stored at 4 °C after volume adjustment. Total Pb concentrations in vegetable samples were measured by inductively coupled plasma-optical emission spectrometry ICP-OES (IRIS Intrepid II XXDL). Method control was performed with concurrent analysis of blank and standard reference materials (tobacco CTA-VTL2, peach leaves (1547)). Metal concentrations (mg kg^{-1}) are expressed on a dry weight (DW) basis.

Pb storage (µg) was calculated by multiplying Pb concentrations ($[Pb]_{plant}$, mg kg^{-1}) by plant DW (mass in g): Pb storage = ($[Pb]_{plant} \times$ DW). Pb transfer kinetic (µg day^{-1}) is the dynamic change of metal storage capacity every day (Pb storage /Exposure duration). Metal translocation factors (TF) were calculated by dividing the metal concentrations in the root tissues ($[Pb]_{roots}$, mg kg^{-1}) by that accumulated in aboveground tissues ($[Pb]_{leaves}$, mg kg^{-1}) (Marchiol et al., 2004; Soda et al., 2012): TF = $[Pb]_{roots}$ / $[Pb]_{leaves}$.

3.1.2.3 Phytotoxicity

After exposure, plants were harvested every 5 days, which were defined as T_5, T_{10} and T_{15} whereas the first day of exposure was defined as T_0. The exposure duration from 1 to 15 days was usually chosen for the study of metal accumulation, as this period allows a significant metal uptake (Capelo et al., 2012; Xiong et al., 2014a).

Plant Biomass was then measured at T_0, T_5, T_{10} and T_{15}: it included the shoot length, number of leaves, fresh and dry weight of leafs and roots, water content and tolerance index (TI) of shoot growth.

Shoot length was measured from the bottom of the shoot to the endpoint of the highest leaves (Fig. 1 ③).

The plants were fresh weighted (FW) and oven-dried to determine the DW, the percentage of water content was calculated according the fresh and dry weight of plants as Eq.1:

$$Water\ content = \frac{FW - DW}{FW} \times 100\% \qquad \text{Eq. 1}$$

TI evaluates the plant tolerance against or susceptibility to the composite effects of metals. According to Mahmood et al. (2007), data of individual growth parameter (G_x) was normalized (G_i) relative to the maximum value (G_{max}) of that parameter in the data set, $G_i = (G_x\ G_{max}^{-1})$. Summing all the G_i's and then divided by the total number of G_i's calculated the $TI = \Sigma(G_x\ G_{max}^{-1})\ n^{-1}$. The TI of shoot growth ranged from 0 to 1, with highly tolerant when it approaches to 1 and having extremely susceptible to the composite effects of heavy metal when it approaches to 0.

For fatty acid composition analysis, one cm^2 of fresh young leaf (secondary developed leaf) was collected for every sample and then immediately placed in screw-capped tubes containing 500 µL methanol acidified with 2.5% H_2SO_4 solution. Samples were stored at 4 °C before analysis. Fatty acid analysis and identification were performed according to Le Guédard et al. (2008 and 2012a) and Schreck et al. (2013): leaf samples stored in acidified methanol were heated to 80 °C for 1 h and then 1.5 mL of H_2O and 0.75 mL of hexane were added after cooling. Fatty acid methyl esters (FAMEs) were extracted into hexane by vigorous shaking and a two-phase system was established by centrifugation (1500 g, 5 min). Separation of FAMEs in the hexane phase was performed by gas chromatography (Hewlett Packard 5890 series II) on a 15 m × 0.53 mm Carbowax column (Alltech) with flame ionization detection. The temperature ramp was performed (Le Guédard et al., 2009): the initial temperature of 160 °C was held for 1 min, followed by a 20 °C min^{-1} ramp to 190 °C and a second ramp of 5 °C min^{-1} to 210 °C, and maintained for 6 min. FAMEs were identified by comparing their retention times with standards (Sigma Chemical, St. Louis, MO, USA).

In general, the lipid composition of leaf consisted of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and alpha linolenic acid (C18:3). Lipid composition was dominated by polyunsaturated fatty acids mainly represented by linolenic acid (C18: 3) which accounted for 39.6-49.0% and 37.9-51.8% of the total fatty acid content, respectively in lettuce and cabbage in our study.

The work done by Le Guedard et al. (2009 and 2012a) showed that the fatty acid ratio (Eq.2) in plant leaves has the indication characteristics of metabolic effect after the plant exposure to metals and has been used to diagnose the impact of foliar metal uptake by

Schreck et al (2013). Moreover, the Z index, defined as the product of three fatty acid concentration ratios (C16:1/C16:0, C18:3/C18:0 and C18:1/C18:2, Eq.3) with different rates (1, 0.57, and 0.23 respectively), can be used when exploring the relationships between uptake and phytotoxicity (Schreck et al., 2013). Thus, change in the ratio of lipids in leaves can be used as a biomarker of stress when plants are exposed to PbO PM by foliar way.

$$Fatty\,acid\,ratio = \frac{C18:3}{C18:0 + C18:1 + C18:2} \times 100\% \quad \text{Eq.2}$$

$$z = \left(\frac{C16:1}{C16:0}\right) \times \left(\frac{C18:3}{C18:0}\right)^{0.57} \times \left(\frac{C18:1}{C18:2}\right)^{0.23} \quad \text{Eq. 3}$$

Gas exchange parameters: net photosynthesis (Pn) and stomatal conductance (gs), were recorded simultaneously using a portable infrared gas analyzer (LI-COR 6400 XT) for estimating the influence of metal stresses on photosynthesis. The infrared gas analyzer system (IRGAs) was equipped with a clamp-on leaf chamber that possesses 6 cm^2 of leaf area. According to Majer and Hideg (2012), IRGAs measure the reduction in transmission of infrared wavebands caused by the presence of CO_2 between the radiation source and a detector. The reduction in transmission is a function of the concentration of CO_2. Thus the measurements of gas exchange are based on the differences in CO_2 and H_2O in an air stream that is flowing into the leaf cuvette (reference cell) compared to the air stream flowing out of it (sample cell). The rate of CO_2 uptake (µmol m^{-2} s^{-1}) is used to assess the rate of photosynthetic carbon assimilation, while the rate of water loss (mol H_2O m^{-2} s^{-1}) is used to assess the rate of transpiration and stomatal conductance.

Gas exchanges measurements were performed under irradiance at 425 µmol m^{-2} s^{-1} and at the temperature of 23±2 °C. During the measurement, humidity was fixed at 65%, CO_2 concentration was maintained at a constant level of 380 µmol mol^{-1} using a LI-6400-01 CO_2 injector with a high pressure liquid CO_2 cartridge source.

3.1.2.4 Statistical analysis

Total Pb concentrations in plants and the measured physiological parameters were subjected to analysis of variance (ANOVA), using Duncan's test at $p < 0.05$. The significances of the treatment effect were investigated by the use of 2-way ANOVA (duration × concentration). Data were tested for normal distribution and homogeneity of variance before the ANOVA analysis. The relationships between exposure conditions and different phytotoxicity parameters and among the parameters were analyzed by principal component analysis (PCA), the «ρ» stand for correlation coefficient between different parameters. Statistical analyses were carried out on the mean of 4 replicates for each exposure condition. Results are represented as mean ± SD (standard deviation).

3.1.3 Results

3.1.3.1 Foliar metal uptake and translocation towards the roots in different plants and treatments

A significant Pb absorption, up to 3932 and 2039 mg kg^{-1} DW respectively for lettuce and cabbage (Fig. 2a) was observed. The uptake of metal by the two vegetables was very high compared to control even during the first 5 days of exposure. From T_5 to T_{15}, the metal concentration increased rapidly in lettuce for L_{10} and L_{250} treatment. Pb concentration was almost constant for C_{10} and C_{250} treatments from T_5 to T_{10}. Lettuce showed a stronger metal absorption capacity than cabbage.

The total Pb storage was variable and differed between the two plants during the exposure period (Fig. 2b). However, the final storage capacity at T_{15} was almost the same in lettuce and cabbage (1000 µg for L_{10} and C_{10}, 3000 µg for L_{250} and C_{250} respectively). The most significant increase of metal storage was observed from T_{10} to T_{15} in both plants (L_{250} and C_{250}).

The kinetic of Pb transfer increased in the first 5 days, from 0 to 23 µgPb·day^{-1}, 48 µgPb·day^{-1}, 67 µgPb·day^{-1} and 85 µgPb·day^{-1} for L_{250}, L_{10}, C_{10} and C_{250} respectively. In the second period of 5 days (from T_5 to T_{10}), the uptake rate was constant in L_{10}, C_{10} and C_{250}, but it significantly increased in L_{250} from 23 µgPb·day^{-1} to 112 µgPb·day^{-1}. In the last 5 days (from T_{10} to T_{15}), transfer kinetics were significantly high in the highest PbO exposure group:

465 µg·day^{-1} for L_{250} and 420 µg·day^{-1} for C_{250} (Fig. 2c). The transfer kinetic is positively related with Pb storage (ρ=0.985 in lettuce and ρ=0.934 in cabbage) and Pb concentration (ρ=0.955 in lettuce and ρ=0.973 in cabbage) (Tables 2 and 3 in SI). Finally, the foliar transfer kinetics was quite similar in the two vegetables and the absence of saturation phenomenon was noticed (L_{250} and C_{250}).

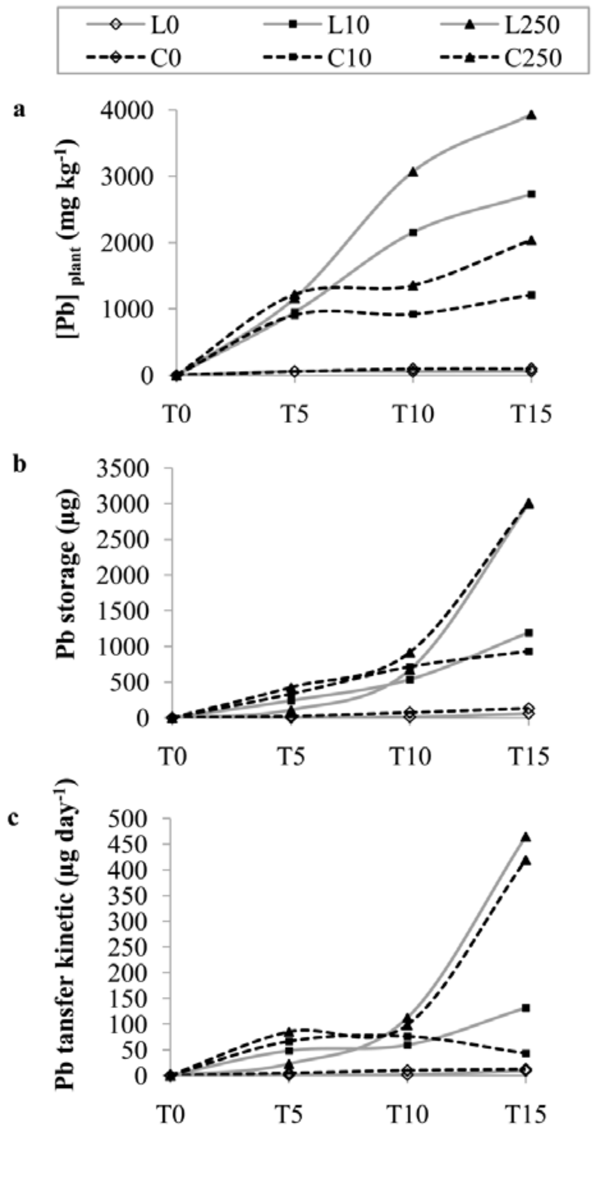


Fig. 2 (a) Pb concentration ([Pb] $_{plant}$, mg kg^{-1}), (b) Pb storage (µg) and (c) Pb transfer kinetic (µg day^{-1}) in lettuces and cabbages exposed to PbO by foliar way (L0, L10 and L250 (and C0, C10 and C250) correspond to respectively 0, 10 and 250 mg of PbO deposited on lettuce (cabbage)) to various exposure durations (T0, T5, T10 and T15). Values are expressed as the mean of 4 replicates for each treatment.

Moreover, metal translocation factors (TF) from leaves to roots ranged from 0.06 to 0.21 were observed (Table 1). TF values were high during the first 5 days, and then decreased with exposure duration.

Table 1 Mean Pb concentrations (mg kg^{-1} of DW) in roots and leaves of lettuces and cabbages and translocation factors (TF) from leaves to roots. T_5, T_{10} and T_{15} correspond respectively to 5, 10 and 15 days of exposure. L_0, L_{10} and L_{250} (and C_0, C_{10} and C_{250}) correspond respectively to 0, 10 and 250 mg of PbO deposited on lettuce (cabbage) leaves. The different letters indicate significant differences among the treatments at $p < 0.05$.

	Mean Pb concentrations and TF								
	T_5			T_{10}			T_{15}		
	Roots	Leaves	**TF**	Roots	Leaves	**TF**	Roots	Leaves	**TF**
L_0	60.4 ± 1.8^{a}	59.9 ± 0.8^{a}	**1.01**	57.9 ± 0.6^{a}	54 ± 1.7^{a}	**1.07**	62.7 ± 0.5^{a}	54 ± 0.4^{a}	**1.16**
L_{10}	333.1 ± 11.3^{b}	1568 ± 44.6^{c}	**0.21**	475.6 ± 15.7^{b}	3829 ± 27^{e}	**0.12**	310.2 ± 12.7^{b}	5149.4 ± 328.5^{f}	**0.06**
L_{250}	359.4 ± 14.4^{b}	1962.2 ± 34.3^{cd}	**0.18**	443.8 ± 15.1^{b}	5695.8 ± 108.1^{f}	**0.08**	472 ± 11^{b}	7392.3 ± 264.7^{g}	**0.06**
C_0	54.9 ± 1.8^{A}	56.5 ± 1.6^{A}	**0.97**	93.2 ± 5.3^{A}	96.2 ± 5.5^{A}	**0.97**	99.3 ± 3^{A}	94.1 ± 2.6^{A}	**1.06**
C_{10}	201 ± 5.1^{B}	1613.4 ± 38^{C}	**0.12**	282.8 ± 7.1^{B}	1558 ± 59.3^{C}	**0.18**	169.5 ± 6.0^{A}	2290 ± 92.4^{D}	**0.07**
C_{250}	237.2 ± 10.7^{B}	2202.6 ± 60.4^{D}	**0.11**	164.8 ± 7.9^{A}	2541.9 ± 34.2^{DE}	**0.06**	411.2 ± 16.4^{B}	3667.1 ± 139.5^{F}	**0.11**

3.1.3.2 Growth parameters for exposed plants

The shoot growth of lettuce and cabbage exposed to PbO are presented in Fig. 3. Lead phytotoxicity caused a significant reduction of shoot growth (in comparison to the control): about 49.9% and 23.6% respectively for lettuce (the more sensitive) and cabbage. In lettuce, the shoot growth in L_0 was about 0.55 cm (T_5), 0.875 cm (T_{10}) and 1.125 cm (T_{15}). While in L_{250}, shoot growth was reduced to 0.175 cm (T_5), 0.3 cm (T_{10}) and 0.4 cm (T_{15}) and the TI of lettuce dropped from 100% (control) to 31.8% (T_5), 34.3% (T_{10}) and 35.6% (T_{15}) respectively. The Pb phytotoxicity on shoot growth was dose and exposure time dependent (Fig. 3a).

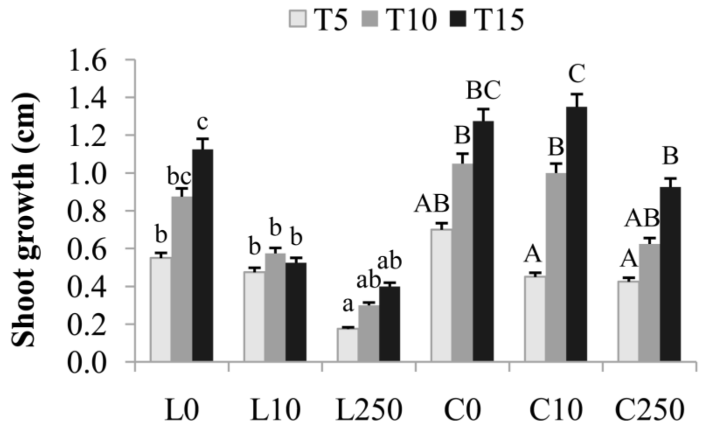
In cabbage, the decrease of shoot growth was initially quick: during the first 5 days, a significant reduction was observed (from 100% (C_0) to 64.3% (C_{10}) and 60.7% (C_{250})). But, after 10 days, the decrease was quite moderate (Fig. 3b). The PCA analysis showed that shoot growth of lettuce and cabbage is negatively related to PbO exposure quantity (ρ = - 0.781 and -0.539 respectively for lettuce and cabbage) (Tables 2 and 3 in SI), which demonstrates the high Pb toxicity to shoot growth.

The aerial biomass is presented in Fig. 3c. A significant difference of aerial biomass compared to control was only notable for T_{15} in lettuce and for C_{250}. The aerial biomass is negative significantly correlated to exposure quantity in cabbage (ρ =-0.353).

The water content ranges from 81% to 89% for lettuce and 67-85% for cabbage, no significant differences were found among all treatments.

Obviously, cabbage acquired a better shoot growth and aerial biomass than lettuce might indicates a better tolerance of cabbage. At the end of the two week of experiments, all the plants were alive. The shoot growth and aerial biomass of the two plants is positively correlated to exposure duration (shoot growth: ρ = 0.341 and 0.771 respectively for lettuce and cabbage; aerial biomass: ρ = 0.909 and 0.88 respectively for lettuce and cabbage), permits plant growth even under high PbO stress (Fig. 6, Tables 2 and 3 in SI).

a



b

Tolerance index (TI) %						
	T5		T10		T15	
	Lettuce	Cabbage	Lettuce	Cabbage	Lettuce	Cabbage
0(mg)	100	100	100	100	100	100
10(mg)	86.4	64.3	65.7	95.2	46.7	105.9
250(mg)	31.8	60.7	34.3	59.5	35.6	72.6

c

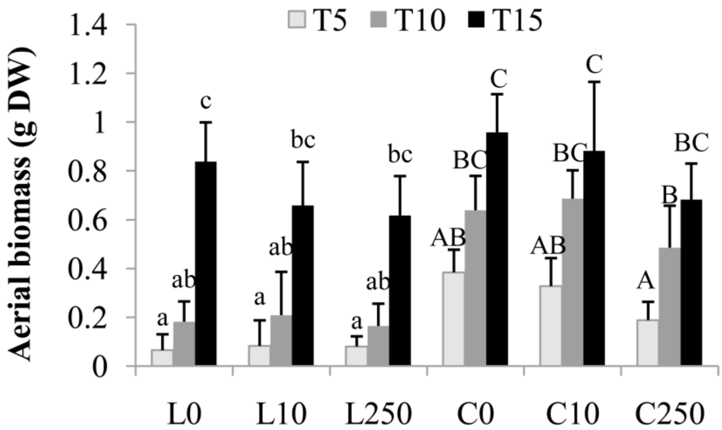


Fig. 3 (a) Shoot growth, (b) tolerance index (TI) % and (c) aerial biomass (DW) of lettuce and cabbage exposed to PbO by foliar way (L0, L10 and L250 (and C0, C10 and C250) correspond to respectively 0, 10 and 250 mg of PbO deposited on lettuce (cabbage)) to various exposure durations (T5, T10 and T15). The values are expressed as the mean of 4 replicates for each treatment (±SD), the different lowercase (capital) letters indicate significant differences at p <0.05.

3.1.3.3 Gas exchanges at the leaf level after exposure with PbO particles

Fig. 4 presents the results of Pn and gs. Photosynthesis activity showed a significant reduction after PbO exposure with different durations. In lettuce, at T_5, Pb caused a decrease of 25% and 58% in Pn respectively for L_{10} mg and L_{250}. For T_{10}, a 30% decrease in Pn was observed for L_{250}, no significant difference was found between L_{10} and control (L_0). Finally for T_{15}, 18% and 26% of reduction were found respectively for L_{10} and L_{250}. The effect of the different levels of PbO exposure on Pn was more pronounced (linear) for T_5 compared to T_{10} and T_{15} (Fig. 4a).

The cabbage showed a different trend: Pn rates decreased significantly (about 50%) between control (C_0) and PbO treatments (C_{10} and C_{250}). No significant change was observed between the C_{10} and C_{250} (Fig. 4a).

The gs value presented a notable decrease in the cabbage leaves atT_{10} and T_{15} (Fig. 4b). However, in lettuce leaves, gs appeared to be stable over time. But, slight drop was observed for L_{250} (Fig. 4b).

In all treatments, Pn was strongly correlated with exposure quantity (ρ =-0.644 and -0.666 respectively for lettuce and cabbage), shoot growth (ρ =0.807 and 0.458 respectively for lettuce and cabbage) and gs (ρ =0.653 and 0.418 respectively for lettuce and cabbage) (Tables 2 and 3 in SI).

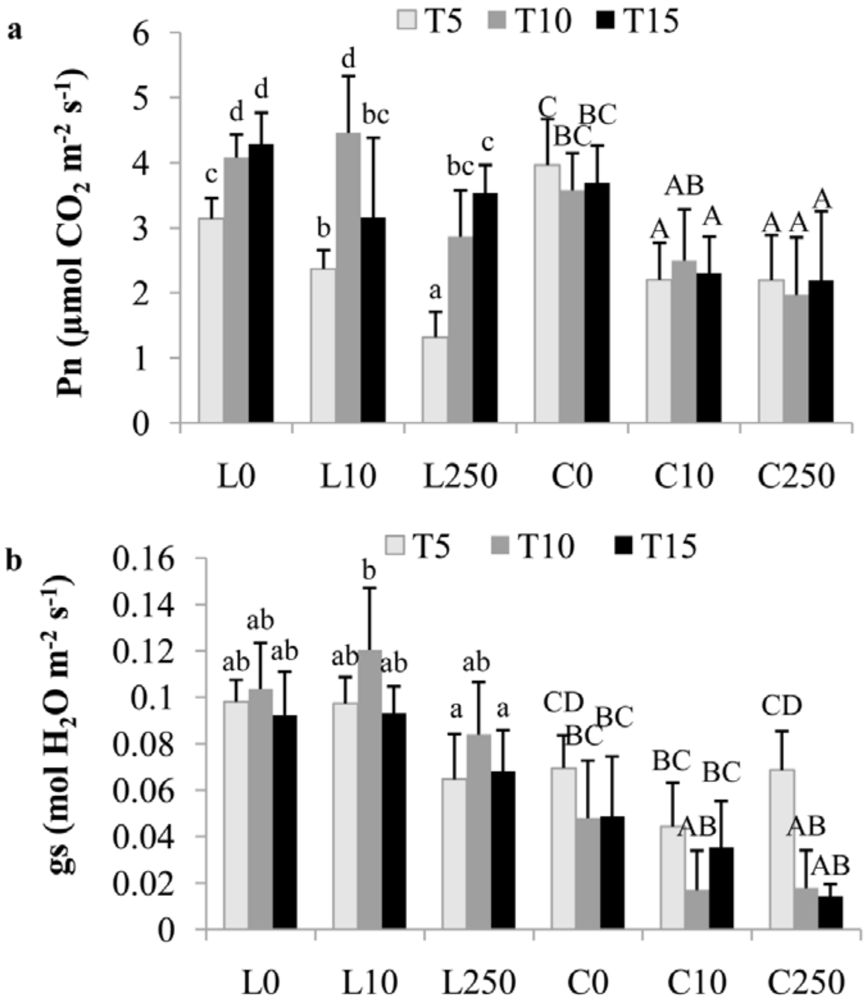


Fig. 4 Measurement of gas exchange ((a) Pn: net photosynthesis, (b) gs: stomatal conductance) at the leaf level of lettuce and cabbage after 5,10 and15 days (T5, T10 and T15) of PbO exposure by foliar way (L0, L10 and L250 (and C0, C10 and C250) correspond to respectively 0, 10 and 250 mg of PbO deposited on lettuce (cabbage)). The values are expressed as the mean of 4 replicates (±SD), the different lowercase (capital) letters indicate significant differences at p <0.05.

3.1.3.4 Effects on fatty acid composition

Fig. 5 presents the effect of Pb concentrations and exposure duration on fatty acids composition in plant leaves. The values of foliar fatty acid ratio showed no significant difference compared to control in both species after PbO exposure, except a slight variation for C_{10}. However, the value of foliar lipid biomarker decreased with the exposure duration (ρ = - 0.776 and - 0.651 respectively for lettuce and cabbage) (Tables 2 and 3 in SI).

The percentage of C18:3 also showed no significant correlation with PbO treatments, but it was negatively correlated with exposure duration (ρ = - 0.813 and - 0.768 respectively in lettuce and cabbage). Statistical analyses highlighted a positive correlation between the fatty acid ratioand the percentage of C18:3 in both vegetables (correlation coefficient ρ = 0.939 and 0.867 for respectively lettuce and cabbage (Tables 2 and 3 in SI).

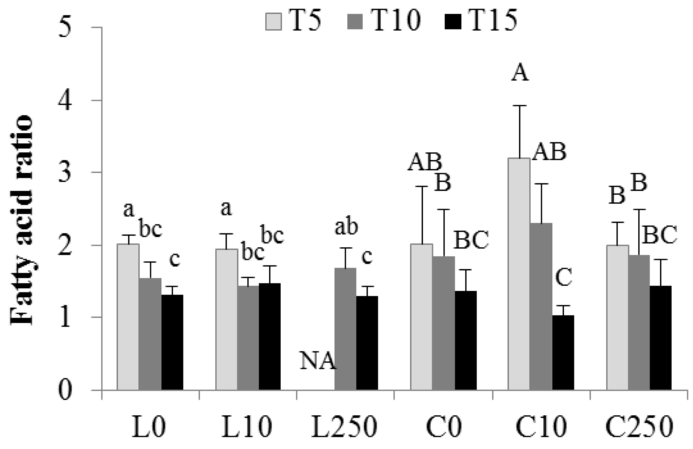


Fig. 5 Mean available values of fatty acid ratio (C18: 3 / (C18: 0 + C18: 1 + C18: 2)) in lettuce and cabbage exposed to PbO by foliar way (L0, L10 and L250 (and C0, C10 and C250) correspond to respectively 0, 10 and 250 mg of PbO deposited on lettuce (cabbage)) to various exposure durations (T5, T10 and T15). Values are expressed as the mean of 4 replicates (±SD), the different lowercase (capital) letters indicate significant differences at p <0.05. NA: Not available.

3.1.3.5 Relationship between exposure conditions, metal accumulation and phytotoxicity

Results of PCA of lettuce and cabbage are shown in Fig. 6. For lettuce, the first two principal components accounted for 82.1% of the total variance. The first axis of the PCA (PC1=50.3% of total variance) was mainly associated with exposure duration, plant DW, Pb storage, aerial biomass, transfer kinetic and Pb concentration, in decreasing order of importance, as revealed by examination of the correlation coefficient matrix. The PC1 mainly represents the biomass and the metal accumulation with the time. The second axis of the PCA (PC2 = 31.8% of total variance) was mainly associated with shoot growth, gs, Pn. Detailed examination of PCA correlation coefficient matrix provided additional information on parameters associations in plants (Table 2 in SI).

For cabbage, the first two principal components accounted for 75.6% of the total variance (eigen value > 1). The first axis of the PCA (representing 39.8% of the total variance) was mainly associated with plant DW, aerial biomass, exposure duration, shoot growth, in decreasing order of importance, as revealed by the examination of the correlation coefficient matrix. The first principal component mainly represent the growth parameters with the time. The second axis of the PCA (PC2 = 35.8% of total variance) was mainly associated with transfer kinetics, Pb concentration, Pb storage, exposure quantity, Pn, gs. Detailed examination of PCA correlation coefficient matrix provided additional information on parameters associations in plants (Table 3 in SI).

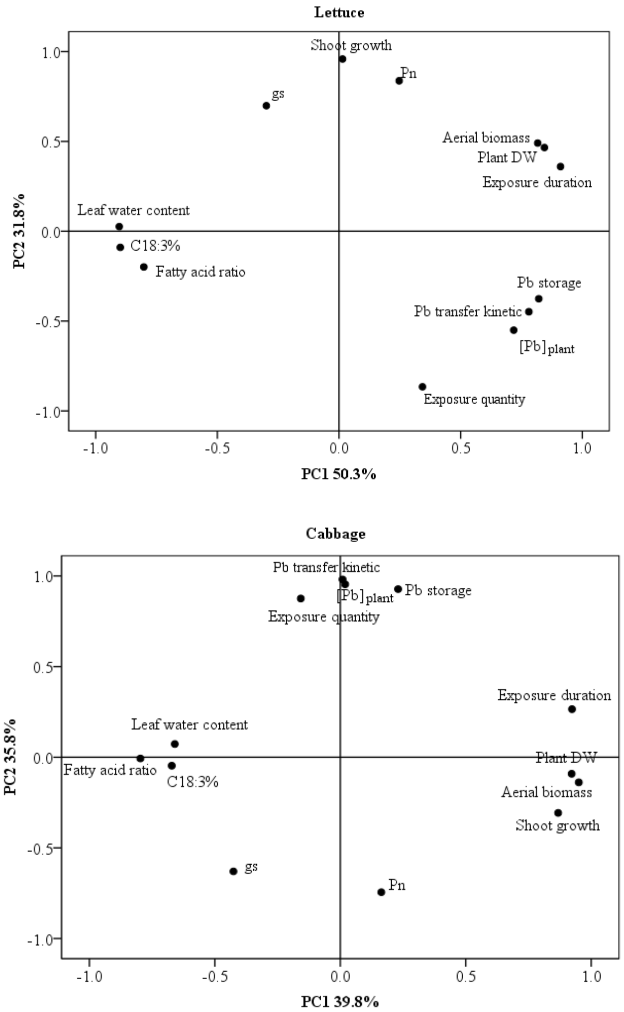


Fig. 6 The first two principal component of the PCA (Principal Component Analysis) on 13 variables (Exposure quantity, Exposure duration, Pb concentration ([Pb]plant), Pb storage, Pb transfer kinetic, Shoot growth, Aerial biomass, Plant DW, Leaf water content, Pn, gs, C18:3% and Fatty acid ratio in lettuce and cabbage after exposed with PbO PM.

3.1.4 Discussion

3.1.4.1 Metal uptake in different plants and treatments

3.1.4.1.1 Pb concentrations and storage capacity in plants

According to Hu et al. (2011) or Schreck et al. (2012a), significant Pb accumulation was observed in plant shoots even after washing. It was positively correlated with the exposure quantities and duration, and negatively correlated with morphological parameters of plant growth.

Pb concentration is higher in lettuce than in cabbage, but the Pb storage in the first 10 days was higher for cabbage than lettuce. There are two possibilities to explain these results. 1-the thin and broad leaves of lettuce allowed quickly metal absorption, at the same time, lettuce grows slowly due to metal phytotoxicity, leading to a high Pb concentration. 2-cabbage grows faster and acquired a bigger biomass than lettuce with dilution effect. But the higher shoot growth and aerial biomass permit cabbage to store more metal per day. Rico et al. (2011) demonstrated that the uptake, translocation, and accumulation of PM depend on the plant species and the life cycle stage of plant. The total storage capacity (in T_{15}) is almost the same in lettuce and cabbage. This may be related to the high metal concentration in lettuce and high biomass of cabbage (as storage capacity depends on both metal concentration and the plant biomass).

Lead transfer kinetic did not show a linear trend whatever the PbO amount deposited on the leaf surface and the plant species. These results strongly suggest that in some extent, the vegetables have defense mechanisms to limit Pb uptake during growth processes. The defense mechanisms may influence metal foliar uptake between T_5 and T_{10} since during this period Pb transfer kinetic was moderate. However, from T_{10} to T_{15}, Pb transfer kinetic strongly increased in L_{250} and C_{250}, suggesting some detoxification mechanisms. The existence of efficient storage and regulation/detoxification mechanisms allows the survival of vegetables under high accumulated metal concentrations (Phillips and Rainbow 1988). Therefore, the change of transfer kinetic might be a process of accumulation (T_0 to T_5), regulation (T_5 to T_{10}) and adaptation (T_{10} to T_{15}) in vegetables.

Moreover, plants exposed to 500 mg of PbO were also tested in this study with lettuce (L_{500}) and cabbage (C_{500}), no significant difference was found in comparison with 250 mg of PbO exposure for cabbage, we suggested there are adaption and saturation in case of high PbO treatment. For example: (i) the biomass (shoot growth: 0.93cm for C_{250} and 0.73cm for C_{500}; aerial biomass: 0.68g for C_{250} and 0.62g for C_{500}) and gaseous exchange (Pn: 2.2 μmol.CO_2 m^{-2} s^{-1} for C_{250} and 1.9 μmol.CO_2 m^{-2} s^{-1} for C_{500}; gs: 0.01 mol H_2O m^{-2} s^{-1} for C_{250} and C_{500}) showed no significant difference between 250 mg / 500 mg of PbO treatment($p>0.05$); (ii) the Pb concentration is not always linear to exposure quantity: in cabbage the Pb concentration even decreased for C_{500} (5173 mgPb kg^{-1}) by comparison to C_{250} (5606 mgPb kg^{-1}) at T_{15}. However, this dose is not really environmental and concerns only extreme situations or a few of highly polluted contexts. Concerning the mechanisms involved in metal transfer via foliar application, Xiong et al. (2014a) concluded that biogeochemical transformations occurred on the leaf surfaces. Internalization through the cuticle or penetration throughout the stomata apertures (Barber, 2004) are therefore proposed as the two major mechanisms involved in foliar uptake of PM. Thus, particle sizes as well as solubility are therefore parameters that appear to strongly control their physical transfer through leave surface (Eichert et al., 2008; Larue et al., 2012). If most of the PbO particles were in the assumed <10 μm diameter size range (original PbO size exposure on leave surface) that direct cuticular uptake would be minimal (cuticular pores are around 2nm diameter by Uzu et al.(2011)), and penetration through stomata openings could be the main uptake pathway.

In our previous study, CdO (< 1 μm) and Sb_2O_3 (< 1 μm) PM were observed near or inside stomata apertures of cabbage after foliar exposure which indicated the foliar transfer of metal(loid)s by stomata (Xiong et al., 2014a). The changes to the particle and/or vegetable leaves induced by atmospheric gaseous pollutants (i.e. O_3,NO_2,SO_2…), which are commonly found in urban or industrial troposphere (Cicek and Koparal 2004; Gandois et al. 2010; Chaparro-Suarez et al. 2011; Terzaghi et al. 2013), could favor the foliar transfer of metal(loid)s containing particles. However, Goix et al. (2014) observed by scanning electron microscopy that PbO particles could form aggregates, thus particles may be trapped on the leaf surface of plants due to leaf hairs and cuticular wax cover (Sæbø et al., 2012). Moreover, phyllosphere organisms release inorganic and organic compounds possessing acidifying, chelating and/or reductive abilities and may play an essential role in element mobilization and uptake at the leaf surface (Michaud et al. 2007). Metal concentrations and speciation in leaves could be modified by interactions on the phyllosphere between PM and microbes. Changes of temperature and humidity may transform PM at the leaf surface after contact with the leaf (Uzu et al., 2010; Schreck et al., 2012a). Pb solubilization (even if it maybe low) could also be a potential mechanism for plant uptake.

3.1.4.1.2 Translocation factor (TF)

In agreement with previous studies from Xiong et al. (2014b), lead translocation from the leaves to roots was observed. Erenoglu et al. (2002) or Zhang et al. (2013) also concluded inorganic elements (zinc, copper, iron and manganese) translocation and phloem Zn transport from leaves to roots was demonstrated by Haslett et al. (2001).

However, as not essential element, the TF of Pb is from 0.06 to 0.21, which is much lower than the essential Zn element (Amadi and Tanee, 2014; Yoon et al., 2006).

3.1.4.2 Phytotoxicity of deposited PM enriched in PbO

3.1.4.2.1 Impact of Pb-enriched PM on plant growth

Shoot growth and aerial biomass decreased in both plant species after PbO exposure in a dose dependent manner. Lead-induced severe growth reduction in plants can be due to nutritional disturbances and probably to a direct Pb toxic effect. Lead phytotoxicity is proven in several previous studies, notably for morphological and growth parameters (Foucault et al., 2013; Shahid et al., 2014d). Capelo et al. (2012) showed a decrease of root and shoot growth in *Lactuca sativa L.* after Pb exposure. Abbas and Akladious (2012) reported Pb-induced significant decrease in all growth parameters: plant height (86.2%), root length (89.2%), number of leaves (96.9%), fresh weight of shoots (93.5%), fresh weight of roots (85.9%), dry weight of shoots (84.7%) and dry weight of roots (81.4%). Lead-induced decrease in plant growth parameters can be due to (i) inhibition of Calvin cycle, (ii) disruption in plastoquinone and carotenoid synthesis, (iii) reduced activities of delta aminolevulinic acid dehydratase and ferredoxin NADP+ reductase, (iv) interference in electron transport chain, (v) inadequate CO_2 concentration due to stomatal closure, (vi) replacement of vital bivalent cations by Pb and (vii) distorted chloroplast ultrastructure owing to Pb affinity for S- and N-ligands of protein (Pourrut et al., 2011, 2013; Shahid et al., 2012, 2014c).

