Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur Arabidopsis halleri en co-culture avec Salix viminalis

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THESE DE DOCTORAT
DE L'UNIVERSITE DU LITTORAL COTE D'OPALE

Ecole Doctorale Sciences, Technologie, Santé (UPJV, EDSTS, ED 585)

Doctorat de Biotechnologies agroalimentaires, science de l'aliment

Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec *Salix viminalis*

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Thèse présentée à ***l'Ecole Doctorale en Sciences Technologie et Santé (ED 585)"*** *et soutenue à l'INERIS le* le 20 mai 2021

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Remerciements

Je tiens à remercier Michel MENCH et Damien BLAUDEZ pour avoir aimablement accepté de réviser mon manuscrit de thèse, me donnant ainsi la possibilité d'améliorer mon travail. Je veux également remercier les autres membres du jury, Nausicaa NORET, Catherine SIRGUEY et Frédérique CADIERE pour l'intérêt qu'ils ont porté à mon étude.

Je tenais à remercier particulièrement Valérie BERT, ma directrice de thèse à l'INERIS sans qui je ne serai pas là aujourd'hui. Merci de m'avoir rappelé après le master 2 pour me proposer ce sujet de thèse fort passionnant. Merci pour tes nombreux conseils, corrections et ta disponibilité en toutes circonstances.

Je remercie également Anissa LOUNES-HADJ SAHRAOUI, ma directrice de thèse, Professeur à l'Université du Littoral Côte d'Opale (ULCO), responsable de l'équipe Interactions Plantes-Champignons (IPCR) à l'Unité de Chimie Environnementale et Interactions sur le vivant (UCEIV), avec qui j'ai été ravi de travailler et d'explorer les microorganismes du sol. Merci pour ta disponibilité et tes conseils tout au long de cette thèse.

Je tiens également à remercier Martine RAMEL, responsable du pôle Risk pour son accueil chaleureux et pour avoir fait en sorte que tout se passe bien à l'INERIS. Mes remerciements vont également à Rodolphe GAUCHER, responsable de l'unité Technologies Propres et Economie Circulaire (TPEC) qui s'est également beaucoup investi pour que cette thèse se déroule dans les meilleures conditions.

Je remercie tous mes collègues du couloir bleu, pour la bonne humeur qui règne toujours dans ce couloir, les pause-café, les gâteaux, les discussions scientifiques (ou non). Un big-up particulier aux 4 fantastiques et la surfeuse 😊 (ils se reconnaitront). Un merci particulier à Fabrice RICHEZ technicien de l'équipe pour toute l'aide apportée que ce soit sur le terrain ou en laboratoire. Merci également à Pauline MOLINA pour avoir remplacé Fabrice quand il n'était pas là et pour les broyages de tronc.

Merci, à tous les stagiaires (Alexandre, Manon, Coralie, Samuel, Mathéo, Océane et Aurélien) ayant participé à mes travaux de thèse en particulier Samuel TEILLAUT, j'espère que ton début de thèse se passe bien, Océane DESANNAUX pour ton implication dans mes dernières expériences menées dans ce contexte si particulier. Merci à tous les stagiaires et thésards de l'unité qui ont su animer le bureau des stagiaires/Thésards. Merci également à tous les thésards et stagiaires des autres unités de l'INERIS qui ont su mettre une ambiance à l'INERIS et à Creil.

J'ai également eu l'opportunité de collaborer avec d'autres équipes de l'INERIS, merci à Hugues BIAUDET et Arnaud PAPIN et les techniciens, Yoan et Farid, de l'équipe RESA qui m'ont accueilli dans leur laboratoire pour les analyses des éléments traces par ICP.

Je remercie tous mes collègues du Laboratoire UCEIV pour leur accueil à Calais très chaleureux. Merci à Joël FONTAINE de t'être impliqué dans mes travaux de thèse. Merci à Maryline pour les trajets de la gare au labo 😊, Corinne et Béatrice pour votre bienveillance. Merci aux 3 soleils du bureau Benoit, Fred et Natacha, merci de votre bonne humeur et de votre aide dans les manips. Merci à tous les doctorants et Post-doctorants (Hacène, Mélodie, Robin, Alice, Nour, Thierry, Aline, Yuko, Amandine et Renata) de l'ambiance au bureau et des sorties à Calais.

Merci à Nausicaa NORET de m'avoir accueilli au laboratoire d'écologie végétale et Biogéochimie de l'université libre de Bruxelles pour les analyses de glucosinolate. Merci en particulier à Sophie LORENT de m'avoir formé à l'extraction et aux analyses métabolomiques.

Enfin, mes remerciements s’adressent à tous mes proches, amis (la team Lilloise) et ma famille, pour leur soutien et leurs encouragements. Big-up à Eleo et Marc pour me supporter tous les jours au téléphone et pour les pauses à Chicago merci de m’avoir fait « godfather » du petit Luca 😊.

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Liste des abréviations

ADEME Agence De la transition écologique

AGPL Acides Gras liés aux PhosphoLipides

AMF Arbuscular Mycorrhizal Fungi

ANOVA ANalysis Of Variance

CCI Chlorophyll Content Index

CEC Capacité d'Echange Cationique / Cation Exchange Capacity

CEM Champignons EctoMycorhiziens

CMA Champignons Mycorhiziens à Arbuscules

DHA Dehydrogenase Activity

DW Dry Weight

ET/TE élément trace/ trace element

FDA Fluorescein diacetate Activity

GLS Glucosinolate

ICP-MS Inductively Coupled Plasma Mass Spectrometry

ICP-OES Inductively Coupled Plasma Optical Emission Spectrometry

INERIS Institut National de l'Environnement industriel et des RISques

MO/OM Matière Organique/Organic Matter

PLFA Phospholipid fatty acid

TTCR Taillis à Très Courte Rotation

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Publication 1 Grignet A, de Vaufleury A, Papin A, Bert V. Urban soil phytomanagement for Zn and Cd in situ removal, greening and Zn-rich biomass production taking care of snail exposure. *Environ Sci Pollu Res Int.* 2020 Jan ;27(3):3187-3201. Doi :10.1007/s11356-019-06796-2. Epub 2019 Dec 14. PMID : 31838670.

Publication 2 Grignet A, Lounès-Hadj Sahraoui A, Fontaine J, Papin A, Bert V. Phytoextraction of Zn and Cd with *Arabidopsis halleri* and *Salix viminalis*: a focus on nitrogen fertilization and mycorrhizal inoculation as a means of increasing biomass. Soumis à Environ Sci Pollu Res Int.

Publication 3 Grignet A, Lounès-Hadj Sahraoui A, Desannaux O, Fontaine J, Papin A, Bert V. Evaluating TE-polluted soil function with biological indicators in a phytomanagement context

Publication 4 Grignet A, Noret N, Teillaut S, Lorent S, Papin A, Souard F, Bert V. Fertilization to improve Zn and Cd phytoextraction of *A. halleri* and potential role of TE and glucosinolates against herbivore attack

Publication 5 Grignet A, Teillaut S, Zdanevitch I, Papin A, Gaucher R, Bert V. Valorization options of the biomass produced on a TE phytomanaged site: what to do with *Salix viminalis* and plant colonizers?

Introduction

En Europe, on dénombre plus de 2.8 millions de sites contaminés en 2013, dont 35% par des éléments traces (ET) (Panagos et al. 2013). En France, 9170 sites et sols pollués ou potentiellement pollués ont été référencés en 2021, dont 983 sites sont localisés en région Hauts-de-France, la classant deuxième en nombre de sites pollués après la région Rhône-Alpes. Ces sites appellent à des mesures de gestion afin d'éviter les risques sanitaires et environnementaux (Géorisques, 2021).

En France, la gestion des sites et sols pollués se base sur la gestion des risques en fonction de l'usage, défini suivant les techniques disponibles et les coûts économiques. La méthodologie de gestion des sites et sols pollués a été actualisée en avril 2017, après une dizaine d'années de mise en œuvre afin de tenir compte des retours d'expériences, des évolutions réglementaires et des pratiques. Elle encourage l'utilisation des meilleures technologies disponibles, prend en compte les outils de diagnostic devenus opérationnels et les nouvelles méthodes issues de la recherche (MEEM 2017).

La pollution des sols par les ET constitue une menace pour l'environnement et la santé humaine. Le terme ET regroupe à la fois les éléments métalliques ainsi que d'autres éléments toxiques qui font partie de la famille des métalloïdes (arsenic, sélénium) (Nieboer and Richardson 1980). Certains ET sont des oligo-éléments (cuivre, chrome, nickel, zinc) indispensables pour le fonctionnement cellulaire mais à de très faibles concentrations (Hooda 2010; Kabata-Pendias 2010). Ils interviennent comme cofacteurs enzymatiques pour former des métalloprotéines. Les éléments les plus souvent utilisés comme cofacteurs enzymatiques sont le magnésium, le zinc et le fer (Waldron et al. 2009). D'autres ET (plomb, mercure, cadmium) n'ont aucune fonction biologique connue et sont très toxiques même à de faibles concentrations (Waldron et al. 2009) Afin de réduire les risques liés à la pollution, la gestion des sols pollués pas des ET passe par différentes techniques, classées en deux grandes catégories : les procédés physicochimiques (lavages chimiques ou par agents tensio-actifs, stabilisation physico-chimique¸ désorption thermique, incinération) et biologiques (bio lixiviation, bio-immobilisation, bioremédiation…) (ADEME 2008). Les techniques biologiques ont l'avantage de pourvoir être utilisé in situ sur de grandes surfaces, de préserver la structure, et les propriétés du sol contrairement aux techniques traditionnelles (physico-chimiques).
Le sol étant une ressource considérée comme non renouvelable, les méthodes de remédiation biologique *in situ* sont de plus en plus privilégiées (Keesstra et al. 2016).

L'utilisation des phytotechnologies sur certains terrains pollués pourrait être un mode de gestion pertinent sur le plan technique, économique et environnemental (Bert V et al. 2012). Parmi les différentes techniques développées au cours des dernières décennies, la phytoextraction est basée sur l'utilisation de plantes accumulatrices ou hyperaccumulatrices à croissance rapide et/ou à forte biomasse. La valorisation de la biomasse enrichie en ET dans des filières non alimentaires permettrait une requalification de ce foncier et une augmentation des surfaces disponibles pour la production de biomasse énergétique ou de matières premières renouvelables. Cette approche qui allie phytotechnologies et valorisation de la biomasse est désignée sous le terme de phytomanagement (Evangelou and Deram 2014). Le concept de phytomanagement se concrétise progressivement grâce au suivi des essais sur le long terme (Kumpiene et al. 2014; Quintela-Sabarís et al. 2017) et l'étude des débouchés possibles pour les biomasses produites (Chalot et al. 2012; Delplanque et al. 2013; Bert et al. 2017; Deyris et al. 2018).

Pour alimenter les connaissances sur la phytoextraction en condition réelles, un essai de phytoextraction couplée à la production de biomasse enrichie en Zn et en Cd a été mis en place en 2013 par l'INERIS en collaboration avec l'Agglomération Creil Sud Oise dans le cadre de son projet de renouvellement urbain. Cet essai, initialement mis en place à Montataire dans le cadre du projet PHYTOAGGLO (ADEME 2013-2017) se poursuit dans le projet EXTRA-Zn (ADEME 2017-2020) dans lequel s'intègre ma thèse. Les objectifs de ces projets sont d'acquérir des connaissances sur les itinéraires culturaux d'*Arabidopsis halleri* et de *Salix viminalis* et d'améliorer les performances de phytoextraction d'ET de ces deux espèces. Nous avons fait l'hypothèse que l'utilisation de pratiques agronomiques permettrait d'augmenter les concentrations en ET et/ou la production de biomasse (performances de phytoextraction) comme cela a déjà été démontré chez d'autres (hyper)accumulateurs tels que *Noccaea caerulescens, Sedum alfredii* et *Alyssum murale* (Li et al. 2003; Ji et al. 2011; Jacobs et al. 2018a). Les résultats préliminaires acquis dans le cadre du projet PHYTOAGGLO ont permis de montrer que les deux espèces se développaient correctement, sans signe de toxicité, sur un sol pollué par les ET à pH basique. Une accumulation en Cd et en Zn largement supérieure aux valeurs normales pour le Cd et physiologique pour le Zn, malgré le pH basique du sol a par ailleurs été mise en évidence. La co-culture de *A. halleri et S. viminalis* en inter-rangée peut permettre d'augmenter l'extraction du Zn et du Cd du sol. L'application du fertilisant (NPK) sur les parcelles d'arabette de Haller a permis de montrer des concentrations en Zn nettement supérieures à celles mesurées en l'absence de fertilisant. Enfin, le risque de dispersion des ET

dans l'environnement a été suivi *via* l'engagement d'escargots *in situ* sur des parcelles d'arabette de Haller et en laboratoire à travers un test de nourrissage (feuilles d'arabette de Haller ou de saule enrichi en ET versus feuilles d'arabette de Haller ou de saule non enrichi en ET). Les feuilles de saules étant peu consommées et les escargots encagés sur le site, n'ayant accès qu'à une faible diversité de plante en comparaison à des conditions naturelles, les résultats ont montré un risque de dispersion des ET dans l'environnement faible mais non négligeable. Ces différents résultats, obtenus dans le cadre de mon stage de Master 2, ont fait l'objet d'une publication (Grignet et al. 2020) et ont permis de dégager des perspectives de recherche qui sont au cœur de ma thèse.

Mon projet de thèse PHYTOEXCO, « étude des performances de phytoextraction du zinc et du cadmium de l'hyperaccumalteur *Arabidopsis halleri* en co-culture avec le saule », cofinancé par l'ADEME et la région Hauts-de-France (2017-2020), s'inscrit dans le cadre du projet Extra-Zn et repose sur le site phytomanagé existant de Montataire (Oise, France). Il vise à étudier les performances de phytoextraction du Zn et du Cd par l'hyperaccumalteur *Arabidopsis halleri* en co-culture avec le saule des vanniers (*Salix viminalis*).

Cette thèse est structurée en 6 chapitres. Le Chapitre 1 est une introduction générale présentant l'état de l'art sur les connaissances nécessaires au déroulement de la thèse ; à la fin de ce chapitre, les hypothèses de travail et les questions abordées au cours de la thèse sont exposées. Les chapitres 2, 3, 4, 5 et 6 présentent les résultats obtenus. Ces chapitres sont organisés en publications. Cette thèse s'achève avec une discussion et une conclusion générales sur l'ensemble des résultats obtenuset ouvre sur des perspectives.

Chapitre 1 : Etat de l'art

1) Disponibilité et comportement des ET dans le continuum sol - plantes

1.1 Facteurs affectant la disponibilité des ET dans le sol

Dans le sol, les ET se présentent sous différentes formes chimiques (spéciation). En solution, ils peuvent se présenter sous forme d'ion libre ou de complexe (organique ou inorganique) (Schneider and Nguyen 2011). Les cations métalliques comme le Cd et le Zn ont la capacité d'interagir avec toute particule organique ou inorganique chargée négativement. Ces interactions vont jouer un rôle important dans la biodisponibilité des ET et dans leur toxicité. Les facteurs influençant la mobilité des ET sont le pH, le potentiel d'oxydo-réduction (Eh) et la capacité d'échange cationique (CEC) (Leschber and Worthington 1984; Kabata-Pendias 2010) (Tableau 1).

Tableau 1 Facteurs influençant la mobilité des ET dans le sol (d'après Leschber and Worthington., 1984)

		Transfert très faible	Transfert faible	Transfert moyen	Transfert élevé	Transfert très élevé
pH	neutre-alcalin	Cr, Hg, Cu, Ni, Co	Pb, Zn, Cd, Tl, As			Se, Mo
	Acide	Se, Mo		Cr, As, Cu, Pb	Zn, Cd, Hg, Co, Ni, Tl	
Eh	Oxydant	Cr	Pb	Cu, Co, Hg, Ni, Zn, Cd, As	Mo, Se	
	Réducteur	Cr, Hg, Cu, Se, Mo, Cd, Pb, As	Zn, Co, Ni			
CEC	Elevée	Cu, Ni, Pb	As, Co, Cr, Hg, Ni, Tl, Cd, Mo, Se, Zn			
	Faible				As, Co, Cr, Hg, Ni, Tl, Cd, Mo, Se, Zn	Cu, Ni, Pb

CEC= capacité d'échange cationique
Eh= potentiel d'oxydo-réduction.

Le pH est le facteur affectant le plus la mobilité et le transfert des ET dans le sol. Il joue un rôle important dans les réactions de sorption, désorption, complexation et précipitation (Kabata-Pendias 2010). Un pH acide favorise la mise en solution des ET (figure 1). En effet, ces derniers entrent en compétition avec les protons H^+ (issu par exemple de la respiration microbienne) pour s'adsorber aux charges négatives des colloïdes (Kabata-Pendias 2010). L'affinité plus importante des protons H^+ aux charges négatives des colloïdes va entrainer un relargage des ET dans la solution de sol. Au contraire,

un pH basique va réduire la mobilité des ET (figure 1) dans la solution de sol. A pH > 8 les ET sont peu disponibles (Kabata-Pendias 2010). Le pH élevé va favoriser la précipitation des ET avec des carbonates, des hydroxydes, des phosphates, des sulfates particulièrement lorsque la concentration en ET est importante (Wong et al. 2007). Le Zn et le Cd sont deux éléments fortement impactés par le pH. A pH basique, leurs biodisponibilités sont faibles.

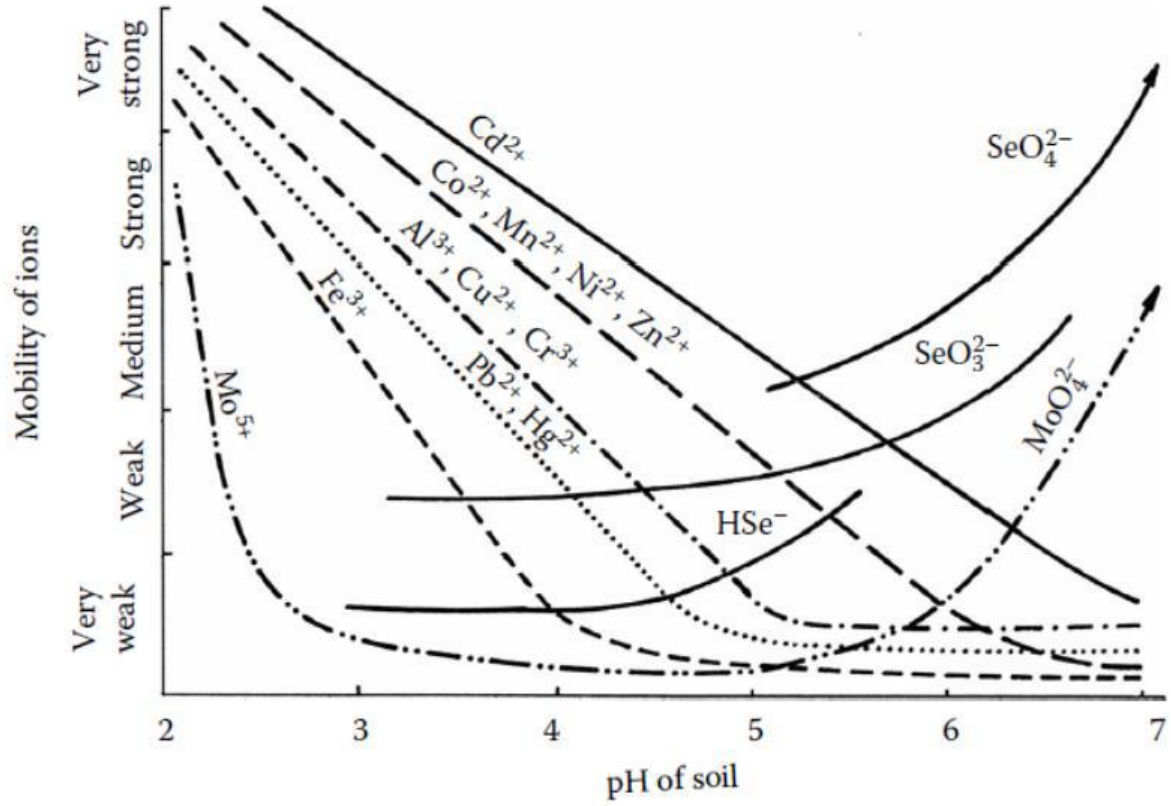

Figure 1Représentation de la mobilité des ET en fonction du pH (Kabata-Pendias, 2010)

La matière organique (MO) et les carbonates sont des phases de sorption dominantes des ET du sol et vont également avoir une influence sur la mobilité. Le Cd^{2+} ayant un rayon ionique et une configuration électronique similaires au Ca^{2+}, l'adsorption du Ca^{2+} engendre une adsorption du Cd^{2+} sur les minéraux argileux (McBride 1980). La MO possède une surface réactionnelle chargée négativement lui permettant d'adsorber facilement les cations comme le Cd et le Zn (Kabata-Pendias 2010). Dans les sols basiques, la matière organique dissoute (MOD) est un facteur important dans l'augmentation de la mobilité du Zn, grâce à la formation de complexes solubles MOD-Zn (Wong et al. 2007). La mesure de la sorption des ET sur des composants du sol est la CEC. Une CEC élevée va traduire une mobilité plus faible des ET et donc une assimilation des cations moins importante par les plantes.

La disponibilité des ET dans le sol va également être affectée par la présence de microorganismes (tels que les bactéries et les champignons). Certains de ces microorganismes ont la capacité d'excréter des molécules organiques (sidérophores, acides organiques) afin de complexer les ET (Kidd et al. 2009; Antoniadis et al. 2017).

1.2 Absorption des ET chez les espèces végétales

L'absorption des ET par les espèces végétales va fortement dépendre de la forme chimique de ces derniers dans la solution de sol (Kabata-Pendias and Pendias 2001), ainsi que de l'espèce végétale et de son cycle de vie. Le prélèvement de nutriment et d'ET à partir du sol se fait à partir des poils absorbants des racines et peut se faire par deux voies : « la voie symplasmique » et « la voie apoplasmique » (Fig 2) (Barber 1995; Morel 1997; Campbell and Reece 2005). La « voie symplasmique » est une absorption au sein de la cellule végétale. Les ET pénètrent à l'intérieur des cellules racinaires à travers la membrane plasmique grâce à des transporteurs membranaires (canaux, pompes à protons, transporteurs tels que les OligoPeptide Transporters (OPT) (Yen et al. 2001). Cette absorption est dépendante de l'élément donné, la compétition entre ET et celle avec des cations majeurs (Ca^{2+} par exemple) affectent ce processus (Hart et al. 2002; Wang et al. 2011). La « voie apoplasmique » n'est pas une absorption, c'est un processus passif et non sélectif contrôlé par la diffusion ou par le flux de la transpiration de la plante (Marschner 2011). Les ET s'accumulent dans l'espace intracellulaire et peuvent également pénétrer à l'intérieur des cellules par voie symplasmique (Morel 1997). Une fois à l'intérieur des cellules racinaires, les ET sont stockés ou transportés des racines vers les feuilles par les vaisseaux du xylème. Le stockage des ET va se faire principalement dans les vacuoles (Prasad and Hagemeyer 1999).

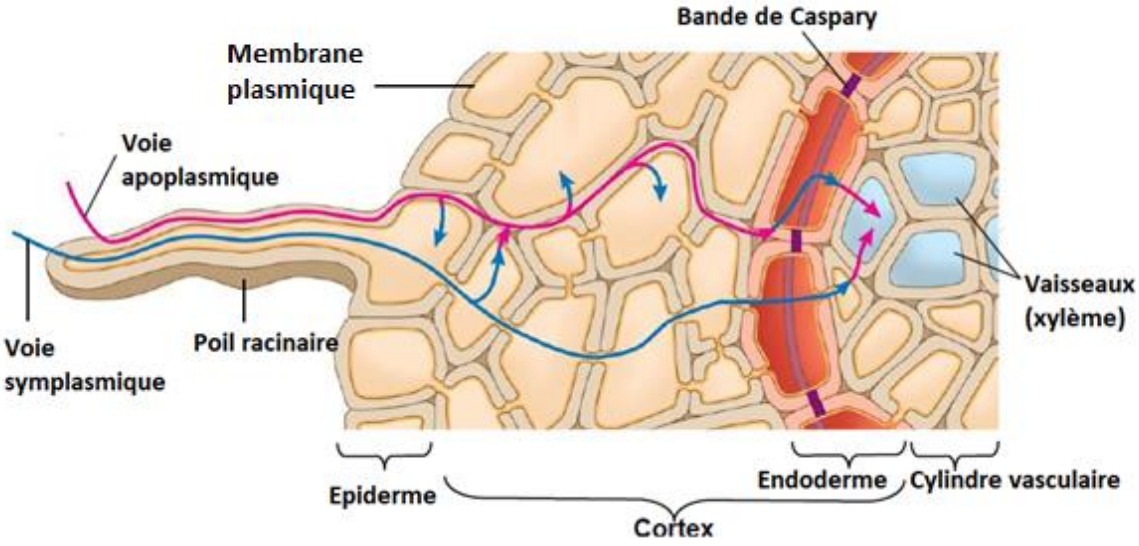

Figure 2Voies de transport apoplasmique et symplasmique dans les racines des végétaux (modifié, d'après Campbell and Reece, 2005)

1.3 Phytotoxicité des ET chez les végétaux

La phytotoxicité des ET se traduit généralement par une réduction de la croissance et l'apparition de symptômes foliaires comme des chloroses et des nécroses (Reichman 2002; Nagajyoti et al. 2010). Les ET vont agir au niveau physiologique dans les processus de photosynthèse, de nutrition, de transpiration et du bilan hydrique. Ils conduisent également à la formation d'espèces réactives de l'oxygène (ERO) entrainant un stress oxydatif. Les ERO sont à l'origine de la dégradation des lipides, des protéines et des acides nucléiques (Shanker et al. 2005; Clemens 2006; Loix et al. 2017). Les ET vont diminuer la photosynthèse par une diminution de la synthèse de chlorophylle et une modification du rapport chlorophylle a et b (Clijsters and Van Assche 1985). Les ET induisent également un stress hydrique par l'augmentation de la résistance stomatique et la perte de la turgescence (Prasad 2011). L'entrée d'eau dans les racines est également réduite par l'inhibition de la croissance des poils absorbants et la réduction de la perméabilité des membranes due à un renforcement de la subérisation et de la lignification (Menon et al. 2007).

1.4 Stratégies de tolérance des végétaux face au ET

Afin de résister aux ET, les plantes ont mis en place différents mécanismes de tolérance. La tolérance aux ET est définie comme étant la capacité d'une plante à survivre et à se reproduire sur des milieux toxiques pour la plupart des autres plantes, en raison de la présence dans ces milieux d'un ou plusieurs métaux en concentration importante (Antonovics et al. 1971; Macnair and Baker 1994). Trois stratégies de base ont été proposées pour expliquer la tolérance des plantes aux ET (Figure 3) :

- L'indication, processus par lequel les plantes contrôlent faiblement leur assimilation en métaux, et dans ce cas, l'accumulation interne reflète la concentration trouvée dans des sols, Cette stratégie est retrouvée chez *Taraxacum officinale* (Czarnowska and Milewska).

- L'exclusion consiste à stocker les ET dans les parties racinaires (Wei et al. 2005; Krämer 2010). On retrouve cette stratégie chez *Arabidopsis arenosa*, *Silene vulgaris*, *Zea mays* par exemple (Brewin et al. 2003; Seregin et al. 2003; Nadgórska-Socha et al. 2013).

- L'accumulation vise à concentrer les ET dans les parties aériennes de la plante et notamment dans les vacuoles des feuilles (pour éviter les dommages cellulaires). On peut distinguer au sein des accumulateurs, les hyperaccumulateurs (Brooks 1998). Une plante est dite hyperaccumulatrice quand la proportion en ET représente au moins 1% en poids sec de la biomasse végétale pour le Zn et 0.01% pour le Cd (Reeves et al. 2000; Becher et al. 2004). On peut retrouver cette stratégie chez *Arabidopsis halleri* et *Noccaea caerulescens* (Meerts and Van Isacker 1997; Bert et al. 2000).

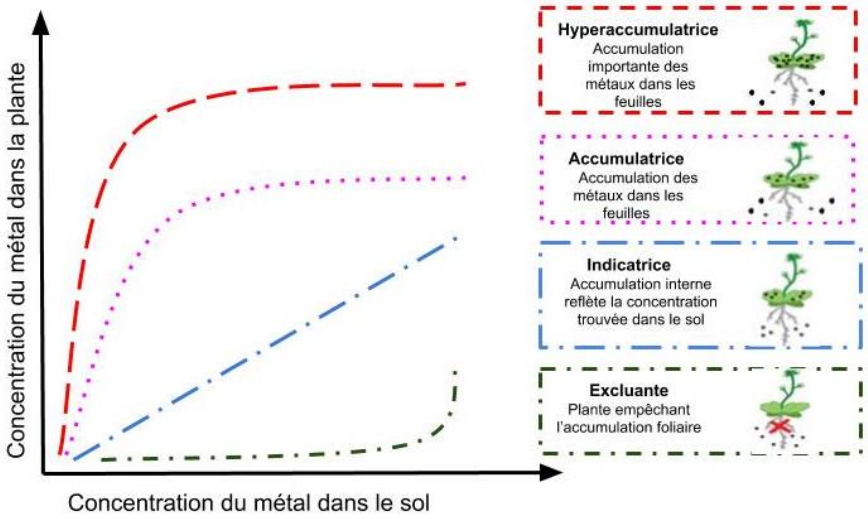

Figure 3 Stratégies rencontrées chez les espèces végétales en réponse aux ET du sol (modifié, d'après Baker, 1981)

L'hyperaccumulation reste une stratégie de réponse relativement rare. En effet, 721 espèces appartenant à 52 familles de plantes terrestres ont été identifiées comme hyperaccumulatrices d'ET (Reeves et al. 2018). La majorité d'entre elles sont des hyperaccumulatrices de Ni (75%) (Baker and Brooks 1989; Brooks 1994; Reeves et al. 2000; Ellis and Salt 2003; Sors et al. 2005; Milner and Kochian 2008). L'hyperaccumulation du Zn et du Cd est majoritairement observée dans une famille, celle des *Brassicaceae*, bien que l'on n'ait pas d'explication à cela (Reeves et al. 2018).

2) Le phytomanagement : méthode de génie écologique pour la gestion des sites et sols pollués

Le phytomanagment est un terme récent qui recouvre un ensemble de techniques de génie écologique utilisant les végétaux pour gérer et valoriser les sites et sols pollués (Evangelou and Deram 2014; Pandey and Bauddh 2019). Le phytomanagement regroupe des techniques basées sur l'utilisation de plantes capables d'extraire (phytoextraction), d'immobiliser (phytostabilisation), ou de dégrader des polluants (phyto/rhizodégradation) (Mench et al. 2010). La phytoextraction et la phytostabilisation sont deux techniques particulièrement bien adaptées au ET. Ces écotechnologies répondent à des enjeux environnementaux et socio-économiques et s'inscrivent dans une dynamique d'économie circulaire. L'installation d'un couvert végétal contribue à restaurer la qualité et les fonctions d'un sol (biologique et physico-chimique) (Touceda-González et al. 2017; Vezzani et al. 2018). De plus, l'utilisation de végétaux sur des sols fortement dégradés, et qui ne peuvent être exploités pour l'alimentation, peut permettre de

produire de la biomasse renouvelable et utilisable en énergie et/ou comme matière première. L'utilisation d'un couvert végétal permet également de limiter la dispersion des ET par le vent, la lixiviation et le ruissellement (Vangronsveld et al. 2009; Mench et al. 2010).

2.1 La phytoextraction

La phytoextraction (Figure 3) est une technique qui utilise des végétaux *in situ* qui accumulent et stockent les ET dans leurs parties aériennes récoltables et permettent une réduction partielle des concentrations en ET dans le sol. Les espèces végétales utilisées en phytoextraction sont soit des accumulateurs présentant une croissance rapide et une forte biomasse (ex : saule) soit des hyperaccumulateurs (ex : *A. halleri*) présentant de fortes concentrations en ET et une plus faible biomasse (Mench et al. 2010).

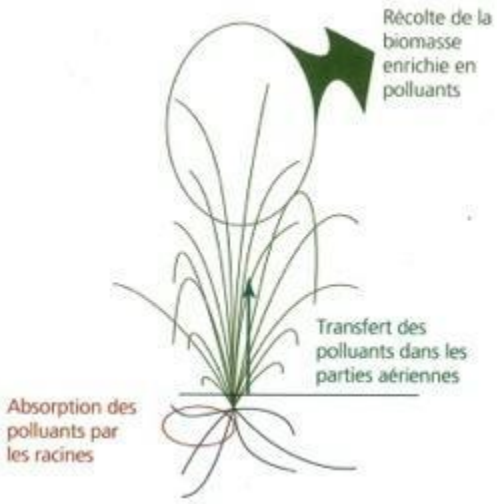

Figure 4 schéma de fonctionnement de la phytoextraction *(Bert V et al. 2012).*

2.2 Amélioration de la phytoextraction

Le potentiel de phytoextraction (production de biomasse et/ou accumulation des ET) peut être amélioré par différentes stratégies (fig 5). Les agents chélatants naturels ou chimiques ont largement été testés dans les années 1990-2000 pour augmenter la biodisponibilité des ET du sol et leur absorption par les racines des plantes (González et al. 2014). Les chélatants chimiques comme l'EDTA (acide ethyldiaminetetraacetique) utilisé pour augmenter l'accumulation foliaire du Pb, sont persistants dans l'environnement (Blaylock et al. 1997) et phytotoxiques à forte dose (Zaier et al. 2010). Les chélatants naturels comme l'acide citrique sont biodégradables, peu phytotoxiques, permettent d'augmenter l'accumulation des ET et montrent

des effets positifs (baisse du pH et augmentation de la mobilité des ET de la solution du sol, accumulation plus importante de ET dans les végétaux) à court terme (Chen et al. 2003b; Wu et al. 2003). L'amélioration génétique a été envisagée, mais ne constitue pas une piste privilégiée par les scientifiques car elle est complexe. En effet, cette stratégie demande d'améliorer à la fois les capacités de tolérance et d'accumulation de l'espèce végétale qui font appel à de nombreux gènes dont ceux impliqués dans le transport et la séquestration des ET tels que les *ZIP* (zinc regulated transporteurs), *MTP* (Metal Transport Protein), *HMA* (Heavy Metal ATPases), familles de gènes codent pour des protéines de transport chez les Brassicaceae (Kärenlampi et al. 2000).

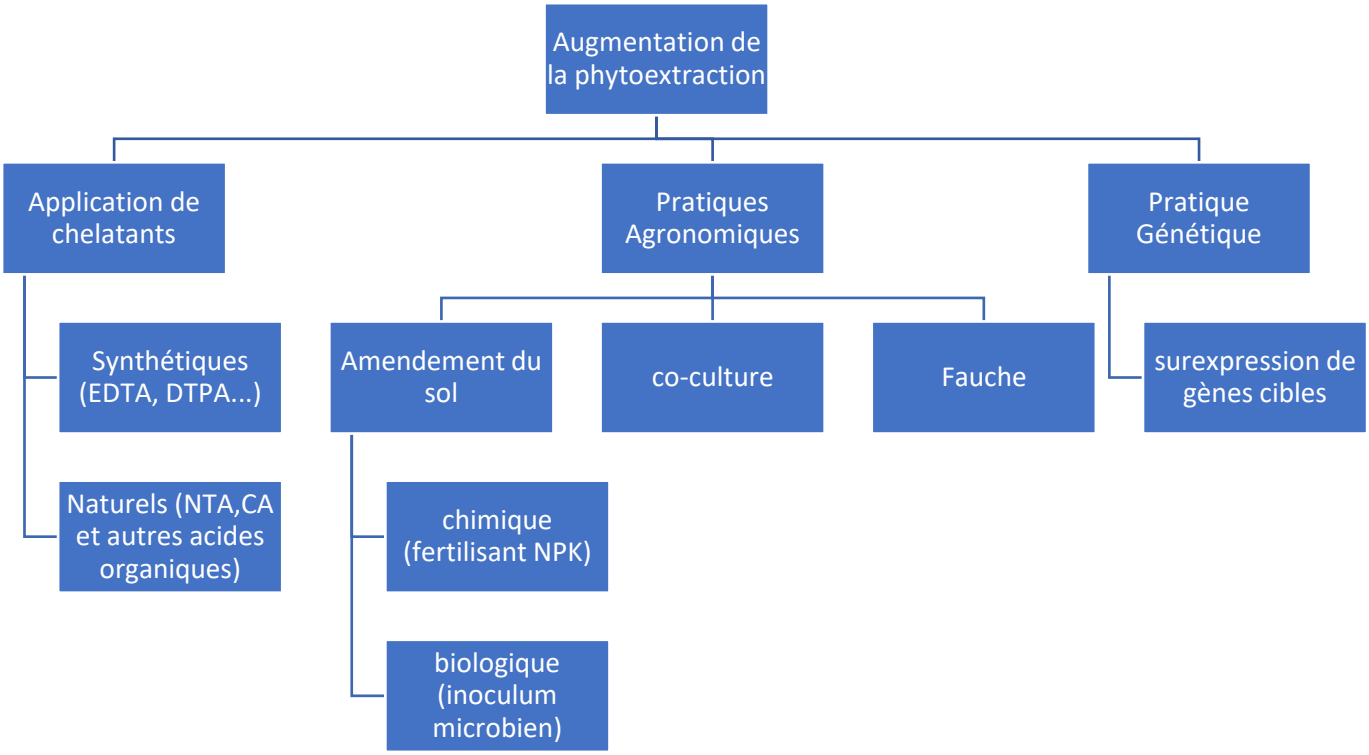

Figure 5 Stratégies pour augmenter la phytoextraction (EDTA = acide ethyldiaminetetraacetique, DTPA= diethylenetriamenepentaacetate ; NTA= 'acide nitrilotriacétique, CA = acide citrique) d'après (Sheoran et al. 2016)

L'intérêt des pratiques agronomiques comme la fauche, la co-culture, l'application de fertilisants, la rotation des cultures, la densité de plantations, l'utilisation d'amendements biologiques (inoculums microbiens à base de bactéries et/ou champignons mycorhiziens), afin d'augmenter le potentiel de phytoextraction de plantes (hyper)accumulatrices a été démontré dans plusieurs études (Whiting et al. 2001b, a; Sell et al. 2005; Marques et al. 2007b; Wang et al. 2007; Wieshammer et al. 2007; Vangronsveld et al. 2009; Jiang et al. 2010; Ji et al. 2011; Kidd et al. 2015; Li et al. 2016; Jacobs et al. 2018a, b; Grignet et al. 2020).

La culture de plusieurs espèces, habituellement deux (co-culture), permet d'augmenter le potentiel d'extraction des ET (ex : augmentation de la quantité de métal par une exploration racinaire plus importante) (Whiting et al. 2001a, b; Jiang et al. 2010; Kidd et al. 2015; Deng et al. 2016). L'utilisation d'espèces (hyper)accumularices en combinaison avec des espèces fixatrices d'azote permet d'augmenter les concentrations en métal de l'espèce accumulatrice par l'acidification de la rhizosphère de l'espèce fixatrice d'azote (Hinsinger et al. 2003). La méthode de co-culture et les espèces choisies ont également un rôle très important dans l'augmentation du potentiel de phytoextraction. En effet, la culture en mélange ou en inter-rangée influence les concentrations en ET. La distance entre deux espèces (hyper)accumulatrices en co-culture est très importante. En effet, lorsque *A. halleri* est co-cultivée en pot avec *Salix caprea* les concentrations en ET et les biomasses des deux espèces sont plus faibles que lorsqu'elles sont cultivées séparément (Wieshammer et al. 2007). Ces résultats sont également observés sur le terrain, quand *A. halleri* est cultivée au pied de *S. viminalis*. Dans ce cas, les concentrations en Zn chez *A. halleri* sont significativement plus faibles que lorsque les deux espèces sont cultivées en inter-rangées, les auteurs faisant l'hypothèse d'une compétition racinaire pour les nutriments, l'eau et les ET (Grignet et al. 2020).

L'utilisation du fertilisant NPK, et notamment l'azote, permet une augmentation de la biomasse et une augmentation des concentrations en ET chez les végétaux (Bennett et al. 1998; Schwartz et al. 2002). La forme chimique de l'azote absorbé par la plante peut directement influencer le pH et donc la mobilité des ET (Tang and Rengel 2003). L'azote peut être absorbé par les plantes sous forme NH_4^+, NO_3^- ou sous la forme neutre d'urée ($(NH_2)_2CO$). L'urée peut également être hydrolysée sous forme NH_4^+. Lorsque la plante prélève le NH_4^+, un déficit en anions se crée à l'intérieur de la cellule racinaire forçant l'expulsion de H^+ et créant une acidification du milieu. Au contraire lorsque la plante prélève le NO_3^- un déficit en cations se crée à l'intérieur de la cellule racinaire forçant l'influx de H^+ du sol et créant une augmentation

du pH du sol. La forme chimique de l'azote apporté doit alors être choisie avec précaution afin d'augmenter ou de réduire le transfert des ET. L'utilisation du fertilisant NPK chez les hyperaccumulateurs (*Alyssum bertolonii*, *Streptanthus polygoloides* et *Noccaea caerulescens*) a été largement testée en condition contrôlée (hydroponie et tests en pots) (Bennett et al. 1998; Monsant et al. 2008; Xie et al. 2009). L'augmentation de la biomasse peut être supérieure à 100% avec un apport en azote de 150 mg N Kg^{-1} (Monsant et al. 2008; Xie et al. 2009). Un apport plus modéré d'azote (31 mg N Kg^{-1}) diminue cette augmentation de 15 à 30%. L'utilisation de fertilisant NPK chez *Alyssum murale,* hyperaccumulateur de Ni, a montré une augmentation de la biomasse (de 2 fois) sans avoir d'effet sur les concentrations en Ni foliaire (Bani et al. 2007, 2015). Chez des hyperaccumulateurs de Zn et de Cd, l'utilisation de NPK en condition de terrain n'a été testée uniquement dans deux études chez *N. caerulescens* et *A. halleri* (Jacobs et al. 2018a; Grignet et al. 2020). Chez *N. caerulescens,* l'application de 30mg N Kg^{-1} augmente la biomasse de 1,5 fois et les concentrations en Zn et Cd restent inchangées par rapport au témoin (Jacobs et al. 2018a). Chez *A. halleri,* l'ajout de fertilisant NPK équivalent à 30mg N Kg^{-1} augmente significativement les concentrations en Zn et en Cd après 1 mois d'application. La biomasse, quant à elle, reste inchangée par rapport au témoin sans NPK (Grignet et al. 2020).

La fauche successive est une stratégie couramment utilisée en agriculture conventionnelle afin de promouvoir la croissance des végétaux (augmentation de la biomasse) (Hamlin and Barker 2006; Vamerali et al. 2014). Peu d'études ont été conduites sur des plantes accumulatrices (Ji et al. 2011) et hyperaccumulatrices (Li et al. 2016; Grignet et al. 2020). Chez les hyperaccumulateurs comme *Arabidopsis halleri,* il a été démontré que l'application de deux fauches successives était possible et permettait d'augmenter la biomasse et les quantités en ET exportés (Li et al. 2016; Grignet et al. 2020).