Generally, cabbage presents a better growth compared to lettuce suggesting an higher tolerance to Pb transfer and toxicity. Indeed, cabbage leaves possess a waxy coating: the epicuticular waxes are dense and are an important barrier to ion and water movement across the cuticle (Adams et al., 1989). In contrast, lettuce has thin and broad leaves surfaces (less cuticle thickness) which may be more sensitive to metal contamination. In general, leaves with a lot of epicuticular wax are more hydrophobic, resulting in less contact between the surface of cuticle and the metals. Tolerance of cabbage may be an adjustment of plant to the metal stress (Shahid et al., 2013b). Finally, all plants were alive and the biomass was increased with exposure duration ($T_{15} > T_{10} > T_5$), which is in agreement with the study of Lamhamdi et al. (2011). Thus, the plant can maintain the basic growth, even under high Pb pollution.

The PCA analysis showed that the lettuce shoot growth and aerial biomass are positively correlated to Pn (ρ=0.807, 0.508 respectively), the shoot growth of cabbage is significantly positively correlated to exposure duration (ρ = 0.771) and Pn (ρ = 0.458), suggesting the potential interactions between net photosynthesis and shoot growth.

3.1.4.2.2 Impact of Pb-enriched PM on the photosynthetic activities

Both lettuce and cabbage showed a decline in Pn and gs value due to PbO foliar exposure. Photosynthesis is quite sensitive to Pb toxicity, and both *in vivo* and *in vitro* photosynthetic CO_2 fixations are affected by Pb (Sheoran & Singh, 1993; Pourrut et al., 2013). Lead-induced toxicity to photosynthesis can be due to multiple effects of PM. The immediate effect is on stomata closure followed by chloroplastic changes (Pourrut et al., 2011). Lead may also affect photosynthesis indirectly via ROS production (Shahid et al., 2012). An enhanced accumulation of endogenous H_2O_2 was detected after treatment of *Lemna trisulca* L. with lead (Samardakiewicz et al., 2015). The results of Pb longterm exposure are the reduction of leaf growth, the decrease of photosynthetic pigments, the alteration of chloroplast structure and the decrease of enzyme activities for CO_2 assimilation (Austruy et al., 2013). Larger metal agglomerates found nearby closed stomata, integrated into the surface wax or on the leaf surface (Birbaum et al., 2010) can decelerate plant net photosynthesis mainly via limiting stomata opening (the decrease of photon acquisition and CO_2-uptake).

3.1.4.2.3 Impact of Pb-enriched PM on the leaf fatty acid composition

In our *ex situ* experiment, there is a slight decrease in fatty acid ratio under high PbO treatment, but no significant effect was observed among the three different groups (0, 10 and 250 mg of PbO deposited). A similar phenomenon was observed by Nouairi et al. (2006) who reported that no significant change in the total fatty acid composition was observed in *Sesuvium portulacastrum* leaves under Cd treatment. Schreck et al. (2013) also found that after 1 and 4 weeks of field exposure in the presence of soil and air Pb contamination (among various metal(loid)s in lower quantities), the fatty acid ratio was not significantly decreased. The results of Le Guédard et al. (2012a) showed that fatty acid ratio is significantly negatively correlated with Ni and Cr, but not with other metal levels (Cd, Cu, Pb and Zn). Generally, the level of trienoic fatty acids that not change with Pb pollution may indicate a plant tolerance (Kodama et al., 1994; Khodakovskaya et al., 2006; Dominguez et al., 2010) to oxidative stress.

By contrast, in their study conducted on a metallurgical landfill, Le Guédard et al. (2012b) showed that Pb and Cr leaf contents were anti correlated to the C18:3/(C18:0 + C18:1 + C18:2) ratio in *Populus nigra* leaves. Our results on *ex situ* experiment could be explained by differences in experimental and exposure conditions. Actually, in our study, Pb was used as a mono-metal, contrary to *in situ* studies in which multiple metal(loid)s were introduced and it was applied to only favor the foliar pathway. Furthermore, we chose young leaves for fatty acid analysis, which may be less contaminated when compared to old leaves which had been exposed for a longer time. Finally, metal speciation, exposure duration and above all contamination sources (soil or atmosphere), and transfer pathways (by root or leaf) can also affect the lipid biomarker (Schreck et al., 2013).

Nevertheless, the variability of our Z values (Fig. 7 in SI), index involved in case of foliar exposure, suggested that Pb can still induce a disturbance on the fatty acid composition. This may be a plant response to oxidative stress and ROS formation (Shahid et al., 2012). PCA results showed that fatty acid ratio decreased with the exposure duration: the change of fatty acid ratio is dependent on the percentage of C18:3. The α-linolenic acid (C18:3) is primarily associated with plastid lipids in leaves, notably in the chloroplasts, suggesting an inhibition, by the metal, of the plastidial lysophosphatidylcholine acyltransferase catalyze acylation when lipids are imported from endomembranes to plastids in eucaryon (Akermoun et al., 2002). The decrease of the C18:3 content with exposure duration may be also related to direct reaction of ROS with unsaturated lipids and responsible of alteration of chloroplast membrane structures, like photosystems, leading to an inhibition of photosynthetic activities (Austruy, 2013). Lead-induced toxicity to lipid membrane via ROS production is well established in literature (Shahid et al., 2014a).

3.1.4.3 Relationship between exposure conditions, metal accumulation and phytotoxicity

PCA results showed that PbO treatment is significantly negatively correlated with shoot growth, gaseous exchanges (Pn and gs), and were not significantly negatively correlated with lipid biomarker (Tables 2 and 3 in SI). These negative correlations, although insignificant, suggested an impact of Pb particles on the composition of membrane lipids in the leaves and a metabolic stress after exposure to Pb particulates.

3.1.5 Conclusions and Perspectives

This study highlights the influence of plant species on lead foliar transfer and phytotoxicity, with some possible adaptation mechanisms as well as the related processes.

Our results could be applied for biomonitoring atmospheric pollution, and assessing vegetable contamination especially when they are cultivated (in farms or kitchen gardens) near industries or urban areas. Actually, populations living in such areas are exposed to high atmospheric quantities of PM enriched with metal(loid)s and can therefore incur health risks by consuming polluted plants. Educational projects such as "Réseau-Agriville" (Educational resources platform on urban agricultures to widely educate citizens about the parameters influencing vegetable quality and diffuse advices to reduce people exposure to pollutants) are therefore essential in order to favor the ecological transition at the global scale.

Furthermore, bioaccessibility experiments performed under different levels of soil and air pollution could help to build a database of metal(loid)s bioavailability values for humans in a context of health risk assessments.

Acknowledgments

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Supporting information

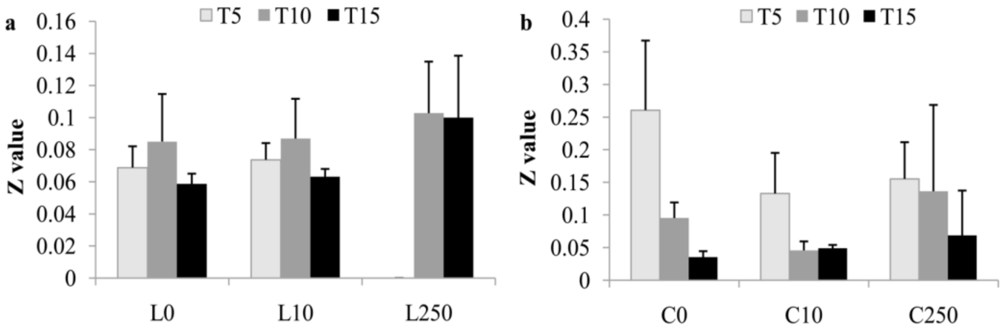

Fig. 7 Z values of (a) lettuces and (b) cabbages exposed to PbO by foliar way (L0, L10 and L250 (and C0, C10 and C250) correspond to respectively 0, 10 and 250 mg of PbO deposited on lettuce (cabbage)) to various exposure durations (T5, T10 and T15). The values are expressed as the mean of 4 replicates. The data of T5 exposed to 250mg PbO (L250) is not available.

Table 2 Correlation coefficient matrix on 13 variables (Exposure quantity, Exposure duration, Pb concentration, Pb storage, Pb transfer kinetic, Shoot growth, Aerial biomass, Plant DW, Leaf water content, Pn, gs, C18:3% and Fatty acid ratio) in lettuce after exposed with PbO PM.

	Exposure quantity	Exposure duration	Pb concentration	Pb storage	Pb transfer kinetic	Shoot growth	Aerial biomass	Plant DW	Leaf water content	Pn	gs	C18:3%	Fatty acid ratio
Exposure quantity	1	0	0.689**	0.53 *	0.584*	-0.781**	-0.153	-0.11	-0.306	-0.644*	-0.74**	-0.289	-0.182
Exposure duration		1	0.5*	0.622*	0.565*	0.341	0.909**	0.92**	-0.786**	0.561*	0.017	-0.83**	-0.776**
Pb concentration			1	0.912**	0.955**	-0.537*	0.242	0.282	-0.637*	-0.126	-0.383	-0.459	-0.369
Pb storage				1	0.985**	-0.353	0.414	0.46	-0.713**	0.006	-0.311	-0.577*	-0.499*
Pb transfer kinetic					1	-0.416	0.346	0.393	-0.685**	-0.034	-0.351	-0.505*	-0.423
Shoot growth						1	0.481	0.473	-0.038	0.807**	0.61*	-0.072	-0.16
Aerial biomass							1	0.995**	-0.764**	0.508*	-0.027	-0.81**	-0.707**
Plant DW								1	-0.786**	0.512*	-0.041	-0.824**	-0.727**
Leaf water content									1	-0.172	0.403	0.761**	0.59**
Pn										1	0.653*	-0.155	-0.292
gs											1	0.312	0.101
C18:3%												1	0.939**
Fatty acid ratio													1

Notes: The significant level was provided at $p < 0.05$ (*) and $p< 0.01$ (**).

Table 3 Correlation coefficient matrix on 13 variables (Exposure quantity, Exposure duration, Pb concentration, Pb storage, Pb transfer kinetic, Shoot growth, Aerial biomass, Plant DW, Leaf water content, Pn, gs, C18:3% and Fatty acid ratio) in cabbage after exposed with PbO PM.

	Exposure quantity	Exposure duration	Pb concentration	Pb storage	Pb transfer kinetic	Shoot growth	Aerial biomass	Plant DW	Leaf water content	Pn	gs	C18:3%	Fatty acid ratio
Exposure quantity	1	0	0.812**	0.72**	0.867**	-0.539*	-0.353	-0.23	0.074	-0.666**	-0.246	-0.216	-0.096
Exposure duration		1	0.276	0.502*	0.257	0.771**	0.88**	0.882**	-0.426	-0.013	-0.667**	-0.484	-0.651*
Pb concentration			1	0.919**	0.973**	-0.2	-0.082	-0.058	0.142	-0.577*	-0.593*	0.012	0.022
Pb storage				1	0.934**	-0.04	0.107	0.147	0.024	-0.52*	-0.714**	-0.078	-0.158
Pb transfer kinetic					1	-0.268	-0.119	-0.072	0.089	-0.642*	-0.582*	-0.046	0.001
Shoot growth						1	0.934**	0.833**	-0.489	0.458	-0.318	-0.338	-0.53*
Aerial biomass							1	0.946**	-0.525*	0.296	-0.43	-0.496	-0.598*
Plant DW								1	-0.387	0.316	-0.388	-0.532*	-0.639*
Leaf water content									1	0.118	0.103	0.72**	0.709**
Pn										1	0.418	0.091	-0.089
gs											1	-0.084	0.04
C18:3%												1	0.867**
Fatty acid ratio													1

Notes: The significant level was provided at $p < 0.05$ (*) and $p < 0.01$ (**).

Table 4 Nutrient solution according to the phases of RHIZOtest® (ISO 16198).

	Chemicals	Concentrations
Germination solution (Pre-culture period)	$CaCl_2 \cdot 2H_2O$	0.6mM
	H_3BO_3	2 μM
Hydroponic growth solution (Pre-culture period)	KH_2PO_4	0.5 mM
	KNO_3	2 mM
	$Ca(NO_3)_2 \cdot 4H_2O$	2 mM
	$MgSO_4 \cdot 7H_2O$	1 mM
	$CuCl_2 \cdot 2H_2O$	0.2 μM
	H_3BO_3	9.98 μM
	$MnCl_2 \cdot 4H_2O$	2 μM
	$ZnSO_4 \cdot 7H_2O$	1μM
	$Na_2MoO_4 \cdot 2H_2O$	0.05 μM
	NaFe(III) (EDTA)	0.1 mM
Incubation solution (Test culture period)	KH_2PO_4	0.05 mM
	KNO_3	2 mM
	$Ca(NO_3)_2 \cdot 4H_2O$	2 mM
	$MgSO_4 \cdot 7H_2O$	1 mM

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3.2 Foliar uptake and phytotoxicity of copper oxide nanoparticles and consequences for sustainable urban agricultures

Forewords

Lead introduced serious demage on vegetables in the previous study, unlike lead, **copper is known as essential trace elements** for the normal healthy growth of plants as cofactors of various proteins (Hänsch and Mendel, 2009) and for most natural soils, deficiency for Cu and Zn seems to be a far larger problem than potential excess (Pilon et al., 2009). However, antropogenic activities elevated metal concentration in soil and atmosphere both by agricultural and industries activities. Relatively high Cu levels (>80 mg kg^{-1} soil) have been recorded in some natural environments, mainly due to the prolonged use in agriculture of Boedeaux mixture ($Ca(OH)_2$+$CuSO_4$), which led to strong increase of Cu concentration: 200–500 mgCu kg^{-1} in the vineyard soils in France (Brun et al., 1998). In the atmosphere, the mean Cu concentration can reach high levels especially in urban and/or highly industrial areas. For instance, 914 ngCu m^{-3}, 813 ngCu m^{-3} and 1550 ngCu m^{-3} were measured in Guangzhou (China) (Lee et al., 2007) , Xi'an (China) (Zhang et al., 2002) and Gandhinagar (India) (Kumar et al., 2001) respectively. Cu has a particularly narrow beneficial range for the growth and development of the plant and becomes toxic after a limited concentration (An, 2006).

Moreover, limited toxicity data on metal oxide nanoparticles are available (Fahmy and Cormier, 2009).

Therefore, in the present study, the impact of nano-CuO (< 50nm) on lettuce (*Lactuca sativa* L.) and cabbage (*Brassica oleracea* L *var. capitata cv. Snowball*) under foliar exposure with a range of increasing Cu concentrations and exposure durations was therefore evaluated. Copper total concentrations measured within leaves and roots allowed estimating kinetic of Cu transfer and storage within plants. Vegetable biomass and gas exchange at leave surface were determined in relation with copper uptake kinetic, localization and chemical speciation studied by SEM-EDX and EPR analysis.

The major concern in this study is the potential transfer of metal(oid)s contaminants within vegetables *via* foliar transfer, when ultrafine particulate matters can be entrapped by the adaxial cuticular waxes, and/or may penetrate into the plant tissue through stomata during the atmosphere-plant gaseous exchanges (Larue et al., 2014; Schreck et al., 2012b).

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Foliar uptake and phytotoxicity of copper oxide nanoparticles and consequences for sustainable urban agricultures

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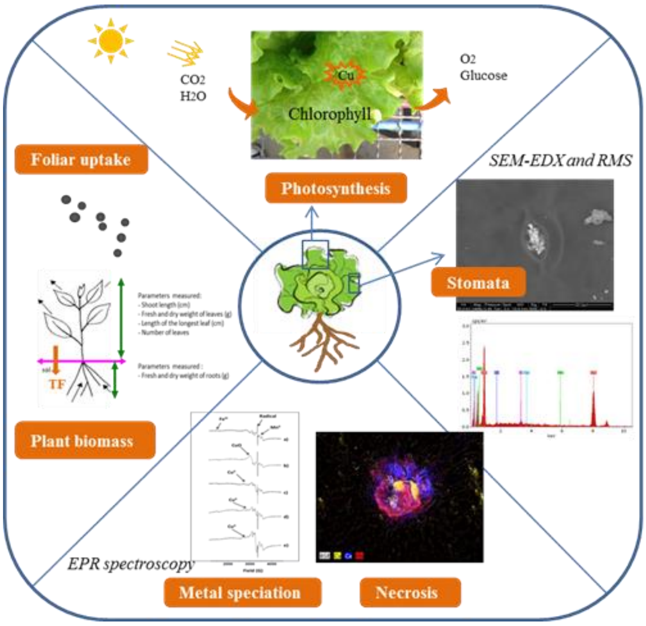
Abstract

Urban agriculture furnishes fresh local vegetables to the populations at the global scale. However, atmosphere fine particulate matters enriched with metals such as copper (widely used in agriculture and industrial processes) can contaminate cultivated vegetables in various urban areas, and induce phytotoxicity. Leafy vegetables were therefore exposed 5, 10 or 15 days at different nano-CuO (< 50 nm) concentrations (0, 10 or 250 mg Cu per plant) by foliar way. Vegetable biomass and gas exchange at leaf surface were determined in relation with copper uptake kinetic, localization and speciation studied by microscopy (SEM-EDX), Raman spectroscopy (RMS) and electron paramagnetic resonance (EPR).

High foliar Cu uptake occurred after nano-CuO exposure (T_{15}): 3773 and 4448 $mgCu.kg^{-1}$ DW, respectively for lettuce and cabbage. Cu translocation from leaves to roots was observed and transfer kinetic suggested a plant Cu regulation process. Decrease of shoot

growth (51%), plant weight (for high doses), net photosynthesis and water content were observed after Cu exposure. Moreover, necrotic Cu-enriched areas were observed by SEM-EDX near deformed stomata with Cu-enriched openings (10-20µm) and EPR analysis evidenced Cu speciation biotransformation in leaf tissues. Finally, a potential health risk associated with consumption of vegetables contaminated by nanoparticles was highlighted.

Abstract art



Key words: Cu nanoparticles; foliar uptake; urban agriculture; transfer kinetic; microscopy; spectroscopy; phytotoxicity; health risk.

3.2.1 Introduction

Urban agriculture has emerged with the rapid development of the cities at the world scale. But, cultivated plant quality can be degraded by air pollution due to different anthropogenic activities[1–4]and human metal(loid)s exposure through ingestion of polluted vegetables[5]could be induced. Actually, in urban areas, metal(oid)s can transfer within vegetables both *via* root exposure to contaminated soils[6] and foliar transfer[7,8]. The atmosphere-to-plant way is recently considered as a significant contamination way of vegetables in urban areas[9–12]. Nowadays, urban atmosphere is largely enriched in particulate maters (PM) of micronic and submicronic (including nanoparticles) sizes, due to the use of more effective filters and high temperature processes in the industry [13]. These ultrafine particles are minor components of the total mass of emitted PM, but they are very reactive owing to their high specific area[14] and can be transported over large distances into the environment[15]. Then, environmental pollution by nanoparticles (NPs) has recently led to numerous studies focused on copper, iron, nickel and cesium oxides[16–19].

Copper is an essential plant micronutrient which can be used as an antifungal and/or fertilizer agent in agriculture[20–22]. But, high Cu concentrations can induce (eco)toxicity[20,23,24]. Cu phytotoxicity is linked to reactive oxygen species (ROS) generation[25]; necrosis, chlorosis, reduced seed germination and root development are observed[26–28]. Moreover, excess Cu interacts with some key enzymes, proteins and carbohydrate metabolism in plants, which are involved in photosynthesis, lipids metabolism and water status[27,29], resulting in strong inhibition of plant growth[23]. Atmosphere Cu concentration can reach high level in urban and/or highly industrialized areas. For instance, 914 ngCu m^{-3}, 813 ngCu m^{-3} and 1550 ngCu m^{-3} were respectively measured in Guangzhou (China),[30] Xi'an (China) [31] and Gandhinagar (India) [32]. But, the effects of nano-CuO is still not well explored, particularly in the case of foliar exposure.

Thus, in the present study, the impact of nano-CuO on lettuce (*Lactuca sativa* L.) and cabbage (*Brassica oleracea* L.) under foliar exposure with a range of increasing NPs concentrations and exposure durations was therefore evaluated. Total Cu concentrations measured within leaves and roots allowed estimating kinetic of Cu transfer and storage within plants. Microscopy and spectroscopy techniques (optical microscope, Scanning Electronic Microscope coupled with Energy Dispersive X-ray microanalysis (SEM-EDX) and Raman microspectrometry (RMS)) provided the distribution feature of particles on leave surfaces as well as physiological effect due to the nano-CuO exposure. Finally, Cu transfer, phytotoxicity and human health impact were discussed in relation with its speciation in plant leaves, determined by electron paramagnetic resonance (EPR) analysis.

3.2.2 Materials and methods

3.2.2.1 Experimental conditions and set up

Lettuce and cabbage were chosen because: (1) they are widely cultivated by farmers and gardeners, and they are commonly eaten by inhabitants[33,34] and sold in markets around the world, (2) they have short life cycles and large leaves[8,35] and thus, are highly suitable for foliar exposition studies, (3) they have previously been studied for foliar metal transfer [36,37].

Foliar transfer experiments were performed using Rhizotest® devices (ISO/CD 16198)[38,39] in controlled chamber with a day/night temperature range of 25±2 °C (16 h) / 20±2 °C (8 h), a 65±5 % of relative humidity and a light intensity of 425±50 photons μmol m^{-2} s^{-1}. Lettuce and cabbage seeds were surface-sterilized with 6% H_2O_2 for 10 min, rinsed 3 times in deionized water and then put in constant temperature incubator for germination (one week). After germination, plants were grown for two weeks under hydroponic conditions. Then, three-weeks vegetables were transferred to unpolluted soil and exposed to nano-CuO particles (CAS 1317-38-0, particle size<50 nm, specific area 12.55 m^2 g^{-1},[40] water insoluble, Sigma-Aldrich) on their leaf surfaces with an applicator brush according to Xiong et al.[11] A geotextile membrane was placed on soil surface to avoid soil-root transfer of CuO[11,41].

Plant leaves were exposed to three different concentrations of nano-CuO: a control condition without any metal input (0 mg), 10 mg CuO inputs and 250 mg CuO inputs. We define lettuces and cabbages exposed to 0, 10 and 250 mg of nano-CuO respectively as L_0, $L_{10,}$ L_{250} and C_0, C_{10}, C_{250}. Foliar expositions were performed for 5 (T_5), 10 (T_{10}) and 15 (T_{15}) days, the first day of exposure was defined as control (T_0).

3.2.2.2 Metal accumulation in plant tissues

The preparation of samples for determining metal transfer and accumulation within plants was disposed elsewhere[10,11,17,42] and is briefly summarized here. After harvest, roots and shoots were separated and washed with deionized water to remove deposited particles[8,10]. Plant tissues were oven-dried at 40°C for 3 days, weighted, grounded and then sieved to < 250 μm particle size. Mineralization of plant samples was then performed by acidic digesting 0.125g dry weight (DW) of homogenised samples (Digiprep® instrument) with 1:1 mixture of HNO_3 and H_2O_2 at 80˚C for 4h. After filtration, Cu levels within plant tissues were quantified by inductively coupled plasma-optical emission spectrometry (ICP-OES, IRIS Intrepid II XXDL). Method control was performed with concurrent analysis of blank and standard reference materials (Virginia tobacco leaves, CTA-VTL-2, ICHTJ).

The Cu storage (µg) within plant tissues was calculated by multiplying Cu concentrations ($[Cu]_{plant}$, mg kg^{-1}) by plant DW (mass in g) as Eq.1:

$$Cu_{Storage} = [Cu]_{plant} \times DW \qquad \text{Eq.1}$$

We defined a kinetic transfer of Cu corresponding to an accumulation dose per day in function of exposure duration. The Cu transfer kinetic (µg day^{-1}) during T_0 to T_5, T_5 to T_{10} and T_{10} to T_{15} were respectively calculated as Eq.2:

$$Cu_{Transfer\ kinetic} = Cu_{Storage} / \text{exposure duration (day)} \qquad \text{Eq.2}$$

The exposure duration is 5 days for all phases.

Metal translocation factors (TF) were calculated by dividing the metal concentrations in root tissues ($[Cu]_{roots}$, mg kg^{-1}) by metal concentrations in leaves ($[Cu]_{leaves}$, mg kg^{-1}) [43] as Eq.3 :

$$TF = [Cu]_{roots} / [Cu]_{leaves} \qquad \text{Eq.3}$$

3.2.2.3 Phytotoxicity parameters

CuO phytotoxicity was assessed through plant biomass and gas exchange determination on four plants per treatment. Plant biomass was measured at T_0, T_5, T_{10} and T_{15}, including shoot length, number of leaves, fresh and dry weight of shoots and roots, and water content. Shoot growth was expressed as the increased shoot length in T_5, T_{10} and T_{15} as a comparison with T_0. The fresh plants were weighted (FW) and oven-dried to determine the DW. Water content (water %) was calculated according to the fresh and dry weights as Eq.4 :

$$water\% = \frac{FW - DW}{FW} \times 100\% \qquad \text{Eq.4}$$

Gas exchange parameters, net photosynthesis (Pn) and stomatal conductance (gs) were recorded simultaneously using a portable infrared gas analyzer (LI-COR 6400 XT) equipped with a clamp-on leaf chamber that exposed 6 cm^2 of leaf area. Humidity was fixed at 65 %, CO_2 concentration was maintained at a constant level of 380 µmol mol^{-1} using a LI-6400-01 CO_2 injector with a high pressure liquid CO_2 cartridge source. Gas exchange measurements were performed under irradiance 425µmol m^{-2} s^{-1} and at 23±2 °C.

3.2.2.4 Characterization of particles on contaminated leaves surface

The adaxial leaf surfaces were observed using optical microscope and SEM-EDX microanalysis. The optical images were recorded with an Olympus BX 41 microscope equipped with a ×100 (N.A 0.9) objective.

SEM-EDX measurements were carried out using a Quanta 200 FEI instrument equipped with a Quantax EDX detector to characterize the morphology of the leaf surfaces and the particles-phyllosphere interactions. The apparatus was operated in low-vacuum mode (~133 Pa) at 20 kV and with a pressure of 0.98 Torr. The dry leaves were fixed on a carbon substrate without any further treatment before analysis as described previously[8].

Additionally, the particle composition was characterized using RMS. The measurements were performed using a Raman HR 800 UV (HORIBA), equipped with a ×80 Mitutoyo (N.A. 0.55) objective and a solid MDB 266 system laser beam (Coherent Laser Group) emitted at 266 nm. The excitation using a UV laser (266nm) was necessary to probe the surface of leave, since many biologically important molecules such as chlorophyll have an intense fluorescence emission when excited in the visible range (400 nm $< \lambda <$ 800 nm)[10].

3.2.2.5 Cu speciation within lettuce leaves

Copper speciation in lettuce leaves contaminated by nano-CuO particles was studied using EPR in continuous waves (CW). EPR spectroscopy experiments were performed at a fixed microwave frequency, the X-band (9.6 Ghz) using a Brüker ELEXSYS E500 spectrometer in CW. The microwave power and amplitude modulation were respectively set to 100 mW and 4G. The spectra were collected at room temperature on contaminated leaf directly inserted in a 8 mm quartz tube. The g-factor, which is a constant of proportionality, was determined in order to have information on the Cu properties. When there is a hyperfine structure, it is possible to determine the hyperfine constant A which corresponds to the distance between two consecutive stripes. The accuracy of the determination of EPR parameters was ±0.005 and ±0.0005 for g values of metal ions and radicals, respectively. The accuracy of the estimation of the hyperfine splitting constant was $\pm 10^{-4}$ cm^{-1}. For pure powder CuO nanoparticles, typical EPR spectrum showed three equally spaced peaks (4 are expected but one is overlapping with perpendicular component) related to the hyperfine coupling of Cu^{2+} electron spin (S= 1/2) with its nuclear spin (I=3/2). The parallel components $g_{//}$ that corresponds to non-degenerated eigenvalue and $A_{//}$ were determined from the spectrum, the values were 2.337 and 179 10^{-4} cm^{-1}, respectively. The perpendicular region cannot be precisely determined from experimental spectrum. Speciation of Cu was determined through the comparison of parallel component values deduced from our experimental spectra and literature values. The best method allowing identification of Cu(II) complexes is EPR. Actually, Cu(II) cations exhibit many valuable features for the application of EPR spectroscopy. With its d9-configuration (S= 1/2, I = 3/2), it shows a strong deviation from a regular electron-density distribution. Thus, one can get information about the type of complex formed by an adsorbed species and its geometry. Moreover, paramagnetic species such as radicals can be successfully detected which generally displays one line at the solid state.

3.2.2.6 Statistical analysis

Differences between treatments and control conditions were subjected to analysis of variance (ANOVA), using Duncan's test at $p < 0.05$. Data were tested for normal distribution and homogeneity of variance before the ANOVA analysis. The data are presented as mean ± SD (standard deviation). The correlation between exposure conditions with growth parameters and metal translocation parameters were analyzed by partial correlation analysis and Pearson correlations coefficient. The values of « r » stand for correlation coefficient between different parameters and significant levels were provided with $p<0.05$ (*) and $p<0.01$ (**). Statistical analyses were carried out on means of four replicates (n=4) for each exposure condition.

3.2.3 Results and discussion

3.2.3.1 Foliar Cu uptake and translocation to the roots: kinetic and mechanistic study

Figure 1 shows the total Cu concentrations, storage and transfer kinetic in lettuces and cabbages for various concentrations (L_0, L_{10} and L_{250}; C_0, C_{10} and C_{250}) after 5, 10 and 15 days (T_5, T_{10} and T_{15}) of CuO NPs deposition. A significant Cu absorption up to 3773 and 4448 mgCu kg^{-1} was observed respectively for lettuce and cabbage at T_{15} (Figure 1a). Total Cu concentration showed positive correlation with both exposure quantity (r =0.831 and 0.825 respectively for lettuce and cabbage) and exposure duration (r =0.525 and 0.526 respectively for lettuce and cabbage) (Table 3 in SI). The same trend was also found in Cu storage (Figure 1b). Cu, a nutritive element for plants, appears as relatively well absorbed by plant leaves.

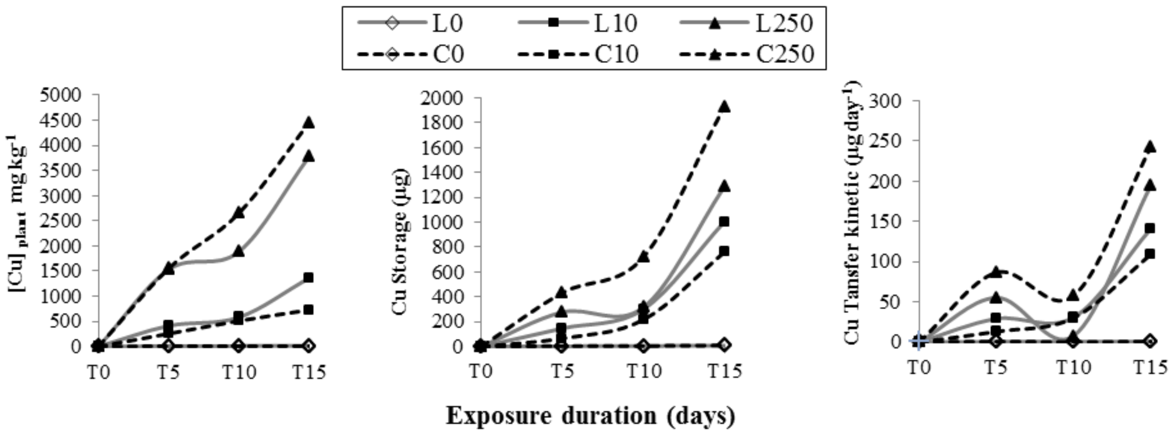


Figure 1 (a) Cu concentration (mg kg^{-1}), (b) Cu storage (µg), and (c) Cu transfer kinetic (µg d^{-1}) in lettuces and cabbages exposed to nano-CuO by foliar way (L_0, L_{10} and L_{250} (and C_0, C_{10} and C_{250}) correspond to respectively 0, 10 and 250 mg of CuO deposited on lettuce (cabbage)) to various exposure durations (T_0, T_5, T_{10}, T_{15}). Values are expressed as the mean of 4 replicates for each treatment.

Concerning the mechanisms involved in foliar Cu transfer, accordingly to previous studies on different metals[8,11,44], the stomatal way appears efficient. Actually, the distribution of NPs on leaf surface as observed by *SEM-EDX and RMS* is given in Figure 2. Most of NPs are deposited as aggregates either on the leave surface (Figure 2a) or in the stomata (Figure 2b). The typical Raman spectra of particles recorded from all aggregates shows a main Raman band at 626 cm^{-1} and is assigned to CuO (see Figure 5 in supporting information

(SI)). In this study, NPs size is less than 50 nm, and NPs are mainly aggregated that exclude the direct cuticle uptake of the particles since cuticle pores are around 2nm diameter [25]. This observation suggests that stomatal openings are likely the main uptake pathway in agreement with Eichert et al.[45] The stomata openings in the epidermis are around 10-20 µm in studied vegetables, which allowed gas exchange and metal accumulation as previously observed by Schreck et al.[46]. In reference to the speciation of copper within plant leaves, Figure 3 displays typical EPR spectra of lettuce leave of control (L_0) and contaminated (L_{10} and L_{250}) plants. The EPR spectrum of L_0 (Figure 3a) exhibits the presence of six equally spaced hyperfine lines with g=2.0238 which are characteristic for Mn(II) ions with high spin state (S=5/2). Between the third and fourth lines of the hyperfine structure, the narrow signal with g~2.0247, typical for radical species, was visible. Such g value for radical indicates an oxygen center species. Moreover, the broad signal with small intensity at g = 4.6627 was distinguished and was characteristic of Fe(III) ions in high state (S=5/2) with rhombic distortion. The elements Mn and Fe(III) are expected in plant tissues while the presence of radicals was commonly reported in EPR studies of plants, including wood substances, humic substances and soils and was attributed to quinoid radicals. This radical formation is promoted by the presence of metals such as Fe or Mn. After T_{15}, the EPR spectrum of leaves from L_0 did not exhibit any significant changes (not shown). The typical EPR spectra of leaves from L_{10} and L_{250} at T_5 and T_{15} are reported in Figure 3 b-e. After T_5 at L_{10} (Figure 3b), the EPR spectrum clearly evidenced a typical EPR signal with g = 2.41 which is similar to the CuO powder spectrum recorded on the same conditions (not shown). The Mn, Fe and radical signals remained unchanged compare to the spectrum of L_0. After T_{15}, the EPR spectrum significantly changed (Figure 3c) and exhibited four equally spaced hyperfine lines typical for copper ions (Cu^{2+}) with $g_{//}$ ~2.348 and $A_{//}$ = 175 10^{-4} cm^{-1}. The EPR spectra of leaves from L_{250} displayed similar spectra (Figure 3d-e) with $g_{//}$ ~2.339 and $A_{//}$ = 174 10^{-4} cm^{-1} for T_5 and $g_{//}$ ~2.353 and $A_{//}$ = 170 10^{-4} cm^{-1} for T_{15}. These values, varying from the value of the free aquo ion (g// = 2.44), were consistent with a four oxygen coordinated organic complex[47] as Cu(II) likely ligated with carbonyl oxygen. The results agreed well with d_{x2-y2} ground state for Cu^{2+} held in inner-sphere complexes with slightly distorted square configuration around the central ion. Actually, the ratio $g_{//}$/ $|A_{//}|$ was reported as an indication of distorsion of a Cu(II) square-planar geometry. In our case the $g_{//}$/ $|A_{//}|$ values varied from 133 to 145 showing different structure distortion related to the CuO to Cu-lignin like structures (Figure 6 in SI, in comparison to the literature). Finally, the decrease of the radical intensity with growth time and the increase of CuO concentrations was conjointly observed with the modification of the Cu(II) and Mn signal intensity. This may reflect an electronic transfer involving Mn together with a Cu(II)-to-Cu(I) reduction. The electron transfer may indirectly evidence the oxidative stress induced by the Cu contamination. However, our EPR data are not sufficient for unambiguous further assignments. The formation of Cu(II)-organic complexes supposes that a CuO internalization and dissolution with biotransformation even though the CuO solubility is low. These Cu(II)-organic complexes are observed when exposure time and dose increase, suggesting that an internalization effect through stomatal openings and then biological processes.