L'ajout d'amendements biologiques peut permettre d'augmenter le potentiel de phytoextraction. On parle alors de phytoextraction aidée ou assistée. Cette technique consiste à optimiser la synergie entre la plante et les microorganismes rhizosphériques bénéfiques (Glick 2003). Cette technique a déjà été décrite dans le cas des pollutions organiques (Van Aken et al. 2004; Barac et al. 2004; Lebeau et al. 2008) mais très peu pour des pollutions métalliques. L'augmentation du potentiel de phytoextraction va alors passer par deux mécanismes complémentaires : (i) l'augmentation de la mobilité des ET dans la matrice sol par la production de biosurfactants tels que la surfactine ou la viscosine produites respectivement par des bactéries comme *Bacillus subtilis* et *Pseudomonas flurorescens* (Herman et al. 1995; Mulligan

et al. 1999, 2001), la production de sidérophores comme la pyoverdine produit par la bactérie *Pseudomonas fluorescens* (Diels et al. 1999; Dubbin and Louise Ander 2003) et la production d'acides organiques comme l'acide citrique produit par le champignon *Aspergillus Niger* (Di Simine et al., 1998 ; Majewska and Kurek, 2005) et (ii) l'augmentation de la biomasse de la plante par l'association avec des rhizobactéries promotrices de la croissance des plantes (Plant Growth Promoting Rhizobacteria, PGPR) (Zhuang et al. 2007) et/ou des champignons mycorhiziens à arbuscules (CMA) et ectomycorhiziens (Khan 2006).

Plusieurs études sur l'utilisation de champignons en bioaugmentation ont montré des effets sur la croissance et/ou sur le transfert des ET contradictoires (Tableau 2). En effet, alors que certaines études montrent des effets bénéfiques pour le potentiel de phytoextaction (augmentation de la biomasse ou des concentrations en ET foliaires, et/ou une tolérance accrue aux ET), d'autres études montrent des effets inverses (diminution des concentrations en ET foliaires), ou aucun effet.

Tableau 2 Quelques exemples d'études de phytoextraction assistée par des champignons mycorhiziens arbusculaires,, ectomycorhiziens et endophytes.

ET	Concentration	Type	Microorganismes	Inoculum	Conditions de culture	Espèce végétale	Effet	Auteurs
Cd		Champignons mycorhiziens à arbuscules (CMA)	*Funneliformis mosseae*	Préparé au Laboratoire	*En Pot Sol non pollué ca*	*Zea mays*	Diminution du transfert vers les parties aériennes	(Chen et al. 2004)
	0,9 mM		*Funneliformis mosseae*	Préparé au Laboratoire	*Pot Sol non pollué stérilisé ca*	*Triticum aestivum*	Augmentation de la concentration racinaire en Cd, pas d'effet sur les parties aériennes	(Shahabivand et al. 2012)
Zn	433- 964 mg. Kg^{-1}		*Rhizophagus irregularis Glomus claroideum*	Préparé au laboratore à partir de souches isolées du site pollué	Pot Sol pollué stérilisé	*Solanum nigrum*	Augmentation du transfert vers les parties aériennes	(Marques et al. 2007a)
	50 et 100 mg/kg		*Funneliformis mosseae*	Nc	Pot Sol non pollué stérilisé *ca*	*Trifolium pratense*	Augmentation de la biomasse	(Bi et al. 2003)
	50, 100 et 300 mg/kg		*Funneliformis mosseae*	Préparé au Laboratoire	Pot Sol non pollué stérilisé *ca*	*Trifolium pratense*		(Chen et al. 2003a)

Cu, Zn	20 µM		*G. intraradices, Glomus. spurcum*	à partir de souches isolées du site pollué	Pot Sol composite ca	*Sorghum bicolor*	Augmentation du transfert vers les parties aériennes	(Toler et al. 2005)
Zn	300 mg. Kg^{-1}		*Funneliformis mosseae* BEG12	Commercial (Biorize) (Dijon, France)	Pot Sol composite Autoclavé ca	*Populus alba, P. nigra*	Pas d'effet sur la translocation, augmentation de la tolérance de la plante	(Lingua et al. 2008)
Cd	1,91 $mg.kg^{-1}$ total 0,003 $mg.kg^{-1}$ extractible	Champignons ectomycorhiziens	*Hebeloma crustuliniforme, Paxillus involutus, Pisolithus tinctorius*	Préparé au laboratoire à partir de souches isolées d'un site non pollué	*Pot* *Sol pollué*	*Salix viminalis, Populus canadensis*	Augmentation du transfert du Cd vers les parties aériennes	(Sell et al. 2005)
	0, 15 et 30 mg. Kg^{-1}		*Rhizopogon roseolus, Suillus bovinus*	Préparé au Laboratoire	*Pot* *Sol forestier* *ca*	*Pinus pinaster*	Augmentation du transfert vers les parties aériennes	(Sousa et al. 2012)
Zn	100 mg. L^{-1}		*Thelephora terrestris*	Préparé au Laboratoire à partir de souches isolées du site pollué	Hydroponie	*Pinus sylvestris*	Augmentation du transfert vers les parties aériennes	(Colpaert and Van Assche 1992)
Cd	1,5 mg/l	Champignon Endophyte	*Piriformospora indica*	Préparé au Laboratoire	*Pot, substrat inerte* *ca*	*Nicotiana tabacum*	Augmentation de la biomasse, diminution des concentrations	(Hui et al. 2015)

							en Cd dans les racines et les feuilles	
Cd	0,2mM		*Piriformospora indica*	Préparé au Laboratoire	*Pot Sol stérilisé ca*	*Arabidopsis thaliana*	Augmentation de la biomasse, augmentation de la tolérance au Cd	(Peskan-Berghofer et al. 2004)
	0,9 mM		*Piriformospora indica*	Préparé au Laboratoire	*Pot Sol stérilisé ca*	*Triticum aestivum*	Augmentation de la résistance et diminution de la concentration foliaire en Cd	(Shahabivand et al. 2012)

CA : contamination artificielle en ET

La majorité des plantes terrestres vivent en symbiose avec des champignons mycorhiziens. Ces champignons vont fournir des éléments nutritifs (notamment N et P) à l'espèce végétale, qui en retour va fournir des composés carbonés issus de la photosynthèse au champignon (Smith and Read 2008; van der Heijden et al. 2015). Il existe différents types de champignons mycorhiziens (Fig. 6) dont les champignons mycorhiziens à arbuscules (CMA) et les champignons ectomycorhiziens (ECM), qui se distinguent par leurs structures et leurs fonctions (van der Heijden et al. 2015).

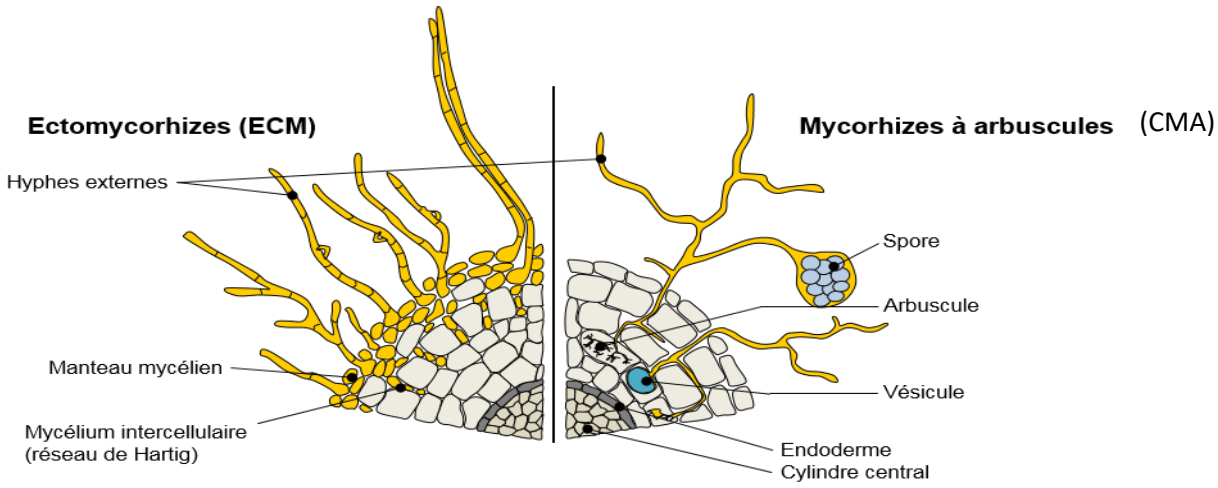

Figure 6 Ectomycorhizes (à gauche) et mycorhizes à arbuscules (à droite) représentées sur une coupe transversale de racine, d'après (Selosse and Le Tacon 1998).

La famille des *Brassicaceae*, regroupant la majorité des hyperaccumulateurs de Zn et de Cd, est réputée non mycorhizable (Demars and Boerner 1996). Cependant, quelques études ont montré que certaines espèces végétales appartenant à la famille des *Brassicaceae* (*Noccaea caerulescens, N. goesingense, N. calaminare, N. cepaeifolium*) peuvent être mycorhizées à certains stades de leurs cycles de développement (Regvar et al. 2003; Hildebrandt et al. 2007). Ces espèces ont été étudiées sur des sites pollués et non pollués. Les racines étaient colonisées par un CMA *Rhizophagus irregularis*. La présence d'arbuscules et de vésicules a été observée (Regvar et al. 2003). Dans cette étude, des pénétrations d'hyphes dans le cortex racinaire d'*A. halleri* issue d'un site pollué ont également été observées. En revanche, aucun arbuscule et aucune vésicule n'ont été observés. A notre connaissance, il n'existe actuellement aucune étude portant sur le statut mycorhizien de l'arabette de Haller. Par ailleurs, aucune donnée n'est disponible sur l'utilisation des inoculums mycorhiziens à base de CMA chez les hyperaccumulateurs dans une perspective d'augmentation du potentiel de phytoextraction.

2.3 Indicateurs d'efficacité du phytomanagement

L'efficacité du phytomanagement a principalement été abordée par des méthodes physico-chimiques (mesure de la réduction et/ou immobilisation des concentrations totales ou extractibles en ET du sol). L'utilisation de bio-indicateurs et de biomarqueurs comme par exemple les activités enzymatiques et l'indice SET (somme des excès de transfert) escargots, afin de mesurer l'impact du phytomanagement sur le sol est en développement (Le Guédard et al. 2017). Les bio-indicateurs sont des outils sensibles qui renseignent sur les risques liés aux transferts et aux impacts globaux des ET sur l'écosystème ainsi que sur l'état écologique des sols (Le Guédard et al. 2017). Il est attendu que le phytomanagement d'un site pollué impacte positivement ces bio-indicateurs et biomarqueurs.

Dans les sols pollués, les paramètres biologiques (activités enzymatiques, biomasse microbienne …) présentent en général des valeurs plus faibles que dans des sols non pollués. Plusieurs études ont montré un effet négatif des ET sur le fonctionnement du sol affectant la croissance, la morphologie (altération des protéines et de l'intégrité membranaire des cellules) et l'activité microbienne du sol (Leita et al. 1995; Renella et al. 2003, 2005; Liao and Xie 2007; Wang et al. 2007; Zhang et al. 2008, 2010; Epelde et al. 2009b; Papa et al. 2010).

Les activités enzymatiques sont considérées comme de bons indicateurs du fonctionnement des sols, qu'ils soient naturels ou anthropisés (Dick and Kandeler 2005; Navnage et al. 2018). Les activités enzymatiques sont des médiateurs et des catalyseurs des plus grandes fonctions du sol comme la décomposition de la MO, le relargage de nutriment inorganique, la fixation d'azote, la nitrification, et ont un rôle crucial dans les cycles du soufre (S) et du phosphore (P) (Dick 1997; Karaca et al. 2010). Parmi ces enzymes, la déshydrogénase (DHA) est l'une des plus sensibles aux changements de qualité et de fertilité du sol (Zhang et al. 2010; Navnage et al. 2018). La biomasse microbienne est un indicateur sensible, robuste et précoce des perturbations d'un sol (ex : modification des pratiques agricoles, contaminations organiques et inorganiques) (Chaussod et al. 1996; Ranjard et al. 2006). Plusieurs techniques sont disponibles afin de quantifier la biomasse microbienne (quantification d'ADN d'un sol ou quantification des acides gras liés aux phospholipides (AGPL)). Plusieurs études ont montré l'impact positif du phytomanagement sur l'augmentation de la biomasse microbienne et les activités enzymatiques du sol (Bouzaiane et al. 2007; Epelde et al. 2009a; Dary et al. 2010; Al Souki et al. 2017).

Les vers de terre sont de bons bionindicateurs de la pollution du sol sont en contact direct avec le sol (Kammenga et al. 2003) et participent à la pédogenèse et au profil du sol. Ils affectent également les propriétés physiques, chimiques et biologiques du sol (Bartlett et al. 2010). Il a été montré que le nombre de vers de terre dans un sol pollué est beaucoup plus faible que dans un sol non pollué (Nahmani and Rossi 2003). Le phytomanagement permet d'augmenter le nombre d'espèces (la diversité) et le nombre d'individus (la richesse) sur un site pollué. En effet, Nahman and Rossi (2003) ont montré par exemple que la plantation d'une peupleraie permettait d'augmenter le nombre d'espèce de vers de terre de 3 à 5 et le nombre d'individus au mètre carré de 12 à 57.

2.4 Filières de valorisation de la biomasse produite sur sols pollués suite à la phytoextraction

La valorisation économique de la biomasse est un enjeu majeur pour le déploiement du phytomanagement. En effet sans filière de valorisation viable et pérenne de la biomasse produite, le phytomanagement n'est pas une technique considérée par les gestionnaires de sites et sols pollués comme une alternative pertinente. Plusieurs propositions de valorisation de la biomasse issue de la phytoextraction ont été étudiées comme la production de bois à des fins énergétiques ou le recyclage des ET contenue dans les feuilles (Conesa et al. 2012; Delplanque et al. 2013; Bert et al. 2017; Deyris et al. 2018). L'évaluation des services écosystémiques rendus par le phytomanagement est en cours et doit être mesurée pour définir sa potentielle plus-value par rapport aux techniques conventionnelles (Rapport Ineris, Applicabilité des phytotechnologies dans la gestion des pollutions des sols, Verneuil-en-Halatte : Ineris-19-180756-1814948-v1.018/12/2019). D'autre part, le contexte réglementaire est un frein, la gestion de ces phytomasses n'étant pas encore considérée dans les réglementations actuelles (Rapport Ineris, Applicabilité des phytotechnologies dans la gestion des pollutions des sols, Verneuil-en-Halatte : Ineris-19-180756-1814948-v1.018/12/2019).

Le taillis à très courte rotation (TTCR) est un mode de culture d'espèce ligneuse à croissance rapide (saule, peuplier) et à forte biomasse. Les arbres sont plantés à très haute densité (10000 à 20000 arbres/ha) et sont, en général, destinés à la production de bois-énergie (Aile 2007). Le bois-énergie été identifié comme un secteur à développer pour répondre à l'une des exigences du Grenelle de l'environnement : en France, 23% de l'énergie produite en 2020 devra être issue de sources renouvelables (LOI n° 2009-967 du 3 août 2009, Grenelle 1). La biomasse produite par

le TTCR peut être vendue au secteur industriel (pour des chaudières industrielles ou collectives) et valorisée en bois énergie par gazéification, pyrolyse ou combustion. La filière bois énergie est une voie de valorisation possible pour les bois récoltés sur sol pollué issus des phytotechnologies (Delplanque et al. 2013). L'utilisation de bois en combustion est soumise à l'article 8 de l'arrêté du 3 aout 2018 qui définit, pour certaines chaudières, des teneurs maximales pour certains éléments dans les combustibles. En absence de réglementation pour le bois issu du phytomanagement, les concentrations en ET de ces bois pourraient être comparées à celles définies pour les combustibles précités (Delplanque et al. 2013).

La chimie verte permettrait de valoriser les métaux contenus dans les feuilles des plantes. Pour cela des procédés d'extraction des ET (Ni, Zn et Mn) ont été mis au point pour former de nouveaux catalyseurs appelés « ecocatalyseurs » (Grison 2015). Au laboratoire, des tests concluants ont été réalisés sur des feuilles de saules et d'arabette de Haller (Deyris et al. 2018). La valorisation du nickel est à ce jour, la plus aboutie pour produire des sels de Ni (Simonnot et al. 2018).

Les autres stratégies de valorisation sont la valorisation des constituants de la biomasse (cellulose, lignine) pour la production de bioéthanol et de biomolécules (Asad et al. 2017). L'utilisation des fibres végétales afin de produire des matériaux composites sont à l'étude, comme par exemple l'utilisation de fibre d'ortie (Jeannin et al. 2020). Le Zn contenu dans la biomasse pourrait également être utilisé en bio-fortifiant dans l'alimentation animale (Anderson et al. 2012).

3) (Hyper)accumulation chez *Arabidopsis halleri* et *Salix viminalis*

3.1 *Arabidopsis halleri*

Arabidopsis halleri (arabette de Haller) est une espèce herbacée de la famille des Brassicaceae L'arabette de Haller est une espèce diploïde (2n=16), pérenne et clonale (Van Rossum et al. 2004). *A. halleri* est largement distribuée en Europe continentale et en Asie (figure 7) où on la rencontre sur sol acide, humide et oligotrophe en montagne ou en plaine (Clapham and Akeroyd 1993; Al-Shehbaz and O'Kane 2002).

Cette espèce est auto-incompatible et entomophile (Bert et al. 2000; Castric and Vekemans 2004; Van Rossum et al. 2004). L'espèce *A. halleri* se divise en deux groupes génétiques distincts séparés par les Alpes (Pauwels et al. 2006). L'arabette de Haller est une espèce pseudométallophyte (Bert et al. 2000), c'est-à-dire qu'elle développe des populations métallicoles et non métallicoles. C'est une espèce hyperaccumulatrice de Zn et de Cd (Bert et al. 2000; Mench et al. 2010).

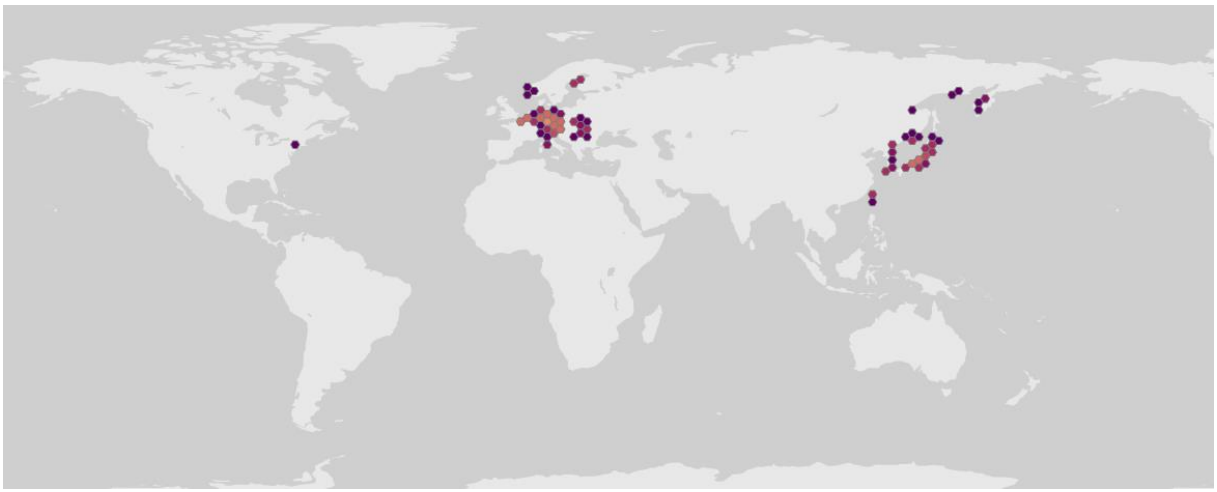

Figure 7 Distribution mondiale d' A. halleri (Arabidopsis halleri (L.) O'Kane & Al-Shehbaz in GBIF Secretariat (2019). GBIF Backbone Taxonomy. Checklist dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2020-02-19.)

Cette espèce forme des petites rosettes qui peuvent assurer la reproduction asexuée grâce à des stolons. Le cycle de vie (Fig. 8) commence par une germination des graines à la fin de l'hiver. Puis une plantule se développe en rosette. Au printemps, elle forme une ou plusieurs hampes florales portant des fleurs blanches. On observe des différences morphologiques et de port entre les plantes de milieu ouvert (prairies) et les plantes de sous-bois. En milieu ouvert, l'appareil végétatif est plutôt réduit avec une simple rosette portant les hampes florales dressées tandis qu'en sous-bois, l'espèce forme des tapis denses (Van Rossum et al. 2004).

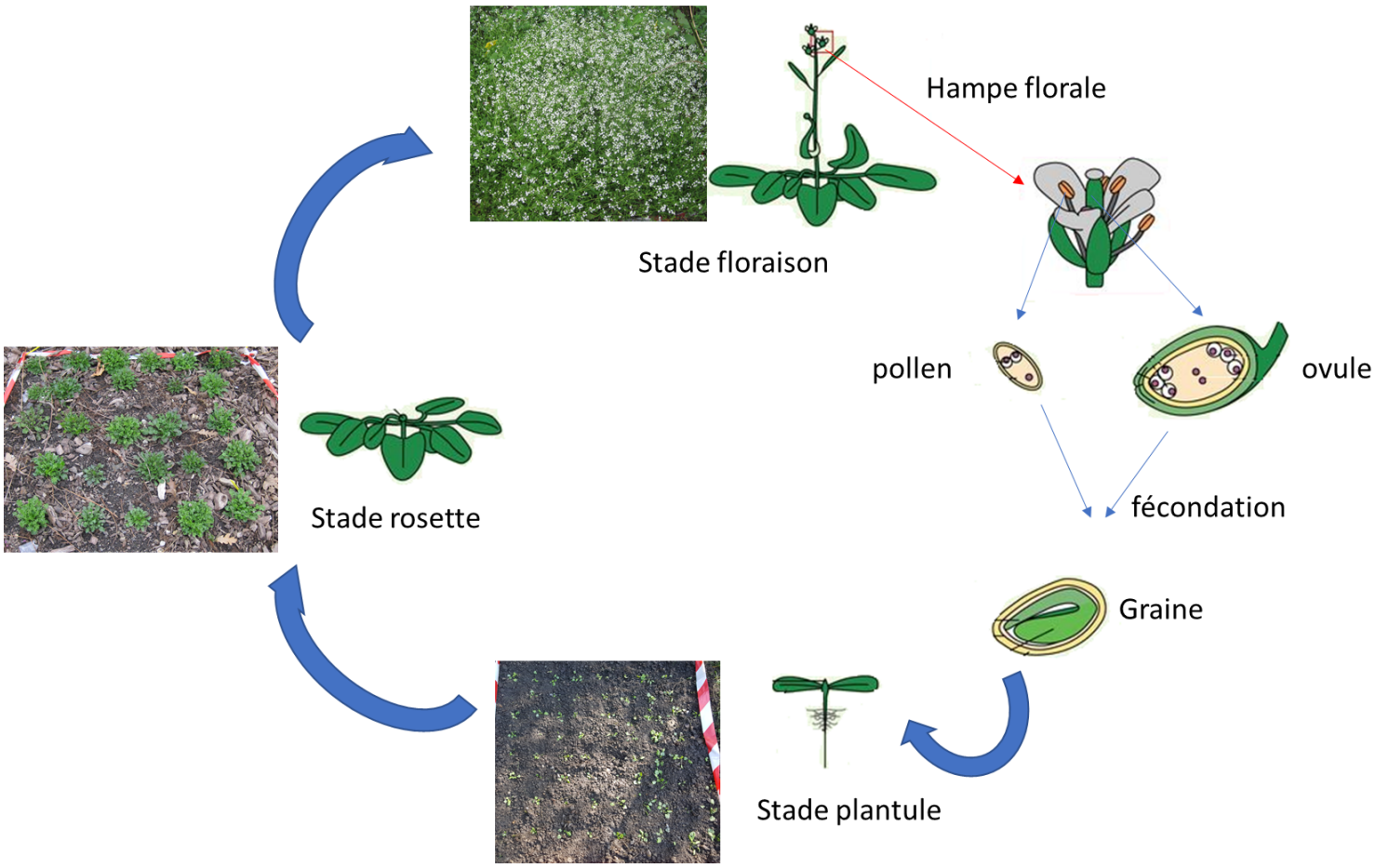

Figure 8Cycle de vie de l'arabette de Haller (d'après Prouteau and Colot, 2005)

3.2 *Salix Viminalis*

Salix viminalis (saule des vanniers) est une espèce ligneuse de la famille des Salicacées, poussant sur milieu humide et des sols acides à neutres (Aile 2009). Les saules sont accumulateurs de Zn et de Cd (Mleczek et al. 2009, 2018) et présentent une croissance rapide (Greger and Landberg 1999). Sur sol pollué, ils présentent également une forte biomasse (Fischer et al. 2005; Mleczek et al. 2010).

3.3 Mécanismes de tolérance et (hyper)accumulation du Zn et du Cd

Depuis plus de 20 ans, *A. halleri* est un modèle pour étudier les mécanismes de tolérance et d'hyperaccumulation (Macnair et al. 1999; Bert et al. 2000, 2002, 2003; Pauwels et al. 2008; Verbruggen et al. 2009; Stolpe et al. 2017b; Uraguchi et al. 2019).

Les plantes qui se sont adaptées aux environnements métallifères sont dites tolérantes et ont la capacité de se développer sur des sols présentant des concentrations en ET anormalement élevées (Macnair 2003).

L'hyperaccumulation d'ET implique trois mécanismes : l'absorption, la translocation et le stockage des ET :

i) Absorption des ET

Les protéines de la famille des ZIP (Zinc-regulated transporter, Iron-regulated transporter Proteins ; Kramer et al., 2007) et MTP (metal tolerance protein), codées par les gènes *MTP1, ZIP9, Nramp3* sont surexprimées dans les racines de *A. halleri.* Ces protéines sont des transporteurs membranaires des cellules du cortex de l'endoderme et du péricycle. Elles permettent un plus grand influx racinaire, limitent le stockage dans les vacuoles racinaires et permettent un transport accru vers le xylème (Lombi et al. 2002; Verbruggen et al. 2009; Corso et al. 2018).

ii) Translocation des ET

La translocation implique des ATPase membranaires contrôlées par le gène *HMA4* (Heavy metal ATPase 4) qui est majoritairement exprimée dans les cellules du xylème et du parenchyme racinaire ce qui permet un chargement du xylème accru (Courbot et al. 2007; Hanikenne et al. 2008; Mishra et al. 2017).

iii) Stockage des ET

Le stockage des ET dans les feuilles implique des transporteurs de type MTP (Metal Transport Protein) (Dräger et al. 2004). En particulier, le gène *MTP1* code pour un transporteur vacuolaire dont les transcrits chez *A. halleri* et *N. caerulescens* sont beaucoup plus importants que chez *A. thaliana* (Assunção et al. 2001; Gustin et al. 2009). Le Cd et le Zn sont principalement stockés dans le mésophylle des feuilles sous forme de ligands Zn-acide organique (malate et citrate)

(Zhao et al. 2000; Schneider et al. 2013) et Cd- acide organique (malate ou citrate) (Huguet et al. 2012; Isaure et al. 2015).

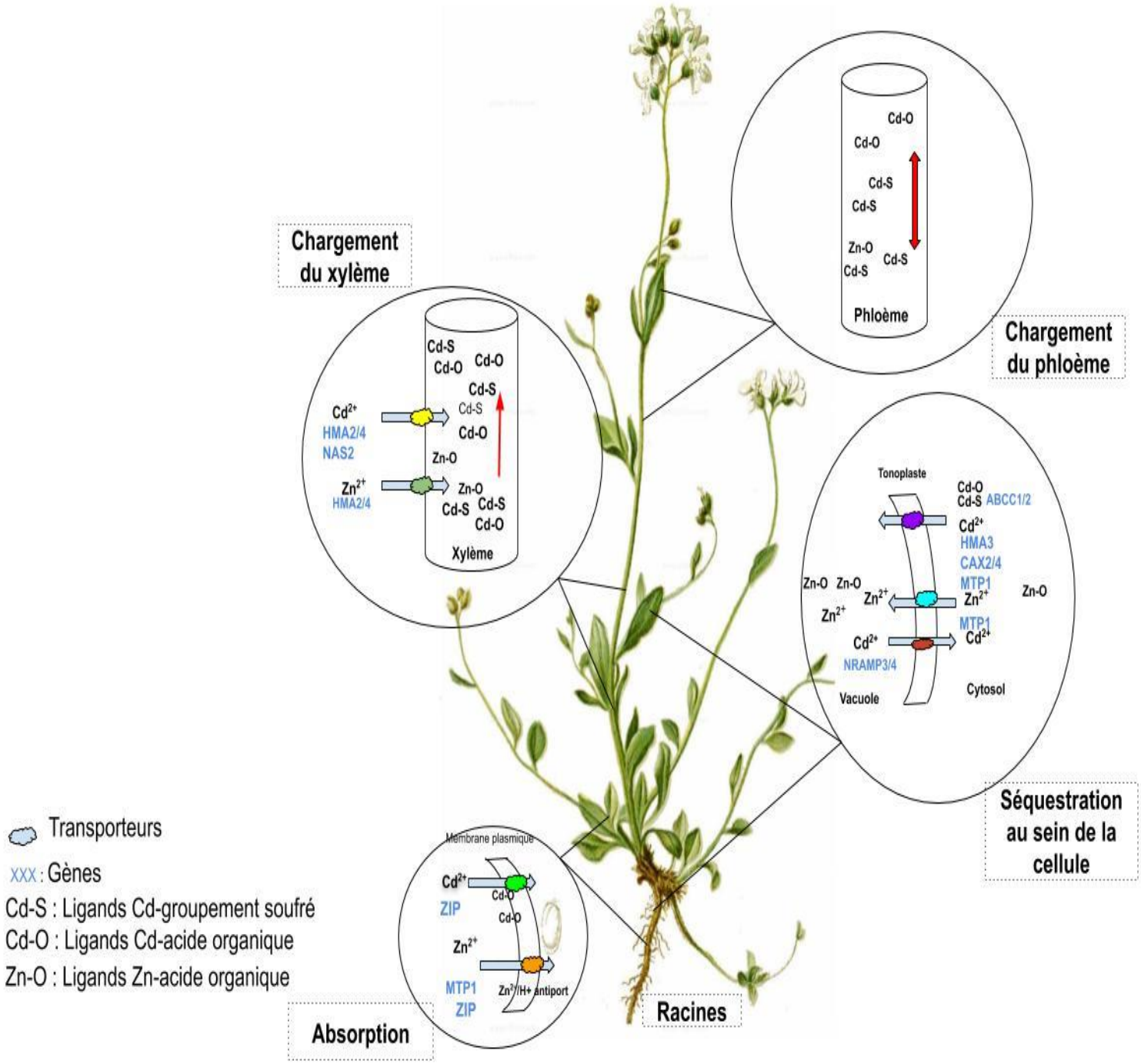

Figure 9 Schéma récapitulatif des mécanismes de séquestration et de transport chez A. halleri. Les ligands Cd-S et Zn-O seraient principalement retrouvés dans le phloème et le xylème.

(D'après : (Zhao et al. 2000; Küpper et al. 2000; Cosio et al. 2006; Vollenweider et al. 2006; Mendoza-Cózatl et al. 2011; Verbruggen et al. 2013; Isaure et al. 2015)).

3.4 L'hyperaccumulation des ET comme mécanisme de défense contre les herbivores?

Plusieurs hypothèses ont été proposées afin d'expliquer l'hyperaccumulation d'ET. La plus testée est celle en lien avec la protection contre les herbivores et les agents pathogènes (Huitson and Macnair 2003; Noret et al. 2004, 2007; Boyd 2007). En effet, la défense contre les herbivores pourrait être due à : (i) un effet direct des ET : les fortes concentrations en ET dans les tissus foliaires sont toxiques chez les herbivores et les agents pathogènes (Martos et al. 2016) (ii) un effet indirect des ET : les ET peuvent être un signal de stress et jouent le rôle de médiateur dans les autres mécanismes de défense (Poschenrieder et al. 2006; Fones and Preston 2013). Les hyperaccumulateurs de Zn et de Cd proviennent majoritairement de la famille des Brassicaceae (Reeves et al. 2018). Cette famille se caractérise par la présence constitutive de glucosinolates (GLS) (Fahey et al. 2001). Les GLS sont considérés comme les molécules organiques majoritaires intervenant dans la défense contre les herbivores dans la famille des Brassicaceae Plusieurs études ont recherché une relation entre les concentrations en GLS et en ET (Tolrà et al. 2001; Noret et al. 2004, 2007; Foroughi et al. 2014; Asad et al. 2015; Stolpe et al. 2017b). Les résultats de ces études montrent une relation complexe et divergente. En effet l'accumulation de métaux peut entrainer une baisse (Tolrà et al. 2001; Asad et al. 2015; Stolpe et al. 2017b), une augmentation (Foroughi et al. 2014), ou un aucun effet (Noret et al. 2007) sur la production des GLS. Les autres hypothèses possibles seraient une meilleure résistance au stress hydrique et l'allélopathie, c'est-à-dire un enrichissement en ET de son milieu après la senescence afin de limiter la compétition avec les autres plantes (Macnair 2003).

Afin de savoir si les espèces hyperaccumulatrices utilisées dans un projet de phytomanagement présentent un risque de dispersion des ET dans l'environnement, un bio-indicateur d'accumulation, l'indice SET (somme des excès de transfert) escargots, peut être utilisé. Les escargots sont de bons indicateurs de la contamination des milieux car ils se nourrissent de végétaux, de sol et d'humus (Berger and Dallinger 1993; Pauget and de Vaufleury 2015). De plus, ils sont facilement récoltables et identifiables. A ce jour, une seule étude impliquant l'utilisation de l'indice SET sur un site phytomanagé a été réalisée (Grignet et al. 2020). Dans cette étude, il a été montré que l'utilisation de *S. viminalis* et *A. halleri* présentaient un faible risque de dispersion des ET dans l'environnement mais que celui-ci n'était pas nul.

3.5 Phytoextraction avec A. halleri et S. viminalis

Bien qu'*A. halleri* soit un modèle pour l'étude et la compréhension de l'hyperaccumulation du Zn et du Cd, très peu d'études ont été réalisées sur ses capacités de phytoextraction contrairement à *N. caerulescens*. Seules trois études ont été conduites *in situ* en parcelles. Deux se sont déroulées sur le même dispositif expérimental (parcelle expérimentale de 1 m², Bedfordshire, Royaume-Unis) (Baker et al. 1994; McGrath et al. 2006) et ont conduit à classer l'arabette de Haller comme un candidat moins performant que *N. caerulescens.* En 2020, Grignet et al. ont démontré l'intérêt d'utiliser l'arabette de Haller sur milieu pollué en conditions a priori non optimales (pH basique) pour la phytoextraction.

4) Objectifs de la thèse et hypothèses de travail

Les hypothèses suivantes ont été émises :

1) l'utilisation de pratiques agronomiques permettrait d'augmenter le potentiel de phytoextraction via l'augmentation de la production de biomasse et/ou d'accumulation des ET dans les parties aériennes.

Nous avons donc testé différentes pratiques agronomiques dont l'ajout d'amendements chimiques (fertilisant NPK) et/ou biologiques à base de champignons mycorhiziens, la co-culture et la fauche.

Les questions posées sont :

1- Quels sont les effets de l'ajout du fertilisant NPK sur la biomasse et/ou les concentrations en Zn et en Cd chez l'arabette de Haller ?
2- Combien de fauches successives peut-on réaliser sur l'arabette de Haller sans réduire ses performances de phytoextraction ?
3- Quels sont les effets de la co-culture d'espèces (hyper)accumulatrices – *A. halleri* et *S. viminalis* - sur les concentrations en ET foliaires de ces espèces, extractibles du sol et la biomasse produite ?
4- L'ajout d'un amendement biologique à base d'inoculum mycorhizien permet-il d'augmenter les performances de phytoextraction du saule ?
5- Quel est le statut de mycorhization spontanée ou naturelle de l'arabette de Haller, espèce végétale de la famille des Brassicaceae, en fonction de ces différents stades de développement sur le site d'étude ? Peut-on induire sa mycorhization dans des conditions contrôlées ? Si oui, quels sont les effets de la mycorhization sur ces performances de phytoextraction ? Si non, peut-on utiliser d'autres champignons endophytes ?

2) le phytomanagement d'un sol pollué améliorerait le fonctionnement biologique du sol.

Le fonctionnement biologique du sol a été évalué par le suivi d'indicateurs microbiens (biomasse bactérienne et fongique et activités enzymatiques microbiennes) et par la mise en œuvre de tests écotoxicologiques (dénombrement des vers de terre) en fonction des modalités de culture (sol pollué non végétalisé, sol végétalisé par les saules seuls, l'arabette seule, ou la co-culture). Les paramètres agronomiques (CEC, C/N, MO …) de la parcelle ont également été évalués.

Question posée : Quels sont les effets des modalités de culture appliquées au site d'étude sur ces indicateurs biologiques ?

3) l'accumulation d'ET chez l'arabette de Haller serait une stratégie qui lui permettrait de résister aux attaques d'herbivores.

Les feuilles enrichies en ET peuvent présenter un risque de dispersion des polluants dans l'environnement (Grignet el al. 2020). Les facteurs affectant l'appétence (concentration en ET, glucosinolates (GLS)) de l'arabette de Haller ont été mesurés).

Questions posées :

- Existe-t-il une corrélation négative entre les concentrations en Zn et celles en GLS dans les parties aériennes des arabettes de Haller métallicoles ?
- Existe-t-il un trade-off entre concentration en Zn et GLS dans la défense des arabettes de Haller face aux herbivores ? et peut-on le discriminer ?
- Le soufre contenu dans le NPK peut -il être assimilé par la plante et augmenter le taux de GLS et par conséquent les défenses de la plante ?

4) Le bois de saule est peut charger en Zn et Cd par rapport au feuille et peut être utilisé en bois énergie (combustion, pyrolyse), les plantes colonisatrices présente sur le site sont soit peu charger et ne présente pas de gestion particulière (compostage, méthanisation) soit fortement chargé et peuvent être utilisé en écocatalyseur.

Questions posées :

- quels devenir pour les phytomasses produites ?
- La filière bois énergie est-elle la bonne voix de valorisation pour les bois de saules ?
- Le cultivar utilisé accumule les ET dans le bois ?
- Présence de plante accumulatrice dans les adventices ? quelle gestion pour ces phytomasse ?

RESULTATS ET DISCUSSIONS

Chapitre 2 : Phytomanagement d'un sol urbain pour l'extraction in situ du Zn et du Cd : utilisation des (hyper)accumulateurs *Arabidopsis halleri* et *Salix viminalis* et réflexions sur les services écosystémiques et les risques associés.

Ce chapitre est présenté sous forme d'un article qui a été publié dans « Environmental Science and Pollution Research » en janvier 2020 (volume 27, page 3187–3201). Il est disponible via le lien https://doi.org/10.1007/s11356-019-06796-2.

La phytoextraction est une technique de dépollution partielle des sols contaminés. Elle se base sur la capacité de certaines espèces végétales à transférer significativement les ET du sol vers leurs parties aériennes. Les candidates à la phytoextraction sont soit des plantes accumulatrices à forte biomasse comme le saule et le peuplier soit des plantes hyperaccumulatrices à faible biomasse comme *Arabidopsis halleri* et *Noccaea caerulescens*. Très peu de données sont disponibles sur les itinéraires techniques des hyperaccumulateurs. La nécessité d'apprendre à les cultiver *in situ* dans des contextes réels de pollution a été mis en évidence dans plusieurs études afin de pouvoir les utiliser en phytoextraction (McGrath et al. 2006; Sirguey et al. 2006; van der Ent et al. 2015; Jacobs et al. 2017, 2018a, b). Les itinéraires techniques des plantes accumulatrices sont mieux documentés. En effet, ces plantes ont déjà été utilisées dans plusieurs études *in situ* (Mleczek et al. 2009, 2010, 2018; Delplanque et al. 2013; Larsen et al. 2014). La synergie entre espèces hyperaccumulatrice et accumulatrice à forte biomasse n'a jamais été testée sur le terrain.

La phytoextraction peut être augmentée par l'utilisation de certaines pratiques agronomiques comme la fauche, la co-culture, l'application de fertilisants, comme cela a été démontré dans plusieurs études (Whiting et al. 2001a; Sell et al. 2005; Marques et al. 2007a; Wang et al. 2007; Wieshammer et al. 2007; Vangronsveld et al. 2009; Jiang et al. 2010; Ji et al. 2011; Kidd et al. 2015; Li et al. 2016; Jacobs et al. 2018a, b). Par exemple, Jacobs et al. (2018a) a montré que la biomasse de *N. caerulescens* était augmentée en présence de fertilisant azoté et qu'en synergie avec une forte densité de plantation, l'efficacité de la phytoextraction était multipliée par 1,5. Dans ce contexte, nous nous sommes demandés si l'utilisation combinée d'espèces hyperaccumulatrice et accumulatrice à forte biomasse pouvait augmenter leur potentiel de phytoextraction.

Les objectifs de cette études étaient d'évaluer le potentiel de phytoextraction de l'arabette de Haller en co-culture avec Salix viminalis et d'augmenter ce potentiel par des pratiques agronomiques (Fauche, co-culture, fertilisant NPK). Cette étude a été réalisé sur le dispositif in situ du projet ExtraZN (figure 1).

Les résultats ont montré qu'en présence de fertilisant (NPK), les concentrations en Zn *d'A. halleri* sont deux fois supérieures à celles mesurées en l'absence de fertilisant. De plus, la biomasse est plus homogène. Des résultats analogues ont été trouvés pour le Cd (augmentation et homogénéité des concentrations). Malgré un pH du sol basique entraînant une faible mobilité du Zn, *A. halleri* présente des concentrations en Zn qui sont celles définissant l'hyperaccumulation (> 10000 mg kg^{-1}, (Baker and Brooks 1989)). Contrairement au Zn, l'accumulation du Cd chez *A. halleri* varie en fonction de son cycle de développement, les concentrations les plus élevées étant retrouvées au stade rosette. La diminution de la

concentration en Cd aux stades floraison et fructification peut être attribuée à un effet de dilution, la minéralomasse (biomasse x concentration) étant constante entre les stades rosette et floraison. Nous avons démontré qu'*A. halleri* pouvait être fauchée à plusieurs reprises et que la fauche stimulait sa repousse. En comparaison de parcelles où elle est cultivée en présence ou en absence de NPK, la biomasse est multipliée par 3, ce qui confirme que la fauche est une méthode qui promeut la croissance des espèces végétales, y compris celle d'hyperaccumulateur, et qui permet d'augmenter leur biomasse (Hamlin and Barker 2006; Li et al. 2012; Vamerali et al. 2014). Bien que peu nombreux, les essais réalisés sur les hyperaccumulateurs montrent l'effet positif d'une double fauche sur l'augmentation de leur biomasse et des concentrations foliaires (Ji et al. 2011; Li et al. 2016).