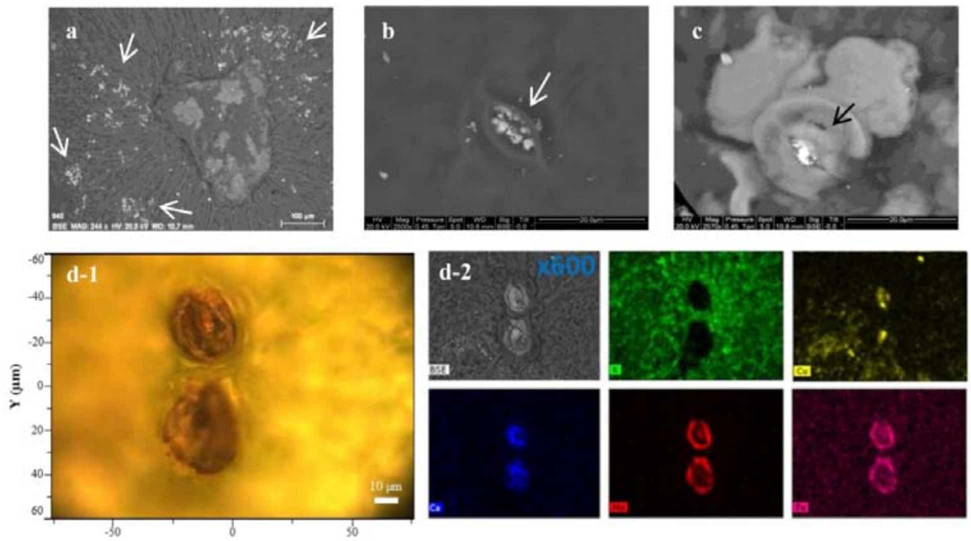


Figure 2 Optical observation and SEM-EDX analysis of CuO NPs on lettuce leaf surfaces. (a) Cu aggregation in lettuce leaf surface; (b) Back-scattered electrons (BSE) image of stomata contaminated with Cu without necrosis; (c) BSE image of stomata contaminated with Cu within a necrosis; (d-1) optical image of necrosis and (d-2) necrosis area enriched with Cu, Ca, Mn, Fe of lettuce L_{250} at T_{10}.

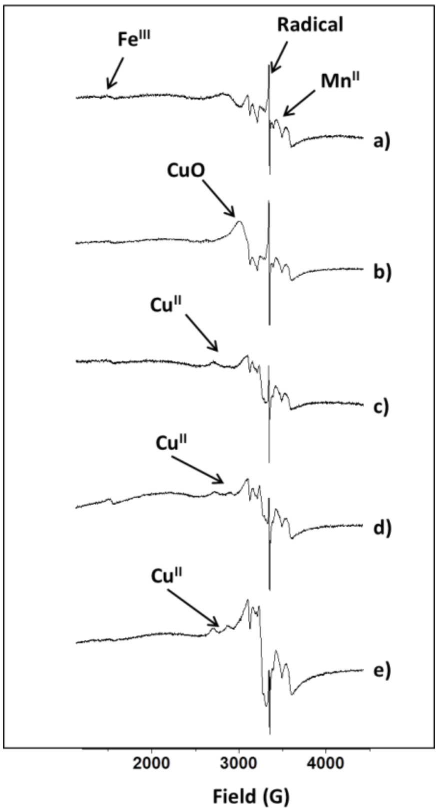


Figure 3 Experimental EPR spectra of L_0 (a); L_{10} at T_5 (b) and T_{15} (c); L_{250} at T_5 (d) and T_{15} (e). Lettuce exposed with nano-CuO (0 mg, 10mg and 250 mg) is defined respectively as L_0, L_{10}, L_{250} and exposure duration (5 and 15 days) is defined as T_5 and T_{15} respectively.

Cu transfer kinetic did not show a linear trend whatever the CuO amount deposited on the leaf surface and the plant species (Figure 1c). These results strongly suggest that in some extent, the vegetables have defense mechanisms to limit Cu uptake during growth processes. These defense mechanisms may influence metal foliar uptake between T_5 and T_{10}. Actually, during this period, Cu transfer kinetic strongly decreased. Cu is an essential element and it's not surprising that reparation mechanisms occur at moderate concentrations of Cu within tissues. Indeed, plants have a complex network of metal trafficking pathways in order to appropriately regulate Cu homeostasis in response to environmental Cu level variations[23]. To our knowledge, the change of transfer kinetic is a process of accumulation (T_0 to T_5), regulation (T_5 to T_{10}) and adaptation (T_{10} to T_{15}) in vegetables. Previous studies showed that regulation processes could be correlated with the activation of plant defense mechanisms for scavenging oxidative stress caused by excessive Cu[48,49]. The existence of efficient storage and regulation/detoxification mechanisms allows the survival of organisms under high accumulated metal concentrations.[50]

Table 2 (in SI) summarizes metal concentrations in leaves, roots and translocation factors (TF). Cu concentrations in roots and leaves were highly related in both species (r=0.630, $p<0.01$ in lettuces; r=0.793, $p<0.01$ in cabbages). By foliar pathway, plant leaves accumulated high Cu amounts and translocated one part to roots (TF ranges 0.04 to 0.33, Table 2 in SI) certainly through vascular system of the plants [51]. Similar translocation factors were previously found for lettuces contaminated by Pb (TF = 0.06) and Cd (TF = 0.28) [43]. TF values are significantly higher in cabbages than in lettuces ($p<0.01$). Indeed, the uptake, translocation, and accumulation of PM depend on metal type and the applied dose, the plant species and its life cycle stage, soil properties and growth conditions [52,53].

3.2.3.2 Nano CuO phytotoxicity: effects and mechanisms involved

Nano-CuO foliar application affected the relative shoot growth rates of vegetables (Table 1). For lettuces, the growth of L_{10} and L_{250} is comparable to L_0 even if a growth reduction (5%~26%) is observed. For cabbage, all the treatments induced a shoot growth reduction varying between 12% and 51%. The most notable decrease was observed for C_{250}. However, no significant decrease of shoot growth was observed whatever the considered vegetable. The average plant biomass (DW) was reduced when the plants were exposed to the highest CuO doses, significantly for L_{250} at T_{15} (Table 1), but L_{10} showed no significant damage to lettuce.

Table 1 Shoot growth and plant biomass (DW) of lettuces and cabbages exposed to nano-CuO by foliar way (L_0, L_{10} and L_{250} (and C_0, C_{10} and C_{250}) correspond to respectively 0, 10 and 250 mg of CuO deposited on lettuce (cabbage)) to various exposure durations (T_5, T_{10} and T_{15}). The values are expressed as the mean of 4 replicates ± standard deviation.

Vegetables	Exposure duration	NPs concentrations	Shoot growth (cm) (Reduction in shoot growth (%))	Plant DW (g) (Reduction in plant DW (%))
Lettuce	T_5	L_0	0.48±0.19 ab [1]	0.22±0.04 AB [2]
		L_{10}	0.35±0.1 (26%) a	0.35±0.04 AB
		L_{250}	0.38±0.08 (21%) ab	0.18±0.04 (17%) A
	T_{10}	L_0	0.5±0.07 ab	0.41±0.04 AB
		L_{10}	0.53±0.05 ab	0.44±0.1 AB
		L_{250}	0.48±0.09 (5%) ab	0.17±0.02 (59%) A
	T_{15}	L_0	0.85±0.13 abc	0.60±0.1 CD
		L_{10}	0.73±0.23 (15%) abc	0.69±0.15 D
		L_{250}	0.7±0.1 (18%) abc	0.32±0.08 (47%) AB
Cabbage	T_5	C_0	0.7±0.16 abc	0.44±0.17 AB
		C_{10}	0.48±0.19 (32%) ab	0.26±0.05 (41%) AB
		C_{250}	0.58±0.12 (18%) abc	0.30±0.08 (32%) AB
	T_{10}	C_0	1.05±0.30 abc	0.68±0.23 BCD
		C_{10}	0.75±0.38 (29%) abc	0.41±0.07 (40%) AB
		C_{250}	0.8±0.27 (24%) abc	0.40±0.17 (41%) AB
	T_{15}	C_0	1.28±0.30 c	0.89±0.27 CD
		C_{10}	1.13±0.50 (12%) bc	0.98±0.19 D
		C_{250}	0.63±0.15 (51%) abc	0.49±0.24 (45%) ABC

[1]The different lowercase letters are the significant difference of shoot growth at $p<0.05$.
[2]The different capital letters are the significant difference of plant DW at $p<0.05$.

Actually, low doses of CuO (10 mg) promoted plant DW for lettuces. This hormesis effect (stimulation of growth under mildly toxic stress) have previously been detected for *Lactuca sativa* L. when soils are contaminated by Pb[54] and Cd[55]. Low Cu quantity favors plant growth in lettuce, as well as Cu is an essential micronutrient for plants[56,57]. The metal-induced antioxidant defenses are key parameters in hormetic responses[58]. Nevertheless, Cu may become phytotoxic above its threshold level, which may vary with plant species[56]. Reduction in plant biomass for crops (cucumber, sorghum, sweet corn and wheat) grown under excess of Cu was also previously observed[56,5956,59]. Finally, plants grew even under excess nano-CuO deposition in our study, suggesting adaptation or metabolic responses to Cu toxicity.

In lettuce, the water content varied from 76 to 89% and remained consistent in all the treated plant groups contrary to cabbage for which a significant decrease of water content was observed with nano-Cu increase. At T_{15}, water % was 60% and 35% respectively for C_{10} and C_{250}, when 78% was measured for C_0. This decrease in water content was significantly correlated to exposure duration and plant species (Table 3 in SI). We notice that vegetable species influence plant biomass, water content and TF in leaves. Cabbages acquired higher shoot growth and plant weight than lettuces suggesting a higher tolerance of cabbage to Cu stress. The morphological characteristics of plants (the difference cuticle thickness between lettuce and cabbage) could be an explanation for these observations. The higher biomass of cabbage also resulted in a higher metal concentration in C_{250} which permitted it to store more Cu per day.

All the samples maintained the photosynthesis activity during the exposure duration, except for C_{250}. The values of Pn and gs of C_{250} at T_{15} were significantly decreased, these damages were consistent with the low water content in this group. Photosynthesis activity decreased significantly after nano-CuO application on plant leaves, particularly in T_{10} and T_{15} for both vegetables (Figure 4). For lettuce, mean Pn values ranged from 5.13 to 6.19 µmol m^{-2} s^{-1} for control (L_0), while a significant decrease was observed for L_{10} and L_{250} with measured values respectively from 2.46 to 4.42 µmol m^{-2} s^{-1} and from 2.77 to 3.32 µmol m^{-2} s^{-1}. For cabbage, the Pn values are comprised between 4.45 and 5.52 µmol m^{-2} s^{-1}, 2.01 and 3.85 µmol m^{-2} s^{-1}, 0 and 4.6 µmol m^{-2} s^{-1}, respectively for C_0, C_{10} and C_{250}. The stomatal conductance (gs) values also decreased with increasing nano-CuO doses. There is no significant correlation between Pn/gs and exposure duration in lettuces (p>0.05), but for cabbages, Pn and gs were both reduced with exposure quantity and exposure duration. Photosynthesis activity was perturbed by high Cu exposure, and had shown more sensitivity to metal contamination than shoot growth and plant DW. Kuzminov et al.[60] also reported a high impact of Cu on photosynthetic processes, and net photosynthesis rates decreased with increasing Cu concentrations in *Glaucium flavum* Crantz[61]. Indeed, Cu plays a key role in photosynthetic and respiratory electron transport chains[23]. The greatest damage caused by Cu results from the inhibition of light reactions in photosynthetis process[62].

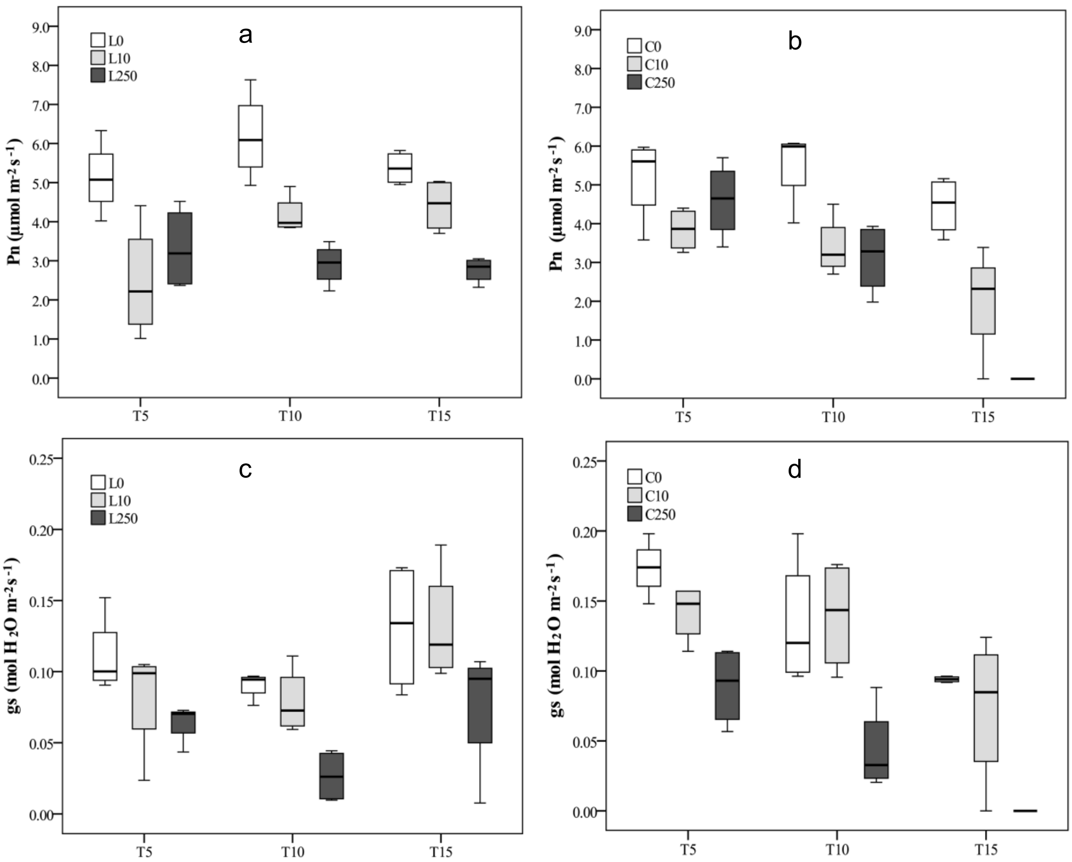


Figure 4 Measurement of net photosynthesis (Pn) (a,b) and stomatal conductance (gs) (c,d) of plants as a function of exposure duration (T_5, T_{10} and T_{15}) and nano-CuO content (0, 10 and 250 mg) deposed on leaves (L_0, L_{10} and L_{250} (and C_0, C_{10} and C_{250}) correspond to respectively 0, 10 and 250 mg of CuO deposited on lettuce (cabbage)).

In our experiment, the low net photosynthesis is caused by Cu both at the surface of plant leaves and inside the plants perturbing the electron transfer chain. Actually, EPR results clearly evidenced the direct relationship between quinoid radical, Mn decreasing and Cu(II) concentration increase. The radical decrease accompanied by the decrease of the Mn signal intensity reflect the photosynthetic inhibiting effect of Cu(II) since Cu^{2+} inhibited photosynthetic evolution through the replacement of Mn ions[63]. On leaf surface, stomata can be blocked by aggregation of nano-CuO powder, which may affect the exchange of O_2, CO_2 and H_2O. Decrease in stomatal conductance during the whole period of exposure can reduce the absorption of CO_2 for producing essential nutrients for its development. Water content was dropped in cabbage as excess Cu can operate as stress factor and may deteriorate the plant water balance[64]. The potential electron transfer involving conjointly Mn and Cu(II)-to-Cu(I) reduction by EPR analysismay indirectly evidence the oxidative stress induced by the CuO contamination. Furthermore, nano-CuO particles on the leaf surface may also disturb the function of chloroplasts on light absorption by affecting their photon capture capacity and then affect photosynthesis via overproduction of reactive oxygen species (ROS)[65]. Major components of the thylakoid membrane are also susceptible to free radical attack[66].

Cu phytotoxicity can cause necrosis, chlorosis and stunting[28]. Necrosis was effectively observed in the leaf surface, even for low doses of deposited NPs. Nevertheless, the number of necroses increased with the CuO doses. Some stomata containing Cu were evidenced in many necroses areas (Figure 2c) which could indicate a possible necrotic phenomenon induced by CuO particles. The X-ray mapping of this typical feature showed Cu in the center of the stomata, whereas Ca, Fe and Mn were concentrated on the stomata edges (Figure 2d). K was observed on the leaf excepted in the necrotic stomata. Ca and Mn accumulation in necrosis was also commonly observed[10]. The orifice of the stomata contained Ca that appeared to be generated by the leaf tissue death. Potassium was missing in necrosis area suggesting that a change in nutrient homeostasis could also be responsible for the observed symptoms. Using algal cells, De Filippis (1979) demonstrated the ability of metals to induce potassium leakage, damage to the permeability barrier of the cell, closely corresponding with their sulfhydryl reactivity [67]. As a consequence, the plasma membrane could not repolarize under severe metal toxicity because K^+ uptake is blocked and the inhibition of proton pumping results in massive K^+ leakage [58]. The Raman spectra recorded in the stomata showed a typical Raman bands at 1058 cm^{-1} which is typical of stretching modes of CO_3^{2-} (Figure 5 in SI) and was attributed to Na/Cu carbonate i.e. $Na_2Cu(CO_3)_2.3H_2O$. The precipitation of carbonates within necrotic areas enriched in metal particles was already described (Uzu et al., 2011). The formation of this secondary phase likely resulted in the modification of CuO particles during the necrosis process. With 250 mg NPs application, the phytotoxicity was more evident since necrosis was even present on veins (not shown).

Generally, nanomaterials toxicity is most often associated with the increased reactivity and surface area, ROS formation, aggregation and adsorption to cell walls, and release of toxic ions[40,68,69]. Cu exhibited a high treat score with high cytotoxicity and high oxidative potential, despite its essential character.[40] Actually, Cu can transfer electrons to molecular oxygen to form reactive superoxides ($O_2^{\bullet -}$) and to H_2O_2 to form hydroxyl ($HO^{\bullet}$),[70] resulting in oxidative damage to cellular components lipids, proteins and nucleic acids[71].

Another important Cu function (under +1 oxidation state) is to bind to small molecules like O_2 as a ligand, bind to cell components such as DNA or the sulfhydryl, carboxyl or imidazole groups of proteins, and may modify their activities[16].

3.2.3.3 CuO NPs transfer to vegetables and consequences for human health

Previous studies reported that leaf washing had been experimented to separate surface contaminants that are only deposited and metals internalized in leaf tissues[17,72]. But, even with washing procedure, NPs can stay in the plant entrapped by the cuticle, stick to the leaf epidermis or adhere to epicuticular waxes. Thus, human health risk should be considered in case of vegetable ingestion even after washing. According to Goix et al.[40], the health impacts of Cu could be attributed to both its solubility and redox properties. Indeed, in human body, free Cu causes toxicity, as it generates ROS[73]. The cytotoxic effects of Cu to human liver and lung cells was reported by Arnal et al[74]. Fahmy and Cormier[75] had also reported that nano-sized CuO toxicity in human lung cells is higher than micron-sized CuO or other metal-based NPs.

To assess the sanitary risk induced by polluted vegetable (after foliar transfer) ingestion, the estimated daily intake of pollutant (EDI, µg kg^{-1} day^{-1}) by edible parts ingestion can be calculated by Eq.5:

$$EDI = \frac{[\mathrm{Cu}]_{\mathrm{leaves}} \times IR}{BW} \quad \text{Eq.5}$$

The ingestion rate (IR) of vegetable is 13 g DW day^{-1} per person (body weight (BW) of an average adult: 60kg) according to the report of Sharma et al.[76], and was used to calculate EDI here. The determined EDI values are then compared to the tolerable daily intake (TDI, µg kg^{-1} day^{-1}) which was expressed as the quantity of pollutant ingested each day (µg) in function of kg BW: 40 µg kg^{-1} day^{-1} for Cu [77]. The maximum allowable daily intake of contaminated plant (MDI) without exceed the TDI was therefore calculated as below (Eq.6): MDI (g day^{-1}) = TDI (µg kg^{-1} day^{-1}) ×BW (kg) / $[\mathrm{Cu}]_{\mathrm{leaves}}$ (mg kg^{-1}) Eq.6

The calculated EDI and MDI values of the exposed lettuce and cabbage are presented in Table 4 in SI. The EDI of control plants (L_0 and C_0) are all below the TDI (40 µg kg^{-1} day⁻

^{1}), however, the EDI of exposed group (L_{10}, L_{250}, C_{10} and C_{250}) ranges from 86 to 1797 µg kg^{-1} day^{-1} and then seriously exceed the TDI, even if no visible morphologic damage were found for L_{10} and C_{10}. The MDI of L_{10}, L_{250}, C_{10} and C_{250} ranges from 0.3 to 6.7 g DW day^{-1}, this value can be easily reached by adults ingesting vegetables. Therefore, we concluded that vegetable leaves can accumulate pollutants and induce serious sanitary risk when high amounts of PM are measured in the atmosphere.

Finally, high Cu concentration was highlighted in vegetable leaves by kinetic study of CuO NPs-leaf interactions. Shoots growth and weight, water content and net photosynthesis used as phytotoxicity endpoints after 5, 10 or 15 exposure days permitted to demonstrate the phytotoxicity of Cu NPs. Actually, leaf necroses and deformed stomata enriched with CuO NPs aggregation were observed on leaf surface by SEM-EDX analysis. Metal speciation changes were observed by EPR analyses with possible bio-physicochemical transformations process. The possible pathway of metal through stomata is also suggested. Cu seems to be stored within plant leaves through Cu(II)-organic complex formation as it was demonstrated using EPR. A significant metal accumulation and phytotoxicity induced by CuO NPs after foliar application was highlighted: among the mechanisms causing phytotoxicity, oxidative stress is always a main factor. Both exposure dose and time are important risk factors for plant health when PM can interact with leaves. Moreover, in the current context of discussions on nanoparticles regulation, our study provides helpful information about the reactivity and dynamics of these nano-substances in the environment at the global scale, and especially in urban areas.

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Supporting information

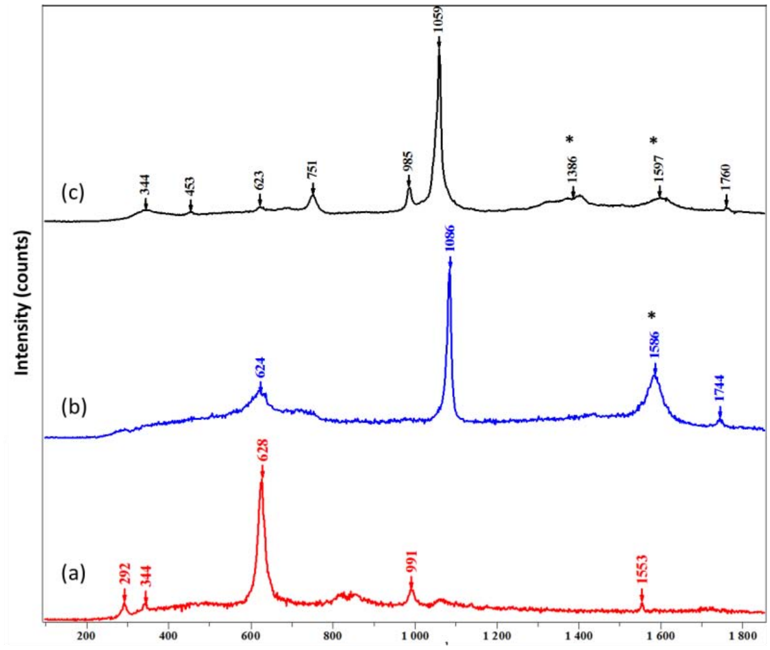

*Figure 5 Typical Raman spectra obtained from RMS analysis of particles on the lettuce leaf surface identified as (a) CuO; (b) $CaCO_3$ and (c) $Na_2Cu(CO_3)_2.3H_2O$ (** indicates Carbon impurities*).*

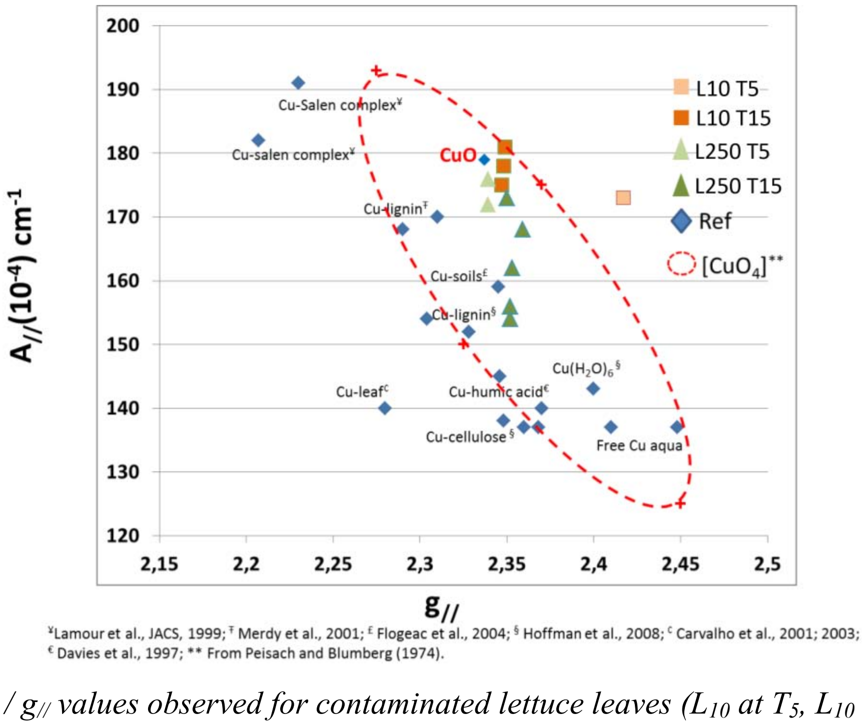

Figure 6 $A_{//}$ / $g_{//}$ values observed for contaminated lettuce leaves (L_{10} at T_5, L_{10} at T_{15}, L_{250} at T_5, L_{250} at T_{15}) and compared to the literature.

Table 2 Mean Cu concentrations ± standard deviation (mg kg^{-1} of DW) in leaves and roots of lettuces and cabbages as function of exposure duration (T_5, T_{10} and T_{15}) and nano-CuO content (0 mg, 10mg and 250 mg) deposed on leaves, and the corresponding translocation factor (TF). Pearson correlations coefficient are also given.

Vegetables	Exposure duration	Exposure quantity	$[Cu]_{leaves}$ (mg kg^{-1})	$[Cu]_{roots}$ (mg kg^{-1})	TF	Pearson correlations coefficient [1]
Lettuce	T_5	L_0	14.3±0.95 [a 2]	3.86±1.29 [A 3]	/	0.630 **
		L_{10}	669.31±154.8 [ab]	154.16±34.19 [BC]	0.23	
		L_{250}	2731.67±510.7 [cd]	338.32±43.02 [DE]	0.13	
	T_{10}	L_0	14.91±4.61 [a]	8.84±3.96 [A]	/	
		L_{10}	1065.99±595.65 [ab]	96.88±60.86 [AB]	0.11	
		L_{250}	3314.75±1328.01 [d]	459.42±80.41 [E]	0.16	
	T_{15}	L_0	15.57±6.26 [a]	8.79±4.4 [A]	/	
		L_{10}	2611.92±563.7 [cd]	114.56±16.43 [ABC]	0.04	
		L_{250}	6165.61 ±2025.27 [e]	238.18±76.04 [CD]	0.04	
Cabbage	T_5	C_0	6.79±2.43 [a]	12.35±1.78 [A]	/	0.793 **
		C_{10}	396.19±140.4 [ab]	116.56±11.72 [ABC]	0.33	
		C_{250}	2752.28±641.6 [cd]	358.91±84.87 [DE]	0.14	
	T_{10}	C_0	6.32±2.37 [a]	8.26±0.86 [A]	/	
		C_{10}	848.61±215.32 [ab]	172.34±107.9 [BC]	0.20	
		C_{250}	2826.73±1444.64 [cd]	386.02±241.7 [E]	0.17	
	T_{15}	C_0	7.01±2.16 [a]	13.78±3.34 [A]	/	
		C_{10}	1386.47±623.65 [b]	68.56±27.66 [AB]	0.06	
		C_{250}	8292.27±1641.89 [e]	603.68±96.46 [F]	0.08	

Notes:
T_5, T_{10} and T_{15} correspond respectively to 5, 10 and 15 days of exposure.
L_0, L_{10} and L_{250} (and C_0, C_{10} and C_{250}) correspond to respectively 0, 10 and 250 mg of CuO desposited on lettuce (cabbage) leaves.
[1]Pearson correlations coefficient and significant level ($p<0.01$ **) of metal concentration in leaves and roots.
[2]The different lowercase letters are the significant difference of $[Cu]_{leaves}$ at $p<0.05$.
[3] The different capital letters are the significant difference of $[Cu]_{roots}$ at $p<0.05$.
/ Not comparable.

*Table 3 Partial correlation coefficients between growth parameters and exposure conditions. The significance level was provided with p<0.05 *, p<0.01 **.*

	Exposure Quantity		Exposure Duration		Vegetable Species
	Lettuce	Cabbage	Lettuce	Cabbage	
$[Cu]_{plant}$	0.831 **	0.825 **	0.525 **	0.526 **	0.010
Cu Storage	0.356 *	0.549 **	0.493 **	0.463 **	0.092
Cu Transfer kinetic	0.453 **	0.745 **	0.562 **	0.613 **	0.091
$[Cu]_{leaves}$	0.814 **	0.810 **	0.540 **	0.526 **	-0.003
$[Cu]_{roots}$	0.829 **	0.852 **	-0.199	0.234	0.162
Shoot growth	-0.116	-0.209	0.540 **	0.317	0.314 **
Plant DW	-0.571 **	-0.291	0.611 **	0.491 **	0.296 *
Pn	-0.557 **	-0.482 **	0.187	-0.591 **	-0.180
gs	-0.567 **	-0.793 **	0.318	-0.735 **	0.137
Water % in leaves	0.128	-0.308	-0.330	-0.659 **	-0.378 **
TF	-0.346 *	-0.503**	0.083	-0.035	0.359 **

Table 4 Estimated daily intake of pollutant (EDI, µg kg^{-1} day^{-1}) and the maximum allowable daily intake of contaminated plant (MDI, g day^{-1}) without exceed the tolerable daily intake (TDI of Cu, 40 µg kg^{-1} day^{-1}).

Vegetable	Exposure Duation	Exposure quantity	EDI (µg kg^{-1} day^{-1})	MDI (g day^{-1})
Lettuce	T_5	L_0	3.1±0.21	168.33±11.15
		L_{10}	145.02±33.55	3.73±0.82
		L_{250}	591.86±110.65	0.91±0.19
	T_{10}	L_0	3.23±1	173.7±55.57
		L_{10}	230.96±129.06	2.67±1
		L_{250}	718.2±287.74	0.82±0.31
	T_{15}	L_0	3.37±1.36	175.29±74.36
		L_{10}	565.92±122.14	0.95±0.18
		L_{250}	1335.88±438.81	0.43±0.17
Cabbage	T_5	C_0	1.47±0.53	395.86±161
		C_{10}	85.84±30.43	6.69±2.49
		C_{250}	596.33±139.02	0.91±0.25
	T_{10}	C_0	1.37±0.51	426.02±171.31
		C_{10}	183.87±46.65	2.97±0.75
		C_{250}	612.46±313.01	0.99±0.39
	T_{15}	C_0	1.52±0.47	373.55±136.45
		C_{10}	300.4±135.12	2.12±1.2
		C_{250}	1796.66±355.74	0.3±0.07

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Chapter 4 Bioaccessibility of process PM enriched with the various metal(loid)s observed in the atmosphere of urban areas - Health risk assessment

Forewords

In the previous chapter, our study concerns the vegetable quantity when exposed with fine and ultrafine particles (including nanoparticles) in foliar level. Foliar lead and copper uptake were demonstrated for vegetables exposed to PbO and nano-CuO PM. For humans health, food consumption had been identified as the major pathway of human exposure to heavy metals, and consuming foodstuff threatens the health of the population (Zheng et al., 2007). The concepts of bioaccessibility are fundamentally important for quantifying the risks that are associated with oral exposure to environmental contaminants (Guerin et al., 2015; Wragg et al., 2011). Therefore, in this chapter we exposed vegetable with atmosphere PM or directly in site, so as to study the bioaccessibility in different conditions (different matrixes, metal species and exposure conditions) and corresponding human health.

Our main objective here (Chapter 4.1) was to highlight the health risk associated with the consumption of vegetables exposed to foliar deposits of PM enriched with the various metal(loid)s frequently observed in the atmosphere of urban areas (Cd, Sb, Zn and Pb). Leaves of mature cabbage and spinach were exposed to manufactured mono-metallic oxide particles (CdO, Sb_2O_3 and ZnO) or to complex process PM mainly enriched with lead. Total and bioaccessible metal(loid) concentrations were then measured for **polluted vegetables** and **the various PM used as sources**. Finally, scanning electronic microscopy coupled with energy dispersive X-ray microanalysis (SEM-EDX) was used to study PM-phyllosphere interactions. The study examined whether foliar uptake varied **depending on the nature of the metal(loid)** and **determined the amount** of pollutants that could be ingested by humans through vegetable consumption.

Taken together, these studies confirm the importance of taking atmospheric PM and bioaccessible metal concentration into account when assessing the health risks associated with ingestion of vegetables grown in urban vegetable crops or kitchen gardens.

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4.1 Foliar uptake and metal(loid) bioaccessibility in vegetables exposed to particulate matter

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Abstract

At the global scale, high concentrations of particulate matter (PM) enriched with metal(loid)s are currently observed in the atmosphere of urban areas. Foliar lead uptake was demonstrated for vegetables exposed to airborne PM. Our main objective here was to highlight the health risk associated with the consumption of vegetables exposed to foliar deposits of PM enriched with the various metal(loid)s frequently observed in the atmosphere of urban areas (Cd, Sb, Zn and Pb). Leaves of mature cabbage and spinach were exposed to manufactured mono-metallic oxide particles (CdO, Sb_2O_3 and ZnO) or to complex process PM mainly enriched with lead. Total and bioaccessible metal(loid) concentrations were then

measured for polluted vegetables and the various PM used as sources. Finally, scanning electronic microscopy coupled with energy dispersive X-ray microanalysis (SEM-EDX) was used to study PM-phyllosphere interactions.

High quantities of Cd, Sb, Zn and Pb were taken up by the plant leaves. These levels depended on both the plant species and nature of the PM, highlighting the interest of acquiring data for different plants and sources of exposure in order to better identify and manage health risks. A maximum of 2% of the leaf surfaces were covered with the PM. However, particles appeared to be enriched in stomatal openings, with up to 12% of their area occupied. Metal(loid) bioaccessibility was significantly higher for vegetables compared to PM sources, certainly due to chemical speciation changes. Taken together, these results confirm the importance of taking atmospheric PM into account when assessing the health risks associated with ingestion of vegetables grown in urban vegetable crops or kitchen gardens.

Keywords : atmospheric particulate matter; foliar uptake; vegetables; metal(loid)s; ingestion bioaccessibility; microscopy.

4.1.1 Introduction

At the global scale, atmospheric pollution by particulate matter (PM) enriched with metal(loid)s is a health risk for populations living in industrial areas and megalopolises (Shi et al. 2012; Juda-Rezler et al. 2011; Zheng et al. 2010; Dmuchowski and Bytnerowicz 2009; Cao et al. 2009). In Europe, the quality standards for ambient air levels of several metal(loid)s were defined in 1999. Directive 1999/30/CE, more recently updated by Directives 2004/107/CE and 2008/50/CE, sets annual limit values at 6, 5, 20 and 500 ng m^{-3} for As, Cd, Ni and Pb, respectively, in the atmosphere. According to Gonzales et al. (2012), limit values for metal(loid)s in the atmosphere are mainly observed in industrial and (sub)urban areas. In France, for 2011, the average anthropogenic airborne PM_{10} emissions were estimated at 3 178 900 kg $year^{-1}$ with 236, 22305 and 419981 kg $year^{-1}$ of Cd, Pb and Zn, respectively (INERIS 2013). PM can be deposited on terrestrial ecosystems (Donisa et al. 2000; Ma et al. 2010; Foucault et al. 2013a; Lévêque et al. 2013) leading to contamination of the soil (Lin and Xing 2007; Lee et al. 2008; Stampoulis et al. 2009; Schreck et al. 2011; Pelfrêne et al. 2013; Foucault et al. 2013b) and plants (Uzu et al. 2010; De Temmerman et al. 2012).