Concernant *S. viminalis*, les concentrations foliaires en Zn et en Cd étaient largement supérieures aux concentrations physiologiques pour le Zn (de 6 à 10 fois) et couramment mesurées pour le Cd (de 7 à 20 fois) chez la plupart des végétaux (Kabata-Pendias 2010). Ce résultat confirme le comportement accumulateur de ces arbres (Vandecasteele et al. 2004; Van Slycken et al. 2013) malgré le pH basique du sol. Une diminution des concentrations a été observée au cours du temps, sans doute due à un effet dilution car la biomasse des arbres, mesurée indirectement par le diamètre et la hauteur, , a augmenté au cours du temps (Klang-Westin and Perttu 2002; Hammer et al. 2003; Wieshammer et al. 2007).

A. halleri a été cultivée entre les rangées de *S. viminalis* et au pied de *S. viminalis*. Cette dernière modalité n'est pas favorable à la phytoextraction du Zn. Un effet de compétition pour cet élément a été mis en évidence comme cela avait été rapporté dans l'étude en pot de Wieshammer et al. (2007). La co-culture de *S. viminalis* et d'*A. halleri* en inter-rangée est une stratégie qui peut donc permettre d'augmenter l'extraction du Zn du sol (Wieshammer et al. 2007).

Afin de déterminer si la consommation de feuilles de *S. viminalis* et d'*A. halleri* par des escargots présentait un risque de dispersion des ET dans l'environnement, nous avons comparé les concentrations dans les viscères d'escargots nourris avec des feuilles plus ou moins enrichies en Zn et en Cd. Au laboratoire, en conditions contrôlées, seuls les escargots en présence *d'A. halleri* peu enrichies en métaux ont vu leur poids augmenter, ce qui montre l'appétence des escargots pour cette condition de plante. Ce résultat confirme l'étude de Stolpe et al. (2017) qui a montré que les feuilles contenant les plus fortes concentrations de Zn et de Cd étaient les moins consommées. Les feuilles des saules quelle que soit leurs concentrations en métaux ne sont pas appétentes pour les escargots. La structure des feuilles de saule et la présence de glucoside phénolique pourraient expliquer ce résultat (Kendall et al. 1996). L'encagement d'escargots *in situ* sur des parcelles avec l'arabette de Haller a mis en évidence une bioconcentration des métaux dans les viscères des escargots et un risque de transfert de ces métaux dans l'environnement faible mais non négligeable.

Environmental Science and Pollution Research (2020) 27:3187–3201
https://doi.org/10.1007/s11356-019-06796-2

RESEARCH ARTICLE

Urban soil phytomanagement for Zn and Cd in situ removal, greening, and Zn-rich biomass production taking care of snail exposure

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Received: 8 March 2019 /Accepted: 16 October 2019 /Published online: 14 December 2019

Abstract

The phytoextraction potential of Arabidopsis halleri (L.) O'Kane & Al Shehbaz and Salix viminalis L. to partially remove Zn and Cd in soil was investigated. In an urban field site, a very short rotation coppice of willows was implemented, and growth parameters were monitored for 3 years. A. halleri was cultivated in the same site with or without fertilizer to improve biomass yield and/or Zn and Cd aerial part concentrations. Effects of harvest and co-cultivation on these two parameters were measured. To determine if willows and A. halleri leaves were risky in case of consumption by a herbivorous invertebrate like the landsnail Cantareus aspersus, metal concentrations of snails fed with Zn- and Cd-enriched and low enriched leaves were compared. Willows and A. halleri grew well on the metal-contaminated soil (1.7 and 616 mg kg^{-1} Cd and Zn, respectively). The A. halleri Zn foliar concentration reached the Zn hyperaccumulation threshold (> 10,000 mg kg^{-1} DW) in the presence of NPK fertilizer and although the soil was alkaline (pH > 8.2). Cd concentration increased with harvest and fertilizer. Cd and Zn foliar concentrations of willows were far above baseline values. Laboratory snails exposure revealed that willow leaves ingestion caused a moderate increase of Cd, Pb, and Zn bioaccumulation in snails compared to the one caused by A. halleri ingestion. The soil and plant metal concentrations were reflected by field snail biomonitoring. This study confirmed the interest of selecting A. halleri and willows to partially remove Zn and Cd in the soil and emphasized their potential usefulness in greening urban contaminated area and producing raw materials for green chemistry while paying attention to the environmental pollutant transfer.

Keywords Phytoextraction . Urban area . Arabidopsis halleri . Salix viminalis . Snailmonitoring . Cd . Zn . Soilremediation

Responsible editor: Elena Maestri

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-019-06796-2) contains supplementary material, which is available to authorized users.

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Introduction

Phytoextraction is an emerging technology on polluted soil remediation and management markets. Phytoextraction could create or enhance ecosystem services in contrast to the extensively used and unsustainable "dig and dump" technology (Hooper et al. 2016), and it could successfully compete with the existing soil remediation technologies in some cases. The sustainable production of non-food biomass on polluted soil could allow the value of such unused land to increase both in monetary terms and in the availability of the land surface for bioenergy production and renewable raw materials. In addition, the use of green and flowering plants on polluted soil could improve the well-being of people by creating aesthetic, green, and recreational areas, while controlling risks and enhancing the natural capital. This new way of thinking phytoextraction gave birth to the phytomanagement concept

Title: Urban soil phytomanagement for Zn and Cd *in situ* removal, greening and Zn-rich biomass production taking care of snail exposure

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Abstract

The phytoextraction potential of *Arabidopsis halleri* (L.) O'Kane&Al Shehbaz and *Salix viminalis* L. to partially remove Zn and Cd in soil was investigated. In an urban field site, a very short rotation coppice of willows was implemented, and growth parameters were monitored for 3 years. *A. halleri* was cultivated in the same site with or without fertilizer to improve biomass yield and/or Zn and Cd aerial part concentrations. Effects of harvest and co-cultivation on these two parameters were measured. To determine if willows and *A. halleri* leaves were risky in case of consumption by a herbivorous invertebrate like the landsnail *Cantareus aspersus*, metal concentrations of snails fed with Zn- and Cd-enriched and low enriched leaves were compared. Willows and *A. halleri* grew well on the metal-contaminated soil (1.7 and 616 mg kg−1 Cd and Zn, respectively). The *A. halleri* Zn foliar concentration reached the Zn hyperaccumulation threshold (> 10,000 mg kg^{-1} DW) in the presence of NPK fertilizer and although the soil was alkaline (pH > 8.2). Cd concentration increased with harvest and fertilizer. Cd and Zn foliar concentrations of willows were far above baseline values. Laboratory snails exposure revealed that willow leaves ingestion caused a moderate increase of Cd, Pb, and Zn bioaccumulation in snails compared to the one caused by *A. halleri* ingestion. The soil and plant metal concentrations were reflected by field snail biomonitoring. This study confirmed the interest of selecting *A. halleri* and willows to partially remove Zn and Cd in the soil and emphasized their potential usefulness in greening urban contaminated area and producing raw materials for green chemistry while paying attention to the environmental pollutant transfer.

Keywords Phytoextraction. Urban area. *Arabidopsis halleri*. *Salix viminalis*. Snail monitoring. Cd. Zn. Soil remediation

Introduction

Phytoextraction is an emerging technology on polluted soil remediation and management markets. Phytoextraction could create or enhance ecosystem services in contrast to the exten- sively used and unsustainable "dig and dump" technology (Hooper et al. 2016), and it could successfully compete with the existing soil remediation technologies in some cases. The sustainable production of non-food biomass on polluted soil could allow the value of such unused land to increase both in monetary terms and in the availability of the land surface for bioenergy production and renewable raw materials. In addition, the use of green and flowering plants on polluted soil could improve the well-being of people by creating aesthetic, green, and recreational areas, while controlling risks and enhancing the natural capital. This new way of thinking phytoextraction gave birth to the phytomanagement concept which in turn gave priority to the land valuation rather than the time-consuming process of trace element (TE) soil removal (Robinson et al. 2009; Evangelou and Deram 2014). Phytomanagement seems thus suitable for a wide range of polluted soils and could be considered as a technically and economically viable strategy, particularly in the case of large soil and low value areas. Phytomanagement could also con- stitute a viable alternative at the smallest scale when soil management time is not a constraint and it could answer to societal expectations, notably in urban areas. Phytoextraction is often known and mentioned by the various operators of the soil remediation and management network but rarely implemented because it is considered as a non-reliable technology due to the lack of being able to give operational and successful clean- up experience feedback in a reasonable time (Bert et al. 2017; Cundy et al. 2016; Robinson et al. 2015).

Phytoextraction could improve management of polluted soil by partially remediating some TE. While field experiments failed to demonstrate the reduction of the total TE con- centration in soil in the time frame of the experiment, phytoextraction was successful in reducing the plant- available TE in soil (Herzig et al. 2014). To reduce soil pollution sources, phytoextraction uses plant species which significantly transfer TE in harvestable plant parts. Among candidates, hyperaccumulating species could be greatly efficient. Indeed, although they generally show low biomass yield, they accumulate TE such as Zn and Cd at inordinate concentrations (10,000 and 100 mg kg^{-1} dry weight for Zn and Cd, respectively) (Reeves and Baker 1999) in their aerial parts. Despite the interest of such plants, few field trials have been implemented to date (McGrath et al. 2006; Jacobs et al. 2017,2018a, b). Other candidates can be TE accumulating species such as crops and trees which are thought to compensate for their low accumulating capacity compared to hyperaccumulators with their high biomass yield. While the phytoextraction ability of such plants was tested in the field (Delplanque et al. 2013), the synergy

between hyperaccumulators and fast-growing trees such as willows as relevant contributors to enhance phytoextraction performances has never been tested under real conditions.

In addition to low biomass yield, the main factor limiting the practical application of phytoextraction with hyperaccumulators is the insufficient knowledge related to their cultivation and domestication, leading to the impossibility of purchasing seeds or seedlings, which is not the case for fast-growing trees (McGrath et al. 2006; Kidd et al. 2015; Jacobs et al. 2018a, b). In this way, agronomic optimization such as the addition of fertilizer to increase the biomass yield and practices that can increase the plant available TE in soil can both contribute to solving phytoextraction limitations.

Arabidopsis halleri (Brassicaceae, formerly *Cardaminopsis halleri* (L.) Hayek) was shown to accumulate more than 10,000 and 100 mg kg^{-1} Zn and Cd respectively when grown on contaminated soil (Dahmani-Muller et al.2000; Bert et al. 2002) which defines it as a Zn and Cd hyperaccumulating species. *A. halleri* is a pseudo-metallophyte, a species able to develop both on TE contaminated and uncontaminated soils. Due to these traits, *A. halleri* has been extensively studied for about 20 years as a model for understanding mechanisms and the evolution of Zn and Cd hypertolerance and hyperaccumulation which uses various expertise and research scales (e.g., Macnair et al. 1999; Bert et al. 2000, 2002, 2003; Huitson and Macnair 2003; Huguet et al. 2012, 2015; Verbruggen et al. 2009, 2013;Gomez- Balderas et al. 2014; Stein et al. 2017; Stolpe et al. 2017; Ahmadi et al. 2018; Uraguchi et al. 2019). To our knowledge, only one field study has been published to date reporting the performance of *A. halleri* in phytoextraction (McGrath et al.2006). Although this field trial demonstrated the Cd and Zn extraction from a metal-contaminated soil, this study concluded that compared to *Noccaea caerulescens* L. (formerly *Thlaspi caerulescens* L., another Zn/Cd hyperaccumulating plant from the Brassicaceae family), the capability of *A. halleri* is lower due to a lower biomass and relatively lower Cd uptake. To our knowledge, no other study has been per- formed to date to explore the possibilities of increasing its biomass and Zn and Cd extraction via agronomic practices.

Salix spp. (Salicaceae) are well-known fast-growing trees with Zn- and Cd-accumulating behavior and consequently are studied for their phytoextraction potential (Van Slycken et al. 2013; Delplanque et al. 2013; Kidd et al. 2015). To date, several studies have been conducted under real conditions assessing the time needed to reduce the TE soil pollution to acceptable regulated values (Kidd et al. 2015). Although generally developing well on metal-contaminated soils, metal ex- traction and biomass yield greatly depend on Salix clones (Maxted et al. 2007). The leaves are the organs that accumulate the metals the most (Maxted et al. 2007). The common practice for Salix cultivation is the short rotation coppice (SRC) which allows wood production for around 24 years. The first

harvest of willows generally occurs after 3 or 4 years then every 3 years hereafter. Calculations performed hypothesizing the collection of leaves, stems, or both, during the time frame of the plantation, pointed out the remediating potential of willows as well as their potential for bioenergy (Delplanque et al. 2013). Phytoextraction of Cd and Zn with *S. viminalis* was efficient in field trials showing significant metal extrac- tion and biomass yield (Hammer and Keller 2003).

Phytoextraction could participate in producing energy with wood and renewable raw matters like Zn from produced bio- mass. The possibility of creating high value products, such as ecocatalysts usable in green chemistry produced from Zn-rich biomass (*A. halleri* and *Salix sp*. leaves), allows the consider- ation of the economic viability of potential value chains based on phytoextraction (Deyris et al. 2018). These new perspectives support the interest in enhancing the knowledge of Zn (hyper)accumulating species, like *A. halleri* and *S. viminalis* in order to use them for such purposes (Kidd et al. 2015).

In the real context, the food chain transfer of pollutants should be considered as a potential non-desired side effect of phytoextraction. Recently, herbivory of *A. halleri* by chewing and sucking herbivores was demonstrated under laboratory conditions where leaves containing the highest Cd and Zn concentrations were the least consumed (Stolpe et al. 2017). Theoretical risk quotients were calculated for willow leaves to assess phytomanagement on brownfields (Enell et al. 2016). To our knowledge, no experimental data were reported in literature to document the potential food chain transfer with willow leaves.

The objectives of the study were to evaluate and improve phytoextraction performances of two Cd / Z n (hyper)accumulators, separately and in a co-cultivation, system using *S. viminalis* and *A. halleri*, focusing on fertilization and harvest management. More precisely, we reported on growth measurement, survival rate, and on Cd and Zn shoot transfer both in the presence and absence of fertilizer, and at various periods of plant development. Based on the ISO standard 15952:2018 which aims at determining the effects of pollutants on juvenile land snail growth and survival, the global metal transfer index was used to assess the Cd and Zn transfer in the trophic chain, i.e., from *A. halleri* and willow leaves to snails. This assessment was performed in laboratory and field conditions. In the latter case, soil exposure was also considered. To our knowledge, this study is the first to report on (hyper)accumulator co-cultivation relationships and bio- monitoring in an urban field study.

The study was part of the PHYTOAGGLO project (“Integration of phytotechnologies in Creil conurbation as metal-contaminated soil management technologies for urban redevelopment”) set up in 2013 in an urban area contaminated mainly by Zn and Cd due to diverse past industrial activities. In addition to the Zn/Cd

extraction objective of the study, the relevancy of *A. halleri* and *S. viminalis* as suitable plants for greening and also for producing Zn-rich biomass for green chemistry was considered.

Material and methods

Site description

Field trials were conducted in the urban area of Montataire (Creil conurbation, Oise, France 49°15′13″N, 2°26′48″E). According to environmental concern and soil management strategy, the Creil conurbation decided to set up a landscaping project on a linear section of 2.5 km along a road which showed both diffuse and hot-spot pollution due to diverse industrial past activities. Metals (Cd, Zn) were the most significant pollutants found in the top-soil. At the polluted points, the soil was excavated to 20 cm depth, moved and replaced at the location where the phytoextraction field trial was implemented (Fig. 1). The top-soil was mechanically homogenized, and waste materials were removed leading to 50 cm thickness of new material. The surface of the site dedicated to the phytoextraction trial was 800 m². The main physicochemical properties of the soil are presented in Table 1.

Table 1Main physico-chemical properties and pseudo-total concentrations of Cd and Zn in the soil of the field site. Values are means ± SD.

Parameters		Phytoagglo
pH-H_2O		8.02 ± 0.24
Pseudo-total (mg Kg^{-1})	Cd	1.66 ± 0.2
	Zn	616.5 ± 248
Clay (%)		14.8
Silt (%)		32.2
Sand (%)		53
Total organic carbon (g Kg^{-1})		36.83 ± 3.69
Organic matter (%)		6.34
C/N		18.48 ± 2.53
CEC (cmol+Kg^{-1})		12 ± 0.65
$CaCO_3$ (%)		21
N (g Kg^{-1})		2 ± 0.17

Experimental design and plant cultivation

144 The site was divided in two field trials, one was devoted to the plantation of willows in co-cultivation with
145 *A. halleri* (650 m²) whereas plots of 1 m² were set up on the rest of the site (150 m²) to test various agricultural
146 practices for improving *A. halleri* biomass production and metal accumulation. In these plots, *A. halleri* seedlings
147 were transplanted at a density of 50 plants per m² (Fig. 1).

148 In May 2013, 350 unrooted cuttings of *S. viminalis* of 1.5 m length bought at a wood nursery close to
149 the field site (Cheille, France, Hardouin Tressage) were planted in very short rotation coppice (VSRC). The design
150 consisted of nine rows with inter-row distance of 1.5 m and 0.6 m spacing between cuttings. *S. viminalis* was
151 selected for its high bio- mass productivity and its ability to accumulate Zn and Cd in its leaves.

152 *A. halleri* seeds came from a metallicolous population (Parc Péru, Auby, Nord, France). Seedlings of *A.*
153 *halleri* were grown in organic compost (Terreau potager bio, Gamm vert) in a culture chamber (20 °C, 70% moisture,
154 12 h day cycles) for 2 months before transplantation to the field. Before trans- plantation, 30 seedlings of *A. halleri*
155 were collected and used as a control. The first transplantation started in 2015 leading to two plots (P1 and P9) (Fig. 1).
156 In 2016, six new plots were set up (P3, P8, P10, P11, P12 and P13) (Fig. 1). In plot P13, *A. halleri* seedlings were
157 transplanted around willows whereas in other plots, *A. halleri* seedlings were transplanted in the space between
158 the willow rows. Before transplantation, 100 g of nitrogen fertilizer (Biogine®) was incorporated in the top-
159 soil (10 cm depth) of plots P3, P8, P10, and P11. Harvest effect was studied on P1 and P9 whereas co-
160 cultivation effect was monitored on P12 and P13.

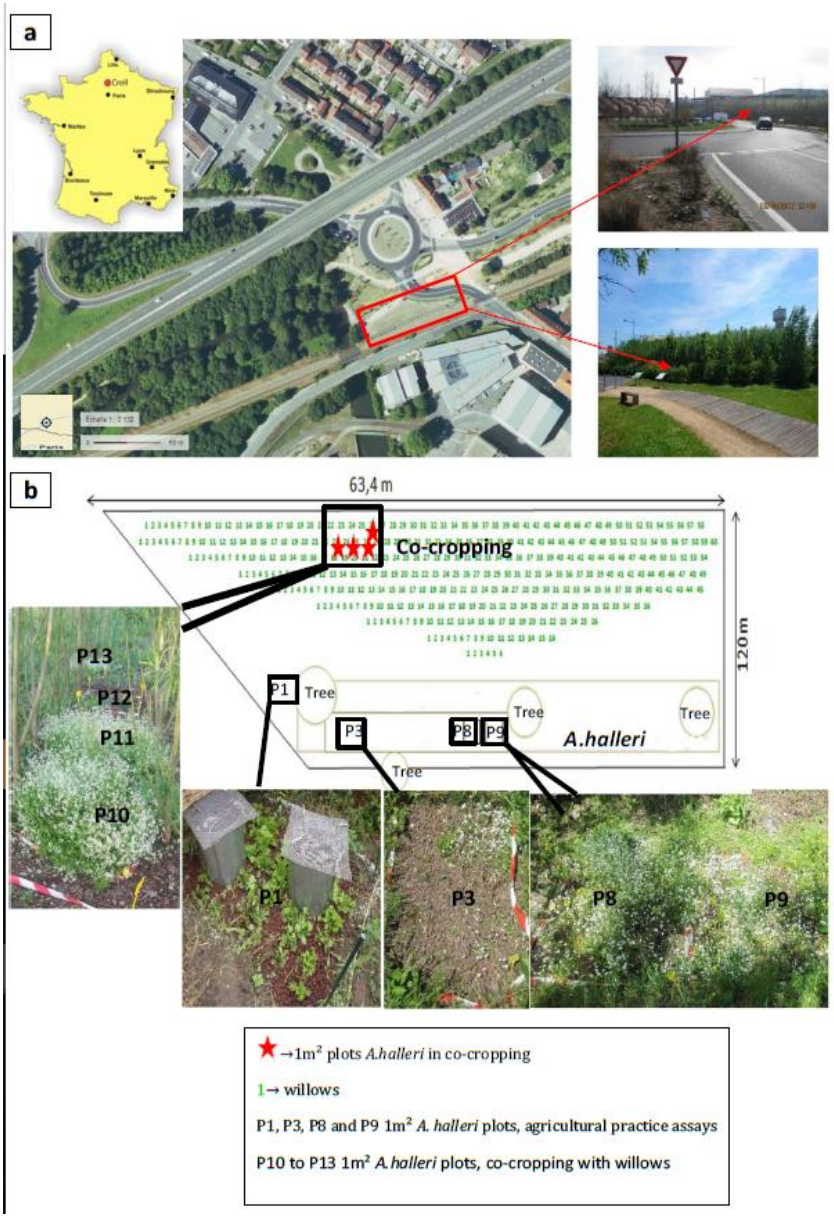

Figure 1 a Location and aerial view of the experimental site. B Experimental design of the field trial and view of A. halleri plots

Soil sampling and analyses

Based on a preliminary pollution diagnosis performed in June 2014 (not shown), soil samples (0–20 cm depth) were collected at 20–30 cm from willow trunks in June 2015 (n = 98), 2016 (n = 98), and 2017 (n = 96). All soil samples were dried at 40 °C in a forced air oven to a constant weight, ground with a grinder (agate mortar, Retsch RM100) and sieved to < 20 mm (Retsch AS 200 digit).

Total concentrations of Cd and Zn were analyzed in the soil samples collected in June2015 and digested (Mars Xpress Soil sampling and analyses CEM) following the NF-EN-13656. Briefly, 0.5 g soil was digested at 180 °C for 20 min with 2 mL of nitric acid (67%), 6 mL of hydrochloric acid (34%), and 2 mL of

hydrofluoric acid (48%) and filtered using 0.45 µm Whatman® filter paper. Zn and Cd concentrations were measured in solutions either by ICP-OES (Agilent 700, Agilent 5100) or ICP-MS (Agilent 7500) depending on soil sample concentrations. One standard reference material was used for analytical quality control (NIST SRM 2710, Montana soil).

To estimate the plant-available fraction of Zn and Cd, selective extraction was performed with 0. 01 M $Ca(NO_3)_2$ solution on soils collected in 2015, 2016, and 2017. Sixty grams dried soil was shaken with 120 mL $Ca(NO_3)_2$ for 48 h in an agitation table. After fil- tration over a Millipore filter (Ø 0.45 µm) and acidification to a pH of 2 for preservation, Zn and Cd in eluates were analyzed either by ICP-OES (Agilent 700, Agilent 5100) or ICP-MS (Agilent 7500).

Soil pH was measured following the NF ISO 10390 (2005) standard. Five grams of 2 mm sieved soils was mixed with 25 mL of distilled water and shaken for 2 h. Water pH was measured after 1 h rest.

Plant sampling and analyses

During the 3 years of monitoring (2015 to 2017), willow leaves were collected each year in June. In addition, in mid- October 2016, fallen leaves of willows were gathered with a leaf blower made available by the green services of the Creil conurbation in a part of the field site, then collected manually and transported in big bags for further weighting and analyses. *A. halleri* aerial parts were collected in the spring and summer 2015 and 2016 which allowed collecting plants at various developmental stages (rosette, flowering, fruiting, and senescence). *A. halleri* samples were collected in plots dedicated to this species (Fig. 1, P1 to P13) to measure potential effects of NPK fertilizer (P1, P8), harvest (P1, P9), and co-cultivation (P12, P13) on its phytoextraction potential. *A. halleri* was harvested on P1 and P9 at the rosette stage, 8 months after transplantation. In May 2016, the rosette was cut the first time at its base which allowed its regrowth. The regrowth was followed by a second harvest 1 month after in August 2016. Plant materials were carefully washed with tap and deionized water before drying at 40 °C until constant weight. Zn and Cd were analyzed in plant samples (0.5 g) after digestion at 180°C for 20 min in 10 mL of nitric acid (67% AnalaR Normapur) and 3 mL of ultra-pure water using a microwave digester (Mars Xpress CEM) by ICP-OES (Agilent 700, Agilent 5100) or ICP-MS (Agilent 7500). One standard reference material was used for analytical quality control (cabbage ‘‘NCS ZC 73012’’, NACIS).

Height and diameter (at 1.30 m) were measured for each living willow during the 3 years of the monitoring (2015 to 2017). Height and diameter increments were calculated as follows:

((Height or diameter − initial height or diameter) / (initial height or diameter)) * 100:

Willow survival rate was determined each year of the monitoring by counting each living willow.

The Chlorophyll Content Index (CCI) which corre- lates with chlorophyll concentration was measured in willow leaves in 2015 using a chlorophyll meter (CCM-200 plus). It was used as an indirect method to evaluate willow nutrient status, N in particular (Parry et al. 2014). Control willows were grown in a growing chamber (20 °C, 70% moisture, 12 h day cycles) in compost (Terreau potager bio, Gamm vert) for 10 weeks. Fifteen trees were monitored in the controlled conditions whereas 99 trees were monitored in the field site. On each tree, three leaves were subjected to analysis.

Cleanup time and Zn/Cd extraction calculations

Concerning willows, cleanup time and Zn/Cd extraction calculations were based on the maximal foliar concentration measured in autumn 2016 and the corresponding biomass (Supplemental Table 3). As the calculations were supported by only one set of empirical data, we hypothesized that foliar concentrations and biomass yield remained constant over time. Concerning *A. halleri*, calculation used the concentrations reported in Fig. 4b (WF1) where the plant cultivated in presence of NPK fertilizer reached the rosette stage and presented the highest Zn and Cd concentrations. The biomass yield was calculated at the same stage by collecting the plants on the 1 m^2 plot (P8) followed by an extrapolation to 1 ha. For the calculations, Cd and Zn concentrations and biomass yield were hypothesized to be annually constant. In both cases, the linear equation proposed by McGrath et al. (2000) was used.

Assessment of Cd and Zn transfer and other potentially toxic elements (PTE) in snails

Seven- to nine-week-old subadult *Cantareus asperses* snails (also known as *Helix aspersa aspersa*) (5.0 g ± 0.30 g) were used for the exposure experiments. In to- tal, 120 snails were used, of which 60 for the laboratory exposure experiment and 60 for the field biomonitoring with microcosms (stainless steel cylinders, 25 cm * 25 cm) (Pauget et al. 2015).

Laboratory exposure

Snails were put in a box (plastic box with ventilation 24 * 10.5 * 8 cm) without soil and fed with fresh leaves of *A. halleri* or *S. viminalis*. Control materials were obtained from *A. halleri* (TE−) cultivated on compost in a growth chamber and from the wood nursery for willow (TE−). As we did not have enough *A. halleri* material in the field site to daily feed snails in laboratory, Cd- and Zn-enriched *A. halleri* (TE+) were collected in a metal-contaminated site in Auby (Bois des Asturies, Nord, France; Gomez-Balderas et al. 2014). This choice allowed exposing snails to *A. halleri* containing Zn and Cd concentrations far above those measured in *A. halleri* control (TE−). *S. viminalis* (TE+) were obtained from the field site. Snails were fed every 2 days with fresh washed *A. halleri* or willow leaves and boxes were cleaned at the same time. Three replicates with five snails in each were used. Time of exposure was 28 days. Snails were weighted once a week. During the exposure time, no mortality was observed. The internal control concentrations of PTEs in snails for laboratory exposure were measured using the snails which grew in the same condition and fed with uncontaminated commercial snail meal (Helixal).

Field exposure in microcosms

Snails were caged on the contaminated site on plot P1 and on compost in the culture chamber (control) for 28 days (Fig. 1). In both cases, 15 snails in each cylinder were exposed from mid-July to mid-August 2015 to the metal-contaminated soil of the field site and to *A. halleri* plants enriched in Zn and Cd (*A. halleri* field site TE+) and compost and *A. halleri* plant less enriched in PTEs (*A. halleri* control TE−) (Table 4). For each condition, two replicates were used. All snails were sampled for metal analysis. Snails were able to feed on soil and vegetation, and thus were exposed via digestive and dermal routes.

Metal analysis

After 2 days of diet (the feces were removed every 24 h), snails were frozen at − 80 °C and dissected after defrosting. Viscera was dried at 60 °C until constant weight. Analysis of Cd, Zn, and Pb in the viscera was performed after digestion at 180 °C for 20 min in 10 mL of nitric acid (67% AnalaR Normapur) and 3 mL of ultra-pure water using a microwave digester (Mars Xpress CEM). Zn, Cd, and Pb concentrations were measured using ICP-OES (Agilent 700, Agilent 5100) or ICP-MS (Agilent 7500). As snails are known as Pb accumulators (Scheifler et al. 2006; Fritsch et al. 2011), this metal, as well as other metals like Cr, Cu, Fe, Mn, and Ni, was

measured in snails to enable the calculation of a more global index transfer (SET) (Supplemental Tables 1 and 2). One standard reference material was used for analytical quality control (cabbage "NCS ZC 73012", NACIS). For each metal, the mean of snail viscera concentrations was compared to the CIRef (CIRef values that corresponded to the upper whisker of the 75th percentile + 1.5 × interquartile range unpolluted plot distribution. They referred to uncontaminated site of the Bioindicators 2 program) (Pauget et al. 2015).

The evaluation of the transfer of metals was calculated using the accumulation quotient (AQ) as follows (Pauget and de Vaufleury 2015):

AQ = C28 days / CIRef or internal concentration of reference:

C28 days is the concentration of the metal in the snail viscera after 28 days of exposure. An abnormal transfer of a metal to a snail was characterized by an AQ > 1 whereas a normal metal transfer was characterized by an AQ ≤ 1. AQ ≤ 1 means that the metal concentration after 28 days of exposure is lower or equal to the CIRef, which means that the snail does not bioconcentrate this element, and the metal transfer from food to snails is low. Consequently, AQ was always adjusted to 1 (to consider a normal transfer) in the next step of the calculation.

The global metal transfer was calculated from the sum of the excess of transfer:

$$SET = \sum AQmetal - 1;$$

where SET represents the global excess of metal transfer calculated from the excess of transfer of each metal (AQmetal-1, where 1 represents a normal transfer). There is no excess of transfer for a particular metal when AQmetal-1 = 0.

Statistical analyses

All data were pre-analyzed by normality tests (Shapiro-Wilk test) and homogeneity of variances (test F). Height and diameter for willows and metal concentrations for plants, soil, and snails were analyzed with ANOVA or test H (Kruskal-Wallis). Post hoc comparisons were performed with Tukey HSD tests. For the comparison of two means (harvest, co-cultivation), a Wilcoxon test was used. All statistics were performed with the R statistical software 3.2.2 (R Core Team).

Results and discussion

Does phytoextraction with willows decrease the Zn/Cd phytoavailability in soil?

To determine the potential impact of willows on Zn and Cd soil phytoavailability, the $Ca(NO_3)_2$ extractable Zn and Cd soil concentrations measured for the 3 years of the study were compared (Table 2). Cd extractable was stable for the three years of the study and represented 0.04% of the Cd total fraction. Zn extractable was the same in 2015 and 2017 and decreased in 2016. Compared to Cd, Zn was more mobile as its extractable fraction represented 0.1 to 0.2% of the total one. These results indicated that a greater fraction of the total Zn than Cd was available for plant uptake and translocation. Nevertheless, these percentages were low which agreed with the alkaline soil pH (pH > 8) of the site, pH being the most important factor regulating the phytoavailability of Zn and Cd and its rate of uptake by plants (Eriksson 1989). Contrary to what was expected, willows did not decrease Zn and Cd soil phytoavailability (Zn: r = 0.20, p value = 0.04; no correlation for Cd). It could be assumed that the metal extractable fraction was reloaded from the less soluble metal pools between the sampling campaigns as already shown (Hammer and Keller 2003).

Table 2 Zn and Cd extractable (0.01M $Ca(NO_3)_2$) concentrations (mg Kg^{-1} dry weight) in topsoil samples over the 3 years monitoring (mean ± SD. Different letters indicate significantly different values at the level of $\alpha = 0.05$).

		2015	2016	2017
Cd	$Ca(NO_3)_2$	0.0007 ± 0.0004	0.0008 ± 0.0004	0.0007 ± 0.0003
	% mobile	0.04	0.05	0.04
Zn	$Ca(NO_3)_2$	0.96 ± 0.85	0.25 ± 0.22	0.81 ± 0.86
	% mobile	0.16 a	0.04 b	0.13 a

Do willows grow well while accumulating Cd and Zn on the metal-contaminated site?

Alteration of willow growth and health could be expected on metal-contaminated site (Morris et al. 2000). In order to mea- sure such alteration, willow height and diameter, CCI, and survival rate were monitored along the study. In addition, the foliar Zn and Cd concentrations were measured to see if they exceeded basal concentrations. The establishment of the wil- low plantation was a success on the metal-contaminated site. Indeed, we observed a significant increase (p value < 0.05) for height and diameter increments throughout the monitoring (Fig. 2). For trees of the same age, growth was better than previously reported on polluted soils (Mleczek et al. 2010). Indeed, the height of *S. viminalis* was 400 cm in Mleczek et al. (2010) vs 664 cm in our study, although metal concentrations in our soil were far above (20 and 3 times more in Zn and Cd, respectively). Results of experiments were however difficult to compare due to different experimental conditions (pots vs field). Several studies showed that the CCI decreased in the presence of a metallic stress (Shi and Cai 2009; Muradoglu et al. 2015). In agreement with the height and diameter results, the CCI did not differ between field site and control willow leaves (averaged CCI were 10.48 and 9.58, respectively; N = 297 and N = 45) suggesting a good health status of willows in both cases though an optimal

N status and chlorophyll pro- duction. Willows showed high survival rate throughout the monitoring (91%, 88%, and 86% for 2015, 2016, and 2017, respectively). Survival rate for *S. viminalis* was just under the one found on unpolluted soil (Beauchamp et al. 2018).

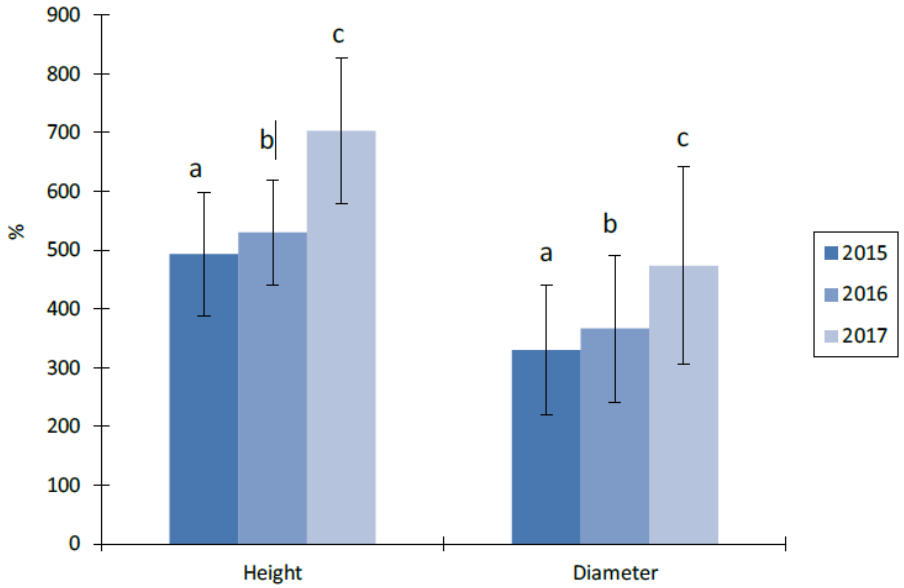

Fig 2 *Salix viminalis annual height and diameter increments (mean ± SD. Different letters indicate significantly different values at the level of* $\alpha = 0.05$*)*

The foliar concentrations in Zn and Cd were higher than the basal values usually measured in willows on unpolluted soils which confirmed the Zn and Cd accumulation behavior of such trees (Fig. 3). These results agreed with many studies which reported that willows accumulated high concentrations of Cd and Zn in leaves in moderate and

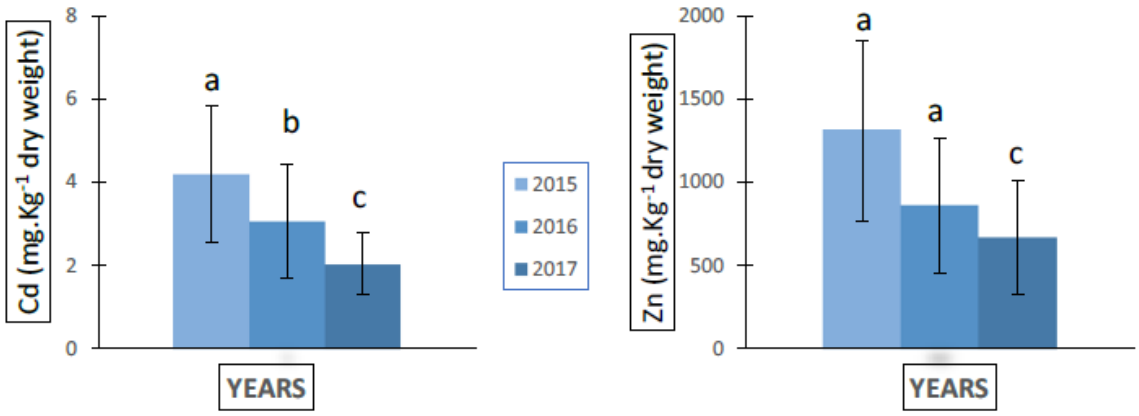

Fig 3 Cd and Zn *Salix viminalis* foliar concentrations over the 3 years of growth (mean ± SD). Significant differences between years are indicated by different letters at the level of = 0. 05

highly polluted sites (Vandecasteele et al. 2004; Van Slycken et al. 2013). As re- ported in various studies on willows (Dos Santos and Wenzel 2007; Wieshammer et al. 2007), foliar Cd and Zn concentrations were strongly correlated

337 ($r = 0.86$, p value = 2.10^{-16}). Throughout the monitoring, a significant decrease in the foliar Zn and Cd concentrations was observed
338 (p value = 0.03, Fig. 3) whereas a significant increase in height and diameter increments was found (Fig. 2) suggesting an increase
339 in willow biomass. A dilution effect due to this biomass increase (Klang-Westin and Perttu 2002) might have occurred as
340 re- ported in several studies in controlled conditions and in situ (Hammer and Keller 2003; Wieshammer et al. 2007). This
341 assumption is supported by the negative correlation between the two indirect parameters of biomass measurement and the Zn an
342 d Cd foliar concentrations (pvalue < 0.05).Nevertheless, this hypothesis should be confirmed in future work by showing a
343 constant value of the metal mineralomass over time (product of mass tissue and concentration). The direct willow biomass
344 measurement could not be performed due to the difficulty of collecting leaves on the whole coppice in spring. Willow leaf Cd
345 concentrations ranged between 2 and 4.8 mg kg^{-1} which agreed with previous work (Klang-Westin and Eriksson 2003; 1 to 7.3
346 mg kg^{-1}) although soil pH and experiment conditions differed (6.5 vs 8.02 in our soil). *S. viminalis* was then considered efficient
347 for Cd and Zn phytoextraction, BCF approximating 2 in the case of Cd and being upper than 1 in the case of Zn (Supplemental
348 Table 3) which confirmed previous data (Klang-Westin and Perttu 2002; Fischerová et al. 2006). As expected, willows did
349 not accumulate Cu and Pb, BCF being lower than 1 (0.1 for Cu and 0.003 for Pb; data not shown).

350
351 Are willows able to clean up Cd and Zn from soil and how long does it take?

352

353 From leaves collected in soil after falling, it was found that 550 and 565 years, respectively, were
354 necessary to decrease the top-soil Cd and Zn concentrations by around half (from1.66 to 1 and 615 to 300 mg kg^{-1},
355 for Cd and Zn, respectively) (supplemental Table 3). Delplanque et al. (2013) calculated a cleanup time of 50 years
356 for Salix "Tora" (*Salix schwerinii* E. Wolf * *S. viminalis* L.) to decrease Cd soil concentration from 2.39 to 2 mg kg^{-1},
357 leaves being collected on the trees during summer and biomass yield being more important than in our case (1.5 to
358 0.5 Mg ha^{-1}, respectively). Consequently, the phytoextraction potential (metal removal per year) of willows Tora for
359 Cd was higher than the one of *S. viminalis* in our case (10 times more). The difference could be explained by the
360 willow type used, *Salix Tora* accumulating more Cd than *S. viminalis* (Mleczek et al. 2009; Beauchamp et al.
361 2018). In another report, 31 and 86 years were necessary to decrease the soil Cd and Zn, from 2.8 to 0.8 and from 650
362 to 150 mg kg^{-1}, respectively, with *S. viminalis* Hammer and Keller 2003). Cleanup time with willows was longer
363 in our case mainly due to lowest Cd and Zn foliar concentrations. This could be explained by the high soil pH
364 which does not promote the extractability of the elements and their transfer to leaves. In these soil conditions, it
365 might be difficult to improve cleanup time. In addition, the time and the practice for willow leave collection were
366 optimal. Indeed, Zn and Cd concentrations were the highest (3.43 vs 3.06 and 1510 vs 856 mg kg^{-1} for Cd and Zn
367 in autumn vs summer, respectively). Leaf collection was easiest after leaf fall than on trees at spring, and it has the
368 advantage to use common materials (leaf blower, material for sucking up leaves) of green services.

369

370 Do and how do agronomic practices increase Zn and Cd phytoextraction of *Arabidopsis halleri*?

371

372 Agronomic practices (NPK fertilizer, harvest, co-cultivation) were assumed to maximize the concentration
373 or/and biomass of *A. halleri* as reported for other hyperaccumulators (Wieshammer et al. 2007; Jacobs et al.
374 2018a; Ji et al. 2011; Li et al. 2016).

375 Accumulation of Cd in *A. halleri* changed according to life cycle (Fig. 4a, right), the highest Cd concentration
376 being ob- served at the rosette stage (Fig. 4a, 1). The Cd concentration decrease observed at the flowering and fruiting
377 stages (Fig. 4a, 2 and 3) could be due to a dilution effect related to growth increase at this stage (Klang-Westin and
378 Perttu 2002). This assumption is supported by the constant value of the metal mineralomass between the rosette
379 and the flowering stages (0.007 ± 0.001 vs 0.006 ± 0.005 kg ha^{-1} $year^{-1}$; p value > 0.05). This result could also be
380 explained by a mechanism of detoxification which would aim to further protect seeds through an internal
381 transport of metals from the green leaves to the old leaves before the leaf-fall (Dahmani-Muller et al. 2000).
382 Although a strong inter-individual heterogeneousness was noticed, Zn concentrations were relatively stable through-
383 out its life cycle (Fig. 4a, left).