Human exposure to airborne contaminants follows two main routes: (i) directly breathing the surrounding air and (ii) food consumption (Cape 2003; Gorna-Binkul and Buszewsky 1997). Many studies investigated air pollution and its impact on human health (Curtis et al. 2006), due to, in particular, infiltration of PM into homes and exposure through hand to mouth contact. However, food represents the main source of human exposure to environmental pollutants: more than 90 % of organic pollutants are absorbed through food, compared to inhalation and percutaneous routes (Fries 1995); this could be the cause of more than 30 % of cancers associated with chronic exposure (Mansour et al. 2009). International and national regulations on food quality have lowered the maximum permissible levels of toxic metals in food items due to increased awareness of the risk posed by these metals to food chain contamination (Radwan and Salama 2006). Vegetables and fruit are the major components of the diet of the world's population; their contamination could therefore have a strong impact on human health. Indeed, numerous studies reported that lead levels in plant tissues can exceed even several hundred times the European threshold: 0.3 mg Pb kg^{-1} fresh weight (Gonzalez-Miqueo et al. 2010). Jassir et al. (2005) reported high levels of metals in vegetables sold in the markets of Riyadh city in Saudi Arabia due to atmospheric deposits, as did Sharma et al. (2008 a and b) for the vegetables from the markets of Varanasi in India. Thus, determining how metal(loid)s carried by PM can be absorbed by vegetables, accumulate and eventually enter the human food chain is a key issue for environmental management and health impacts that remains to be studied.

According to Schreck et al. (2012 a and b), foliar uptake of metal(loid)s could be the main way edible parts become contaminated in polluted areas such as in the vicinity of industrial sites or urban agglomerations. Hu et al. (2011) showed that airborne Pb-rich particles contribute up to 70 % of *Aster subulatus* leaf pollution in a context of high atmospheric pollution (Nanjing, China). However, in contrast to root transfer (Hinsinger et al. 2000; Shahid et al. 2013), foliar metal(loid) uptake has been relatively poorly studied and most studies focused on lead (Hu et al. 2011; Schreck et al. 2012 a and b). Indeed, foliar transfer of inorganic pollutants was mainly studied in the case of radioactive elements, especially following the Chernobyl (1986) and Fukushima (2011) accidents. According to Hurtevent et al. (2013) and Choi et al. (2002), the interception of dry or wet radioactive deposits and further translocation to the edible parts of plants play a major role in food chain contamination. Hurtevent et al. (2013) explained that the behavior of metals during foliar transfer is similar to their analogs among essential elements. They finally concluded that a wide variety of processes can affect foliar transfer: pollutant interception by plants, fixation and penetration through the foliar cuticle, internalization by leaf cells and release into the phloem leading to transportation and distribution within the plant. Uzu et al. (2010) also highlighted the influence of the physico-chemical characteristics of the PM on their reactivity.

Gardening activities which are flourishing all over the world, due to economic conditions and/or their perceived health benefits may sometimes promote human metal(loid)s exposure through ingestion, if the vegetables are grown in polluted urban areas (Hu et al. 2012; Szolnoki et al. 2013; Pandey et al. 2009 and 2012). Thus, to assess the human risk associated with the consumption of vegetables impacted by PM, metal(loid) bioaccessibility needs to be measured (Suruchi and Pankaj Khanna 2011).

The aim of the present study was to investigate the interaction between PM enriched with different metal(loid)s (Cd, Sb, Zn and Pb) and leaves of vegetables commonly cultivated in kitchen gardens (cabbage and spinach). We examined whether foliar uptake varied depending on the nature of the metal(loid) and determined the amount of pollutants that could be ingested by humans through vegetable consumption.

4.1.2 Materials and Methods

4.1.2.1 Characterization of the PM used in the study

Mono-metallic and high purity (99 % minimum) PM oxides (CdO, Sb_2O_3 and ZnO) were purchased from Sigma-Aldrich®. Their physico-chemical characteristics are shown in Table 1. Their mean diameters varied between 0.1 (ultrafine particles) and 1 µm (fine particles) and their specific areas ranged from 4.3 and 25 $m^2\ g^{-1}$. The water solubility of these PM oxides is low due to the particulate status of the metal(loid)s which leads to a very low ionic (dissolved) fraction. However the ZnO particles were much more soluble than the others.

Process PM highly enriched with lead was collected from the furnace of a secondary lead smelter that recycles car batteries, located in the urban area of Toulouse, south-western France (43°38'12'' N, 01°25'34'' E). According to Uzu et al. (2009; 2011 a and b), this process PM contains particles with the following diameter distributions: 9%, 50 %, 20 % and 21 % in the $PM_{>10}$, PM_{10}, $PM_{2.5}$ and PM_1 fractions, respectively. Lead is the major element at 33 % of the total metal content and its speciation was found mainly to be PbS, $PbSO_4$, $PbO.PbSO_4$ and PbO. Some minor elements were also measured: Cd (0.2 %), Sb (0.55 %) and Zn (0.2 %).

Table - 1 Physico-chemical properties (PM size, specific areas, water solubility, purity and molecular weight) of mono-metallic particles: CdO, Sb_2O_3 and ZnO.

	CAS	PM size (µm)	Specific area (m² g^{-1})	Water solubility, 20°C (mg L^{-1})	Purity (%)	MW (g mol^{-1})
CdO	1306-19-0	< 1 (PM_1)	4.3	0.227	99.5%	128.41
Sb_2O_3	1309-64-4	< 0.25 (PM_1)	15.6	0.017	> 99.9%	291.52
ZnO	1314-13-2	< 0.1($PM_{0.1}$)	15-25	1,600	> 99%	81.39

4.1.2.2 Experimental set-up for foliar exposure to PM

In order to produce reliable results, the different steps of the experimental set-up were stringently pre-tested and are carefully described in the following section. Foliar metal(loid) uptake was compared for spinach (*Spinacia oleracea*) and cabbage (*Brassica oleracea*) in controlled experiments. These two short life-cycle leafy vegetables, with large and smooth leaf surfaces are often cultivated in kitchen gardens and consumed all over the world (Sæbø et al. 2012; Pandey et al. 2012). After germination, the plants were grown for two weeks in a phytotron in control unpolluted soil: light, temperature and humidity were controlled throughout the experiment as reported by Schreck et al. (2012b). A geotextile membrane was placed on top of the soil to protect it from PM fallout and thus to avoid soil contamination and metal transfer via root uptake, as described by Uzu et al. (2010), Schreck et al. (2012 a, 2012b) and Hurtevent et al. (2013).

Cd, Sb and Zn were chosen because of their frequent occurrence in the atmosphere of urban areas (Gonzales et al. 2012); lead was used only as a control element as it was previously studied by Uzu et al. (2010). Leaf surfaces were therefore exposed to mono-metallic commercial species as oxides (CdO, Sb_2O_3 and ZnO) or process PM mainly enriched with lead, as previously studied by Schreck et al. (2012 a). Vegetable leaves were exposed to a total quantity of 300 mg of PM, to be comparable to field experiments conducted by Schreck et al. (2013) which measured PM fallouts of 325 µg cm^{-2} $week^{-1}$ in an industrial area. For three weeks, a fraction of the total PM was deposited onto the leaves of each studied plant, with five replicates per treatment: every week, 100 mg of PM were homogeneously deposited with an applicator brush onto the entire leaf surface which had been previously moistened with a hand sprayer. Note that the brush was only used to deposit the PM onto the leaf surfaces without spreading. The solution volume for a 1 m^2 plot was set at 100 mL which according to Hurtevent et al. (2013) corresponds to 0.1 mm of rainfall. This volume, at the lower end of the range of the water storage capacity of a wheat canopy (Kinnersley et al. 1997), was low enough to prevent runoff from foliage. The homogeneity of the solution distribution on the leaves and reproducibility of the technique were confirmed in previous tests. To avoid artifacts during our experiments, no surfactants were used (these are sometimes used to favor particle dispersion). In order to determine the precise quantity of pollutants deposited on the leaf surfaces, the applicator brush was rinsed with 20 mL distilled water after PM deposition. The rinse water was then centrifuged and after removing the supernatant, the particles were mineralized with aqua regia, and the metal(loid) concentrations measured. Thus, the final PM amounts really deposited on leaf surfaces were determined and are presented in Table 2. This procedure therefore allowed the exact amount of PM deposited on the leaves to be calculated so that the interaction between PM and leaves could be simulated in quite a realistic way. Indeed, we used this method because deposit of dry PM with an applicator brush without pre-wetting can mechanically bring the particles into the leaf and favor coarse particle uptake whereas fine particles are mainly present in a wet deposit. After exposure, plants were harvested and shoot biomass was measured.

Table - 2 Total metal(loid) (Cd, Sb, Zn and Pb) contents in plant shoots (mg kg^{-1} of Dry Weight) and calculated global enrichment factors (GEF) in relation to the total quantities of PM(mg) (CdO, Sb_2O_3, ZnO and process PM) deposited on the leaf surface .

PM type ► *studied pollutant*	Total PM deposit (mg)	*Metal(loid)s concentrations in plant shoots (mg kg^{-1} of DW)* and calculated GEF					
		Spinach			**Cabbage**		
		Controls	Exposed	**GEF**	Controls	Exposed	**GEF**
CdO► *Cd*	243.30 ± 6.50	0.80± 0.10	317.30± 10.40	**396.63**	0.30± 0.10	59.20± 4	**197.33**
Sb_2O_3► *Sb*	242.60 ± 4.90	5.50± 1.10	276.30± 26.40	**50.24**	2.50± 0.70	199.50 ± 7	**79.80**
ZnO► *Zn*	236.40 ± 8.2	25.50 ± 6	144.20± 16.30	**5.65**	15.80± 2	73.30± 6	**4.64**
Process PM► *Pb*	238.10 ± 9.70	6.10± 2	485.50± 14.80	**79.59**	3.50± 0.70	214.20± 5	**61.20**

4.1.2.3 Total metal(loid) content in plant leaves

Only aerial parts of the plants were studied to focus on the edible leaves and shoots and the involved health risks After harvest at maturity, the vegetable shoots were washed twice (Birbaum et al. 2010; Uzu et al. 2010) in order to reproduce the scenario of consumption by humans. For PM desorption all the leaves were washed, first in running tap water for 30 s and then in two baths of deionized water for 1 min. The leaves were then oven-dried at 40 °C for 72 h, weighed and ground and sieved to < 250 µm particle size.

The plant samples were mineralized in *aqua regia* (mixture of ¼ HNO_3 and ¾ HCl) at 80 °C for 4 h (0.125 g per sample) with a Digiprep® instrument from SCP Science producer, which is a block digestion system allowing fast and uniform plant sample digestion. The Cd, Sb, Pb and Zn concentrations were then measured by inductively coupled plasma-optical emission spectrometry ICP-OES (IRIS Intrepid II XXDL) as described in Schreck et al. (2012 a and b). Five replicates were performed for each sample and the control consisted of blank samples subjected to the same treatment (mineralization and assay). The detection limits for Cd, Sb, Zn and Pb were 0.2, 0.2, 2.2 and 0.3 µg L^{-1} respectively, whereas the limits of quantification were 0.4, 0.4, 3 and 0.4 µg L^{-1} respectively (Schreck et al. 2012 a and b).The accuracy of the acid digestion and the analytical procedures was verified using reference materials: Virginia tobacco leaves, CTA-VTL-2, ICHTJ and TM-26.3 certified reference material (CRM) from the National Water Research Institute, Canada. The concentrations in Cd, Sb, Zn and Pb measured in the CRM were within 98-101 % of the certified values.

The global enrichment factor (GEF) was calculated as the ratio between the pollutant concentrations (mg kg^{-1} DW) in leaves of exposed plants compared to the controls (Ngole 2011; Chopra and Pathak 2013).

4.1.2.4 *In vitro* bioaccessibility of metals and metalloids after plant ingestion

Gastric bioaccessibility (GB) was measured for PM and on exposed vegetables, using the adapted Barge Unified protocol (Cave et al. 2006; Uzu et al. 2011; Foucault et al. 2013b). This test consists in a three step extraction procedure to simulate the chemical processes occurring in the mouth, stomach and intestine compartments using synthetic digestive solutions. The temperature was maintained at 37 °C throughout the extraction procedure. The composition of the solutions and the extraction test procedure were described by Wragg et al. (2011). Dried and sieved particles or vegetable samples (0.6 g) were mixed with 9 ml of saliva (pH 6.5) and shaken for 5 min. Then 13.5 mL of gastric solution (pH 1.0) was added to the suspension. The pH of the solution was reduced to 1.2 using HCl if necessary. The suspension was mixed using an end-over-end rotation agitator at 37 °C for 1 h. The pH of the suspension was checked to be in the range of 1.2–1.6. The stomach phase was extracted by centrifuging the suspension at 3000 g for 5 min. Metal(loid) concentrations in the gastric phase solution were measured by ICP-OES. These gastric bioaccessibility tests were also carried out on reference material obtained from foliar uptake experiments on lettuce exposed to atmosphere particles as described by Uzu et al. (2011). The total lead and cadmium concentrations (mg. kg^{-1}, DW) measured in the lettuce leaves were 138±5 and 1.8±0.05, respectively. The percentage of bioaccessible lead and cadmium compared with the total metal concentrations measured in the edible plant parts were 40±3% and 69±3%, respectively. Values given are the means of ten replicates (± SD).

Bioaccessibility results were expressed as the percentage of the initial total metal(loid) content in PM or vegetables dissolved during the bioaccessibility assay. For each sample, tests were carried out on five replicates.

4.1.2.5 Scanning Electronic Microscopy coupled with Energy Dispersive X-ray microanalysis (SEM-EDX)

Environmental SEM-EDX measurements using a Quanta 200 FEI instrument equipped with a Quantax EDX detector were carried out to investigate the morphology of the leaf surfaces, elemental distribution in metal-enriched areas and to study particle-phyllosphere interactions. Leaves were dried and fixed on a carbon substrate without any further preparation before analysis (Schreck et al. 2012a). The apparatus was operated in low-vacuum mode (~133 Pa) at 20 kV. The leaf coverage rate by particles was estimated by image analysis of back scattering emission images (BSE). ImageJ software was used for image analysis to estimate the surface occupied by the particles and thus, the distribution of the particles on the leaf surfaces. Fixed parameters were kept similar for image analysis of all BSE images. Binary image creation (black and white image) from BSE images gave the total number of particles on the defined area as well as the corresponding surface area of the particles. The recovering rate was estimated by calculating the ratio between the total area covered by particles and the total surface of the field defined in the image, i.e the total leaf surface. In order to be representative, five images of about 230×210 µm² by leaf were systematically recorded, giving a total surface of 48.3 mm² for each leaf. Finally, the coverage rates of stomata by particles were also estimated using a similar procedure. The coverage rate was calculated on a selected area of the BSE image which only included stomata.

4.1.2.6 Statistical analyses

Total and bioaccessible metal(loid) concentrations were subjected to statistical analysis. An analysis of variance (ANOVA), using the software Statistica, Edition ’98 (StatSoft Inc., Tulsa, OK) was performed on the measured values. Results were expressed as mean ± SD (standard deviation). Data were tested for normal distribution and homogeneity of variance before the ANOVA. Statistical analyses were carried out on means of five replicates for each exposure condition. Significant differences (p-value < 0.05) were determined using the LSD Fisher test.

4.1.3 Results and Discussion

4.1.3.1 Total metal(loid) concentrations measured in plant shoots after PM exposure and washing

Total metal(loid) (Cd, Sb, Zn and Pb) concentrations in plant shoots after PM exposure (CdO, Sb_2O_3, ZnO and process PM) and washing are shown in Table 2. In the table, deposits are expressed as total PM (mg): 243.30, 242.60, 236.40 and 238.10 for CdO, Sb_2O_3, ZnO and process PM respectively. These total PM quantities correspond to the following quantities of metal(loid)s: 213 mgCd, 202.2 mgSb, 189.9 mgZn and 101.3 mgPb. Even after thorough washing, metal(loid) concentrations were five to 400 times higher ($p < 0.05$) in exposed plant leaves than in control samples. When the vegetables were exposed to reference mono-metal PM enriched with Cd, Sb or Zn, very high quantities of these metal(loid)s were taken up via foliar transfer, without biomass loss or symptoms of macroscopic phytotoxicity which could have been a visual sign to the consumers that the vegetables were possibly contaminated. Indeed, no significant biomass changes were observed between the different treatments: the average dry biomasses of shoots (for the 5 replicates) were 4.5 ± 0.2 g and 1.5 ± 0.1 g respectively for cabbage and spinach. Previous field studies on lettuce exposed to process PM showed that the washing procedure removes a maximum of 25 % of total lead-rich particles (Schreck et al. 2012 a, 2012 b). This might suggest that PM become embedded in the cuticular wax of lettuce as demonstrated previously (Schreck et al., 2012). According to De Temmerman et al. (2012), in celeriac leaves exposed to PM roughly 25 % of Cd which accumulated in dust deposits could be removed by thoroughly washing the leaves. They concluded that metal(loid)s which had accumulated inside the leaves of celeriac and carrot may have been transferred from roots. Atmospheric lead deposits) are the most important source of contamination on the aerial parts of leafy vegetables (Voutsa et al. 1996) and this is also the dominating pathway for As (Larsen et al. 1992).

The highest metal concentrations in the leaves were for lead (485.5 and 214.2 mg kg^{-1} of DW in spinach and cabbage, respectively). These values obtained for controlled experiments are in agreement with those of Uzu et al. (2010) for lettuce exposed for six weeks in the courtyard of a battery recycling facility: the average Pb concentration in washed leaves after exposure for 43 days to atmospheric fallout was 335±50 mg kg^{-1} DW. Cadmium concentrations were the most different between spinach and cabbage: the Cd concentration in spinach was 5.4 times higher than in cabbage (317 and 59.2 mg kg^{-1} of DW respectively). Zinc accumulated the least in the vegetables: 144.2 and 73.3 mg kg^{-1} of DW in spinach and cabbage, respectively. The Zn concentration in the exposed vegetables was only five to six times higher than in control samples. This result can be attributed to: (i) the essential nature of this element and its presence in higher quantities than other pollutants in the control plants and (ii) the physico-chemical properties of the ZnO powder. Indeed, according to data from table-1, compared to the other particles, ZnO are the finest and most water-soluble: washing certainly removed these PM more efficiently from leave surfaces. We also observed that quite high levels of the emerging pollutant antimony also accumulated in the vegetable leaves: 276 and 199 mg kg^{-1} of DW for spinach and cabbage, respectively. This is an interesting finding especially because very few data are available concerning its impact on human health. Several other studies previously highlighted the strong influence of the nature of the metal present in the PM on its foliar uptake (Schreck et al. 2013; De Temmerman et al. 2012). Thus, overall our results confirmed the foliar uptake of various metal(loid)s as previously shown by Schreck et al. (2012a) in a field study of the same process PM. These authors reported concentrations of 122, 29, 1.7 and 1.4 mg kg^{-1} of DW for Pb, Zn, Cd and Sb, respectively, for lettuce exposed to industrial fallout of Pb-enriched process PM,(see 2.1 section: Pb: 33 % of the total metal content; Cd: 0.2 %; Sb: 0.55 %; Zn: 0.2 %).

In addition to the total lead concentrations, in the present study we also measured total Cd, Sb and Zn concentrations in plants exposed to process PM, in controlled conditions. Metal(loid)s concentrations were: (i) for spinach: 4, 20 and 53 mg kg^{-1} of DW for Cd, Sb and Zn, respectively and (ii) for cabbage: 2, 18 and 28 mg kg^{-1} of DW for Cd, Sb and Zn, respectively. Compared to the total concentrations measured in the plants exposed to the mono-metallic and high purity commercial PM (CdO, Sb_2O_3 and ZnO), (see table-2), these concentrations are comparatively higher in terms of ratios between the quantities of metal(loid)s initially in PM sources and those taken up by the vegetables. This phenomenon is certainly due to a saturation phenomenon such as that described for cadmium by Lovy et al. (2013).

Finally, regardless of the pollutant studied, total metal(loid) concentrations measured were 1.4 to 5.4 times higher in spinach than in cabbage leaves, suggesting that the capacity for metal absorption and accumulation differs in these two vegetables. The use of calculated global enrichment factors (GEF; taking into account the control conditions and vegetable biomasses) to compare the foliar uptake of the different metal(loid)s, confirmed that spinach effectively accumulates higher quantities of Cd, Zn and Pb than cabbage. However, the reverse effect was observed for Sb (GEF = 79.80 for cabbage > GEF = 50.24 for spinach). These phenomena highlight the likely effect of plant morphology and anatomy in the uptake mechanisms involved, as previously underlined by Schreck et al. (2012) for lead absorption. Indeed, plant architecture, leaf density, leaf inclination angle, as well as factors such as the morphological characteristics of the leaf lamina and leaf area affect PM retention (Qiu et al. 2009). The very high GEF values for Cd confirm its strong interaction with plants in the context of air pollution (Radwan and Salama 2006).

4.1.3.2 Microscopy observations of spinach and cabbage leaves

SEM-EDX analysis of PM-exposed cabbage and spinach leaves highlighted that particles were distributed all over all the leaf surfaces. For all the metal(loid)s, both plants, and regardless of the nature of the deposited PM, the coverage rate for particles on the leaf surfaces was estimated at approximately 2 %. The low percentage of particles found on the leaf surface is consistent with the washing procedure performed after harvesting. However, the coverage rate estimated for stomata alone varied from 4 to 12 % depending on particle type. For process PM, the stomata recovering rate was 4%. This value obtained in controlled experiments is comparable to those obtained for field experiments with lettuce (Uzu et al. 2010; Schreck et al. 2012 a; Schreck et al. 2013). For CdO, Sb_2O_3 and ZnO particles, the coverage rates of stomata are 7 %, 12 % and 1 %, respectively. Electronic images clearly showed that stomatal openings in both species were filled with particles (Figure-1). This observation is consistent with the differences found between the coverage rates of leaves and stomata. The particulate concentrations within stomatal apertures appeared to depend on particle composition and/or size, the highest values were observed for Sb_2O_3 (< 1µm) and to a lesser extent CdO (< 1 µm) whereas less ZnO (< 0.1µm) particles were found in the stomata. Actually, the presence of particles on opening stomata is directly correlated to the solubility of the metal oxides in water (at 20°C) reported in Table-1. The solubility varied from ZnO > CdO > Sb_2O_3 resulting in a lower proportion of ZnO and more Sb_2O_3 on stomata. Several reports (Kozlov et al. 2000; Eichert et al. 2008; Schreck et al. 2012 a) found that particles may penetrate inside the leaf through stomatal apertures. Thus, particle size as well as solubility are therefore parameters which appear to strongly control their physical transfer through stomatal apertures (Eichert 2008; Larue 2012). We also observed different morphologies in cabbage leaves, which may have affected particle uptake. In cabbage leaves, the cuticular wax appeared too thick for particles to induce any morphological changes at the leaf surface, even in the presence of a large quantity of particles. No necrotic areas were observed on cabbage leave surfaces whereas necrosis formation was frequently encountered for contaminated lettuce (Uzu et al., 2011). Also in contrast to observations of lettuce leaves, no chemical transformation of the metal-rich particles (regardless of the PM origin) was observed at the leaf surface. This suggests that for cabbage plants chemical processes may occur only inside the leaf tissues which likely include the influence of epicuticular wax on particle transfer (Schreck et al. 2013).

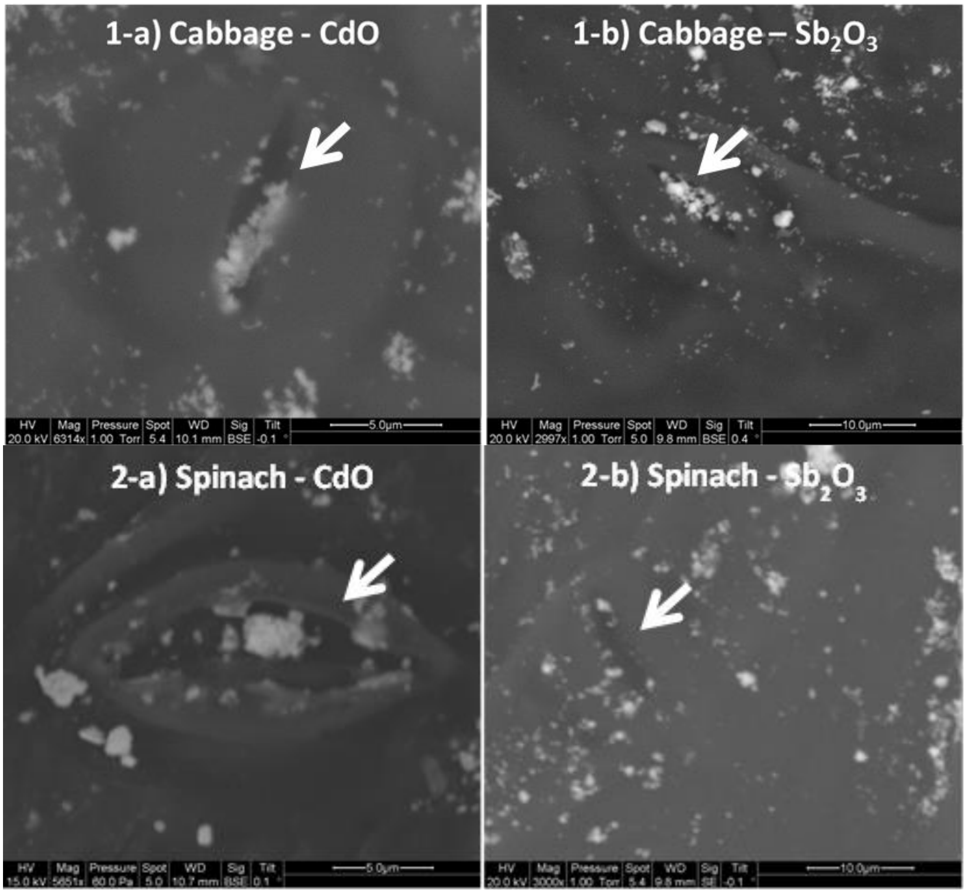

Figure - 1 SEM-EDX observations of cabbage (1) and spinach (2) leaves exposed to PM deposits of CdO (a) or Sb_2O_3 (b) (arrows mark the stomatal aperture).

Sæbø et al. (2012) suggested that leaf properties such as hair and waxes were important traits regarding PM accumulation. Nevertheless, complementary studies (Schreiber

2005; Birbaum et al. 2010; Schreck et al. 2012 a) have shown that other uptake pathways can occur such as diffusion across the cuticle, or internalization through the cuticle wax via various processes such as endocytosis. The cabbage cuticle is relatively thick, with various lipophilic waxes, conferring to the cabbage leaf its properties of water repletion (Levitt 1986). However, according to Zangi and Filella (2012), as yet little is known about the mechanisms involved in metal uptake, transport routes through the cell membrane and bioaccumulation in plant leaves. Phyllosphere organisms release inorganic and organic compounds possessing acidifying, chelating and/or reductive abilities, and may play an essential role in element mobilization and uptake at the leaf surface (Michaud et al. 2007; Nannipieri et al. 2008). Finally, changes to the particle and/or vegetable leaves induced by atmospheric gaseous pollutants (i.e. O_3, NO_2, SO_2…), which are commonly found in urban or industrial troposphere (Cicek and Koparal 2004; Gandois et al. 2010; Chaparro-Suarez et al. 2011; Terzaghi et al. 2013) could favor the foliar transfer of metal(loid)s-containing particles.

4.1.3.3 Metal(loid) bioaccessibility in plant shoots compared with process particles

The GB of metal(loid)s in spinach and cabbage exposed to PM are reported in Figure 2. The GB means ranged from 13.9 to 98 % for Cd ≈ Zn > Pb > Sb for spinach and Cd > Pb ≈ Zn > Sb for cabbage. In comparison, the lead bioaccessibility measured for lettuce exposed for one month in field experiments by Schreck et al. (2012b) was 45 %. The GB of Zn and Sb were significantly higher in spinach than in cabbage. Indeed, the bioaccessible fraction was larger in spinach than cabbage, with 39.8 and 13.9 % for Sb and 98 and 81.7 % for the Zn respectively. This suggests that leaf morphology not only plays a role in metal(loid) uptake and/or influences the solubility properties of the species enriched within particles but also, affects metal bioavailability in plant tissues once subjected to human digestion fluids. Speciation changes could also be involved as previously observed by Uzu et al. (2011).

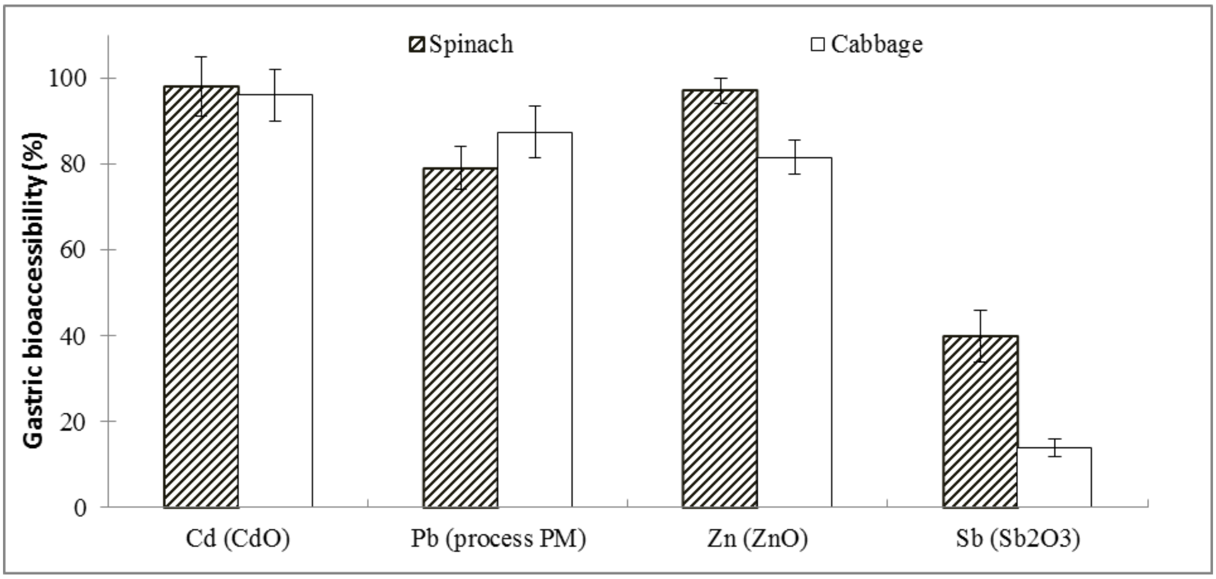

Figure - 2 Gastric bioaccessibility of metal(loid)s in spinach and cabbage leaves exposed to PM.

The GB of metal(loid)s followed the same trend in PM sources as in polluted vegetables, with Cd showing the highest values and Sb the lowest. The GB values for PM samples are shown in Table 3, the following sequence was observed: Cd (9.8 %) > Zn (8.9 %) > Pb (0.7 %) > Sb (0.2 %). These GB values concerning PM with high amounts of metal(loid)s are relatively low compared to those of metal(loid)s in soils (using the UBM protocol) reported in the literature. Indeed, compilation of previous soil GB analyses gives a range of 24-86 % for Cd, 12-60 % for Pb, 1.5-12 % for Sb and 6.9-68.5 % for Zn (Wragg et al. 2011; Barsby et al. 2012; Denys et al. 2009). The very high metal(loid) concentrations in process and commercial PM (ranging from 425,600 to 875,399 mg kg^{-1}) could saturate the gastric solution during the in vitro test (Appleton et al. 2013) as in the human digestion system. Nevertheless, due to the high metal(loid) contents in PM, very high bioaccessible pollutant quantities were observed. Several simultaneous phenomena could explain the higher bioaccessibility of metal(loid)s in the plant matrix than in PM sources: (i) speciation changes of the metal(loid)s after interaction with the leaves; (ii) preferential uptake of the smallest fraction and consequently the most bioactive particles during the PM-leaf interaction; (iii) the influence of saturation phenomena on the bioaccessibility.

Table - 3 Total concentrations and bioaccesibility of metal(loid)s in the different PM.

Studied PM	Total element concentration in PM (mg kg^{-1})	Element concentration in bioaccessible fraction (mg kg^{-1})	Gastric bioaccessibility (%)
CdO	875399	85856	9.8
Sb_2O_3	833562	1447	0.2
ZnO	803415	71120	8.9
Process PM enriched with Pb	425600	2965	0.7

In order to assess human exposure following ingestion of vegetables contaminated due to foliar transfer, daily intake (DI, µg d^{-1}) can be estimated from the measured vegetable pollutant concentrations (µg kg^{-1}) and daily vegetable consumption rates (kg d^{-1}). Daily vegetable consumption is generally obtained from field studies such as those carried out by Sharma et al. (2009). They observed foliar metal pollution of vegetables in and around an Indian city and studied the associated risk of exposure to the metals. Formal interviews conducted in the urban areas of Varanasi showed that the average daily consumption of fresh vegetables per person (body weight of an average adult: 60 kg) was around 77 g of FW or 13 g of DW. The following equation (Eq. 1) is generally used to calculate the daily intake of pollutant (Cui et al. 2004; Sharma et al. 2009; Swartjes 2011; Okorie et al. 2012):

Daily intake of pollutant (DI, µg d^{-1}) = (vegetable pollutant concentration × Daily vegetable consumption). (Eq. 1)

The determined DI values are then compared to tolerable daily intake (TDI, µg kg^{-1} d^{-1}) expressed as the quantity of pollutant ingested each day (µg) as a function of kg body weight: 0.15, 1, 0.4 and 300 µg kg^{-1} d^{-1} for Pb, Cd, Sb and Zn, respectively (US EPA 2007; Winter-Sorkina et al. 2003; Baars et al. 2001; WHO, 2008; Nathanail et al. 2009). TDI values are quite low for Cd, Pb and Sb, considered as harmful elements in comparison with that of Zn, an essential element. In our study, the maximum measured concentrations due to foliar transfer in the case of cabbage were: 59, 199, 73 and 214 mg kg^{-1} of DW for Cd, Sb, Zn and Pb respectively. The maximum daily quantities of vegetables consumed to reach the TDI can therefore be calculated with Eq. 2:

Daily vegetable consumption (kg plant per kg body weight and per day)

= TDI (µg kg^{-1} d^{-1}) / vegetable pollutant concentration (µg kg^{-1}) (Eq. 2)

The maximum daily quantity of vegetables exposed to PM that can be consumed without exceeding the TDI were therefore calculated using the concentration values measured for the exposed cabbage and 60 kg for the body weight of an average adult. These values are presented in table 4. As the mean dry biomass of shoots was 4.5 ± 0.2 g for one cabbage, the TDI can be reached easily for Cd, Sb and Pb, in contrast to Zn. Furthermore, Sharma et al. (2009) concluded that the average daily consumption of vegetables per person (body weight of an average adult: 60 kg) was around 13 g of DW (77 g of FW). Due to the high GB of Cd, Pb and Zn in cabbage, there is only a negligible decrease in the DI if only the bioaccessible fraction is taken into consideration. The same trend, however, was not observed in the case of Sb (13.9 % for cabbage and 39.8 % for spinach). Finally, as concluded by Sharma et al. (2009), the foliar transfer of pollutants can therefore lead to significant health risks when high amounts of PM are measured in the atmosphere. In the present study, we have demonstrated high amounts of pollutants can accumulate in the leaves of vegetables.

Table - 4 Calculated maximum daily quantities of plants exposed to PM (g d^{-1}, dry weight) that can be consumed without exceeding the TDI.

Cabbage	Spinach	Pollutant
1.01	0.19	Cd
0.12	0.09	Sb
245.57	124.83	Zn
0.04	0.02	Pb

4.1.4 Conclusions and perspectives

This study had shown that when exposed to PM deposits, the edible leaves of vegetables such as spinach and cabbage, not only take up Pb, but also other metal(loid)s such as Sb, Cd and Zn . Thus, there could be a human health risk if vegetables that are grown in industrial or urban areas exposed to high atmospheric quantities of PM enriched with metal(loid)s are consumed. Indeed, even after thorough washing, the vegetable leaves were still significantly contaminated by foliar uptake of particles; this is highly influenced by the plant morphology and physiology (cuticular waxes, stomata numbers…) and the physico-chemical properties of the metal(loid) species and particles (size, composition, solubility). Stomata openings are particularly enriched with pollutants, suggesting that this is a preferential uptake pathway. The gastric bioaccessibility of metal(loid)s was significantly higher in vegetables than in PM sources and depends on the nature of the pollutant with Cd being particularly bioavailable.