384 After 1 month of growth with NPK fertilizer, a significant increase of Zn and Cd concentrations in *A. halleri*
385 was measured although the plant biomass did not increase (Fig. 4b). Compared to the condition without NPK fertilizer
386 (WOF), the Zn and Cd concentrations were roughly doubled (Fig. 4b, WF1). Highest Zn and Cd concentrations
387 were reached at the rosette stage (Fig. 4b, WF1) which agreed with previous results (Fig. 4a, 1). After 2 months (Fig.
388 4b, WF2), the biomass of *A. halleri* tended to increase although not significantly showing a large individual heterogeneity
389 for this parameter. The Cd con- centration decreased (Fig. 4b, WF2, right) while the Zn and Cd mineralomass did not
390 differ. This result might suggest a long- term effect on the biomass and a short-term effect on the metal concentrations
391 of the NPK fertilizer. Many studies reported that N-fertilizer increased shoot biomass and enhanced TE extraction or
392 foliar concentrations (Ebbs et al. 1997; Bennett et al. 1998; Schwartz et al. 2003; Jacobs et al. 2018a). The beneficial
393 effect of the NPK fertilizer on the Zn and Cd accumulation in the case of *A. halleri* was demonstrated.

394 *A. halleri* was considered as a Zn/Cd hyperaccumulator, i.e., a plant species able to accumulate more than 0.01% Cd
395 and 1% Zn in its shoot dry biomass (Verbruggen et al. 2009; Bert et al. 2002).

396 In our field study, *A. halleri* in the presence of NPK fertilizer reached the threshold value for Zn as more
397 than 10,000 mg kg^{-1} was measured although the plant was not, a priori, in optimal conditions (Fig. 4b, WF1, left). Indeed, this

concentration was not necessarily expected because the soil pH of the field site was alkaline leading to low Zn soil mobility (Table 2).

A. halleri was harvested twice at the rosette stage on P1 and P9 plots (Fig. 1) to study the effect of harvest on regrowth and on Zn and Cd concentrations (Fig. 4c). We demonstrated that *A. halleri* could regrow after a first harvest (1H) and that after a second harvest which occurred after the first one (2H), the plant was capable of regrowth. Both harvests showed similar effects on the biomass and the Zn and Cd concentrations but no significant effects on these two parameters. However, a beneficial effect on the biomass was evidenced as it reached the value of 6 mg kg^{-1} DW whereas it approximated only 1 and 2 mg kg^{-1} DW either in absence or in presence of the NKP fertilizer (Fig. 4a, b, respectively). Thus, we confirmed that harvest was the most common method to promote plant growth and obtain higher biomass (Hamlin and Barker 2006; Li et al. 2000; Vamerali et al. 2014). Few studies reported harvest effects on hyperaccumulators, but, when in existence, they showed a beneficial increase like enhanced shoot biomass and TE extraction when double harvest was practiced (Ji et al. 2011; Li et al. 2016).

Zn and Cd foliar concentrations in *A. halleri* and its biomass were studied when co-cultivated with willows either in inter- rows, i.e., *A. halleri* was cultivated between willow rows (P12, Fig. 1), or in mixture, i.e., *A. halleri* was cultivated at the foot of willows (P13, Fig. 1) (Fig. 4d). Inter-rows co-cultivation positively influenced the Zn accumulation of *A. halleri* (Fig. 4d, P12, left). No effect of co-cultivation was evidenced either for the Cd concentration or for the biomass of *A. halleri*. Few studies reported co-cultivation. This study is the first performed in field conditions. A decrease in Zn accumulation was already reported in a pot experiment when *A. halleri* was co-cultivated with willows (Wieshammer et al. 2007). Our results suggested that a competition for Zn, water, and nutriments occurred when *A. halleri* is cultivated in the close vicinity of willows (Wieshammer et al. 2007). Cultivation in inter-rows with willows seems thus a relevant strategy to maximize the Zn extraction potential of *A. halleri* (Wieshammer et al. 2007).

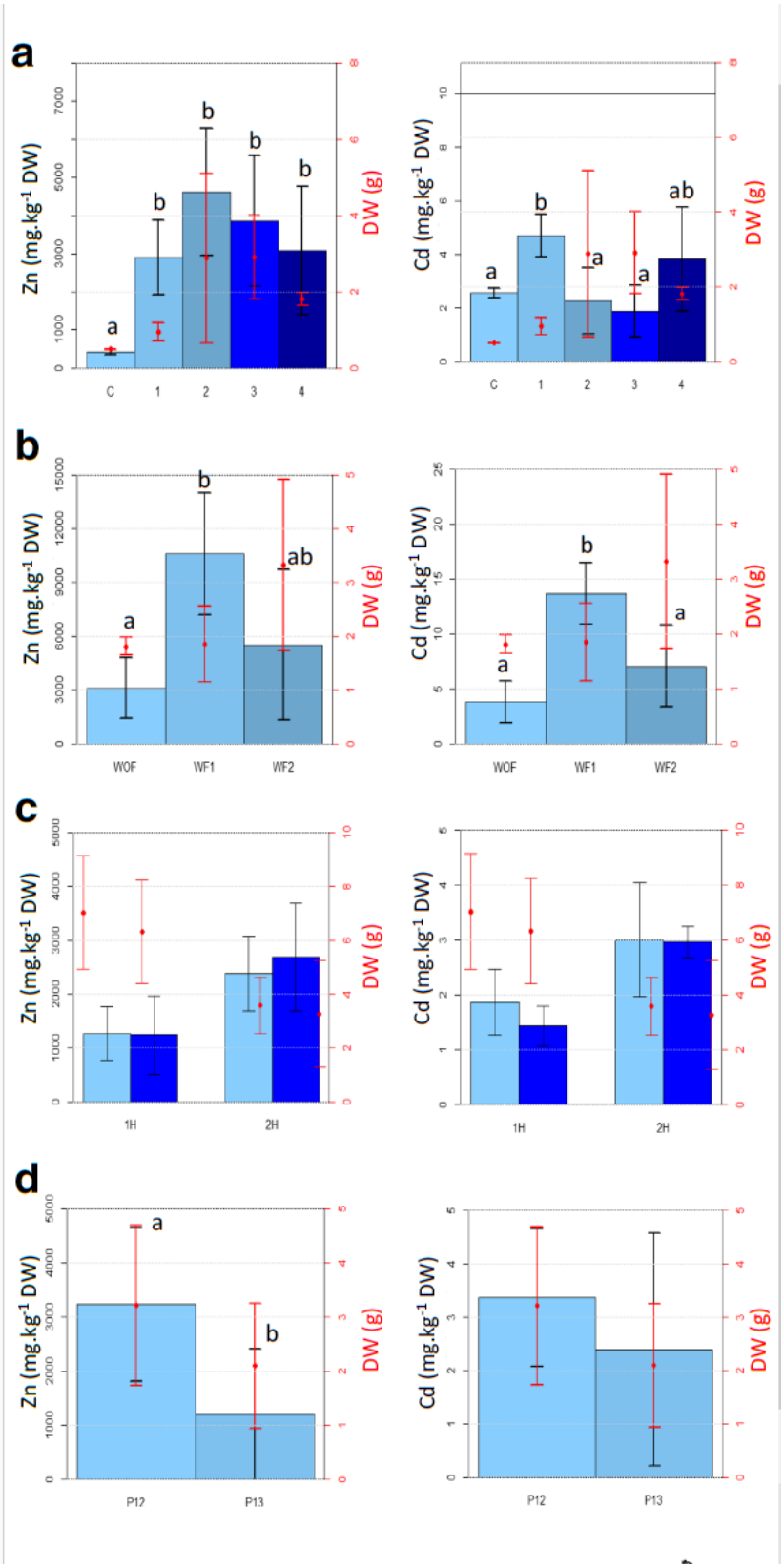

Fig 4 Zn and Cd Arabidopsis halleri concentrations and corresponding dry weight (Mean ± SD). A: values over one life cycle at various development stages (1 to 4) (rosette, flowering, fruiting, senescence). C (control) corresponds to values before transplantation in the field; B: effect of NPK fertilizer (WOF = without fertilizer, WF1 = with fertilizer for 1 month, WF2 = with fertilizer for 2 months); C: effect of harvest (P1 light blue and P9 deep blue) 1H = after a first harvest, 2H= after a second harvest; D: effect of co-cultivation with willows (P12 = inter-rows, P13 = around willows). Significant differences between conditions are indicated by different letters at the level of α = 0.05

Is *A. halleri* able to cleanup Cd and Zn from the soil and how long does it take?

The cleanup time for Cd and Zn was calculated using the concentrations reported in Fig. 4b (WF1) where the plant cultivated in presence of NPK fertilizer reached the rosette stage and presented the highest Zn and Cd concentrations. The biomass yield was calculated at the same stage by collecting the plants on the 1 m² plot followed by an extrapolation to 1 ha. To halve soil Cd and Zn concentrations with *A. halleri*, 39 and 25.5 years, respectively, were necessary (Supplemental Table 4). In our field condition, *A. halleri* was more successful for the extraction of Zn than for Cd. The collection of willow leaves and *A. halleri* allowed a reduction in the cleanup time to 36 years for Cd and 24 years for Zn(Supplemental Table 4). Hammer and Keller (2003) reported a reduction of the cleanup time for the Zn/Cd hyperaccumulator *Noccaea* (ex *Thlaspi*) *caerulescens* depending on its culture (field transplantation vs sowing), best cleanup times being obtained when transplanted (33 and 279 years when transplanted vs 49 and 586 years when sowed for Cd and Zn, respectively). In this work, we did not study direct sowing of *A. halleri*.

The estimation of the biomass yield at the rosette stage in presence of the NPK fertilizer was 3.5 t ha^{-1} (Supplemental Table 4). This yield was far higher than the one reported in the field study of McGrath et al. (2006) with *A. halleri* (0.002 to 0.25 t ha^{-1}) and comparable to the one reported in the field study of Jacobs et al. (2018a) with *N. caerulescens* (3.5 t ha^{-1}) where nitrogen fertilizer was applied (NH_4NO_3). In addition, the Zn concentration was also far higher in our case (10,586 vs 2869 mg kg^{-1} in McGrath et al. (2006)) and slightly lower for Cd (13.6 vs 17.4 mg kg^{-1} in McGrath et al. (2006)) despite an alkaline pH (8 vs 6.5 in McGrath et al. (2006)) and lower total metal concentrations in soil (615 vs 2780 mg kg^{-1} for Zn and 1.66 vs 99 mg kg^{-1} for Cd in McGrath et al. (2006)). Compared to *N. caerulescens*, the Zn concentration was higher in our case (10,586 vs 7500 mg kg^{-1} in Jacobs et al. (2018a)) and lower for Cd (13.6 vs 25 mg kg^{-1} in Jacobs et al. (2018a)). Thus, *A. halleri* appeared as a good candidate for field phytoextraction, biomass yield, and metal foliar concentrations being close to those obtained with *N. caerulescens*. Besides a priori, non-optimal soil pH for Zn and Cd plant removal, we evidenced very encouraging phytoextraction performances of *A. halleri* when relevant agronomic practices were applied (NPK fertilizer addition, rosette stage harvest).

Which ecosystemic services could phytoextracting plants provide?

In addition to the Zn/Cd phytoextraction objective of the study, the relevancy of *A. halleri* and *S. viminalis* as suitable plants for greening and to produce Zn rich biomass for green chemistry was considered based on the development of the species and their Zn concentrations.

Even if no specific health parameters were measured on *A. halleri*, our observations (Fig. 1) suggested that metal- contaminated soil greening with *A. halleri* was successful. Indeed, the hyperaccumulating plant normally developed with a high cover density through its life cycle to produce seeds showing its suitability for the site condition (climatic and soil conditions). Similarly, willows grew well on the metal-contaminated site and did not seem to suffer from competition with *A. halleri*. Both plant species were expected to promote aesthetics providing cultural services. A long-term monitoring and over a larger area to quantify this greening consideration is in progress.

For the first time efficient Zn-ecocatalyst, as raw materials, were produced from *A. halleri* and willow leaves collected on metal-contaminated field sites (Deyris et al. 2018). In this study, the leaves of willows and *A. halleri* contained 3159 and 12,960 mg kg^{-1} of Zn, respectively. Ecocatalyst activity was better when performed with willow than *A. halleri* in spite of its lower Zn concentration. As Zn concentration of willow leaves and *A. halleri* from our site (1309 and 10,586 mg kg^{-1} respectively, in Supplemental Table 3 and Fig. 4b) was close to those of Deyris et al. (2018), we can assume that both species could be processed in green chemistry. This possibility will be verified in future work.

Is the biomass produced at the field site risky in terms of TE transfer in the food chain?

To determine if leaves of willows and *A. halleri* were risky in case of consumption by snails, we compared metal concentrations of snails fed with Zn and Cd enriched leaves (TE+) and non- or lower-enriched ones (TE−).

Laboratory exposure allowed the assessment of the evolution of the weight of snails according to time and diet (Fig. 5).

Snails fed with *A. halleri* TE− gained weight whereas snails fed with *A. halleri* TE+ (p value < 0.5) did not, meaning either that the snails consumed more *A. halleri* TE− than *A. halleri* TE+ and/or that ingestion of contaminated TE+ leaves had caused a toxic effect. A toxic effect due to the ingestion of TE+ was indeed possible, Zn showing a toxic effect from 4000 EC50-Zn (EC50 = 5500 mg kg^{-1}) (Gomot-de Vaufleury 2000). Food avoidance also possibly occurred for snails fed TE+ leaves. Because we did not measure the leaf amount consumed by the snails, we could not separate the effects of differences in the quantities consumed between treatments from the effect caused by difference of meal toxicity. Noret et al. (2007) reported that increased foliar Zn concentration in the metallicolous population of *N.*

caerulescens did not prevent herbivory until 5400 mg Zn kg^{-1} dry matter, above which herbivores would be deterred by Zn. This Zn concentration threshold was exceeded in *A. halleri* TE+ (17,167 mg kg^{-1}, Table 3) but not in *A. halleri* TE− that contained eight times less Zn (2209 mg kg^{-1}, Table 3). Thus, the difference of weight between snails fed with *A. halleri* TE+ and TE− could be explained by the fact that in *A. halleri* TE−, the threshold that could cause snail deterrence was not reached. This would agree with Huitson and Macnair (2003) who did not evidence any difference in consumption when snails were fed with *A. halleri* with various Zn concentrations (< 5000 mg kg^{-1}). In the Brassicaceae family, glucosinolates (GLS) can play a role in the defense against herbivore (Fahey et al. 2001). Nevertheless, the effects of metal accumulation on GLS are complex and di- verse (e.g., GLS concentration was the same in enriched or non- enriched leaves in *N. caerulescens* (Noret et al. 2004) whereas it was lower in enriched leaves than in non-enriched leaves in *A. halleri* (Stolpe et al. 2017)). The relationship between GLS and high Zn, and possibly Cd, in *A. halleri* on snail deterrence could be verified in our case.

For willows leaves, a weight reduction of snails was observed in both conditions (enriched in Zn and Cd or basal concentrations) which could be the result of a reduced palatability of willow leaves to snails (Fig. 5). After 2 days of contact, willow leaves were slightly consumed regardless of the metal concentration. The structure of the leaves of willows and the presence of phenolic glucoside could explain the feeding avoidance (Kendall et al. 1996).

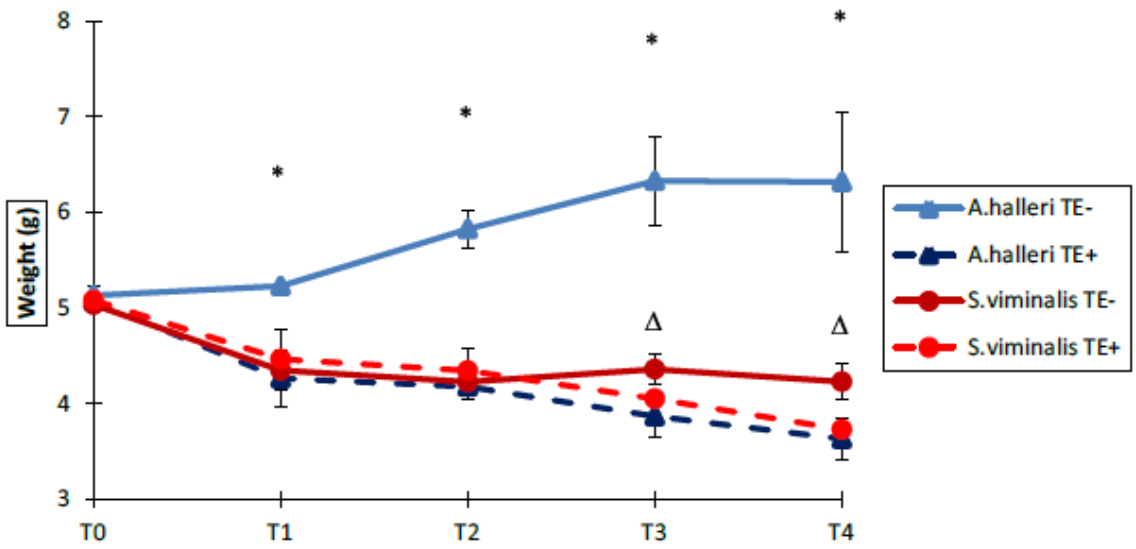

Fig 5 Fresh weight of snails in laboratory during 28 days of feeding exposure (Mean ± SD). TE- = low TE leaves. TE+ = high TE leaves. Significant differences between A. halleri TE- and A. halleri TE+ are indicated by * and significant differences between S. viminalis TE- and S. viminalis TE+ are indicated by Δ, at the level of α = 0.05

The concentrations of TEs in the snail viscera differed according to the feeding source (Table 3). Snails fed with leaves of willows bioconcentrated less TEs than the ones fed with *A. halleri* leaves. Irrespective of the feeding source, the concentrations in the snail viscera for Cd, Pb, and Zn were significantly higher when fed with plants (p values

510 < 0.05) than with control meal. Snails fed with either leaves of willows TE+ or *A. halleri* from Auby (*A. halleri* TE+)
511 showed higher TE concentrations (p values < 0.05) than those fed with *S. viminalis* TE− and *A. halleri* TE−. These
512 results should be related to the metal con- centration of each type of food given to snails, control willow leaves (TE−)
513 presenting baseline Zn, Cd, and Pb concentrations contrary to *S. viminalis* TE+ and *A. halleri* TE− containing less Zn and
514 Cd concentrations than *A. halleri* TE+ (Table 3).

515 For the laboratory test (Table 3), the AQ value for Zn was superior to 2 for all feeding modes and reached 26.12
516 in snail fed with *A. halleri* TE+. In the case of Cd, AQ was lower than 2 when snails were fed with *S. viminalis* TE. The
517 Cd AQ value for snails fed with *A. halleri* was high (20 for TE− and 85 for TE+) compared to non-accumulator plant (Cd
518 AQ do not exceed 3) (Pauget et al. 2015).

519 *Table 3 Cd, Pb and Zn concentrations in internal snail viscera after 28 days of feeding exposure with S. viminalis and A.*
520 *halleri and corresponding S. viminalis and A. halleri foliar concentrations, accumulation quotient and sum of the excess of*
521 *transfer (laboratory exposure). (Mean ± SD). Significant differences between control and treatment are indicated by * and*
522 *significant differences between inter-treatment are indicated by different letters at the level of $\alpha = 0.05$.*

		$mg_{metal}Kg^{-1}$ DW						AQ			SET
modality		Cd		Pb		Zn		Cd	Pb	Zn	
S.viminalis TE-	Snail viscera	1.12 ± 0,22	* a	0.77 ± 0.23	*	649.82 ± 200	* a	1.77	1.96	2.84	3.57
	Plant	0.5		< 0.5		105					
S.viminalis TE+	Snail viscera	1.58 ± 0.38	* b	0.78 ± 0.24	*	824.07 ± 182	* b	2.51	1.97	3.61	5.09
	Plant	5		<0.5		1595					
A.halleri TE-	Snail viscera	12.77 ± 3.39	* a	1.02 ± 0.27	* a	2536.72 ± 731	* a	20.26	2.59	11.10	30.95
	Plant	4		0.5		2209					
A. halleri TE+	Snail viscera	53.89 ± 42.28	* b	19.23 + 15.74	* b	5969.59 ± 2106	* b	85.48	48.97	26.12	157.57
	Plant	259		127		17167					
control		0.63 ± 0.08		0.39 ± 0.13		228.53 ± 28.09					

523
524 Caged in microcosms on the contaminated site (P1, Fig. 1), snails exposed to soil and *A. halleri* (field site TE+)
525 showed TE concentrations in the viscera significantly higher than those measured in the viscera of snails exposed
526 to compost and *A. halleri* control TE− (Table 4). The most worrying concentrations were those of Cd and Zn. In
527 comparison with reference values (CIRef), AQ were the highest for Cd and Zn showing the bioavailability of these
528 elements either in the soil or in the plant to snail. Various experiences of onsite biomonitoring showed
529 concentrations in the viscera of snails from 10 to 270 mg kg^{-1} DW of Cd and from 2000 to more than
530 5500 mg kg^{-1} DW of Zn (Pauget et al. 2012, 2015; Fritsch et al. 2011). In our study, Zn concentrations in snails
531 were higher than those measured in *C. aspersus* (until 5527 mg Zn kg^{-1} soft tissues) on a polluted site (until 7611 mg

Zn kg^{-1} soil) in the presence of *A. halleri* (5264 mg Zn kg^{-1}) (Fritsch et al. 2011). This result could be due to the highest Zn concentration in our plants (*A. halleri* field site TE+ 6327 mg kg^{-1} vs 5264 mg kg^{-1} in Fritsch et al. (2011)). Regarding the AQ, results suggested that *A. halleri* could lead to a risk of metal transfer. However, these modalities of exposure could be considered as a worst-case scenario, snails being in a no-food- choice situation. In natural conditions, snails have a choice of food and the accumulation quotient could be lower.