Further studies are in progress to better assess the health risks linked to ingestion of polluted vegetables via foliar and/or root transfer of different PM using, for example, various sizes or mixtures of pollutants. Various techniques are also currently being used to investigate the mechanisms involved in the interactions between pollutants and the leaf surface, i.e. the ratio between adsorption and absorption, localization in the plant and the speciation changes. Our results highlighted the importance of plant species and cultivar, and thus, opened up new avenues of research to reduce the foliar transfer of pollutants.

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4.2 Lead and Cadmium Phytoavailability and Human Bioaccessibility for Vegetables Exposed to Soil or Atmospheric Pollution by Process Ultrafine Particles (Influnce of exposure pathways on Bioaccessibilty)

Forewards

The previous study measured bioaccessibllity for polluted vegetables (foliar exposure) and the various PM used as sources. We found the gastro-bioaccessibility of metal(loid)s was significantly higher in vegetables than in PM sources and depends on the nature of the pollutant with Cd being particularly bioavailable. Stomata openings are particularly enriched with pollutants, suggesting that this is a preferential uptake pathway. We conclude that there could be a human health risk if vegetables that are grown in industrial or urban areas exposed to high atmospheric quantities of PM enriched with metal(loid)s are consumed.

In this study near a recycling factory (STCM), we compared bioaccessibility of vegetable exposed with metal respectively by foliar and root pathway. Actually, when plants are exposed to airborne particles, their consumed parts can accumulate metals both through root or foliar transfers. However, there is a lack of knowledge on the influence of plant exposure conditions on human bioaccessibility of metals, while urban gardening activities are actually increasingly developed. Moreover, Pb was the main metal found in the recycling factory emissions (33.4%), and the other metal contents were Cd, Sb, As, Cu, and Zn at 2.7,1.8, 0.09, 0.09, and 0.7%, respectively (Uzu et al., 2011). Pb and Cd are two main heavy metals regulated by European commission for contaminants in foodstuffs (EC regulation, 2001). Therefore, the objective of this study is to compare Pb and Cd human bioaccessibility under foliar and root exposure of vegetables to ultrafine particles. Lettuces, radishes and parsley were therefore exposed to metals-rich ultrafine particles from, either via field atmospheric fallouts or polluted soil. To our knowledge, the human bioaccessibility of metals in vegetables was performed for the first time with the comparison between foliar and root plant exposure.

This report highlight the conditions of plant exposure must be taken into account for environmental and sanitary risks assessment, a significant translocation of Pb isotope from leaves towards the roots were also observed in the case of foliar exposure.

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Lead and Cadmium Phytoavailability and Human Bioaccessibility for Vegetables Exposed to Soil or Atmospheric Pollution by Process Ultrafine Particles

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Abstract

When plants are exposed to airborne particles, their consumed parts can accumulate metals both through root or foliar transfers. However, there is a lack of knowledge on the influence of plant exposure conditions on human bioaccessibility of metals, while urban gardening activities are actually increasingly developed. Lettuces, radishes and parsley were therefore exposed to metals-rich ultrafine particles from recycling factory, either via field atmospheric fallouts or polluted soil. Both total lead (Pb) and cadmium (Cd) concentrations in consumed parts of plants and human bioaccessibility, were then measured. Moreover Pb translocation phenomena throughout the plants were studied using Pb isotopic analysis. The Pb and Cd bioaccessibility measured for consumed parts of the different polluted plants was significantly higher for root exposure (70% for Pb and 89% for Cd in lettuce) in comparison

to foliar exposure (40% for Pb and 69% for Cd in lettuce). That difference in metal bioaccessibility could be linked to the metal compartmentalization and speciation changes in relation with exposure conditions. Metal nature strongly influences the measured bioaccessibility: Cd presents higher bioaccessibility in comparison to Pb. Moreover, in the case of foliar exposure, a significant translocation of Pb from leaves towards the roots was observed. To conclude, the type of pollutant and the way of exposure significantly influences the phytoavailability and human bioaccessibility of metals especially in relation with contrasted phenomena involved in rhizosphere and phyllosphere. The conditions of plant exposure must therefore be taken into account for environmental and sanitary risks assessment.

Keywords: lead: cadmium; bioaccessibility; way of metal exposure; ultrafine particles,

Abbreviation: General Enrichment Factor (GEF), Bioaccumulation Factor (BF), Translocation Factor (TF), Particulate Matter (PM).

4.2.1 Introduction

At the global scale, atmospheric pollution by particulate matters (with different origins, size distribution and chemical composition) is observed (Salma et al. 2005; Barima et al., 2014). The presence of highly reactive ultrafine particles in urban areas can induce environmental and sanitary risks (Knibbs and Morawska, 2012; Marris et al., 2012; Austruy et al., 2014). Moreover, inorganic elements such as Pb, Cd, As, Cr, Cu… which are mainly emitted from anthropogenic processes at high temperature such as metallurgy, refining, vehicles and exhaust pipe tend to accumulate preferentially in these ultrafine particles (Hieu and Lee, 2010).

Studies in urban areas in the immediate vicinity of industrial activities showed that the levels of airborne particles can be widely affected by emissions from factories (Jang et al., 2007; Fernandez--Camacho et al., 2012). To limit the unwanted emissions of airborne particles from factories, different trapping devices can be used. But, these treatment systems are not always effective especially for fine and ultrafine particles that can escape these sensing devices (Schaumann et al., 2004; Biswas and Wu, 2005) and ultimately pollute the environment (Waheed et al., 2011; Shi et al., 2012).

Particle matter (PM) enriched with Pb are emitted near Pb recycling workplaces. These PM having different sizes (PM_{10}, $PM_{2.5}$, PM_1 and $PM_{0.1}$) are reported to be a source of Pb exposure to workers via ingestion and inhalation. Among these PM, PM10 have adverse effects on the human and environmental health and are therefore the target species of World Heath Organization (WHO, 1987) and the European Union Framework Directive on ambient air quality assessment (European Commission, 1999). However, the micrometric and sub-micrometric PM fractions have very little role to ambient particle mass. Majority of the studies dealing with the characterization of metal-enriched PM in the ambient air provide data regarding quantitative measurements for PM10 fractions with very little on the sub-micronic fraction (Lazaridis et al., 2002). These ultrafine particles are very reactive owing to high specific area and can migrate over long distances in the troposphere (Barrie, 1992). Therefore these particles exhibit a greater effect on the biosphere than coarse particles (Fernandez Espinosa and Rossini Oliva, 2006).

Airborne ultrafine particles enriched with metals can therefore pollute the soils (Shahid et al., 2013a; Lévêque et al., 2013) or the shoots of the cultivated plants (Shahid et al., 2014a,b). Actually, numerous studies highlighted Pb and Cd concentrations in consumable plant parts higher than the European threshold of 0.3 mg Pb kg^{-1} fresh weight for leafy vegetables but 0.1 mg Pb kg^{-1} fresh weight for root crops. For Cd, the maximum levels are 0.2 and 0.1 mg kg^{-1} for leafy vegetables and root crops respectively (Honour et al., 2009; Morman and Plumlee, 2013).

Metals can accumulate in plants both through root (Pourrut et al., 2013; Shahid et al., 2013b) and/or foliar uptake (Schreck et al., 2012a; Yin et al., 2013). The foliar uptake of metals can often be neglected in comparison with the root transfer (Shahid et al., 2012a). But in the case of Pb, which is highly insoluble in soil, significant amount may be taken up by plants via foliar uptake that can affect the plant quality (Hu et al., 2011; De Temmerman et al., 2011). However, only few studies compared the influence of the two ways of metal uptake by plants (Schreck et al., 2013) and none of these studies reported metal bioaccessibility in the polluted vegetables. Actually, it is well known that the context of pollution can influence the metal transfer toward plants (Birbaum et al., 2010; Zangi and Filella, 2012). According to Xiong et al. (2013), in addition to the total metal quantities measured in vegetables, the determination of the bioaccessible fraction using the barge procedure (Foucault et al., 2013) could improve the sanitary risk assessment linked to the ingestion of polluted vegetables.

Lead and Cd phytoavailability and bioaccessibility were therefore studied for the two possible ways of metal uptake by plants using different vegetables species currently cultivated in urban kitchen gardens: *Lettuce, radish and parsley*. Moreover, in the case of foliar plant uptake, the possible Pb transfer from shoot to roots was also studied to evaluate the pollution of root vegetables. Therefore, the objective of this study is to compare Pb and Cd human bioaccessibility under foliar and root exposure of vegetables to ultrafine particles. To our knowledge, the human bioaccessibility of metals in vegetables was performed for the first time with the comparison between foliar and root plant exposure.

4.2.2 Materials and Methods

A schematic view of the experiment is presented by the Fig.1.

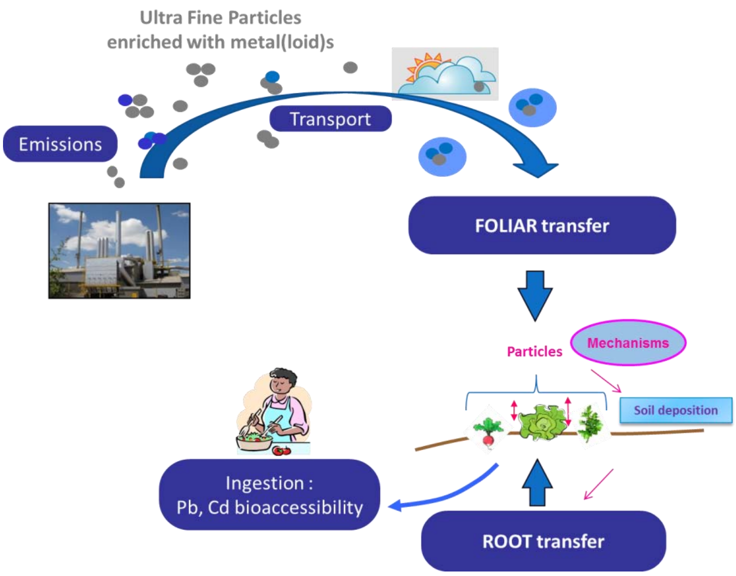

Fig. 1 Schematic view of root and foliar interactions between vegetables and ultrafine particles enriched with metals and measure of human Pb and Cd bioaccessibility.

4.2.2.1 Study of Pb and Cd Uptake by Vegetables under Foliar and Root Transfers

Separate foliar and root metal plant uptake experiments were performed using three different vegetable species: (i) lettuce *(Lactuca sativa)*, one of the most frequently self-grown and human consumable leafy vegetable (Harsha et al., 2013), (ii) radish (*Raphanus sativus*), a root vegetable (Goyeneche et al., 2013) and (iii) parsley (*Petroselinum crispum*), a herb used to give fragrance to different food products (Doğru and Erat 2012). In this study, pot experiment is preferred over field experiments due to the ability to control the experimental conditions and confounding variables.

For the foliar transfer experiments, forty five pots, containing one plant each and 4 kg of unpolluted soil, were placed for 6 weeks in the courtyard of a secondary Pb smelter that recycles car batteries and is located in South-West France, in the peri-urban area of Toulouse (43°38’12’’ N, 01°25’34’’ E). The annual emission of particulate matter (PM) from the smelter is 328 kg (Schreck et al. 2012b). These emitted PM have been previously characterized by Uzu et al. (2011a). The size distribution of PM is mainly comprised in the 1-100 μm size range (89% in volume fraction) with 9%, 50%, 20% and 21% in the PM>10, PM_{10}, $PM_{2.5}$ and PM_1 fractions, respectively. Lead is the major element in these atmospheric fallouts with 33% of the total metal content and the second major metal is Cd at 2.7%. Lead speciation in PM was found to be mainly PbS, $PbSO_4$, $PbO.PbSO_4$ and PbO (Uzu et al., 2011a).

During the vegetable exposure experiments, Pb concentration in industrial atmospheric fallouts was controlled in the smelter courtyard by collecting bulk depositions in Owen gauges (Taylor and Witherspoon, 1972), following the procedure described by Schreck et al. (2012b) and Gandois et al. (2010). Metal concentrations of Pb and Cd in atmospheric fallouts were respectively 450 ± 7 and 0.8 ± 0.1 mg $m^{-2}.month^{-1}$.

Here, the plants were only exposed to atmospheric fallouts by placing a geotextile membrane on the top of the unpolluted soil (25.5 ± 1.6 mgPb and 0.45 ± 0.05 mgCd kg^{-1} of dried soil) to avoid soil contamination and metal transfer via root uptake, as described by Uzu et al. (2010) and Schreck et al. (2012a, 2012b).

For the root transfer experiments, twenty-five plants of each species were individually cultivated for 6 weeks under an unpolluted atmosphere in 5 L pots containing each 4 kg of polluted soil (2000 mgPb and 1.2 mgCd kg^{-1} of dried soil) collected from the smelter's site. The soil was therefore contaminated by the same atmospheric PM fallouts as previously described for foliar metal transfer. The physico-chemical properties of the soil were: pH_{water} = 8.5, CEC = 6.9 $cmol^{(+)}$ kg^{-1}, amounts of soil organic matter and carbonates ($CaCO_3$) were respectively: 6 and 4 g kg^{-1}. Light, temperature, humidity and pluviometry were controlled during all the experiment as reported by Schreck et al. (2012b).

4.2.2.2 Plant Analysis

After six weeks of exposure, 10 plants of each species from the two experiments were harvested, and fresh biomasses were measured. A two-step washing method with deionized water was performed for shoots of plants (Birbaum et al., 2010; Uzu et al., 2010). First in running tap water for 30 s and then in two baths of deionized water for 1 min.

Total Pb and Cd concentrations in plant parts were then measured after acidic mineralization as described by Arshad et al. (2008). After oven-drying for 72 h at 40 °C, plant samples were mineralized with a Digiprep instrument from Science producer. 0.125 g of each plant sample was digested by 5 ml of aqua regia (mixture of 1/4 HNO_3 and 3/4 HCl) + 2 ml of H_2O_2 at 55 °C for 25 min and then at 80 °C for 4 h. After dilution in ultra-pure water and syringe filtration (0.45 mm), the Pb and Cd concentrations were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES: IRIS Intrepid II XXDL). Ten blanks were submitted to the same treatment (mineralization and assay) for control. Each sample was analyzed in triplicate. The accuracy of measurements was checked using reference materials: Virginia tobacco leaves, CTA-VTL-2, ICHTJ and TM-26.3 certified reference material from the National Water Research Institute, Canada. The concentrations found were within 97-101% of the certified values for all measured elements (Schreck et al., 2012a).

4.2.2.3 General Enrichment Factor (GEF), Bioaccumulation Factor (BF) and Translocation Factor (TF)

The general enrichment factor (GEF) has been calculated to determine the degree of metal accumulation in leaves or roots of plants grown on contaminated site (with soil or atmosphere transfer) with respect to uncontaminated sites (Ngole, 2011; Chopra and Pathak, 2013). Thus, GEF was calculated as a ratio of metal concentration in plant leaves grown on contaminated site and on control site. The GEF method calculation normalizes the measured metal on the contribution of natural resources of metal content. An internal normalization was applied with a known background element such as Mg element.

The bioaccumulation factor (BF) was determined as a ratio of metal concentration in consumable plant part and metal concentration in contaminated soil. Then, BF is an indicative factor of root transfer.

The translocation factor (TF) was determined as a ratio of metal concentrations in root/leaves in the case of foliar exposure and leaves/roots in the case of root exposure.

4.2.2.4 In Vitro Bioaccessibility of Pb after Plant Ingestion

Gastric bioaccessibility (GB) measurements were performed on plants after exposure period, using the adapted Barge Unified protocol (Cave et al., 2006; Foucault et al., 2013). The test consists of a three-step extraction procedure using synthetic digestive solutions to simulate the chemical processes occurring in the mouth, stomach and intestine compartments. Temperature was maintained at 37 °C throughout the extraction procedure. The composition of the solutions and the extraction test procedure were described by Wragg et al. (2011). Dried and sieved particles or vegetable samples (0.6 g) were mixed to 9 ml of saliva (pH 6.5) and shaken for 5 min. Then 13.5 mL of gastric solution (pH 1.0) was added to the suspension. The pH of the solution was reduced to 1.2 using HCl if necessary. The suspension was mixed using an end-over-end rotation agitator at 37 °C for 1 h. The pH of the suspension was checked to be in the range 1.2–1.6. The stomach phase was extracted by centrifuging at 3000 g of suspension for 5 min. Lead concentrations in the gastric phase solution were measured by ICP-OES. Bioaccessibility results are expressed as the percentage of the total metal in vegetables. For each sample, tests were carried out on five replicates.

4.2.2.5 Study of Pb Translocation from Leaves to Roots of Radish and Lettuce by Isotope Tracing

The Pb translocation phenomenon (from exposed leaves to roots) in the case of lettuce and radish leaves exposed to airborne PM was subsequently considered by the Pb isotope tracing. Isotopic signatures analysis ($^{206/207}$Pb, $^{208/206}$Pb and $^{208/207}$Pb) of Pb in PM particle sources, control and exposed plants (radish and lettuce) and soil (growing media) was performed with an ICP-MS quadripolaire (Elan 6000, Perkin-Elmer) apparatus.

4.2.2.6 Statistical Analysis

The data obtained were analyzed for differences between treatments using an analysis of variance (one-way ANOVA). Statistical analysis was carried out using the software Statistica, Edition'98 (StatSoft Inc., Tulsa, OK, USA). A Fisher's LSD test was used to determine the level of significance (p-value < 0.05) against the control.

4.2.3 Results

4.2.3.1 Characterization of PM

The studied process PM comes from channelled emissions of a battery recycling plant. They were size-segregated (micronic and sub-micronic particles) and investigated to correlate their speciation and morphology with their root transfer towards lettuce by Uzu et al. (2009). According to XRD and Raman spectroscopy results, the two fractions presented differences in the amount of minor lead compounds like carbonates, but their speciation was quite similar, in decreasing order of abundance: PbS, $PbSO_4$, $PbSO_4$.PbO, a-PbO and Pb^0. Morphology investigations revealed that $PM_{2.5}$ contained many Pb nanoballs and nanocrystals which could influence lead availability (Uzu et al., 2011b).

4.2.3.2 Total Metal Concentrations in Plant Leaves and Roots under Foliar or Root Exposures

For the two ways of transfer, high metal concentrations were measured in different plant parts of lettuce, radish and parsley as presented in Table 1. The maximum allowable concentrations of Pb and Cd in commercial vegetables are respectively 3mgPb.kg^{-1} DW and 2mgPb.kg^{-1} DW (Honour et al., 2009; Morman and Plumlee, 2013). The washing procedure only removed maximum 30% of total Pb in accordance to previous studies on lettuce exposed to similar conditions (Schreck et al., 2012a, 2012b). The metal concentrations measured in vegetables significantly differ in function of exposure type (foliar or root), metal nature (Pb or Cd), plant type (lettuce, radish or parsley), as well as plant part type (roots or leaves). For foliar exposure, metal concentrations were higher in leaves, whereas for root exposure metal contents were higher in roots of all plant species. The accumulation of Pb in plant consumable parts was higher than Cd for all plant species under both types of pollution exposure. The accumulation of Pb and Cd in leaves or roots did not follow a common trend for three plant species under two types of pollution exposure. Under both foliar and root exposure, parsley accumulated the highest levels of Pb in leaves. In case of roots, Pb contents were higher in radish under foliar exposure and in lettuce for root exposure. The highest level of Cd in leaves was accumulated in lettuce under both type of pollution exposure. Similarly in roots, Cd levels were the highest in radish under both root and foliar exposure.

Table 1 Total lead and cadmium concentrations (mg. kg^{-1}, DW) measured in leaves and roots of the vegetables in function of metal exposure type: foliar exposure (1-A) or root exposure (1-B). Values are given as a mean of ten replicates (± SD).

1-A: Foliar Exposure	**Lettuce**		**Radish**		**Parsley**	
	Control	Polluted	Control	Polluted	Control	Polluted
[Pb]Leaves	0.6 ± 0.07	138 ± 5	0.7 ± 0.08	176 ± 21	1.5 ± 0.5	310 ± 18
[Pb]Roots	0.8 ± 0.03	8 ± 0.5	0.9 ± 0.02	15 ± 0.9	N.D.	N.D.
[Cd]Leaves	0.1 ± 0.07	1.8 ± 0.05	0.2 ± 0.08	1.6 ± 0.08	0.4 ± 0.05	0.9 ± 0.05
[Cd]Roots	0.1 ± 0.07	0.5 ± 0.05	0.2 ± 0.07	0.8 ± 0.06	N.D.	N.D.

N.D. indicates Not Detected

1-B: Root exposure	**Lettuce**		**Radish**		**Parsley**	
	Control	Polluted	Control	Polluted	Control	Polluted
[Pb]Leaves	0.6 ± 0.07	41 ± 3.5	0.7 ± 0.08	36 ± 7	1.5 ± 0.5	193 ± 18
[Pb]Roots	0.8 ± 0.03	125 ± 11	0.9 ± 0.02	81 ± 0.9	N.D.	N.D.
[Cd]Leaves	0.1 ± 0.07	0.8 ± 0.03	0.2 ± 0.08	0.6 ± 0.07	0.4 ± 0.05	1 ± 0.05
[Cd]Roots	0.1 ± 0.07	2.3 ± 0.05	0.2 ± 0.07	2.8 ± 0.04	N.D.	N.D.

N.D. indicates Not Detected

4.2.3.3 General Enrichment (GEF), Bioaccumulation (BF) and Translocation (TF) Factors, and Bioaccessibility in Plant Leaves

Table 2 reports metals (Pb and Cd) enrichment and bioaccumulation factors for the three plant species for foliar or root exposures. The GEF and BF are higher for leaves in case of foliar application whereas under root metal transfer GEF and BF are high for roots. This shows that there exists very low transfer/movement of metals inside the plants and generally the metals tend to accumulate at the site of entrance (root or leaves). TF is higher under root exposure compared to foliar exposure. The TF is higher for Cd compared to Pb showing that Cd is more mobile than Pb inside the plants. Moreover, for the root exposure, BFs were low, and a higher Pb content (more than 2-fold) was also observed in parsley leaves compared to lettuce leaves.

Table 2 General enrichment (GEF), bioaccumulation (BF) and translocation (TF) factors in plants after 6 weeks of metal exposure: foliar exposure (2-A) or root exposure (2-B).

2-A: Foliar Exposure	Factors	Lettuce	Radish	Parsley
		Lead (Pb)		
Pb in leaves	GEF	230	251	207
Pb in roots	GEF	10	17	N.D.
Pb in leaves	BF	0.07	0.09	0.16
Pb in roots	BF	0.00	0.01	N.D.
TF-Pb (Leaves→Roots)	TF	0.06	0.09	N.D.
		Cadmium (Cd)		
Cd in leaves	GEF	18	8	2
Cd in roots	GEF	5	4	N.D.
Cd in leaves	BF	1.50	1.33	0.75
Cd in roots	BF	0.42	0.67	N.D.
TF-Cd (Leaves→Roots)	TF	0.28	0.50	N.D.

N.D. indicates Not Detected

2-B: Root exposure	Factors	Lettuce	Radish	Parsley
		Lead (Pb)		
Pb in leaves	GEF	68	51	129
Pb in roots	GEF	156	90	N.D.
Pb in leaves	BF	0.02	0.02	0.10
Pb in roots	BF	0.06	0.04	N.D.
TF-Pb (Leaves→Roots)	TF	0.33	0.44	N.D.
		Cadmium (Cd)		
Cd in leaves	GEF	8	3	3
Cd in roots	GEF	23	14	N.D.
Cd in leaves	BF	0.67	0.50	0.83
Cd in roots	BF	1.92	2.33	N.D.
TF-Cd (Leaves→Roots)	TF	0.35	0.21	N.D.

N.D. indicates Not Detected

Table 3 Percentages of bioaccessible lead and cadmium in comparison with the total metal concentrations measured for the edible plant parts for both foliar (3-A) and root (3-B) exposures.

3-A: Foliar Exposure	**Lettuce leaves**	**Radish roots**	**Parsley leaves**
Bioaccessibility for Pb (%)	40 ± 3	35 ± 2	28 ± 2
Bioaccessibility for Cd (%)	69 ± 3	58 ± 3	56 ± 3

3-B: Root exposure	**Lettuce leaves**	**Radish roots**	**Parsley leaves**
Bioaccessibility for Pb (%)	70 ± 4	61 ± 5	53 ± 3
Bioaccessibility for Cd (%)	89 ± 5	73 ± 3	65 ± 4

As observed in Table 3, gastric Pb and Cd bioaccessibility measured for polluted lettuce leaves was significantly higher for root exposure (lettuce: 70% ± 4.5) in comparison to foliar exposure (lettuce: 40% ± 3). Moreover, under both types of pollution exposure ways, gastric bioaccessibility is higher for Cd than Pb for all the three vegetable species. Differences were also observed in function of plant species: lettuce > radish > parsley.

4.2.3.4 Lead Translocation from Leaves to Roots with Measure of Isotope Signature

Lead isotope signatures data are regrouped in table 4. Translocation of anthropogenic Pb from the leaves to the roots of lettuces and radishes was observed. Indeed, the isotopic signature of Pb in plant roots of the plants exposed by foliar fallouts of particles is comparable to that of particles sources and significantly different to that of the unexposed control samples. These isotopic results are in agreement with those of total Pb concentrations measured in roots of radishes and lettuces after foliar transfer.

Table 4 Lead isotopic signatures ($^{206/207}Pb$, $^{208/206}Pb$ and $^{208/207}Pb$) for: (i) source particle; (ii) lettuces (leaves, central nervures and roots) and radishes (roots) after foliar exposure.

Lead isotopic signatures	$Pb^{206/207}$	$Pb^{208/206}$	$Pb^{208/207}$
Lettuce leaves			
Control	1.146	2.102	2.400
Exposed	1.144	2.120	2.423
Lettuce nervures			
Control	1.149	2.106	2.419
Exposed	1.143	2.118	2.422
Lettuce roots			
Control	1.147	2.109	2.421
Exposed	1.145	2.13	2.419
Radish root			
Control	1.148	2.108	2.421
Exposed	1.144	2.125	2.425
Source particle	**1.144**	**2.119**	**2.424**

Maximum SD ± 0.003

4.2.4 Discussions

4.2.4.1 Emission of PM from industry

Mbengue et al. (2014) determined the size distribution of potentially toxic trace metals in atmospheric particulate matter (PM) of urban–industrial area. They concluded that metals emitted by high-temperature processes are preferentially present in the fine (submicronic) and ultrafine fractions, whereas natural and mechanical processes release coarser metallic particles (Ny and Lee, 2011). Their results imply that industrial emissions might have high levels of metal enrichment in the submicron fraction (PM0.29–1). However, Choel et al. (2006) reported a fast evolution (within a few minutes) of metal-rich particles (Pb and Zn) during their transport from the pollution plume to the nearby residential area (at approximately 2 km), highlighting that the nano scale of PM can be effectively modified in the environment. Moreover, the particle and/or vegetable leaves changes induce by atmospheric gaseous pollutants, which are commonly found in urban or industrial troposphere (Terzaghi et al. 2013) could favor the foliar transfer of metal(loid)s-containing particles.

4.2.4.2 Influence of Exposure Conditions on the Metal Bioaccessibility

Lead and Cd bioaccessibility measured for the polluted consumed plant parts (leaves for lettuces and parsley and roots for radishes), was significantly higher for root exposure in comparison to foliar exposure. In the case of root transfer, a first step of Pb metal solubilization is needed to permit their root absorption from the soil solution. But, in the case of foliar transfer, a part of Pb can be integrated to lettuce cuticle by endocytosis (Schreck et al., 2012a) without chemical change. As the source particles present a low bioaccessibility (0.7% and 8% respectively for Pb and Cd); a first hypothesis to explain that difference in bioaccessibily could be a greater absorption of solid particles in the case of foliar transfer comparatively to the root transfer (absorption of soluble compounds).

The present study suggests that Pb speciation in plants depends on exposure conditions and plant species. Therefore, taking into account the compartmentalization and speciation of metallic contaminants in edible plants might considerably improve the accuracy of human health risk assessment.

Recently, Schreck et al. (2014) investigated the consequences on lead compartmentalization and speciation in plant leaves of the way of exposure (foliar or root). The pollution context was the same as for the present study: atmospheric smelter particles. Using a combination of microscopic and spectroscopic techniques (electron microscopy, laser ablation, Raman microspectroscopy and X-ray absorption spectroscopy), they showed that Pb localization and speciation are influenced by the way of exposure (root or shoot pathway) and the plant species. Moreover, they highlighted the PM changes potentially occurring in the phyllosphere or rhizosphere and then in the plant (with possible bio-transformations).

Dealing with the mechanisms involved, the foliar transfer of inorganic pollutants was mainly studied in the case of radioactive elements. According to Hurtevent et al. (2013), the interception of radioactive dry or wet deposits and further translocation to the edible parts of plants have been shown to play a major role in food chain contamination. Numerous processes could be involved: fixation and penetration through the foliar cuticle, internalization by leaf cells and release into the phloem leading to transportation and distribution within plant. Larue et al. (2014) studied in controlled experiments, the uptake of engineered nanoparticles (NPs) Ag-NPs in Lactuca sativa after foliar exposure and their possible biotransformation and phytotoxicity. Evidence for the entrapment of Ag-NPs by the cuticle and penetration in the leaf tissue through stomata, for the diffusion of Ag in leaf tissues, and oxidation of Ag-NPs and complexation of $Ag^{(+)}$ by thiol-containing molecules was concluded. However, the mechanisms of foliar transfer of NPs and solutes are still poorly understood. Two pathways have been proposed, for hydrophilic compounds via aqueous pores of the cuticle and stomata, and for lipophilic ones by diffusion through the cuticle (Schreck et al., 2012). Similar associations with the cuticular wax and stomata were observed on lettuce leaves after a field foliar contamination to Pb-rich nano- and microparticles (Uzu et al., 2010). Larue et al. (2014) observed NPs inside the sub-stomatal chamber and inside cells of the plant suggesting that endocytosis might be a route of entry for NPs.

Solubilization-crystallization process at the leaf surface can induce transformations of micrometer and submicrometer scale PM exposed to humidity (Falgayrac et al., 2013) and to biochemical compounds present in the phyllosphere (Rajkumar et al., 2012). Stomata containing some particles in the stomatal openings showed some deformations of the guard cells. PM may be trapped on the leaf surface of plants, due to leaf hairs and wax cover (Sæbø et al., 2012) or be included in stomata apertures, depending on their size (Eichert et al., 2008). However, according to Zangi and Filella (2012), mechanisms involved in metal uptake, transport routes through the cell membrane and bioaccumulation in plant leaves are still little known.

The <u>root transfer</u> of inorganic pollutants was widely studied. The influence of the physico-chemical characteristics of the PM on their reactivity was highlighted by Uzu et al. (2010). The influence of rhizosphere activity on the status of metals in the substrate was evidenced in many studies (Rajkumar et al., 2012). After root uptake, metals are transferred by apoplasmic pathway or symplastic transport across the root cortex to plant storage tissues with different ways depending on the considered plant and metal (Pourrut et al., 2011). The relationship between metal speciation and bioaccessibility by ingestion for humans is well recognized in soils (Pascaud et al., 2014).

For polluted soil matrix, the relationship between metal compartmentalization, speciation and bioaccessibility is recognized (Kalis et al., 2013; Batista et al., 2011; Shhaid et al., 2014). Lead solubilization in soil and uptake by plants vary significantly with its chemical speciation in soil solution (Shahid et al., 2011). Han et al. (2012) reported that the bioaccessible Pb form was significantly correlated to the organic, carbonate and Pb Fe-Mn oxides fractions. This is consistent with the fact that these fractions are unstable in an anoxic and reducing environment such as the gastrointestinal track (Marschner et al., 2006). Finally the higher bioaccessibility observed for Cd in comparison with Pb is consistent with previous results in soil matrix (Foucault et al., 2013).

The differences observed for metals bioaccessibility in function of the exposure conditions could first be induced by the differences of bio-physicochemical conditions in rhizosphere in comparison with phyllosphere and secondly to the possible biotransformation of particles in plant cells. In the case of root exposure, the influence of the rhizosphere activity on the status of metal(loid)s in the substrate was evidenced in many studies (Jouvin et al., 2012; Rajkumar et al., 2012). In addition, rhizosphere is a place of intense microbial activity and excretion of various inorganic and organic compounds (Shahid et al., 2012b,c). In the case of foliar transfer, particles may be trapped on the leaf surface of plants, due to leaf hairs and wax cover (Sæbø et al., 2012) and depending on their size (Eichert et al., 2008, Schreck et al., 2012a). Metal-containing particles deposited on the phyllosphere are submitted to changes in humidity, temperature, physico-chemical conditions. Moreover, the phyllosphere hosts an intense microbial activity and is a place of excretion of various inorganic and organic compounds (Timms-Wilson et al., 2006).

4.2.5 Conclusions and Perspectives

For the first time, the influence on metal accumulation in consumable plant parts and human bioaccessibility under different exposure conditions (atmospheric *versus* soil) was highlighted. That phenomenon could be due to changes in metal compartmentalization and speciation under different modes of metal uptake. Human bioaccessibility of metals also varies with the type of plant, applied P amendment and metal. Field surveys are therefore essential in polluted areas where people cultivate vegetables, in order to better understand the scenarios of human risk exposure to metals. Experiments performed under different levels of soil and air pollution could help to build a database of bioavailability values of metal in plants used in the context of health risk assessments.

Further studies are in progress to better explore the link between metals compartmentalization and speciation in plants and their bioavailability and (eco)toxicity. Microbiological and speciation studies could bring more relevant information to better understand the phenomena involved in the complex phyllosphere and/or rhizosphere interfaces.

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Chapter 5 Soil-Vegetable-Air quantity and Human health assessment near waste incinerator and highway

Forewords

Control experiment in green house is a good way to study the metal foliar transfer mechanism. It permits to focus on the studied paremeters and control other influcing factor. That condition also can avoid the influence of light, temperature, climate, rainfall and wind.

However, evaluation of metal transfer in greenhouse is sometimes misleading since it can overestimate/underestimate metal uptake by plants because roots and leaves are confined to contaminated soil or deposited PM. Therefore, field studies can provide more accurate information on the uptake of metals by plants in real scenarios. Also, the soil pH, background value, microorganisms, climate, vegetable species can always influence the metal uptake. Factors such as direct exposure to sunlight can influence metal uptake and might not be seen in laboratory studies (De Leon et al., 2010) and rainfall can influence the wet deposition of atmosphere particles and foliar metal uptake.

Therefore, in this study, the quality of cultivated consumed vegetables in relation to environmental pollution was investigated in urban and peri-urban areas that host the majority of people. A field study was conducted on two different sites near: a waste incinerator (A) and a highway (B) in order to evaluate the fate of metals in urban soil-plant-atmosphere systems with their consequences on human exposure.

This study can be used to assess environmental and sanitary risk, biomonitoring regulated industries with leafy vegetables and communicate with stakeholders, so as to decrease human exposure to metal(loid)s.

Actually, this study has received the finance support form international Xu Guangqi Programme (32155SC, 2014) “Global analysis (metals and organic pollutants) of the quality for vegetables cultivated near waste incinerator: environmental and sanitary risk assessment”. The object is described as bellow (Fig. 1), which aims to better understand the potential impact of fine particles emitted by a waste incinerator onto the human health and its close environment.

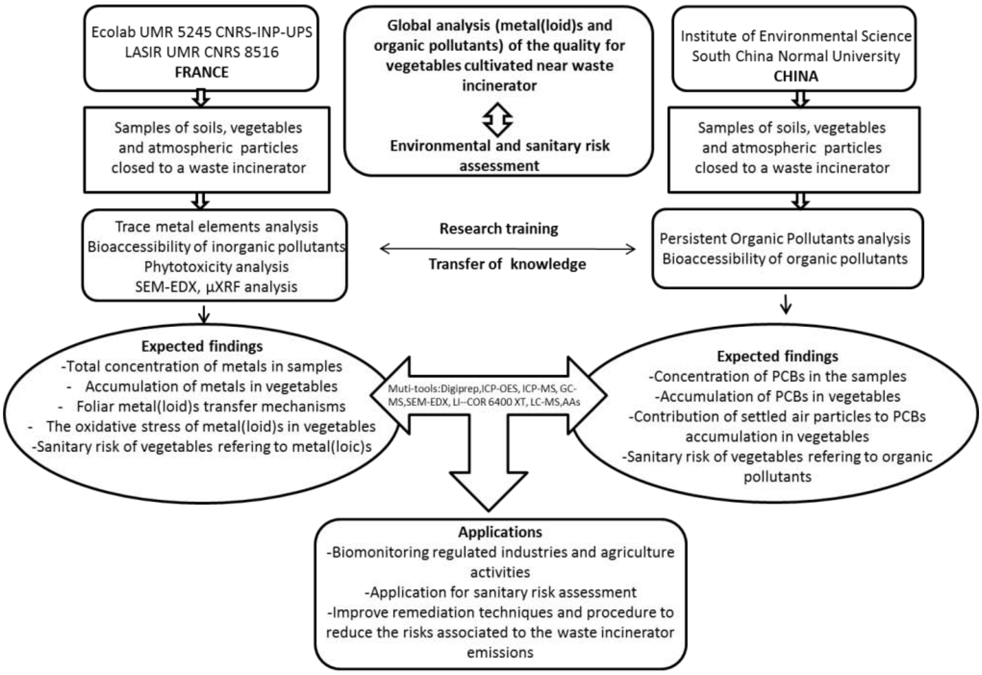

Fig.1 Xu Guangqi project.

►This chapter is presented in a form of a submitted article to Environmental Geochemistry and Health (under review since June 2015).

Metal bioaccessibility measures to improve human exposure assessment: field study of soil-plant-atmosphere transfers in different urban areas.

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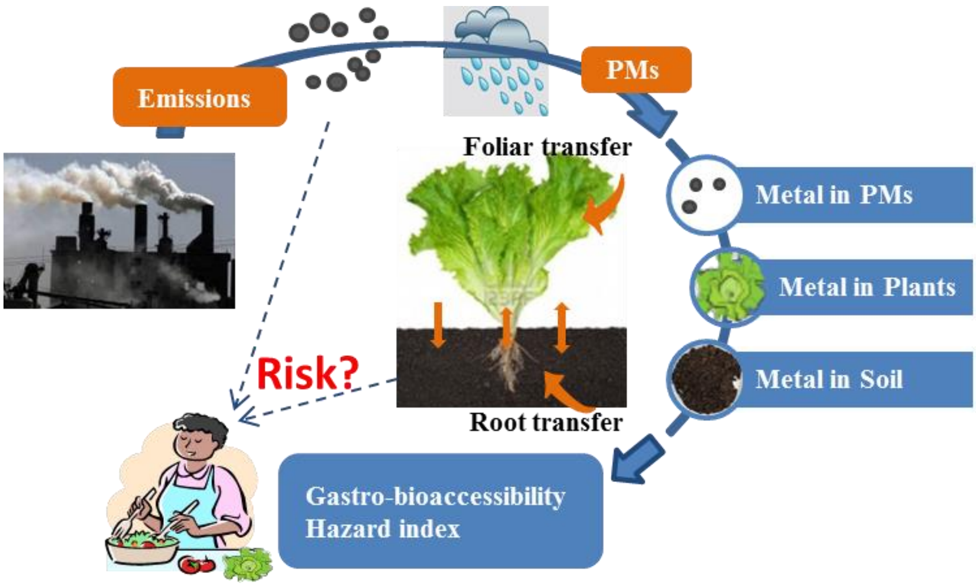
Abstract

The quality of cultivated consumed vegetables in relation to environmental pollution is a crucial issue for urban and peri-urban areas hosting the majority of people at the global scale. In order to evaluate the fate of metals in urban soil-plant-atmosphere systems and their consequences on human exposure, a field study was therefore conducted on two different sites near: a waste incinerator (site A) and a highway (site B). Metals were investigated in soil, settled atmospheric particulate matters (PM) and vegetables, and risk assessment was performed using both total and bioaccessible metal concentrations for vegetables.

Total metal concentrations of PM were (mg kg^{-1}): (site A) 417 Cr, 354 Cu, 931 Zn, 6.3 Cd and 168 Pb; (site B) 145 Cr, 444 Cu, 3289 Zn, 2.9 Cd and 396 Pb. Several total soil Cd and Pb concentrations exceeded China Environmental Quality Standards. For both sites, significant metal enrichment from atmosphere to leaf vegetables (correlation between Pb concentrations in PM and leaves: r=0.52, p<0.01) was observed. Total Cr, Cd, and Pb concentrations in vegetables were therefore above or just under the maximum limit levels in foodstuffs of China and European Commission Regulation. Moreover, high metal bioaccessibility (60-79%, with maximum value for Cd) was observed. Bioaccessible hazard index was above 1 only for site B, due to moderate Pb and Cd pollutions induced by highway. In contrast, site A was considered as relatively safe for urban agriculture.

Keywords: bioaccessibility, metals, urban areas, waste incinerator, roads, vegetable quality, settled particulate matters, human exposure

Graphic abstract



5.1 Introduction

For several industrialized countries, the emission of metal(loid)s rich airborne fine and ultrafine particulate matters (PM) from industrial activities have largely declined due to regulatory changes (Schreck et al. 2013). Actually, for some countries (Eastern Mediterranean and South-East Asia), which produce and export worldwide large quantities of items, regulation on air quality is increasing gradually, but currently maximum threshold levels of PM emitted into the environment are relatively high (annual average PM_{10} concentrations levels is 70 μg/m^3) when compare to the World Health Organization (WHO) guideline level (20 μg/m^3) (WHO, 2014a,b). PM are monitored within the framework of European regulations for air quality (96/62 / EEC; 99/30 / EC, 2003/0164 / COD). Some metals (Cd, Pb, Ni, As, and Hg) are classified as "Substance of Very High Concern" (REACH Regulation) and their presence in fine particles (even nano metric particles) makes them highly toxic. The emission of metal enriched PM from industries can cause contamination of soils (Han et al. 2009; Lévêque et al. 2014; Li et al. 2014) and plants (Fernández Espinosa and Rossini Oliva 2006; Uzu et al. 2014; Zhou et al. 2014). For humans, in addition to the health risk induced by inhalation of particles, the consumption of contaminated plants (Douay et al. 2008; Dumat et al. 2006; Xiong et al. 2014) and ingestion of contaminated soil (Pascaud et al., 2014) are the two main routes of exposure included in the evaluation of health risks. Although many studies attributed the contamination of plants via soil-plant transfer, it has recently been demonstrated that 25 to 40% of plant metal content can be derived from leaf transfer coming from industrial particles (Nowack and Bucheli 2007; Schreck et al. 2012). Therefore, in addition to knowing soil-plant transfer of metal, atmosphere transfer is equally important for risk assessment.

Nowadays, significant amounts of PM (PM_{10}, $PM_{2.5}$ and PM_1 – rich in As, Cd, Cr, Cu, Ni, Pb, Zn...) are emitted by several anthropogenic activities such as waste incineration and road traffic. These PM are observed in urban areas with concentrations exceeding sometimes the European regulations thresholds (Bu-Olayan and Thomas 2009; Harrison and Yin 2010; Moreno et al. 2010; Shahid et al. 2013; Zhang et al. 2005). Waste incinerator is considered as a sustainable practice for reducing waste volume and recovering its energy to produce electricity and district heating (Buonanno and Morawska, 2014). The use of waste-to-energy incinerators to manage waste was advocated by Chinese government due to their high efficiency, minimal land requirement and significant impact in terms of reducing solid mass (Wan et al. 2015; Zhang et al. 2014). According to Roberts and Chen (2006), the UK national waste strategy requires increased incineration with energy recovery. However, this policy is unlikely to gain wide public acceptance unless governments find ways to clearly communicate on the risks involved to address public anxiety (Roberts and Chen, 2006). In order to better understand public health implications of incineration, it’s essential to evaluate the magnitude of the risks involved. Indeed, accordingly to Sharma et al. (2013), incinerators release a wide variety of pollutants (ultra-fine particulate matters enriched with metal(loid)s, polychlorinated biphenyls (PCBs), dioxin, acid gases, nitrogen oxides …), depending on waste composition (Hu et al. 2003; Zeng et al. 2014; Zhang et al. 2014). These emissions can lead to health deterioration such as higher incidence of cancer (Elliott et al. 1996), respiratory symptoms, congenital abnormalities or hormonal defects. Thus, there is a dire need to adopt newer, widely accepted, economical, and environment-friendly technologies for incineration. On the other side, in urban areas, road traffic is also an important source of PM from tires, wear of brakes, other vehicle components, exhaust emissions, and re-suspended road dust (Pagotto et al. 2001; Johansson et al. 2009; Sternbeck et al. 2002).

The analysis and control of the pollution, information to the public and advices to reduce human exposure is therefore a crucial issue. The present study was conducted to investigate vegetable, rhizosphere soil, and atmospheric PM for two different urban areas: (site A) near a waste incinerator and (site B) near a highway. The study of the soil-plant-atmosphere systems aims to assess the environmental and sanitary risks due to PM fallouts enriched with metal(loid)s in the case of consumption of contaminated vegetables by humans. The ultimate purpose is to propose regulation monitoring for industries, road traffic, and agricultural activities as well as improve procedures to reduce the sanitary risk associated to atmosphere PM emission.

5.2 Materials and Methods

5.2.1 Sampling and sample processing

Vegetables, rhizosphere soils, and settled atmospheric PM were collected at two sites from Guangzhou (China). Site A is located less than 0.5 km away from a waste incinerator, which has an area of 101778 m^2 and daily refuse processing capacity of 1040 t, representing about 1/7 of local household refuse. This incinerator is running since 2006 and is the only waste incinerator in Guangzhou. Site B is a cultivated collective garden near a highway located in Guangzhou Higher Education Mega Center (urban area, more than 30 km away from the incinerator). Vegetables grown at both sites are supplied to local markets for human consumption. Vegetables species which were collected from the two sites are given in Table 1. Three vegetables were sampled for every species and each plant sample was prepared in order to obtain three analytical replications (9 pseudo-replicates). Rhizospheric soil is defined as the soil adhering to roots and directly influenced by root exudates (Hinsinger, 2001). Rhizosphere soils were sampled after separation from bulk soil by carefully shaking the root system. Rhizosphere soil was preferred over bulk soil in order to better assess metal(loid) soil-root transfer (Uzu et al., 2014). According to Zeng et al. (2014), air particles settled on cement surfaces located in the vegetable garden may represent air particles deposited on the vegetable leaves. Therefore, settled PM were sampled by collecting dust from the cement surface of house roof/window at each sampling site with a vacuum cleaner. All samples were stocked into polythene zip-lock bags.

Afterward, vegetables were separated into root and shoot parts, and both were fresh weighted. In order to assess sanitary risks, vegetable were thoroughly washed according to human consumption procedure to remove air born dust and soil particles (Birbaum et al. 2010; Uzu et al. 2010; Xiong et al. 2014). Samples were first rinsed with running tap water for 30 s before being immersed in two baths of deionized water for 1 min and rinsed another time with deionized water (Hong et al. 2014; Xiong et al. 2014). Soils and plant tissues were oven-dried (48 h; 50 °C), ground and 250 µm sieved. PM were also sieved by a 250 µm mesh. This choice is supported by the fact that 250 µm of particles are directly available for ingestion by children (Gron and Andersen 2003).

Table 1 Vegetable species collected at site A and site B.

	Common name	Scientific name	Edible part of vegetable
Site A	Leaf lettuce	*Lactuca sativa* (L.) var. *longifolia*	Leaf
	Water spinach	*Ipomoea aquatic* Forssk.	Leaf
	Leafmustard	*Brassica juncea* (L.) Czern Coss.	Leaf (or Root)
	Purslane	*Portulaca oleracea*L.	Leaf
	Welsh onion	*Allium fistulosum* L.	Leaf
	Bitter lettuce	*Cichorium endivia* L.	Leaf
Site B	Water spinach	*Ipomoea aquatic* Forssk.	Leaf
	Welsh onion	*Allium fistulosum* L.	Leaf
	Amaranth	*Amaranthus mangostanus* L.	Leaf

5.2.2 Metal(loid) concentration in plant tissues and soil samples

Plant and soil samples were mineralized with a Digiprep® instrument from SCP science producer, which is a block digestion system allowing fast and uniform samples digestion. Vegetables (0.125 g per sample) were mineralized in a 1:1 mixture of HNO_3 and H_2O_2 at 80˚C for 4h (Uzu et al., 2014). Soil and PM (0.5 g per sample) were digested by *aqua regia* (1:3 mixture of HNO_3 and HCl) at 80˚C for 1h30min, supplemental with H_2O_2 (30%) and digested at 80˚C for 4h (modified from Foucault et al. 2013). Metal(loid)s concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS, X Series II, Thermo Electron). The accuracy of measurements was checked using reference materials: (i) Virginia tobacco leaves for plant tissues, CTA-VTL-2 and (ii) Swiss loess soil for soil samples, RTH 912. Detection limits for Cr, Cu, Zn, Cd, and Pb were respectively 0.1, 1.3, 2.2, 0.2, and 0.3 $\mu g.L^{-1}$.

5.2.3 In vitro bioacessibility measurement

Gastric bioaccessibility measurements were performed on vegetables samples, using the adapted Unified BARGE Method (Cave et al. 2006; Foucault et al. 2013; Uzu et al. 2011). This test consists in a three steps extraction procedure to simulate the chemical processes occurring in the mouth, stomach, and intestine compartments using synthetic digestive solutions. Temperature was maintained at 37 °C throughout the extraction procedure, as in human bodies. The composition of the solutions and the extraction test procedure were described by Mombo et al. (2015) and Wragg et al. (2011). Dried vegetable samples (0.6 g) were mixed with 9 ml of saliva solution (pH 6.5) and shaken for 5 min. Then 13.5 mL of gastric solution (pH 1.0) was added to the suspension. The pH of the solution was adjusted to 1.2 using HCl (37%) or NaOH (1M) if necessary. The suspension was mixed 1h using an end-over-end rotation agitator. The pH of the suspension was checked to be in the 1.2–1.6 range. The stomach phase was extracted by centrifuging the suspension at 3000 g for 5 min. Metal(loid)s concentrations in the gastric phase solution were measured by ICP-MS. For each sample, tests were carried out in triplicates.

5.2.4 Non-carcinogenic risk assessment

To assess the sanitary risk of vegetable ingestion, the estimated daily intake (EDI, µg kg^{-1} day^{-1}) of edible parts were calculated by Eq.1:

$$EDI = \frac{C_{metal} \times IR}{BW} \quad \text{Eq.1}$$

where C_{metal} is the measured metal concentration of leaves based on fresh weight (mg kg^{-1} FW). We work with fresh weight because we are interested by health exposure in the case of fresh vegetable ingestion. BW is the average body weight of the exposed individual (58.6 kg), which is the mean BW value of Chinese adult (62.7 kg and 54.4 kg for males and females, respectively) (Wang et al. 2011). IR is the ingestion rate (345 g day^{-1}) in urban area of China (Wang et al. 2005). Non-carcinogenic hazards through vegetable ingestion are characterized using the hazard quotient (HQ). It is defined as the quotient of the chronic daily intake, or the dose divided by the toxicity threshold value, which refers to the tolerable daily intake (TDI, μg kg^{-1} day^{-1}) of a specific chemical.

$$HQ = \frac{EDI \times EF \times ED}{TDI \times AT} \quad \text{Eq.2}$$

EF is the exposure frequency (350 day $year^{-1}$), ED is the exposure duration (70 years), equivalent to the average lifetime (USEPA 1989), and AT is the averaging time for non-carcinogens (ED×365 day $year^{-1}$). TDI was expressed as quantity of pollutant ingested each day (μg) in function of kg BW: 1500 μg kg^{-1} day^{-1} for Cr (USEPA 1997), 40, 300 and 1μg kg^{-1} day^{-1}, respectively for Cu, Zn and Cd (USEPA 2003), 3.5 μg kg^{-1} day^{-1} for Pb (JECFA 1993). To assess the overall potential for non-carcinogenic effects posed by more than one chemical, a Hazard Index (HI) approach has been applied. For a mixture of five metals in the present study, the hazard index was calculated from Eq. 3: $HI = \sum_{i=1}^{5} HQ$ Eq.3

Exposed population is assumed to be safe if the HI value is less than one, otherwise, adverse health effects may occur (Wang et al. 2005). The maximum allowable daily quantities of plant consumed were also calculated to give suggestion to the local resident. The maximum allowable daily plant intake (MDI) to reach the TDI was therefore calculated as below (Eq.4):

MDI (g day^{-1}) = TDI (μg kg^{-1} day^{-1}) ×BW (kg) / C_{metal} (mg kg^{-1}) Eq.4

In addition, the metal bioaccessibility in consumed parts of vegetables were incorporated into risk assessment to establish more realistic measures according to Pelfrêne et al. (2013) and Uzu et al. (2014). The bioaccessible estimated daily intake (BEDI) and bioaccessible hazard quotient (BHQ) were calculated by multiplying, respectively, the EDI and HQ values by their corresponding bioaccessibility. Bioaccessible maximum allowable daily plant intake (BMDI) was calculated by dividing the MDI by their corresponding bioaccessibility. The corresponding bioaccessible hazard index (BHI) was calculated with BHQ according to Eq. 3.

5.2.5 Statistical analysis

Total and bioaccessible metal(loid) concentrations were subjected to statistical analysis. Analysis of variance (ANOVA) using SPSS 18.0 software was performed on measured values. Data were tested for normal distribution and homogeneity of variance preceding the ANOVA. Statistical analyses were carried out on means of 9 replicates for each plant species, soil and PM sample. Mean and range values were used to assess contamination levels of metals in soil, PM and plants. Significant differences (p-value < 0.05) were determined using the LSD Fisher test, Duncan's test, and Student's t-test. The relation of metal concentration in soil and PM with that in plants was investigated using linear regression, and principal component analysis (PCA). The correlation coefficient of different parameters was expressed with r values.

5.3 Results and Discussion

5.3.1 Total metal concentrations in rhizosphere soils and PM

Metal concentrations in rhizosphere soil from two studied sites are listed in Table 2. The regulation values for metals in contaminated soils are listed in Table 3. Metal concentrations of settled atmospheric PM in the two sites are listed in Table 6 in supporting information (SI). All data were expressed on a dry weight (DW) basis.

5.3.1.1 Rizosphere soil samples

Metal concentration in rhizosphere soil varies with vegetable species even in the same field (Table 2): significant higher Cd concentration was detected in rhizosphere soil of purslane (1.41 mgCd kg^{-1}) and bitter lettuce (1.56 mgCd kg^{-1}) on site A, significant higher Zn concentration was detected in rhizosphere soil of bitter lettuce (185 mgZn kg^{-1}) on site A, significant higher Pb concentration was measured in rhizosphere soil of leaf lettuce (45.1 mgPb kg^{-1}) and leaf mustard (40.2 mgPb kg^{-1}) on site A and in amaranth (53.4 mgPb kg^{-1}) on site B. Inversely, significant lower metal concentrations were found in rhizosphere soil of water spinach (site A) and purslane (except Cd). Vegetable effect is significant for Cd, Zn, Cu and Pb (in decreasing order). In previous study from Shahid et al. (2014), significant difference between rhizosphere soils of various species were also observed; a lower-metal concentrations were found in the rhizosphere soil of mustard than pea. Rhizosphere microbial populations and type and quantity of root exudates (i.e. organic acids) vary with plant species and might therefore differently influence rhizosphere soil characteristics (Austruy et al. 2014; Bergqvist et al. 2014; Columbus and Macfie 2015; Shahid et al. 2012). Moreover, significantly positive correlation was found between metal concentration in roots and in soil for Cd ($r=0.53$, $p<0.01$), Cu ($r=0.50$, $p<0.01$) and Zn ($r=0.34$, $p<0.05$), no significant relation was found for Cr and Pb. This indicates that a higher Cd, Cu, and Zn concentration in soil results in a higher metal root accumulation. In reverse, the plant influence metal uptake in the rhizosphere: plant growth can elevate As accumulation by the effect of organic acids (Bergqvist et al., 2014).

Total metal concentrations measured in soil (mg.kg^{-1}) ranged between: (site A) 30.2 - 41.4 Cr, 5.8-14.9 Cu, 57-195 Zn, 0.22-1.91 Cd and 25-55 Pb and (site B) 39.8-50.1 Cr, 17.2-24.9 Cu, 68.0-102.8 Zn, 0.02-0.30 Cd and 35.6-54.2 Pb. Generally, Cr and Cu concentrations in all soil samples were below China Environmental Quality Standards for Soils (CN EQSS) (GB15618-2008, 150 mgCr kg^{-1} and 50 mgCu kg^{-1}). Zn concentration in site A (ranging from 57 to 195 mgZn kg^{-1}) was close to China EQSS but still safe for green food production (MAC SGF) (NY/T391-2000, 200 mgZn kg^{-1}). Similarly, some rhizosphere soils were slightly contaminated by Pb (25-55 mgPb kg^{-1}), but still under or close to the limits of safe green food production (NY/T391-2000, 50 mgPb kg^{-1}). Cd concentration at site A (0.22 to 1.91 mg kg^{-1}) significantly exceeded China EQSS (GB15618-2008, 0.3 mgCdkg^{-1}) and the maximum allowable Cd concentrations in soil for green food production areas (NY/T391-2000, 0.3 mgCd kg^{-1}). The China EQSS were set according to soil pH values, considering the average soil pH 5.6 (with high metal solubility in soil) in Guangdong provinces (Guo et al., 2011), therefore the EQSS with pH 5.5~6.5 were used (Table 3).

Table 2 Measured metal concentration (mean value and range of 9 pseudo-replicates) in rhizosphere soil samples from two sites (mg kg^{-1} DW).

Site A						Site B					
Soil samples	Cr	Cu	Zn	Cd	Pb	Soil samples	Cr	Cu	Zn	Cd	Pb
Leaf lettuce	38.5 bc[1]	13.0 b	150 b	0.66 bc	45.1 cd	Water spinach	43.9 d	23 d	82.4 a	0.28 ab	45.7 c
	(36.9-41.4)	(10.8-14.8)	(139-163)	(0.55-0.76)	(39.4-55.3)		(40.6-47.7)	(21.9-24.8)	(76.1-92.9)	(0.25-0.30)	(44.6-46.5)
Water spinach	34.4 ab	6.8 a	64.4 a	0.26 ab	25.7 a	Welsh onion	42.4 cd	19.5 c	85.4 a	0.16 a	38.8 b
	(30.6-41)	(5.8-7.5)	(57.2-74.4)	(0.22-0.31)	(24.5-27.4)		(39.8-45)	(17.2-21.8)	(68-103)	(0.02-0.30)	(35.6-42)
Leaf mustard	33 a	12.6 b	132 b	0.84 c	40.2 bcd	Amaranth	49 e	24.8 d	84.6 a	0.11 a	53.4 d
	(31.8-33.7)	(11.4-13.9)	(104-186)	(0.53-1.37)	(37.1-42.3)		(47.7-50.1)	(24.5-24.9)	(82.8-87)	(0.10-0.11)	(52.9-54.2)
Purslane	32.6 a	9.1 a	84.5 a	1.41 d	26.9 a						
	(30.2-34.7)	(8.3-9.8)	(81-90)	(1.06-1.91)	(26.2-27.8)						
Welsh onion	36.5 ab	14 b	143 b	0.56 abc	39.1 bc						
	(35.8-37)	(13.5-14.4)	(137-147)	(0.53-0.57)	(38.3-40.1)						
Bitter lettuce	36.9 ab	13.7 b	185 c	1.56 d	31.4 a						
	(36.1-38)	(12.1-14.9)	(175-195)	(1.34-1.91)	(30.8-32)						

[1]The different lowercase letters highlight significant difference between different rhizosphere soil ($p<0.05$).

Table 3 Regulation values of metals in contaminated soils.

Regulation in different countries	Cr	Cu	Zn	Cd	Pb
CN EQSS [1]	150	50	200	0.3	50
MAC SGF [2]	120	50	200[3]	0.3	50
NYS DEC [4]	11	270	1100	0.43	200
EC SVs [5]	30-150	20-200	60-600	0.3-4	25-200
EC threshold values [6]	50	40	50	0.5	100
France agriculture soil [7]	150	100	300	2	100

[1] China Environmental Quality Standards for Soils (CN EQSS) (GB15618-2008); Grade 2, vegetable land, (pH 5.5~6.5).

[2] China maximum amounts of contaminants in soil for green food production area (MAC SGF); Environmental technical terms for green food production area (NY/T391-2000).

[3] Environmental quality evaluation standards for farmland of edible agriculture products (HJ/T332-2006).

[4] New York State Department of Environmental Conservation -NYS DEC (2006).

[5] European Commission Soil Screening Values (EC SVs), Joint Research Centre, (Carlon, 2007).

[6] Background values in European soils, proposal for threshold values in function of soil pH ($5 \leq pH < 6$)(GAWLIK and BIDOGLIO, 2006).

[7] Limits value for metal concentration in agriculture soil of France. Ministry of Spatial Planning and the Environment (1998).

The United States Environmental Protection Agency (US EPA) and New York State Department of Environmental Conservation (NYS DEC) have set guidelines for determining safety of various land uses based on total soil metal concentrations using standard EPA methods. The soil clean up objectives based on human health risks (NYS DEC 2006) are listed in Table 3. When compared with the guidelines of NYS DEC, Cr (both at site A and Site B) and Cd (at Site A) exceeded the threshold limits (11 mgCr kg^{-1}, 0.43 mgCd kg^{-1}), which may present a potential toxic risk for human health.

In European Union, Soil Screening Values (SVs) are used as quality standards to regulate contaminated sites. Derivation methods of SVs have scientific and political bases; and strongly differ between countries, and SVs numerical values vary consequently (Carlon, 2007). The Europe Commission Soil Screening Values (EC SVs) are summarized in Table 3 according to Joint Research Center (JRC) Scientific and Technical Reports by Carlon (2007). Actually Cr, Cu, Zn, Cd, and Pb concentrations in different countries range, respectively from 30-150 mg kg^{-1}, 20-200 mg kg^{-1}, 60-600 mg kg^{-1}, 0.3-4 mg kg^{-1} and 25-200 mg kg^{-1}. Using the conservative European reference values (minimum values), it can be concluded that soils from sites A and B are contaminated with Cr, Zn, Cd, Pb or Cr, Cu, Zn, Pb, respectively. JRC also proposes threshold values in European soils (EC threshold values) upon the existing database of European countries (Gawlik and Bidoglio 2006). According to these values, it seems important to carry out a monitoring of the evolution of the quality of soil, air, and vegetables near waste incinerators, especially on Cd and Zn pollutant.

Generally, the China EQSS is strict for Cd and inside the range of EC SVs. The China EQSS for agriculture soil are stricter than in France (Table 3), however soil pollution in China is sometimes due to the needs of economic development, the weak consciousness of the public on environmental protection and loose government management/regulation. Thus, besides establishing strict guidelines, education and monitoring are also necessary to improve soil quality.

It is not easy to conclude soil contamination status due to the intersection of different regulation values. The limit values depend on soil pH, texture, parent material, and land use. Soil regulation varies with countries, there is not a union quantity standard for soil, sometimes loose and incomplete standards were used in some countries. In this situation, there is a need for setting a unified standard for contaminants in various matrixes at global scale, no matter the background values of countries. Actually, European Reach (2006) regulation on hazardous chemicals and global harmonized system (GHS) for classification of chemicals replaces various classifications and labels used in different countries in order to fix the same consistent criteria for chemicals at the global level.

5.3.1.2 Settled atmospheric PM

Total measured mg of metal per kg^{-1} of PM were: (site A) 417 Cr, 354 Cu, 931 Zn, 6.3 Cd and 168 Pb; (site B) 145 Cr, 444 Cu, 3289 Zn, 2.9 Cd and 396 Pb. Metal concentration in PM is significantly higher than that in the soil ($p<0.01$). Mean concentrations of Cr, Cu, Zn, Cd, and Pb in PM of site A (Table 6 in SI) are, respectively 11.81, 30.69, 7.37, 7.13, and 4.84 times higher than in soil. At site B, concentrations of Cr, Cu, Zn, Cd, and Pb in PM are, respectively 3.21, 19.79, 39.08, 16.17, and 8.61 times higher than in soil (Table 6 in SI). PM can deposit on soil and aerial parts of vegetables, thus leading to a higher phyto-toxicity after long term exposure. Actually, Cu, Cd, and Pb in the soil (S-Cu, S-Cd, S-Pb) were highly related to the metals in PM (A-Cu, A-Cd, A-Pb), which is in accordance with the hypothesis of soil pollution by incinerator and road traffic atmospheric deposits (Fig.4 in SI). In contrast, concentrations of Cr and Zn in PM (A-Cr, A-Zn, Fig.4 in SI) were little related to rhizosphere soil Cr and Zn concentrations (S-Cr, S-Zn, Fig.4 in SI). This suggests the complementary effect of other pollution sources or complex environmental processes, such as volatilization, leaching, translocation, soil adsorption and complexation, as well as bio-transformation and degradation (Foucault et al. 2013; Shahid et al. 2014; Zeng et al. 2014).

Metal concentrations of PM are significantly higher at site A for Cr (417 mg kg^{-1}) and Cd (6.27 mg kg^{-1}), which may be mainly from the stack emission from the incinerator. These findings are in agreement with the study of Hu et al. (2003), where Cr and Cd originated from the stack emission of incinerator. Compared with site A, mean concentration of Cu (443.62 mg kg^{-1}), Zn (3289 mg kg^{-1}), and Pb (396 mg kg^{-1}) are predominantly elevated at site B. Significant positive correlations were observed between Cu and Zn ($r=0.87$, $p<0.01$), Cu and Pb ($r=0.91$, $p<0.01$) as well as Zn and Pb ($r=0.95$, $p<0.01$) suggesting a similar source. Since site B is near a motor way, higher metal concentration is induced by transport emissions. Indeed, the exhaust emission from both gasoline and diesel vehicles contain variable quantities of Cu, Zn, Pb, and Fe (Hu et al. 2003). Cu and Zn pollution can also come from brake wear emissions, smelting, and coal burning (Johansson et al., 2009; Piatak et al., 2004).

5.3.2 Metals in vegetables

Concentrations of metals in aerial parts and roots of vegetable samples are shown in Table 4. All data were expressed on a fresh weight (FW) basis. Metal concentration in leaves and root are variable between different plants with an order of Zn>Cu>Cr≈Cd≈Pb.

Table 4 Measured metal concentrations (mean value and range of 9 pseudo-replications) in aerial parts and roots of vegetables (mg kg^{-1} FW).

Table 4-A Measured metal concentrations in aerial parts and roots of vegetables from site A (mg kg^{-1} FW).

Site A	Cr			Cu			Zn			Cd			Pb		
	leaf	root		leaf	root		leaf	root		leaf	root		leaf	root	
Leaf lettuce	0.35 ab[1]	0.28 A[2]		0.52 a	0.49 A		3.7 a	4.95 A		0.02 a	0.05 A		0.07 a	0.35 A	
	(0.26-0.48)	(0.19-0.43)		(0.40-0.71)	(0.39-0.58)		(2.89-4.47)	(3.41-6.43)		(0.01-0.02)	(0.05-0.06)		(0.05-0.10)	(0.22-0.51)	
Water spinach	0.36 ab	0.33 AB		0.69 a	1.56 AB		4.2 a	6.31 A		0.03 a	0.11 A		0.11 a	0.34 A	
	(0.31-0.38)	(0.18-0.59)		(0.61-0.79)	(0.86-2.38)		(3.70-4.46)	(3.38-9.10)		(0.03-0.04)	(0.07-0.15)		(0.10-0.11)	(0.20-0.59)	
Leaf mustard	0.25 ab	0.88 BC	**	0.6 a	1.72 AB		6.59 ab	11.5 A		0.05 a	0.15 A		0.11 a	0.92 AB	**
	(0.15-0.38)	(0.44-1.47)		(0.57-0.65)	(1.22-2.26)		(5.84-7.46)	(8.24-16.1)		(0.05-0.05)	(0.12-0.19)		(0.10-0.14)	(0.14-1.36)	
Purslane	0.64 b	0.34 AB		1.42 ab	3.38 B	**	10.4 bc	17.8 B	**	0.06 a	0.55 B	**	0.10 a	0.46 AB	
	(0.21-1.14)	(0.10-0.49)		(1.09-1.70)	(2.69-4.12)		(7.73-12..3)	(9.65-24.8)		(0.05-0.07)	(0.20-0.78)		(0.08-0.13)	(0.20-0.64)	
Welsh onion	0.33 ab	0.25 A		0.49 a	0.85 A		5.54 a	6.16 A		0.02 a	0.09 A		0.04 a	0.48 AB	*
	(0.25-0.46)	(0.15-0.34)		(0.14-0.67)	(0.37-1.68)		(4.13-6.79)	(3.63-9.84)		(0.00-0.03)	(0.07-0.12)		(0.02-0.06)	(0.20-0.88)	
Bitter lettuce	0.28 ab	0.78 ABC	*	0.69 a	2.5 AB	*	5.55 a	22.6 B	**	0.08 a	0.17 A		0.08 a	0.99 B	**
	(0.25-0.31)	(0.46-1.41)		(0.46-0.89)	(1.54-4.10)		(4.04-7.34)	(22.4-22.9)		(0.05-0.11)	(0.12-0.23)		(0.06-0.11)	(0.72-1.31)	
MAC in vegetables[3]	0.5			10			20			0.2			0.3		
EC for vegetables[4]										0.2			0.3		

Table 4-B Measured metal concentrations in aerial parts and roots of vegetables from site B (mg kg^{-1} FW).

Site B	Cr			Cu			Zn			Cd			Pb	
	leaf	root		leaf	root		leaf	root		leaf	root		leaf	root
Water spinach	0.6 ab[1]	1.06 C[2]	*	4.0 c	9.5 D	**	4.64 a	6.16 A		0.11 a	0.20 A		0.39 b	0.51 AB
	(0.36-0.77)	(0.88-1.26)		(3.30-4.41)	(7.49-12)		(4.30-5.01)	(5.98-6.38)		(0.09-0.14)	(0.17-0.25)		(0.16-0.65)	(0.42-0.59)
Welsh onion	0.19 a	0.54 ABC		1.1 ab	5.61 C	**	12.1 cd	6.85 A	*	0.07 a	0.09 A		0.09 a	0.37 A
	(0.01-0.35)	(0.22-0.84)		(0.14-1.94)	(4.03-6.70)		(7.04-16.6)	(6.17-7.56)		(0.03-0.11)	(0.06-0.11)		(0.08-0.10)	(0.27-0.46)
Amaranth	0.25 ab	0.40 AB		1.61 b	1.77 AB		14.8 d	8.05 A		0.47 b	0.18 A	**	0.36 b	0.63 AB
	(0.05-0.58)	(0.38-0.41)		(1.07-2.55)	(1.36-2.43)		(12.2-19)	(5.03-12.8)		(0.35-0.65)	(0.17-0.19)		(0.18-0.70)	(0.57-0.73)
MAC in vegetables [3]	0.5			10			20			0.2			0.3	
EC for vegetables [4]										0.2			0.3	

[1] The different lowercase letters are the significant difference between leaves at $p<0.05$.

[2] The different capital letters are the significant difference between roots at $p<0.05$. The level of significance between leaf and root were provided ($p<0.05$ *, $p<0.01$ **).

[3] China's maximum allowable amounts of contaminants (MAC) in foodstuffs (Cr, Cd, Pb: GB2762-2012); (Cu: GB15199-94); (Zn: GB13106-91)(Cao et al., 2010b).

[4] European Commission regulation (EC) No 466/2001 of 8 March 2001, setting maximum levels for certain contaminants in foodstuffs-Official journal of the European communities.

Cu and Zn are essential trace elements for the healthy growth of plants as cofactors of various proteins (Hänsch and Mendel, 2009) but excess of these elements can induce (eco)toxicity, that's why measured concentrations were compared with reference values. The Cu concentration is significantly higher in purslane of site A, and in water spinach and root of welsh onion site B. Actually, water spinach in site B accumulated the highest Cu concentration (4.0 and 9.5 mg kg^{-1} in leaf and root respectively) among all vegetables. Zn concentration in leaves is higher in purslane (site A, 10.4 mg kg^{-1}), welsh onion and amaranth (site B, 12.1-14.8 mg kg^{-1}) than other vegetables. The highest Zn concentration is in root of bitter lettuce (22.6 mg kg^{-1}). The concentration of Cu and Zn in vegetable samples are all below the China's maximum allowable amounts of contaminants (MAC) in foodstuffs (10 mgCu kg^{-1} and 20 mgZn kg^{-1}) except for bitter lettuce root (22.6 mg kg^{-1}) exceeding the MAC of Zn in foodstuffs (Cu: GB15199-94; Zn: GB13106-91). Overall, Cu and Zn seem not bring toxicity in both sites.

However, these values are higher than the results of metal concentrations in vegetables cultivated in industrial area in Jiangsu (1.69 mgCu kg^{-1} and 5.83 mgZn kg^{-1}), China (Cao et al., 2010b). In view of the usual critical plant toxicity levels (Cu 20-30 and Zn 100-300 mg kg^{-1} DW) and on the basis of a dry matter content of nearly 10 %, Cu may respectively restrict the growth of water spinach in site B.

The metal concentration for Cr (0.19-1.06 mg kg^{-1}), Cd (0.02-0.55 mg kg^{-1}) and Pb (0.04-0.99 mg kg^{-1}) in both aerial parts and roots partly exceeded the regulation of China (0.5 mgCr kg^{-1}, 0.2 mgCd kg^{-1} and 0.3mgPb kg^{-1}, GB2762-2012) and European Commission (0.2 mgCd kg^{-1} Cd and 0.3mgPb kg^{-1}, EC 466/2001). However, the aerial parts of vegetables were not strongly polluted: the measured values are sometimes above but relatively near the reference values. Cr concentrations exceeded the MAC merely in purslane (site A) and water spinach (site B) leaves. Cd concentrations were beyond the MAC solely in amaranth (site B) leaves. The Pb concentration exceeded the MAC only in water spinach (site B) and amaranth (site B) leaves. Notably, Pb concentrations in all vegetable roots were above the MAC, might indicating a health risk by potential translocation towards edible vegetable parts (Page et al., 2006; Sasmaz et al., 2008; Wiseman et al., 2014). The Cd in amaranth was also found higher than other vegetables in the report of Alam et al. (2003). The Cd level during 2004-2013 in spinach and kale in the direct vicinity of incinerators (Netherlands) were within the maximum acceptable Cd level for leafy vegetables (0.2 mg kg^{-1} FW) (van Dijk et al., 2015), which agrees with our study. The maximum level of Cd and Pb set by the Australian and New Zealand Food Authority are 0.1 mg kg^{-1} fresh weight (Kachenko and Singh, 2004). When compared to this standard, there are more vegetable samples that exceeded the limits in our study.

Cu in vegetables is mainly related to soil metal content ($p<0.01$). Zn in vegetable leaves and roots are supposed respectively from PM ($r=0.48$, $p<0.01$) and soil ($r=0.34$, $p<0.01$). The pollution of Cr in all the vegetables is supposed to originate from air pollution because the soil is under the permissible limit of green food production. At site A, Cd in vegetables may come from, both soil ($p<0.01$) and air pollution (settled particulate matters enriched with Cd). At site B, Cd pollution may mainly originate from air pollution as the soil is not contaminated. Lead contamination in vegetables mainly originates from atmosphere deposits ($r=0.52$, $p<0.01$) and as previously observed by Xiong et al. (2014), Pb translocation from leaves toward roots is critical .

Metal accumulation in leaves and root significantly differ, which depends on the metal concentration in soil and PM, plant species and the metal species. For majority of plant species, metals mainly accumulated in root tissues, the level of significance between leaf and root at both sites is provided ($p<0.05$ *, $p<0.01$ **) in Table 4. Metal sequestration in plant root is a well known phenomenon, especially in the case of Pb (Shahid et al. 2014). Indeed, as the nutritive organ of plant, root is the most important pathway for elements absorption of plants. In addition, higher metal concentration in root may also be due to less water content in root, a higher accumulation in root apoplasm and less distribution to other organs. Even summer rainfall (around 225mm, (Zhou et al. 2006)) in Guangzhou can partially wash the PM deposited on leaves, foliar uptake is significant and plays an important role in metal accumulation when plants are exposed to PM (Schreck et al. 2012; Schreck et al. 2013). However, metal in leaves can be diluted and distributed to other parts of the plant (new leaves, stems, etc) due to plant growth.

In general, purslane and bitter lettuce (site A) and water spinach, welsh onion and amaranth (site B) accumulated most metals in total. Purslane and bitter lettuce preferred Zn uptake, water spinach preferred Cu accumulation. Therefore, more attention should be paid in the ingestion of these vegetables (Fig.1a). Leaf lettuce is the least polluted vegetable. Since vegetables were not polluted by Cu and Zn, therefore only metal concentration of Cr, Cd and Pb are shown in Fig. 1b. Leaf mustard, purslane and bitter lettuce at site A, whereas water spinach and amaranth at site B accumulated significant higher non-essential metals (Cr, Cd and Pb) than other vegetables. Considering that only leaves are consumed, the most toxic vegetables are purslane, water spinach (site B) and amaranth.

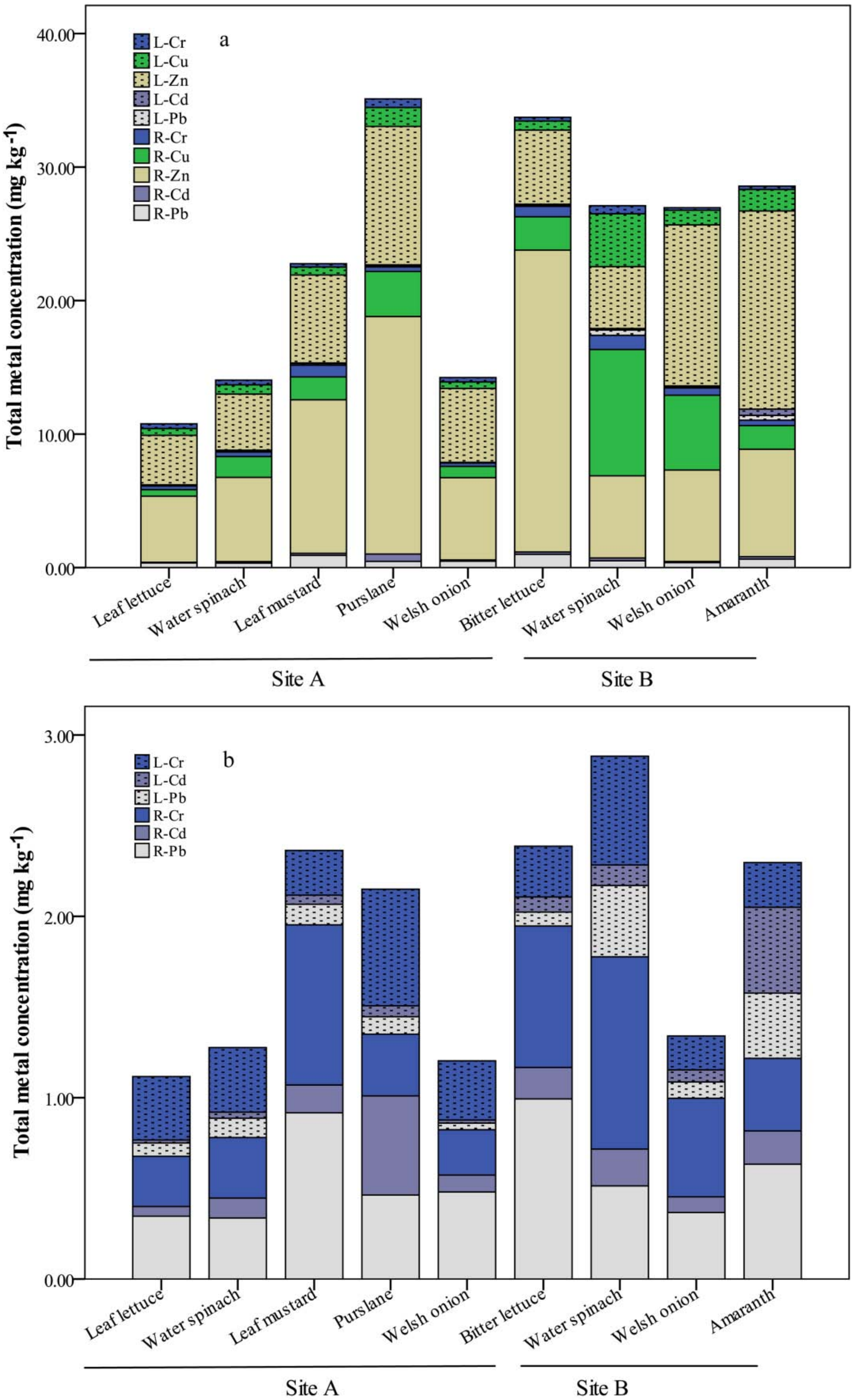


Fig. 1 a) Distribution of metal concentration and b) Distribution of Cr, Cd and Pb concentration in leaf (L) and root (R) of different vegetables from sites A and B.

5.3.3 The relationship between metal in vegetables and their sources

Metal concentrations in leaves are highly related to concentrations in PM ($r=0.78$, $p<0.01$) while metal concentration in roots is mainly related to concentrations in soil ($r=0.69$, $p<0.01$) (Fig.2). This suggests that leaves can uptake metal pollutants from ambient PM deposits in addition to soil. Indeed, agriculture lands in urban areas present a contamination risk through aerial deposition, as these areas are often directly in contact with roads and/or industrial emission (Pierart et al., 2015). Metal accumulation from soil-plant transfer (root uptake) are well documented by previous studies (Austruy et al. 2014; Foucault et al. 2013; Schreck et al. 2013). Atmosphere-plant transfer has also been reported via foliar uptake: CdO, SbO, ZnO, and Pb-PM were accumulated by cabbage and spinach leaves after foliar exposure (Xiong et al., 2014). There was significant foliar lead uptake by lettuce exposed to the atmospheric fallouts of a lead-recycling plant (Uzu et al., 2010). The approximate contributions of airborne $^{206}Pb/^{207}Pb$ into plant leaves were 72.2% and 65.1%, respectively for total suspended particulates and size-segregated aerosols, suggesting that airborne Pb is the most important source for the Pb accumulation in leaves (Hu et al. 2011). Furthermore, fine particles can enter directly into leaves by stomata apertures (Larue et al., 2014; Nair et al., 2010; Samaj et al., 2004; Terzaghi et al., 2013). The mechanisms involved in foliar uptake of various metal(loid)s from atmospheric PM fallout were investigated by Schreck et al. (2012). They reported that internalization through the cuticle or penetration through stomata openings are proposed as the two major mechanisms involved in foliar uptake of PM. Once internalized, metals are transported from the foliage to target organs in the phloem vascular system in a similar manner to photo-synthates.

In such sites, the quality of vegetables depends both on atmosphere and soil quality. When the soil is polluted, roots are mainly enriched with metals in consequence of the soil-plant transfer, but also can be enriched by translocation of metals from leaves. Metal concentration in leaves and roots are significantly related ($r=0.66$, $p<0.001$). Translocation from leaves to root were demonstrated by Xiong et al. (2014). When fine PM are present in the atmosphere, shoots are mainly enriched by atmosphere-leave transfer of metals.

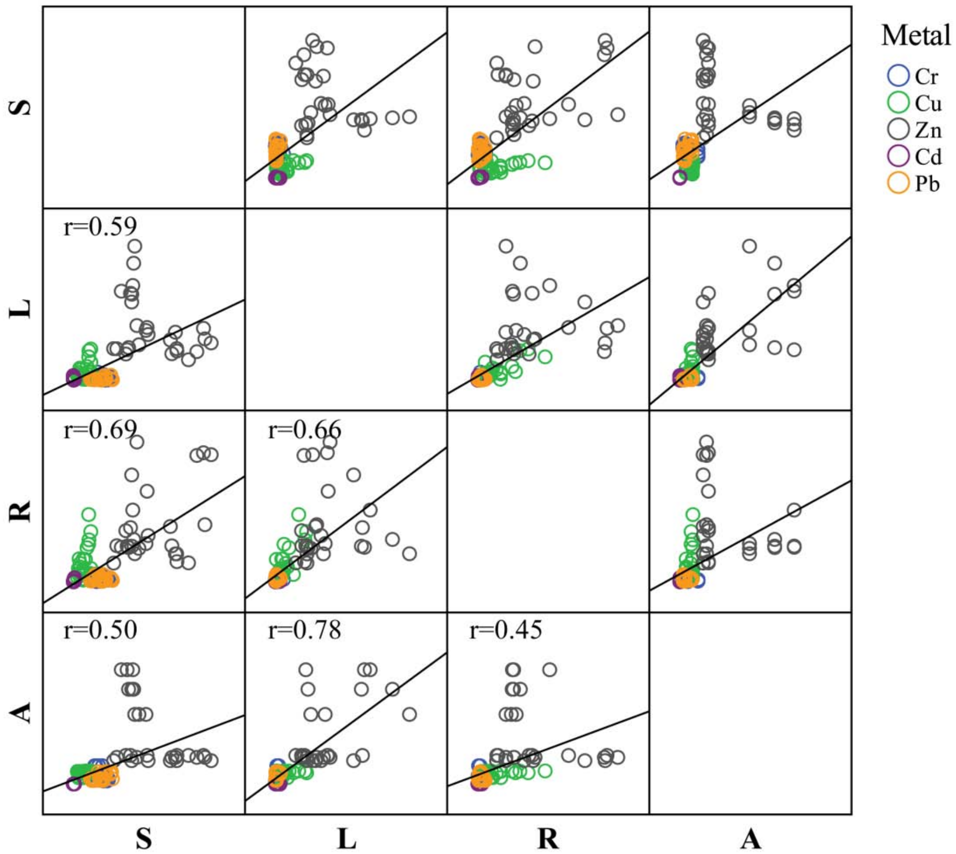


Fig. 2 Scatter plots of metal concentration in settled atmospheric PM (A), rhizosphere soil samples (S), leaves (L) and roots (R). "r" is the correlation coefficient between different variables.

5.3.4 In vitro bioaccessibility of metals and non-carcinogenic risk assessment

Fig.3 presents the bioaccessibility of studied metals after digestion process. No significant differences were observed among studied vegetables and between two sites ($p>0.05$). In comparison with other metals, Cd presents the highest bioaccesibility, with a mean of 79%, followed by Zn (76%), Pb (71%), Cr (64%) and Cu (60%). These results are in agreement with those of Mombo et al. (2015). They also observed that Cd bioaccessibility (66-85%) was higher in comparison to Pb (17-54%) for different vegetables (leek, lettuce, celery, carrot…). But, Pb bioaccessibility in present study (71%) is higher than reported (54%) by Mombo et al. (2015). This difference can be due to variation in Pb speciation induced both by pollution type (recycling factory for Mombo et al.) and bio-transformations inside the plants as a function of plant species (Uzu et al., 2014). One of the factors potentially affecting the bioaccessibility is also the level of contamination in the matrix considered (Guerin et al., 2015). Bioaccessible metals taking into account the total metal concentration in raw vegetables leaves. The bioaccessible concentrations of Cu, Zn Cd, and Pb in gastric phases increased significantly with increasing total concentration whatever is the studied vegetable (R^2=0.95 for Cu, R^2=0.73 for Zn, R^2=0.98 for Cd and R^2=0.73 for Pb respectively, p<0.05). However, the correlation was not found between Cr concentration in leaves and bioavailable parts. Indeed, other physic-chemical parameters also disturb the bioaccessibility during digestion process, including metal speciation changes (Uzu et al. 2011; Xiong et al. 2014).

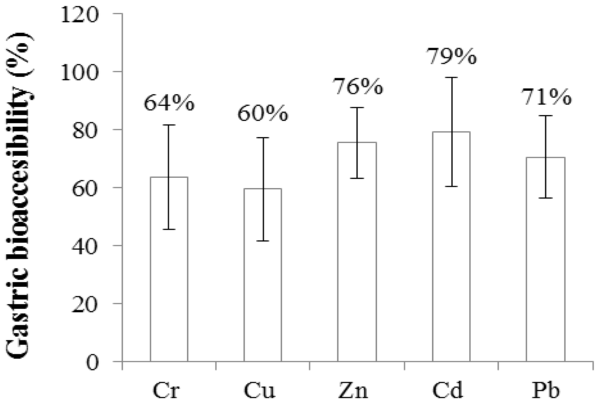


Fig. 3 Gastro-bioaccessibility of studied metals in vegetables (mean ± standard deviation).

Concerning risk assessment, the values of estimated daily intake (EDI), hazard quotient (HQ), and hazard index (HI) of metals in vegetable leaves are listed in Table 5. EDI ranges for adults in site A were, respectively within 1.46-3.8 (Cr), 2.9-8.38 (Cu), 21.80-61(Zn), 0.10-0.49 (Cd) and 0.23-0.66 (Pb) μg kg^{-1} day^{-1}, which did not exceed TDIs. EDI ranges for adults in site B were, respectively within 1-3.54 (Cr), 6.47-23.3 (Cu), 27.30-87.37(Zn), 0.39-2.79 (Cd) and 0.52-2.32 (Pb) μg kg^{-1} day^{-1}, with EDI of Cd in amaranth (2.79 μg kg^{-1} day^{-1}) exceed TDI of Cd (1 μg kg^{-1} day^{-1}). Similarly, the individual HQs of Cr, Cu, Zn, Cd and Pb for all vegetables are acceptable (<1) except that of Cd in amaranth (2.68>1). The trend of EDIs and HQs of vegetable is identical to that of metal concentration in vegetables. Consequently, HIs exceeded 1 in amaranth (3.77>1), and also in water spinach on site B (1.97>1) due to the high HQ of Cu, Cd and Pb. These results firstly highlight the site effect on human health risk, since site B (highway) represent a higher sanitary risk than site A. This is in agreement of the study of Pierart et al. (2015), when urban gardens or arable lands are set up in the direct vicinity of a road, long-term exposure could lead to an increased accumulation of metals in soils, leading to food-chain accumulation, human exposure and in the worst cases, disease. Secondly, these results suggested that health risk associated with metal(loid) exposure is significant only for some vegetables. However, we know that people eat generally a mixture of plants. Actually, in the present work we proposed scenarios exposure in relation with the plant species: it means its ability to absorb more (amaranth) or less (leaf lettuce, welsh onion) metals. The choice of cultivated plant is therefore a good strategy to manage urban agriculture in low or media polluted areas. In addition, we suggest that careful washing before vegetable ingestion can significantly reduce health risk. Our previous field studies on lettuce exposed to industrial PM also showed that the washing procedure removes up to 25 % of total lead-rich particles (Schreck et al. 2012).

Among studied metal(loid)s, HQ of Cd was the highest almost in all vegetables, followed by Pb. Consequently, HI was mainly composed of HQ of Cd (29-71%) and Pb (15-33%) despite their low concentration in vegetables. These results indicate a higher potential health risk of Cd and Pb compared to other metals. The choice of Pb, Cd, and Hg in European regulation for metals pollutant on vegetable is therefore pertinent from a scientific of view. The potential health risk of Cr is the lowest, which almost contribute nothing to hazards, this result may be mainly due to its high oral reference dose (Cr(III),1500 μg kg^{-1} day^{-1}). The TDI of Cr(III) was taken to represent that of Cr in this study because Cr(VI) can be reduced to Cr(III) under the acidic condition in the stomach (De Flora et al. 1997; Wang et al. 2011). The study of Wang et al. (2005) on health risk of metal via vegetable and fish consumption also reported a higher health risk of Cd and lowest risk of Cr in all vegetables.

The maximum allowable daily plant intake (MDI) (g day^{-1}, FW) without exceeding the TDI for the two sites are listed in Table 7 in SI. These values considerably vary between vegetables species. The maximum quantity of amaranth daily ingestion should not exceed 132 g. In addition, bitter lettuce from site A and water spinach from site B also have potential risk if maximum consumption of these vegetables per day exceeded 802 g and 541g, respectively. Therefore, in the aim of protecting local consumers from metal toxicity, they should be moderate while consuming these vegetable species.

Table 5 The values of (bioaccessible) estimated daily intake ((B)EDI), (bioaccessible) hazard quotient ((B)HQ) and (bioaccessible) hazard index ((B)HI) of metals in vegetable leaves. The values in bold indicate high health risk.

Vegetable			Cr	Cu	Zn	Cd	Pb	HI		Cr	Cu	Zn	Cd	Pb	BHI
Site A	Leaflettuce	EDI ($\mu g\ kg^{-1}\ day^{-1}$)	2.05	3.06	21.8	0.11	0.44		BEDI ($\mu g\ kg^{-1}\ day^{-1}$)	0.93	1.46	16	0.11	0.24	
		TQ	0.00	0.07	0.07	0.11	0.12	0.37	BTQ	0.00	0.03	0.05	0.10	0.07	0.26
	Water spinach	EDI	2.09	4.08	24.7	0.19	0.62		BEDI	1.70	1.98	15.7	0.15	0.41	
		TQ	0.00	0.10	0.08	0.18	0.17	0.53	BTQ	0.00	0.05	0.05	0.14	0.11	0.35
	Leafmustard	EDI	1.46	3.54	38.8	0.30	0.66		BEDI	1.17	1.59	29.3	0.24	0.40	
		TQ	0.00	0.08	0.12	0.29	0.18	0.68	BTQ	0.00	0.04	0.09	0.23	0.11	0.47
	Purslane	EDI	3.8	8.38	61.0	0.37	0.56		BEDI	1.97	4.78	45.3	0.29	0.35	
		TQ	0.00	0.20	0.19	0.36	0.15	0.91	BTQ	0.00	0.11	0.14	0.28	0.10	0.63
	Welshonion	EDI	1.92	2.90	32.6	0.10	0.23		BEDI	1.22	2.34	27.1	0.11	0.22	
		TQ	0.00	0.07	0.10	0.10	0.06	0.34	BTQ	0.00	0.06	0.09	0.10	0.06	0.31
	Bitter lettuce	EDI	1.64	4.03	32.7	0.49	0.45		BEDI	1.26	2.12	25.7	0.41	0.36	
		TQ	0.00	0.10	0.10	0.47	0.12	0.80	BTQ	0.00	0.05	0.08	0.39	0.10	0.62
Site B	Water spinach	EDI	3.54	23.3	27.3	0.66	2.32		BEDI	1.65	13.4	21.9	0.53	1.46	
		TQ	0.00	0.56	0.09	0.63	0.63	**1.91**	BTQ	0.00	0.32	0.07	0.51	0.40	**1.30**
	Welshonion	EDI	1.09	6.47	71.0	0.39	0.52		BEDI	0.70	3.50	49.7	0.23	0.31	
		TQ	0.00	0.16	0.23	0.38	0.14	0.90	BTQ	0.00	0.08	0.16	0.22	0.09	0.55
	Amaranth	EDI	1.46	9.50	87.4	**2.79**	2.12		BEDI	0.93	7.20	64.6	**1.96**	1.60	
		TQ	0.00	0.23	0.28	**2.68**	0.58	**3.77**	BTQ	0.00	0.17	0.21	**1.88**	0.44	**2.69**
TDI ($\mu g\ kg^{-1}\ day^{-1}$)			1500	40	300	1	3.5			1500	40	300	1	3.5	

The same trends but with lower values were observed for BEDI, BHQ, and BHI when comparing to EDI, HQ, and HI values of the consumed parts of vegetables (Table 5). Introduction of the bioaccessible metal concentration led to a decrease in calculated risk, which is in consistent with the previous reports on risk assessment with soil ingestion (Li et al. 2014; Luo et al. 2012; Pelfrêne et al. 2013), particles inhalation (Huang et al., 2014), and plant ingestion (Uzu et al., 2014). Indeed, a bioaccessibility adjustment factor may be necessary to accurately assess the potential health risks and avoid overestimation of the risk (Huang et al., 2014). Our results enhance the important of bioaccessible parts of metal with plant ingestion. Accordingly, a higher quantity of vegetable could be consumed daily according to the higher value of BMDI than MDI (Table 7 in SI).

5.4 Conclusions and perspectives

Finally, taking into account all the parameters involved, it can be concluded that sanitary risk linked to ingestion of these vegetables cultivated in urban areas (near incinerator or highway) is relatively low and can be easily decreased by pertinent plant species selection and efficient washing procedure. However, other organic or inorganic pollutants can have potential cumulative risk to human health. The consumption of other food stuffs (i.e. rice), water drinking, soil ingestion, re-suspended solid dusts and aerosol particles inhalation might also introduce pollutants to human body. The joint effect might be chronic to local residents. More attention should be paid to sensitive children (Beccaloni et al. 2013; Chang et al. 2014).

The concentration of Cr, Cd and Pb in studied vegetables partly exceeded the regulation set by China government and European Commission, may due to high metal concentrations in atmosphere PM. Moreover significant metals translocation between leaves and roots was observed, and metal accumulation capacity depended on vegetable species: water spinach accumulates Cu, bitter lettuce root accumulates Zn, and amaranth accumulates higher Cd than other plants. These results emphasize the need for long term monitoring in various urban areas. In particular, atmospheric regulation could gradually integrate new measures when standard methods will be available at reasonable cost and if public space is mobilized for better consideration of environmental and health aspects. Actually, the study of the accumulation of metal(loid)s in the leaves of vegetables grown in nearby factories that emit metals or urban areas with high population density is a crucial health issue. Soil parameters, vegetable species, pollutant nature, and even fertilization way can influence plant contamination. Further research both in field and greenhouses may be performed to better understand soil-plant-atmosphere transfer mechanisms. A communication effort with the population and training on environment and health subject is needed to reduce pollutants exposure. Participatory projects such as “Agriville network” (http://reseau-agriville.com/) have this goal in sharing knowledge related to urban cultures.

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Supporting information

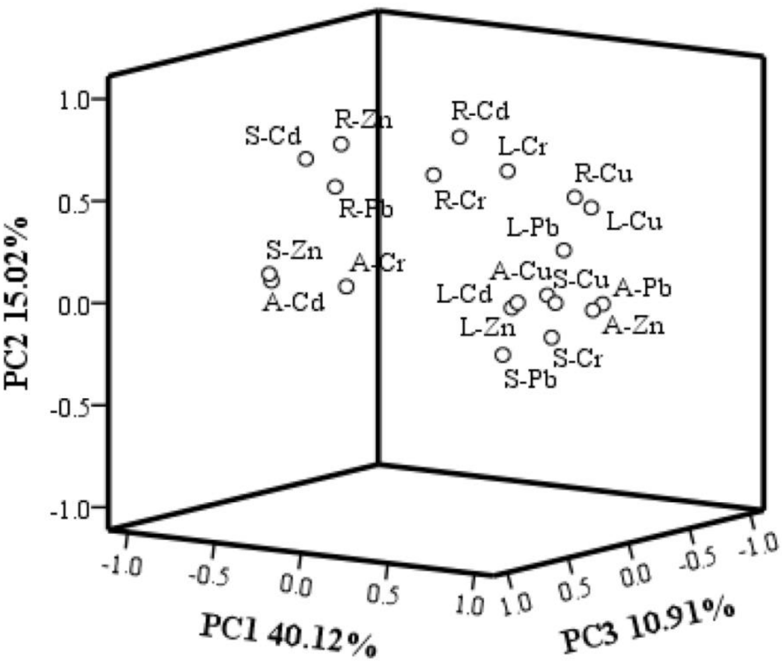

Fig. 4 PCA of metal contents in different media, including settled atmospheric PM (A), rhizosphere soil samples (S), leaves (L) and root (R).

Table 6 Site effects on measured metal concentration (mean value and range of 9 pseudo-replications) in settled atmospheric PM (mg kg^{-1} DW).

	Cr	Cu	Zn	Cd	Pb
Site A	417b[1] (293-650)	354a (231-449)	931a (831-1016)	6.3b (4.78-7.04)	168 a (131-188)
Site B	145 a (114-176)	444 b (404-467)	3289b (2466-4045)	2.9 a (2.60-3.34)	396 b (356-435)

[1]The different lowercase letters are the significant difference between different rhizosphere soil at p<0.05.

Table 7 Calculated (bioaccessible) maximum allowable daily plant intake((B)MDI) (g day-1, FW) without exceeding the TDI in the two sites.

	Vegetable	Maximum daily vegetable leaves consumption (g day^{-1})						Bioaccessible maximum daily vegetable leaves consumption (g day^{-1})					
		Cr	Cu	Zn	Cd	Pb	**Max**	Cr	Cu	Zn	Cd	Pb	**Max**
Site A	Leaflettuce	270853	4797	4902	3182	3012	**3012**	598941	10051	6673	3302	5477	**3302**
	Water spinach	250310	3422	4216	1819	1946	**1819**	306713	7063	6637	2377	2928	**2377**
	Leafmustard	413246	3909	2697	1139	1889	**1139**	517003	8682	3565	1458	3071	**1458**
	Purslane	217770	1703	1765	946	2259	**946**	417696	2987	2378	1223	3637	**1223**
	Welshonion	289448	7802	3311	6676	6126	**3311**	454734	9666	3980	6426	6521	**3980**
	Bitter lettuce	316464	3693	3361	802	2884	**802**	413950	7022	4278	965	3690	**965**
Site B	Water spinach	163463	601	3804	541	708	**541**	350476	1045	4741	669	1120	**669**
	Welshonion	3210254	6821	1652	1163	2342	**1163**	5030148	12608	2359	1976	3913	**1976**
	Amaranth	904671	1676	1228	132	820	**132**	1417529	2210	1662	188	1085	**188**

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Chapter 6 General conclusions and perspectives

Conclusions

Depuis plusieurs décennies, les industries et le trafic routier contribuent de manière significative aux émissions d'éléments traces métalliques dans l'atmosphère, devenant ainsi une menace importante pour la flore, la faune, et la santé humaine. Cependant, avec le développement de l'urbanisation et la crise économique mondiale, les populations ont la volonté de cultiver leur propre nourriture dans les jardins publics, associatifs, ou privés (Galt et al., 2014). En effet, avoir un jardin pour produire des légumes sains est l'un des objectifs souvent évoqué par les jardiniers urbains (Grard et al., 2015). Mais, ces zones sont souvent influencées par des routes et / ou industries (Pierart et al., 2015) . L'agriculture dans les zones urbaines et péri-urbaines est donc une opportunité mais sous réserve de gérer les pollutions. Par conséquent, cette thèse s'est focalisée sur l'agriculture urbaine (légumes potagers en particulier), et notamment les conséquences en termes de phytotoxicité et de santé humaine de la pollution due aux particules émises par une usine de recyclage du plomb, un incinérateur de déchets et le trafic routier urbain. Le transfert des PM, leurs impacts sur le sol, les plantes et les humains ont été étudiés en fonction des propriétés physico-chimiques des éléments considérés, de leur phytodisponibilité et de leur bioaccessibilité gastro-intestinale.

L'étude du transfert et de l'accumulation des métaux dans les feuilles des légumes cultivés à proximité de sites industriels qui émettent des métaux ou de zones urbaines à forte densité de population est une question cruciale de santé. L'évaluation des risques sanitaires nécessite à la fois la caractérisation des particules métalliques qui impactent ces plantes ainsi que l'étude du devenir des métaux dans/sur les feuilles des plantes potentiellement influencé par des transformations bio-physico-chimiques.

Durant ma thèse, j'ai étudié les risques environnementaux et sanitaires de PM atmosphériques enrichies en éléments polluants traces métalliques. L'objectif de la thèse était double:

1) Mieux cerner l'impact des métaux portés par ces PM sur les légumes consommés par les humains.

2) Évaluer le risque sanitaire avec des quotients de risque en utilisant les concentrations totales et bioaccessibles en métaux.

Plusieurs questions scientifiques étaient à l'origine de l'étude scientifique, une approche expérimentale progressive a permis de répondre à nos questions scientifiques, et de développer une méthode globale, pratique et transversale pour comprendre les incidences environnementales et sanitaires des métaux issus des PM. Les principaux résultats obtenus sont présentés ci-dessous.

► Dans l'expérience en conditions contrôlées, notre principal objectif était d'étudier la cinétique du transfert foliaire et la phytotoxicité du Pb et du Cu pour des particules fines de PbO et CuO et des légumes feuilles (laitue et chou) souvent cultivés à l'échelle mondiale. Les légumes peuvent accumuler après absorption foliaire (via les ostioles des stomates notamment) des quantités significatives de métaux apportés par des particules fines et ultrafines. La procédure de lavage pour simuler le scénario de l'ingestion humaine peut éliminer les contaminants de surface adsorbés sur les tissus foliaires. L'accumulation de Pb et Cu dans les légumes feuilles est positivement corrélée avec les quantités d'exposition et la durée, et une corrélation négative avec les paramètres morphologiques de la croissance des plantes. En fait, la phyto-toxicité du plomb peut diminuer la croissance végétale et la biomasse aérienne respectivement de 68% et 50%. La phytotoxicité du cuivre peut diminuer la croissance végétale et la biomasse aérienne respectivement de 51% et 59%. D'autres processus métaboliques (par exemple échange gazeux, profil foliaire en acides gras ...) pourraient également être affectés par la pollution métallique. Une translocation des métaux depuis les feuilles vers les racines a été observée. Les perturbations physiologiques étaient élevées, même au début de l'exposition indiquant la sensibilité des légumes aux métaux. Cependant, les résultats de phytotoxicité indiquent une adaptation des plantes ou des mécanismes de détoxification des métaux. Les deux métaux Pb et Cu ont une haute phytotoxicité pour les légumes (production des ROS). La cinétique de transfert des métaux et de la phytotoxicité par voie foliaire dépend de plusieurs paramètres, et la relation entre les quantités absorbées dans les plantes et la phytotoxicité est donc complexe (non linéaire). Les paramètres de croissance, les échanges gazeux et l'accumulation des métaux étaient différentes entre les deux espèces de légumes feuilles. En effet, cette étude met également en évidence l'influence des espèces végétales, certains mécanismes d'adaptation possibles, ainsi que des processus connexes.

Des particules ultrafines PbO (<10 um) et nano-CuO (<50 nm) ont provoqué des nécroses après interaction avec la surface des feuilles. Ces nécroses foliaires ainsi que des stomates déformés enrichis en agrégats de CuO ont été observés sur la surface de la feuille par SEM-EDX. Des changements de spéciation des métaux ont été observés par analyse RPE et des transformations bio-physico-chimiques semblent possibles. Le transfert des métaux à travers les stomates est également envisagé grâce à l'observation de nano-particules de CuO piégées dans des stomates.

Pris ensemble, ces résultats démontrent que le transfert foliaire est une voie significative pour l'accumulation de polluants, avec des changements morphologiques et physiologiques chez les plantes exposées, et sans lien simple entre l'accumulation de métal et la phytotoxicité. Nos résultats pourraient enfin être appliqués pour la biosurveillance de la pollution atmosphérique, et à l'évaluation de la contamination des légumes cultivés à proximité d'industries, dans des zones urbaines et des jardins potagers. Les populations vivant dans des zones industrielles ou urbaines exposées à des quantités élevées de particules atmosphériques enrichies en métaux peuvent donc être la cible de risques sanitaires si elles consomment des légumes pollués.

► L'ingestion de légumes est en effet une principale voie d'exposition des êtres humains aux polluants. Les concepts de bioaccessibilité et biodisponibilité orale sont fondamentalement importants pour quantifier les risques qui sont associés à l'exposition par voie orale à des contaminants environnementaux. Les résultats ont montré que la nature du métal influe fortement sur la bioaccessibilité mesurée: Cd présente une bioaccessibilité plus élevée en comparaison du Pb. Une translocation significative du Pb à partir des feuilles vers les racines a été observée dans le cas de l'exposition foliaire. En outre, la voie d'exposition influence considérablement la phytodisponibilité et la bioaccessibilité humaine des métaux, en particulier en relation avec des phénomènes contrastés impliqués dans la rhizosphère et la phyllosphère. Ce phénomène pourrait être dû à des changements dans la compartimentation et la spéciation des métaux selon le contexte de pollution. Par conséquent, ces résultats de thèse ont démontré l'importance de renseigner le contexte de pollution pour l'évaluation des risques environnement-santé.

Nous concluons que la bioaccessibilité pour l'homme des métaux varie avec le type de plante, le métal et les conditions d'exposition. Une bioaccessibilité humaine relativement élevée des métaux suggère un risque potentiel pour la santé en cas de consommation régulière de légumes contaminés. Les enquêtes de terrain sont donc indispensables dans les zones polluées où les gens cultivent des légumes, afin de mieux comprendre les scénarios d'exposition aux risques sanitaires. Les expériences réalisées pour diverses conditions de pollution pourraient aider à construire une base de données de valeurs de biodisponibilité de métaux dans les plantes.

► Les études de terrain ont été effectuées pour fournir des informations plus précises sur l'absorption de métaux par les plantes dans des scénarios réels. Nous avons étudié le devenir des métaux dans les systèmes sol-plante-atmosphère avec leurs conséquences sur l'exposition humaine dans diverses zones urbaines aux fortes densités de population. L'étude sur deux sites différents (à proximité d'un incinérateur de déchets et près de l'autoroute) a permis de conclure qu’il n’y avait pas de risque sérieux à proximité de l'incinérateur étudié, mais une exposition conjointe à de multiples polluants à proximité de sites industriels est possible. En outre, un jardin potager près d’une autoroute peut présenter un risque sanitaire certe modéré, mais non nul. Plusieurs stratégies peuvent permettre de réduire les risques en choisissant des espèces de plantes et en améliorant la procédure de lavage. Ces résultats soulignent l'importance d'enquêter sur les polluants dans l'environnement et l'évaluation des risques pour la santé. Il est donc nécessaire de contrôler la pollution, de communiquer avec toutes les parties, de prendre en compte l'anxiété du public et surtout de donner des préconisations pour réduire l'exposition humaine. Ces résultats soulignent la nécessité d'une surveillance sur le long terme des différentes zones urbaines afin de favoriser le développement des villes durables. En particulier, la réglementation concernant la qualité de l’air pourrait intégrer progressivement de nouvelles mesures, lorsque les méthodes standards seront disponibles à un coût raisonnable et si l'espace public est mobilisé pour une meilleure considération des aspects environnement-santé. D’autre part, nos résultats seront utiles pour favoriser la transition écologique actuellement initialisée en Chine afin de concilier objectifs économiques et santé publique.

En conclusion, les recherches développées durant ma thèse apportent des données à la fois pour la communauté scientifique et la société (gestion durable de la pollution). Pour l'étude scientifique, les différents sites d'exposition ont été comparés, les mécanismes de transfert des métaux dans le système sol-plante-atmosphère ont été étudiés. Pour la société, notre étude incite l'espace public à tenir compte du risque environnement-santé (sécurité alimentaire) via la culture raisonnée de légumes : choix des espèces végétales afin d'éviter les espèces qui accumulent; conseils sur la quantité maximum quotidienne de légumes pouvant être ingérés sans dépasser la valeur de seuil de toxicité, etc. Ces résultats peuvent également donner une impulsion et une orientation en matière de santé pour le travail des urbanistes et des professionnels impliqués dans la protection de l'environnement. Le but ultime est de proposer un suivi de la réglementation pour les industries et les activités de l'agriculture, ainsi que l'amélioration des techniques et procédures pour réduire les risques associés à la pollution de l'atmosphère. Cette thèse est donc utile pour la gestion durable des agricultures urbaines et la qualité des légumes cultivés dans les mégapoles à l'échelle mondiale.

Perspectives

1) Ces travaux peuvent être poursuivis avec différents nano polluants. La bio-tansformation semble influencer la spéciation des métaux et leur bioaccessibilité dans les légumes. Il est nécessaire de bien comprendre ce phénomène. En outre, la taille des PM influence l'accumulation foliaire de métaux. Cependant, l’impact de la taille des PM et de la concentration en métaux sur la bioaccessibilité reste complexe.

2) Pour l'évaluation des risques sanitaires, en plus de l’ingestion, la consommation d'autres produits alimentaires (riz par ex.), l'eau, l'ingestion de sol, l'absorption cutanée, la remise en suspension des poussières solides et des particules d'aérosols (inhalation) également introduire des polluants pour le corps humain (Wang et al., 2011). D'autres polluants (polluants organiques, des gaz toxiques ...) sauf en métal conduisent également à risque pour la santé humaine. Une prise en compte plus globale des risques encourus est à envisager.

3) Les effets sur la santé des PM sont encore mal compris, mais une hypothèse est que la plupart des effets néfastes constatés découleraient d’un stress oxydatif, initié par la formation d'espèces réactives de l'oxygène (ROS) dans les cellules affectées (Cho et al., 2005). Les études épidémiologiques dans les zones urbaines ont lié l'augmentation des pathologies respiratoires et cardiovasculaires avec des doses de PM provenant des activités anthropiques (Uzu et al., 2011a). De plus, l'inhalation est une voie importante pour l'homme s’il est exposé à des PM. Le potentiel intrinsèque oxydatif des PM pourrait dès lors être testé par le dithiothréitol (DTT) qui est lié à la production des ROS (Rattanavaraha et al, 2011). Le taux de consommation de DTT est proportionnel à la concentration de l'espèce redox-actif catalytiquement actifs dans l'échantillon; cette analyse cinétique a été appliquée aux gaz d'échappement diesel et échantillons de particules (Rattanavaraha et al., 2011). Cho et al.

(2005) ont démontré que le dosage de DTT peut fournir une bonne mesure de l'activité d'oxydo-réduction de particules en déterminant la formation de radicaux super-oxydes. Malgré de récents progrès dans l'analyse ROS, les composants d'aérosols provoquant la formation de ROS restent peu clairs.

4) Pour le développement et la gestion d’une agriculture durable, il apparait important de renforcer la réglementation et de mettre en place les systèmes d'information géographique (SIG). En outre, les techniques d'assainissement sur le terrain peu coûteuses sont nécessaires pour permettre la culture de récoltes de qualité utilisées pour la consommation humaine. Améliorer les sols cultivés et la qualité des cultures est une priorité forte du Ministère de la protection de l'environnement en Chine (2014). En effet, environ 20% des sols agricoles présentent des problèmes de pollution, dont 7% concernent la pollution en Cd. L'assainissement des sols pollués est aussi nécessaire. Par exemple, le PR Zhian Li du jardin botanique de l'Académie des Sciences de Chine (Guangzhou, Chine) développe une technique d’amélioration synergique de la qualité des récoltes des terres agricoles par la combinaison (wollastonite qui immobilise le cadmium) et fertilisation avec des micronutriments. Enfin, réduire la bioccessibilité métallique à l'aide d’une alimentaire spéciale (telles que les fibres) pourrait aussi être envisagée afin de réduire l'exposition humaine aux polluants.

Conclusions

Since several decenies, industries and traffic significantly contribute to the emission of metal trace elements and became an important threat for both flora, fauna, and human health (Tubek and Tubek, 2008). However, with the development of urbanization and the worldwide economic crisis, people are showing a clear desire to grow their own food in public, associative, or kitchen gardens (Galt et al., 2014). Indeed, having a garden to produce healthy vegetables is one of the objectives highlighted by urban gardeners (Grard et al., 2015). But, these areas are often directly in contact with roads and/or industries (Pierart et al., 2015), the agriculture in urban and peri-urban areas were then threatened both by lessened agricultural land size and reduced production quality. Therefore, this thesis focused on urban agriculture (vegetable gardens) pollution due to particles emitted by lead recycling factory, waster incinerator, and urban road traffic, as well as their transfer and impacts on soil, plants, and human inrelated to their physic-chemical properties, particle size, phytodisposability, phytobioavailability, and bioaccessibility.

The study of the **accumulation and transfer of metals in the leaves of vegetables** grown in nearby factories that emit metals or urban areas with high population density is a crucial health issue. The health risk assessment requires both the **characterization of metal particles** falling near these plants and also the study of the fate of metals in / on plant leaves influenced by possible **bio-physicochemical transformations**.

During my PhD, I investigated the environmental and health risks of PM atmosphere enriched with pollutants. The objective was twofold:

1) Assess the impact of metal(loid)s from PM on vegetables consumed by humans.

2) Evaluate the health risk with hazard quotient of the total and bioaccessible metal concentration.

Several scientific questions were at the origin of the scientific study. A gradual experimental approach has allowed to answer our scientific questions, and to develop a comprehensive, practical, and transversal approach to understand environmental and health impacts of metal(loid)s by atmosphere deposits. The principal results obtained are present below.

► In control experiment, **our main objective** was to study in controlled conditions the kinetic of foliar transfer and Pb/Cu phytotoxicity in the case of foliar exposure to PbO/CuO fine particles for leafy vegetables (lettuce and cabbage). Vegetables can accumulate significant **metal(loid)s by foliar uptake**, fine and ultrafine particles can enter directly into leaves by stomata apertures. **Washing procedure** to simulate human ingestion scenario can remove adherent surface contaminants from leaf tissues. Pb and Cu accumulation in leafy vegetables **correlates positively** with exposure quantities and duration, and negatively correlates with morphological parameters of plant growth. Actually, Pb **phytotoxixity** can decrease the vegetable shoot growth and aerial biomass respectively by 68 % and 50%, Cu phytotoxixity can decrease the vegetable shoot growth and aerial biomass respectively by 51 % and 59 %, other metabolic process (i.e. gaseous exchange, fatty acid composition, etc.) also could be affected by metal pollution. Sinificant translocation from leaves towards roots was observed. Physiological perturbations were **high even at the beginning of the exposure,** which indicates the **sensitivity** of vegetable to metal treatment. However, phytotoxicity results indicated **plant adaptation or detoxification mechanisms** to Pb/Cu foliar exposure with increased exposure duration. Both Pb and Cu have high toxicity to vegetables, even low Cu exposure elevated plant growth. Both their toxicity were supposed by related to **ROS** generation. The **kinetics of metal** transfer and **phytotoxicity** through foliar pathway depended on several parameters and the relationship between absorbed quantities in plants and phytotoxicity is therefore **complex** (not always linear). Growth parameters, gaseous exchanges and metal accumulation were different between the two leafy vegetables. Actually, this study also highlights the influence of **plant species**, some possible adaptation mechanisms as well as the related processes.

Ultrafine PbO (<10μm) and nano-CuO (<50nm) particles caused **necrosis** after interaction with leaf surface. Actually, leaf necrosis and deformed stomata enriched with CuO PM aggregation were observed on leaf surface by SEM-EDX analysis. Metal speciation changes were observed by EPR analysis under possible bio-physicochemical transformations process. The possible pathway of metal transfer through stomata is also suggested, since nano-CuO trapped within stomata was observed.

Taken together, these results demonstrate that **foliar transfer is a momentous pathway** for pollutant accumulation, with both **morphological and physiological changes** in exposed plants, and without simple link between **metal accumulation and phytotoxicity**. Our results could finally be applied for **biomonitoring atmospheric pollution**, and assessing vegetable contamination cultivated near industries, in urban areas and kitchen gardens. Populations living in industrial or urban areas exposed to high atmospheric quantities of PM enriched with metal(loid)s can therefore incur sanitary risks when they consume polluted vegetables.

► Ingestion of vegetables is the main route for human exposed to metal(loid)s pollutants. The concepts of bioaccessibility and oral bioavailability are fundamentally important for quantifying the risks that are associated with oral exposure to environmental contaminants. We firstly investigate the process PM and manufactured mono-metallic particles (Cd, Sb, Zn, and Pb) interaction with leaves of vegetables that commonly cultivated in kitchen gardens (cabbage and spinach), examined whether foliar uptake varied depending on the nature/sources of the metal(loid) and determined the amount of pollutants that could be ingested by humans through vegetable consumption. This result found that a maximum of 2% of the leaf surfaces were covered with the PM, particles appeared to be enriched in stomatal openings, with up to 12% of their area occupied, which suggesting stomata is a preferential uptake pathway. The gastric bioaccessibility of metal(loid)s were significantly **higher in vegetables** and soil (certainly due to chemical speciation changes) than in PM sources and depends on the nature of the pollutant with **Cd being particularly bioavailable**.

We compared Pb and Cd human bioaccessibility of vegetable exposed to ultrafine particles respectively under **foliar and root pathways**. Results also showed that **metal nature** strongly influences bioaccessibility: **Cd** presented higher bioaccessibility in comparison to Pb. A significant **translocation** of Pb from leaves towards the roots was observed in the case of foliar exposure. Moreover, the way of exposure significantly influenced the phytoavailability and human bioaccessibility of metals especially in relation with contrasted phenomena involved in rhizosphere and phyllosphere. This phenomenon could be due to changes in metal compartmentalization and speciation under different modes of metal uptake. Therefore, this report highlights the conditions of plant exposure that must be taken into account for environmental and sanitary risks assessment.

We conclude that human bioaccessibility of metals varies with the type of plant, metal, and exposure conditions. The relatively high metal human bioaccessibility was measured suggesting a potential health risk in the case of regular consumption of contaminated vegetables. Field surveys are therefore essential in polluted areas where people cultivate vegetables, in order to better understand the scenarios of human risk exposure to metals. Experiments performed under different levels of soil and air pollution could help to build a database of bioavailability values of metal in plants used in the context of health risk assessments.

► Field studies were performed to provide more accurate information on the uptake of metals by plants in real scenarios. We investigated the fate of metals in soil-plant-atmosphere systems with their consequences on human exposure in various urban areas. The study with two different sites (near a waste incinerator and near a highway) led to the following conclusions: no serious risk near the studied incinerator with respect to human consumption quality of the investigated vegetables, but the additive exposure to different pollutants is often possible near industrial sites. Moreover, the vegetable garden near the highway presented a moderate sanitary risk. Since local residences must to live in that area, several available strategies should be developed to manage the plants with sustainable way: reduce risk by selecting **plant species** and improve **washing procedure**.

These results highlight the importance of investigating pollutant in environment and assessing health risk. It is therefore necessary to control the pollution, communicate with all stakeholders, address public anxiety and give advices to reduce human exposure. These results emphasize the need for long term monitoring on various urban areas for sustainable development. In particular, atmospheric regulation could gradually integrate new measures when standard methods will be available at reasonable cost and if public space is mobilized for better consideration of environmental and health aspects. Our results are involved in the ecological transition currently initialized in China to reconcile economic goal and public health.

►In conclusion, the researches developed during my PhD carry scientific data both for scientific community and society (sustainable management of pollution) (Figure 1). For scientific study, different exposure sites were compared, the mechanisms of metal transfer in the soil-plant-atmosphere system had been studied, environmental and health risk of PM had been assessed. Therefore bio-monitoring of atmosphere pollution with the sensitive parameters of vegetable is possible with advantages of long-term monitoring of pollution and easy sampling without the use of expensive equipment. For human beings, our study can mobilize public space to take account of the environmental and health risk (food security) with vegetable culture, help famers and gardeners to choose vegetable species in order to avoid hyper accumulation species, and give advice on the maximum amount of daily vegetable ingestion without exceeding the toxicity threshold value. This results/information can also provide health-related impetus and guidance for the work of urban planners and those involved in environment protection. The ultimate purpose is to propose regulation monitoring for industries and agriculture activities, as well as improving techniques and procedure to reduce the risks associated to the atmosphere pollution. This thesis is important for sustainable management of urban agricultures and quality of vegetables cultivated in megacities at the global scale.

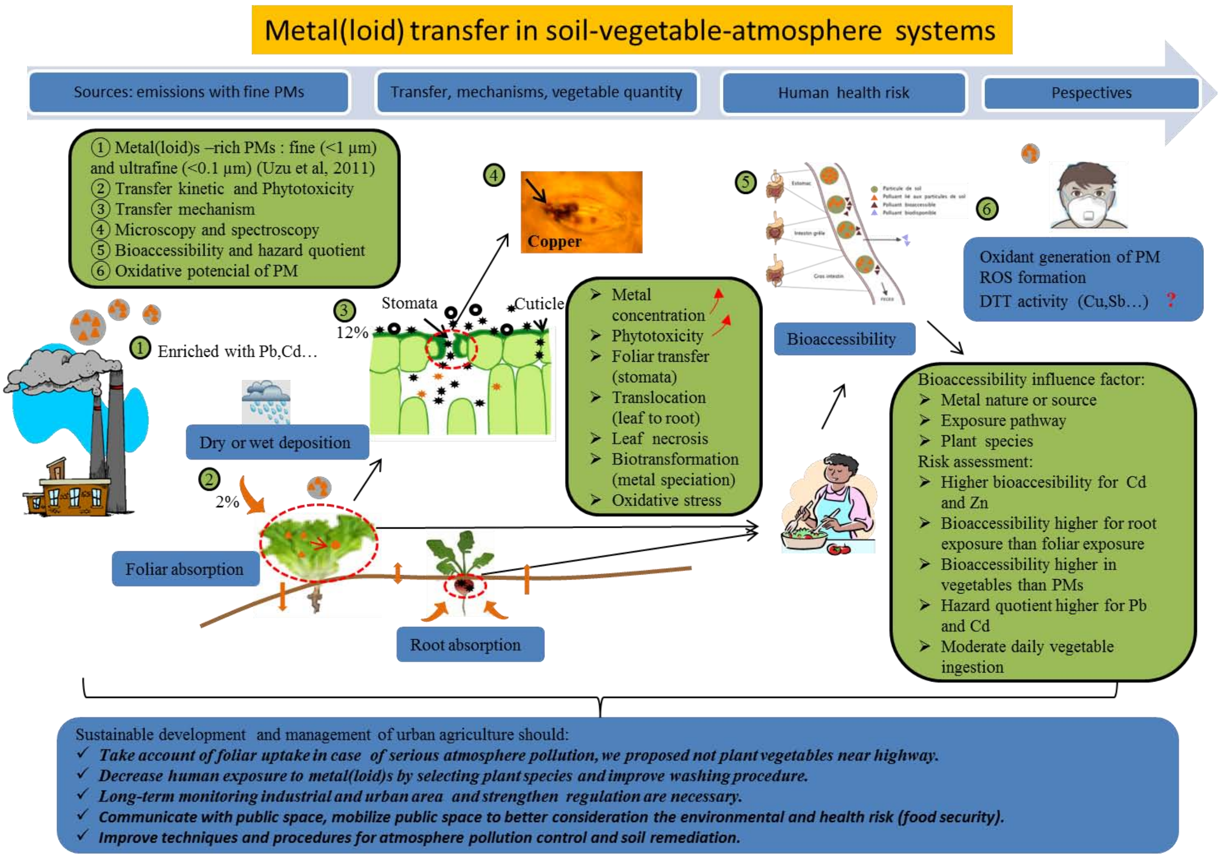

Figure 1 Metal(loid) transfers through plants, soils, and humans : a global approach using various tools to assess their toxicity and bioaccessibility.

Perspectives

Work will continue with both persistent pollutants (historical pollution) and nano particles emerging pollutants.

1) Bio-tansformation seems to influence metal speciation and further impacts metal bioaccessibility of vegetables. It is nessessary to better understand this phenomenon. Moreover, PM size influences the metal foliar accumulation. The impacts of PM size and metal concentration to metal bioaccessibility are not clear yet, and would deserve further investigation.

2) For health assessment, except vegetable ingestion, the consumption of other food stuffs (i.e. rice), water drinking, soil ingestion, dermal absorption, re-suspended solid dusts and aerosol particles inhalation also introduce pollutants to human body (Wang et al., 2011). Other pollutants (organic pollutants, toxic gas…) except metal also lead to health risk to human beings. Complementary study in the future work is then important for mimic more realistic human exposure scenario.

3) PM-related health effects are still incompletely understood. A hypothesis under investigation is that many of the adverse health effects may derive from oxidative stress, initiated by the formation of reactive oxygen species (ROS) within affected cells (Cho et al., 2005). Epidemiological studies in urban areas have linked increasing respiratory and cardiovascular pathologies with atmospheric particulate matter (PM) from anthropic activities (Uzu et al., 2011a). Since inhalation is an important route for human exposed to PM, therefore, to continue this research, we will establish method of health risk assessment with PM inhalation. The Intrinsic **Oxidative Potential the PM** can be test by dithiothreitol (DTT) assay. The dithiothreitol (DTT)-based chemical reactivity is considered a quantitative method for the assessment of the capacity of a PM sample to catalyze ROS generation (Rattanavaraha et al., 2011; Uzu et al., 2011a). In this assay, redox-active chemicals in PM oxidize DTT to its disulfide form and the linear rate of DTT loss is used as a measure of the oxidative capacity of the PM. The rate of DTT consumption is proportional to the concentration of the catalytically active redox-active species in the sample (Figure 2); this kinetic analysis has been applied to diesel exhaust and PM samples (Rattanavaraha et al., 2011). Cho et al. (2005) demonstrated that the DTT assay can provide a good measure of the redox activity of particles by

determining superoxide radical formation. Despite recent advancements in ROS analysis, the aerosol components causing the formation of ROS remain unclear. Therefore, the study of metal(loid) enriched PM would help to find the major driving forces behind for ROS formation.

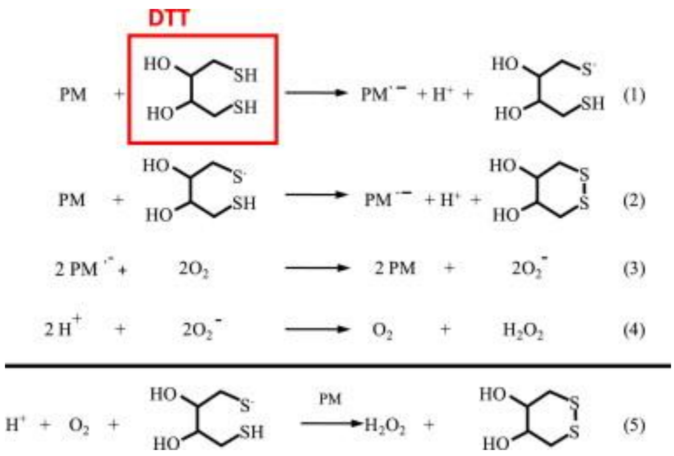

Figure 2 Chemical reaction between DTT and oxygen with PM as a catalyst (Rattanavaraha et al., 2011).

Therefore, the pulmonary toxicity and gastric bioavailability of metal enriched PM will be studied respectively with DTT assay and bioacccessibility. Actually, we already started to research the oxidive potential of emissions from diesel exhaust and wood burn with the modified DTT method that we developed (see Appendix). We found that PM from both diesel exhaust and wood burn emissions have high oxidative potential (high DTT activity), and DTT activity of wood burn emissions is higher that that of diesel exhaust.

4) For sustainable agriculture development, regulation of contaminants needs to be renforced for soil, atmosphere, and foodstuffs, and set up geographic information system (GIS) for sustainable management can be developed. Moreover, efficient and low cost field remediation techniques are needed to permit the culture of quality crops used for human consumption. Improve cultivated soils and crop quality is a strong priority of the Ministry of Environmental Protection in China (2014) as around 20% of farmland soils present pollution problems, with 7% concerning Cd. For instance, PR Zhian Li from South China Botanical Garden, Chinese Academy of Sciences (Guangzhou, China) has developed synergistic improvement of farmland crops quality by combination wollastonite cadmium immobilization and micronutrient fertilization. Finally, reduce metal bioccessibility using special food (such

as fibers) could also reduce human exposure to pollutants in the case of low or media polluted cultivated soils not yet treated for decontamination.

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Appendix

DTT Assay - Human health risks due to PM inhalation (oxidative potential of PM)

Since several years, there is an increasing concern on human health associate with ambient particulate matters (PM) (Cho et al., 2005; Janssen et al., 2014; Li et al., 2009; Uzu et al., 2011a; Yang et al., 2014).

1) Principle. The DTT-based chemical reactivity is considered a quantitative method for the assessment of the capacity of a PM sample to catalyze reactive oxygen species (ROS) generation (Rattanavaraha et al., 2011) , it provides a measure of the intrinsic capacity of particles to catalyze electron transfer between reducing DTT (DTT E_0 = -0.33 V) and oxygen (Figure 1) (Uzu et al., 2011). The electron transfer is monitored by the rate at which DTT is consumed under a standardized set of conditions, and the rate is proportional to the concentration of the catalytically active redox-active species in the PM sample (Rattanavaraha et al., 2011).

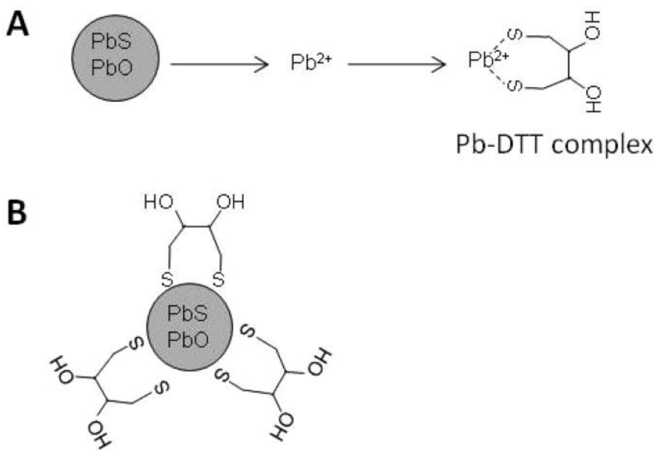

Figure 1 Potential mechanisms of DTT stabilization through complexation with solubilized lead (A), or via PM surfaces (B) (Uzu et al., 2011).

Kumagai et al. (2002) have shown that redox-active compounds catalyze the reduction of oxygen to superoxide by DTT, which is oxidized to its disulfide. The remaining thiol is allowed to react with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), generating the mixed disulfide and 5-mercapto-2-nitrobenzoic acid, which is determined by its absorption at 412 nm (Figure 2).

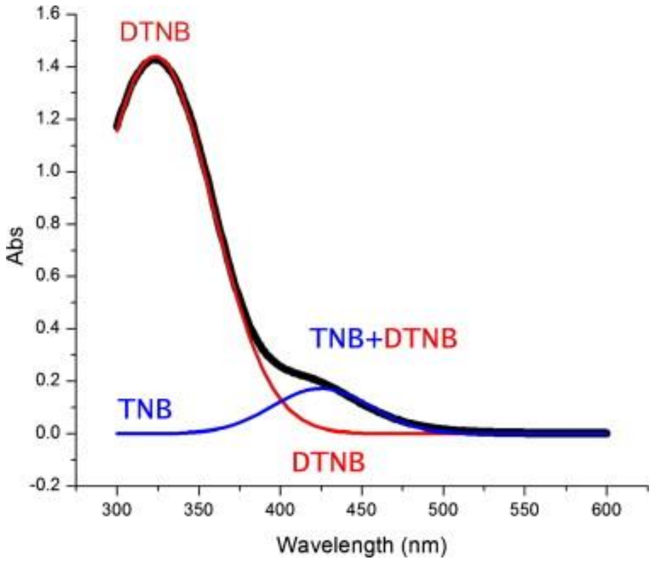

Figure 2 An example of UV–VIS absorption spectrum. Spectra were recorded using a Hitachi U-3300 spectrophotometer. TNB (2-nitro-5-thiobenzoic acid) is determined by its absorption at 412 nm (Rattanavaraha et al., 2011).

2) Experimental procedure

The chemicals used for DTT assay are listed in Table 1. Dithiothreitol (DTT), 5,5'-Dithio-bis-(2-nitrobenzoic Acid)(DTNB), 1,4-Naphthoquinone (1,4-NQ) and ethylenediaminetetraacetic acid (EDTA) were purchased from sigma (St.Louis, MO). All other reagents were of the highest grade available.

Table 1 Chemicals for DTT assay

Chemical name	Molecular formula	CAS NO.	MW (g mol^{-1})
Potassium hydrogen phosphate	K_2HPO_4	7758-11-4	174.18
Potassium dihydrogen phosphate	KH_2PO_4	7778-77-0	136.09
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	60-00-4	292.24
DL-Dithiothreitol (DTT)	$C_4H_{10}O_2S_2$	3483-12-3	154.25
5,5′-Dithio-bis-(2-nitrobenzoic Acid) (DTNB)	$C_{14}H_8N_2O_8S_2$	69-78-3	396.36
1,4-Naphthoquinone (1,4-NQ)	$C_{10}H_6O_2$	130-15-4	158.1534

Notes: All the chemicals used were obtained from commercial sources and were of the highest grade available.

To prepare the stock solution of particle samples, first, 1mg of sample is weighed respectively and added to 10 mL high purity water (18MΩ), then samples are sonicated 15-60 min with Hi-Volume impactor. Therefore a stock suspension of 100 μg ml^{-1} PM is prepared to use for DTT assay. The standard procedure of DTT method for particle samples is modified from Li et al. (2009): first, 1.0 ml of 0.1 M potassium phosphate (pH =7.3, containing 1 mM EDTA) was added to a 1.5 ml test tube (24 well plates) , each sample are prepared triplicate (Table 2A); second, 50 μl of 0.5 mM DTT were added to the test tube; then, 25–200 μl of PM extraction solution were added to the mixture (or external standard: 25 μl of 0.01 mg ml^{-1} 1,4-NQ). After mixing the mixture very well and incubated the test tube at 37 °C for designated duration (15 min, 30 min, 45 min and 60 min), 100 μl of 1.0 mM DTNB were added to the reaction solution and the absorption (Abs) at 412 nm was measured.

For PM collected through filters (teflon and quartz filter), PM samples (1/4 filter) or 1,4-NQ (25μL, 0.1g L^{-1}) were directly incubated with DTT (3mL, 60μm) at 37 ˚C (6 well plates), each sample is prepared on triplicate (Table 2B) . A 3mL of DTT is necessary for completely immersing 1/4 filter. No extraction was performed previously, while during the reaction shake or sonication with an ultrasonic bath was performed to loosen the particle filter sufficiently. At designated time points (15 min, 30 min, 45 min and 60 min), filters were removed from the incubation mixture and the reaction was terminated by the injection of DTNB (50μL, 10mM). The absorbance of the formed 2-nitro-5-thiobenzoic acid (TNB) was measured at 412 nm by Tecan's reader control and data reduction software (infinite F200 PRO TECAN). Actually, to acquire the real absorption of the remaining DTT after the catalytic redox reaction (Abs'), the absorbance at 412 nm should be measured before and after the injection of DTNB, the first measurement is intrinsic absorbance of samples (Abs_i), the second measurement is the final absorbance of all the elements (Abs), therefore Abs' can be express as: Abs'= Abs-Abs_i. In the test, a linear regression of DTT consumption against time can be established.

Table 2 Samples in 24 well (A) and 6 well (B) plates.

A

Bc 1	Bc 2	Bc 3	PC 1	PC 2	PC 3
Sa 1	Sa 2	Sa 3	Sb 1	Sb 2	Sb 3
Sc 1	Sc 2	Sc 3	Sd 1	Sd 2	Sd 3
Se 1	Se 2	Se 3	Sf 1	Sf 2	Sf 3

B

¼ fliter	¼ fliter	¼ fliter
Bc1	Bc1	Bc1

Notes: Bc :blank control; Pc: positive control; S: sample.

3) Data calculation and analysis

Residual DTT, DTT activity and normalized index of oxidant generation and toxicity (NIOG) were used for assessing the oxidative potential of PM in this study.

Residual DTT (nmol) was used to illustrate the remaining DTT after the catalytic redox reaction, a decrease tendency of residual DTT shows an increased DTT consumption.

Residual DTT=Total DTT amount$\times \frac{Abs'}{Abs_0}$, Where Abs_0 is the absorbance due to DTT added in a blank sample. Abs' is the absorbance of the formed TNB as mentioned before.

DTT activity (nmol min^{-1} μg^{-1}) is expressed as the rate of DTT consumption per minute per microgram of sample (Rattanavaraha et al., 2011), based on the average of triplicate readings, resulting in a value expressed as nmol DTT min^{-1} per μg PM.

DTT activity= $\frac{\text{DTT consumption}}{\text{t} \times \text{m}}$, where DTT consumption= Total DTT amount$\times \frac{Abs_0 - Abs'}{Abs_0}$, t is the reaction time (min), m is the PM mass (μg).

In order to standardize reported data obtained from the DTT method, **NIOG** as standardized unit is also presented. According to (Li et al. 2009), NIOG is expressed as the percentage of abs decrease ((Abs_0−Abs′)/Abs_0*100) per minute (t) and per microgram of sample (m), and then normalized by the index of oxidation generation and toxicity (IOG) of 1,4 NQ ($IOG_{1,4-NQ}$), which is used as the external standard:

$$NIOG_{sample} = \frac{IOG_{sample}}{IOG_{1,4-NQ}}, \text{ where } IOG = \frac{Abs_0 - Abs'}{Abs_0} \times \frac{100}{t \times m}.$$

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- Mission to the Austrian Institute of Technology (Austria, in August 2013) for microorganisms analysis of soil (trainning in microbiology).

- Mission to Incheou INHA University (Korea, in 6-14 August 2014) for the analysis of the roots and rhizosphere soils, financing by PHC STAR programme.

- Mission to South China Normal University (China, in 20-29 August and 11-18 December 2014) for the evaluation of pollutant (Cr, Cu, Zn, Cd, Pb) near a waste incinerator, quality analysis of plants consumed around an incinerator, financing by PHC Xu Guangqi programme.

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ORIGINAL PAPER

Foliar uptake and metal(loid) bioaccessibility in vegetables exposed to particulate matter

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Abstract At the global scale, high concentrations of particulate matter (PM) enriched with metal(loid)s are currently observed in the atmosphere of urban areas. Foliar lead uptake was demonstrated for vegetables exposed to airborne PM. Our main objective here was to highlight the health risk associated with the consumption of vegetables exposed to foliar deposits of PM enriched with the various metal(loid)s frequently observed in the atmosphere of urban areas (Cd, Sb, Zn and Pb). Leaves of mature cabbage and spinach were exposed to manufactured mono-metallic oxide particles (CdO, Sb_2O_3 and ZnO) or to complex process PM mainly enriched with lead. Total and bioaccessible metal(loid) concentrations were then measured for polluted vegetables and the various PM used as sources. Finally, scanning electronic microscopy coupled with energy dispersive X-ray microanalysis was used to study PM–phyllosphere interactions. High quantities of Cd, Sb, Zn and Pb were taken up by the plant leaves. These levels depended on both the plant species and nature of the PM, highlighting the interest of acquiring data for different plants and sources of exposure in order to better identify and manage health risks. A maximum of 2 % of the leaf surfaces were covered with the PM. However, particles appeared to be enriched in stomatal openings, with up to 12 % of their area occupied. Metal(loid) bioaccessibility was significantly higher for vegetables compared to PM sources, certainly due to chemical speciation changes. Taken together, these results confirm the importance of taking atmospheric PM into account when assessing the health risks associated with ingestion of vegetables grown in urban vegetable crops or kitchen gardens.

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Lead and Cadmium Phytoavailability and Human Bioaccessibility for Vegetables Exposed to Soil or Atmospheric Pollution by Process Ultrafine Particles

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Abstract

When plants are exposed to airborne particles, they can accumulate metals in their edible portions through root or foliar transfer. There is a lack of knowledge on the influence of plant exposure conditions on human bioaccessibility of metals, which is of particular concern with the increase in urban gardening activities. Lettuce, radish, and parsley were exposed to metal-rich ultrafine particles from a recycling factory via field atmospheric fallouts or polluted soil. Total lead (Pb) and cadmium (Cd) concentrations in of the edible plant parts and their human bioaccessibility were measured, and Pb translocation through the plants was studied using Pb isotopic analysis. The Pb and Cd bioaccessibility measured for consumed parts of the different polluted plants was significantly higher for root exposure (70% for Pb and 89% for Cd in lettuce) in comparison to foliar exposure (40% for Pb and 69% for Cd in lettuce). The difference in metal bioaccessibility could be linked to the metal compartmentalization and speciation changes in relation to exposure conditions. Metal nature strongly influences the measured bioaccessibility: Cd presents higher bioaccessibility in comparison to Pb. In the case of foliar exposure, a significant translocation of Pb from leaves toward the roots was observed. To conclude, the type of pollutant and the method of exposure significantly influences the phytoavailability and human bioaccessibility of metals, especially in relation to the contrasting phenomena involved in the rhizosphere and phyllosphere. The conditions of plant exposure must therefore be taken into account for environmental and health risk assessment.

At the global scale, atmospheric pollution by particulate matter (PM) with different origins, size distribution, and chemical composition is observed (Salma et al., 2005; Barima et al., 2014). The presence of highly reactive ultrafine particles in urban areas can induce environmental and health risks (Knibbs and Morawska, 2012; Marris et al., 2012; Austruy et al., 2014). Moreover, inorganic elements, such as Pb, Cd, As, Cr, and Cu, that are mainly emitted from anthropogenic processes at high temperature (e.g., metallurgy, refining, and engine combustion) tend to accumulate preferentially in these ultrafine particles (Hieu and Lee, 2010).

Studies in urban areas in the immediate vicinity of industrial activities showed that the levels of airborne particles can be widely affected by emissions from factories (Jang et al., 2007; Fernandez-Camacho et al., 2012). To limit the unwanted emissions of airborne particles from factories, different trapping devices can be used, but these treatment systems are not always effective, especially for the fine and ultrafine particles that can escape these sensing devices (Schaumann et al., 2004; Biswas and Wu, 2005) and ultimately pollute the environment (Waheed et al., 2011; Shi et al., 2012).

Particle matter enriched with Pb is emitted near Pb recycling workplaces. Particulate matter of different sizes (PM_{10}, $PM_{2.5}$, PM_1, and $PM_{0.1}$) has been reported to be a source of Pb exposure to workers via ingestion and inhalation. Among these PM groups, PM_{10} has adverse effects on the human and environmental health and is therefore the target species of the World Health Organization (WHO, 1987) and the European Union Framework Directive on ambient air quality assessment (European Commission, 1996). However, the micrometric and submicrometric PM fractions contribute very little to the ambient particle mass. The majority of the studies dealing with the characterization of metal-enriched PM in the ambient air

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Abbreviations: BF, bioaccumulation factor; GEF, general enrichment factor; NP, nanoparticle; PM, particulate matter; TF, translocation factor.

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