The SET indices constitute an alarm regarding transfer of TE in the food chain (Pauget et al. 2015). This index reflects the accumulation of all the measured metals. The SET values found in this study (Tables 3 and 4) are equal or higher than those determined, for example, on a former industrial site mainly contaminated by Cd and Pb (SET = 4, calculated from Pauget et al. (2015)), revealing a non-negligible transfer in the simplified food chain considered here.

In the field condition, the herbivore consumption of *A. halleri*, especially at the beginning of its development, should be followed with caution. Indeed, we observed that the young *A. halleri* were consumed when foliar concentrations in Zn and Cd were low, and this consumption decreased with the age of the plant (personal observations). These observations might suggest that the young leaves would not have enough GLS or Zn/Cd to protect themselves against herbivores.

Table 4 Cd, Pb and Zn concentrations in internal snail viscera after 28 days of soil and plant exposure in the field (A.halleri field site TE+) and in the culture chamber (A. halleri control TE-) in microcosm and corresponding A. halleri foliar concentrations, accumulation quotient and sum of the excess of transfer for each modality. (Mean ± SD). Significant differences are indicated by different letters at the level of $\alpha = 0.05$.

		$mg_{metal}Kg^{-1}$ DW						AQ			SET
modality		Cd		Pb		Zn		Cd	Pb	Zn	
A.halleri Control TE-	Snail viscera	11.63 ± 3.45	a	1.32 ± 0.30	a	1446 ± 420	a	5.12	1	1	4.12
	Plant	9.69		< 0.5		895					
A.halleri field site TE+	Snail viscera	10.19 ± 3.37	a	16.40 ± 6.36	b	6327 ± 2357	b	4.49	1.27	4.25	7.01
	Plant	8.12		2.11		5802					
CIRef		2.27		12.9		1490					

Conclusions

In this study performed on a metal-contaminated soil in an urban context, we demonstrated the potential of *S. viminalis* and *A. halleri* to partially remove soil Zn and Cd. Both species were able to grow in alkaline soil and concentrate Zn and Cd in their aerial parts to significant values, although soil condition was not optimal for phytoextraction. These results emphasize the possibility of using *A. halleri* on a wide range of soils with diverse pH. Fertilization and harvest

559 management of *A. halleri* allowed in- creasing Zn phytoextraction. Similarly, inter-rows co-cultivation with willows
560 seemed a relevant agricultural strategy for increasing Zn phytoextraction of *A. halleri*. Willow leaves and *A. halleri* should
561 contain sufficient Zn to be processed in green chemistry to produce Zn-ecocatalysts (Deyris et al. 2018). In addition to
562 phytoextraction, the possibility of using these Zn (hyper)accumulators for such usage combined with greening of
563 contaminated areas confirmed the need to optimize their cultivation. Long cleanup time evidenced in this study, in particular,
564 for willow but also for *A. halleri*, could be compensated by these environmental benefits.

565 Given that leaves of willows were only lightly consumed by snails in laboratory condition and that caged snails
566 on the studied field plot had access to a lower diversity of plants than in natural conditions, our results suggested that
567 the risk of metal dispersion via the food chain was low but not negligible. In addition, we evidenced a risk in the
568 case of the most Cd and Zn concentrated *A. halleri* leaves, as snails consumed them and bioconcentrated these metals.
569 This pollutant transfer represented a non-desired side effect of phytoextraction which should be controlled to avoid
570 pollutant dispersion in the environment.

571

572 **Acknowledgments**

573 The authors thank the Creil conurbation, and particularly H Coudière (ACSO Agglomération Creil sud Oise) and
574 JL Deremy (Mairie de Montataire), which provided access to the field site and technical support during the entire
575 duration of the project. The authors are also grateful to Lisa Citerne, Sophie Laime, and Fabrice Richez for their
576 technical contribution during the sampling campaigns and chemical analyses. We also thank Susan Fulford and
577 Charlaine Noury, two native English, for the improvement of the text quality. We thank the anonymous reviewers
578 for their comments which greatly help us to improve the manuscript.

579

580 Funding information This work was supported by national funds through PHYTOAGGLO (ADEME - Agence De
581 l'Envrionnement et de la Maîtrise de l'Énergie), convention no. 1072C0045, 2013-2017). A. Grignet was granted by
582 ADEME (no. TEZ17-21) and the Hauts de France Region.

583
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Supplemental Table 1 Ranking of the site by SET according to their risk of metal transfer (laboratory exposure).

	Snail viscera concentrations ($mg_{metal}Kg_{sn}^{-1}$ DW)								accumulation quotient (AQ)								SET
Modality	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn	
S.viminalis TE-	1.12	0.34	156.14	161.83	180.89	0.88	0.77	649.82	**1.77**	1.00	**1.64**	1.00	**1.56**	1.00	**1.96**	**2.84**	**4.78**
S.viminalis phytoagglo TE+	1.58	0.69	158.72	187.78	148.20	1.08	0.77	824.07	**2.51**	1.00	**1.66**	1.00	**1.28**	1.00	**1.97**	**3.61**	**6.04**
A.halleri TE-	12.77	0.41	131.13	172.65	133.56	1.52	1.02	2536.72	**20.26**	1.00	**1.37**	1.00	**1.16**	1.00	**2.59**	**11.10**	**31.49**
A.halleri auby TE+	53.89	0.61	146.89	216.92	170.09	2.09	19.23	5969.59	**85.48**	1.00	**1.54**	1.04	**1.47**	1.00	**48.97**	**26.12**	**158.62**
Control	0.63	1.02	95.38	209.54	115.60	2.40	0.39	228.53									

Supplemental Table 2 Ranking of the site by SET according to their risk of metal transfer (field exposure).

	Snail viscera concentrations ($mg_{metal}Kg_{sn}^{-1}$ DW)								accumulation quotient (AQ)								SET
modality	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn	
A.halleri control TE-	11.63	0.61	144.78	316.79	116.84	2.40	1.32	1446.87	**5.12**	1,00	1,00	**1,51**	1,01	1,00	1,00	1,00	**4,65**
A.halleri phytoagglo TE+	10.19	2.42	149.42	713.24	261.75	3.14	16.40	6327.33	**4.49**	**1,20**	1,00	**3,40**	**2,26**	1,00	**1,27**	**4,25**	**10,88**
CIRef	2.27	2.01	184.70	209.54	115.60	5.17	12.90	1490.00									

Supplemental Table 3 Metal concentration in soil, leaves of *Salix viminalis* and calculated phytoextraction potential. Data are Cd and Zn concentrations in soil and leaves of *S. viminalis* collected in autumn 2016 (mean ± SD), bioconcentration factors (BCF, calculated as metal concentration in leaves over soil total metal concentration), biomass yield (based on leaf fall in autumn 2016), Cd and Zn removal per year and predicted cleanup time supposing a linear extraction expressed as years, specific soil density of 1.500 Kg m^{-3} and depth for remediation of 0.2m. Scenario : cleanup time needed to reduce the soil Cd and Zn concentrations to the half.

Element	Soil concentrations (mg Kg^{-1} DW)	Willow leaves concentrations (mg Kg^{-1} DW)	BCF	Biomass yield (Kg ha^{-1})	Metal removal (Kg ha^{-1} $year^{-1}$)	Cleanup time (year)
Cd	1.66 ± 0.2	4.2 ± 1.6	1.8	579.62	0.0036	550
Zn	615.5 ± 248	1309 ± 490	1.5		1.67	565

Supplemental Table 4 Metal concentration in soil, leaves of *Arabidopsis halleri* and calculated phytoextraction potential (*A. halleri* harvested and co-cropping). Data are Cd and Zn concentration in soil and in *A. halleri* (Fig 4 – WF1, rosette stage in presence of NPK fertilizer), bioconcentration factor (BCF, calculated as *A. halleri* metal concentration over soil total metal concentration), biomass yield, Cd and Zn removal per year and predicted cleanup time supposing a linear extraction expressed as years for *A. halleri* specific soil density of 1.500 Kg m^{-3} and depth for remediation of 0.2m. Scenario : cleanup time needed to reduce the soil Cd and Zn concentrations to the half.

	Element	Soil concentration (mg Kg^{-1} DW)	*A. halleri* concentrations (mg Kg^{-1} DW)	BCF	Biomass yield (Kg ha^{-1})	Metal removal (Kg ha^{-1} $year^{-1}$)	Cleanup time (year)
A. halleri	Cd	1.66 ± 0.2	13.67	1.12	3500	0.051	39
	Zn	615.5 ± 248	10586	2.05		37	25.5
A. halleri and willow leaves	Cd	1.66 ± 0.2			3500 + 580	0.054	36
	Zn	615.5 ± 248				38.67	24

Chapitre 3 : Phytoextraction du Zn et du Cd avec Arabidopsis halleri et Salix viminalis : la fertilisation azotée et l'inoculation mycorhizienne permettent-elles d'augmenter la biomasse de ces espèces ? .

Ce chapitre est présenté sous forme d'un article qui a été soumis dans « Environmental Science and Pollution Research » en décembre 2020.

Un des facteurs limitant l'implantation *in situ* de la phytoextraction avec des hyperaccumulateurs est leur faible biomasse. Le moyen le plus efficace pour augmenter la biomasse des espèces végétales est l'utilisation de fertilisants chimique ou biologique.

Dans le chapitre précédent, nous avons pu démontrer que l'utilisation de fertilisant chimique (fertilisant NPK) permettait d'augmenter le potentiel de phytoextraction de l'arabette de Haller au stade rosette (augmentation significative des concentrations en ET et tendance à l'augmentation pour la biomasse). De nombreux travaux ont montré que l'utilisation de biofertilisant, en particulier des inoculums microbiens à base de champignons mycorhiziens, pouvait avoir un effet bénéfique sur l'installation, la croissance, la tolérance et la survie des plantes en améliorant leur nutrition minérale et hydrique et en leur procurant une meilleure protection contre le stress oxydant induit par de nombreux polluants tels que les ET (Firmin et al. 2015; Labidi et al. 2017). Les champignons mycorhiziens peuvent également faciliter le prélèvement et l'accumulation des ET dans les parties aériennes de la plante en augmentant la densité des racines et le volume de sol exploré par le réseau mycélien (Göhre and Paszkowski 2006; Bhalerao 2013). Cependant, les espèces végétales appartenant à la famille des Brassicaceae sont réputées non mycorhizables (Demars and Boerner 1996). Pourtant, quelques travaux ont montré qu'à certaines périodes de leur cycle de développement et/ou dans certaines conditions, certaines espèces de Brassicaceae, *Noccaea caerulescens*, *T. goesingense*, *T. calaminare*, *T. cepaeifolium*, pouvaient être mycorhizées (Regvar et al. 2003; Hildebrandt et al. 2007).

Ainsi, le premier objectif de ce chapitre était d'étudier l'effet du fertilisant azoté sur l'ensemble du cycle de l'arabette de Haller (rosette, floraison, fructification). Le second objectif concernait l'étude de l'éventuel statut de mycorhization de l'arabette de Haller à ces différents stades de développement en présence et en absence de fertilisant NPK.

Par ailleurs, l'influence de la mycorhization arbusculaire des saules sur sols pollués a été peu explorée. La phytoextraction du Zn et du Cd chez *S. dasyclados* est augmentée grâce à l'inoculation par des champignons ectomycorhiziens qui permet une meilleure croissance des saules et augmente ainsi la biomasse produite (Baum et al. 2006). La capacité de mycorhization des saules semble dépendre des conditions du sol et de la disponibilité de certains nutriments minéraux dont le phosphate et l'azote par exemple.

L'étude des interactions saule-sol-champignons mycorhiziens arbusculaires (CMA) en plein champ et en conditions contrôlées en tenant compte des stades de développement du saule et des conditions physico-chimiques et agronomiques du sol permettrait de définir les possibilités d'utiliser la mycorhization comme une nouvelle stratégie pour augmenter les performances de phytoextraction du saule. L'effet de la mycorhization du saule sur la mobilité des ET du sol à proximité d'*A. halleri* dans un scénario de co-culture n'a, à notre connaissance, jamais été étudié. La mycorhization du saule pourrait augmenter le pool labile d'ET accessible par l'arabette de Haller et ainsi impacter positivement ses capacités phytoextractrices.

Les résultats obtenus nous ont permis de mettre en évidence l'intérêt d'utiliser un fertilisant azoté en condition *in situ* pour augmenter la biomasse de l'arabette de Haller de 50%, ce qui corrobore ce qui a été rapporté chez *N. caerulescens* (Jacobs et al. 2018a). L'utilisation du fertilisant permet également d'homogénéiser les concentrations en Zn foliaire et d'augmenter la concentration en Cd au stade fructification. Ces résultats confirment le bénéfice d'utiliser un fertilisant azoté comme pratique agronomique pour réduire la limitation due à la faible biomasse des hyperaccumulateurs.

Par ailleurs, nous avons montré qu'*in situ*, l'arabette de Haller ne présente pas de mycorhization par des champignons mycorhiziens à arbuscules (CMA) quel que soit son stade de développement et ce aussi bien sur les parcelles amendées ou non avec le fertilisant azoté. Des essais d'induction de la mycorhization de l'arabette de Haller menés dans des conditions contrôlées, en présence d'un inoculum mycorhizien commercial et d'une plante nurse nous ont permis de confirmer le statut non mycotrophe de l'arabette de Haller par les CMA. Cependant, nous avons réussi à montrer que cette Brassicaceae était capable d'être colonisée par un champignon endophyte, *Piriformospora indica.* Confirmant ainsi les résultats de récentes études qui ont montré que ce type de champignon pouvait coloniser également *A. thaliana* (Abdelaziz et al. 2017; Thürich et al. 2018). Le taux de colonisation de *A. halleri* par *P. indica* en conditions contrôlées était de 8%. Ces résultats sont très encourageants et demandent à être d'avantage explorés. En effet, l'utilisation de champignons endophytes tolérants au Zn et au Cd pourrait être une stratégie afin d'augmenter la biomasse et/ou le transfert d'ET chez *A. halleri*.

Par ailleurs, chez le saule, la pré-inoculation par un inoculum mycorhizien commercial à base de CMA a montré un taux de mycorhization à hauteur de 10% dans des conditions contrôlées. Ces résultats sont cohérents avec ce que l'on retrouve dans la littérature. En effet, les saules sont faiblement mycorhizés à l'état naturel que ce soit sur milieux pollués ou non (Van der Heijden 2001; Bissonnette et al. 2010).

Title: Phytoextraction of Zn and Cd with *Arabidopsis halleri* and *Salix viminalis*: a focus on nitrogen fertilization and mycorrhizal inoculation as a means of increasing biomass.

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Abstract

The current work aims to investigate the influence of nitrogen fertilization (N-fertilizer) and fungal inoculation (*Funneliformis mosseae* and *Piriformospora indica*, respectively arbuscular mycorrhizal (AMF) and endophytic fungi) on the phytoextraction potential of *Arabidopsis halleri* (biomass yield and/or aerial part Zn and Cd concentrations) over one life plant cycle. In addition, *Salix viminalis*, which interacts with *A. halleri* on the metal-polluted field site, was inoculated with AMF and ectomycorrhizal fungi to assess their effect on willow growth. The mycorrhizal rates of *A. halleri* and *S. viminalis* were measured *in situ* while the fungal inoculation experiments were carried out under controlled conditions. N-fertilizer was found to increase the biomass of *A. halleri* at rosette, flowering and fruiting stages. N-fertilizer reduced the Zn concentration variability between developmental stages and increased the Cd concentration at fruiting stage. Contrary to willow, *A. halleri* roots did not show AMF colonization at any stage in our field conditions, neither in the absence nor in the presence of N-fertilizer, thus suggesting that *A. halleri* is not naturally mycorrhizal. Induced mycorrhization agreed with this result. However, *P. indica* has been shown to successfully colonize *A. halleri* roots under controlled conditions. This study confirms the benefit of using N-fertilizer to increase the phytoextraction potential of *A. halleri* and identifies *P. indica* as a potential means of improving *A. halleri* phytoextraction performances.

Keywords: phytoextraction, *Arabidopsis halleri*, nitrogen fertilizer, mycorrhization, Cd, Zn

Funding information

This work was supported by national funds through PHYTOAGGLO and EXTRA-Zn projects (ADEME - Agence De l'Environnement et de la Maîtrise de l'Énergie, conventions n° 1072C0045, 2013-2017 and 1972C0007, 2019-2022). This work has also been carried out in the framework of the Alibiotech project which is financed by the European Union, the French State and the French Region of Hauts-de-France. A. Grignet received grants from ADEME (n° TEZ17-21) and the Hauts-de-France Region.

Authors' contributions

This collaboration work was carried out among all the authors. AG performed all the experiments reported in the manuscript, designed outlines, wrote the draft and prepared the figures and tables of the manuscript. VB and AL discussed the original idea of the manuscript with AG. VB wrote some part of the manuscript. VB, AL and JF reviewed the manuscript. AG was assisted by ST for the experimental work and analyses. AP was responsible for the validation of the TE analyses. All authors read and approved the final submitted version of the manuscript.

Acknowledgments

The authors would like to thank the Creil conurbation, and particularly L Raphael Pardon (ACSO – Agglomération Creil Sud Oise) and JL Deremy (Mairie de Montataire), for providing access to the field site and technical support during the entire duration of the project. The authors are also grateful to Fabrice Richez for his technical contribution during the sampling campaigns and chemical analyses. We also thank the Solten company (https://solten.co.uk/en/) for improving the quality of the English text.

Introduction

Phytoextraction is an environmentally friendly green technology in comparison to the physical and chemical remediation techniques used to clean-up contaminated soils. This phytotechnology uses the capacity of hyper(accumulator) plants to extract trace elements (TE) from polluted soils (Krämer 2005; McGrath et al. 2006; Hooper et al. 2016). Contrary to conventional (physicochemical) techniques, it has the advantage of being applicable *in situ* on a large surface and of preserving the structure and microbial properties of the soil (Bert et al. 2017). However, few field trials or commercial operations using phytoextraction have been implemented (Robinson et al. 2006; Jacobs et al. 2017; Grignet et al. 2020). Currently, only the Ni hyperaccumulator *Alyssum sp.* has been developed into a commercial technology (Chaney et al. 2018). Recently, it has been shown that phytoextraction can enable the production of wood for the energy sector and of new raw material formed from aerial biomass rich in TE (Delplanque et al. 2013; Deyris et al. 2018). It has also been demonstrated that some TE, including Zn, accumulated in the foliar biomass of hyperaccumulators can be used in ecocatalysis for industrial chemistry (Grison 2015; Deyris et al. 2018).

One of the main factors limiting the implementation of *in situ* phytoextraction is the fact that most hyperaccumulator species produce low biomass. Several strategies, including agronomic practices, could be tested to increase the biomass production and/or TE accumulation of these plant species and thus their extraction capacity. The most common method used to boost plant growth and obtain higher biomass is fertilization by different chemical or biological amendments. The benefits of adding N-fertilizer have been demonstrated in several studies that report an improvement in biomass and/or TE accumulation in Brassicaceae (Bennett et al. 1998; Xie et al. 2009; Jacobs et al. 2018; Grignet et al. 2020). For example, Jacobs et al. (2018) showed that using N-fertilizer increases the biomass of *Noccaea caerulescens* by more than 50%. Mycorrhizal fungi have long been thought to usefully improve plant growth and tolerance to TE, biomass yield and/or TE shoot accumulation (Bi et al. 2003; Hui et al. 2015; Toler et al. 2005; Marques et al. 2007; Lebeau et al. 2008, Phanthavongsa et al. 2017; Wani and Gopalakrishnan 2019). However, most of these experiments were carried out in pots under controlled conditions rather than in the field (Colpaert et al. 2011). Plant species belonging to the Brassicaceae family are known to be non-mycorrhizal by arbuscular mycorrhizal fungi (AMF) (Harley and Harley 1987; Demars and Boerner 1996). However, some studies have shown that *N. caerulescens, N. goesingense, N. calaminare* and *N. cepaeifolium* can be colonized by AMF at some stages of their development (Regvar et al. 2003; Hildebrandt et al. 2007). Furthermore, several studies have shown the beneficial effect of the endophytic fungus, *Piriformospora indica*, on plant growth under TE stress (Padash et al. 2016; Hui et al. 2015; Nanda and Agrawal 2018) and its ability to

colonize Brassicaceae species such as *Arabidopsis thaliana* (Thürich et al. 2018; Abdelaziz et al. 2017, Veiga et al. 2013).

Arabidopsis halleri, of the Brassicaceae family (formerly *Cardaminopsis halleri* (L.) Hayek), is well-known as a Zn and Cd hyperaccumulator (Bert et al. 2000, 2002, 2003; Verbruggen et al. 2009, 2013). Despite this property, very few field studies have reported the performance of this plant in phytoextraction (McGrath et al. 2006; Grignet et al. 2020). For the first time at field scale, Grignet et al. (2020) demonstrated the beneficial effect of the NPK fertilizer on the Zn and Cd concentrations in *A. halleri* at the rosette stage. In addition, the authors showed that this fertilizer has a positive effect on the biomass yield at the rosette stage, the yield results being comparable to those reported in the field for *N. caerulescens* (Jacobs et al. 2018). To the best of our knowledge, the effect of N-fertilizer on *A. halleri* biomass over its entire life cycle has never been reported. Moreover, no data are available on the ability of mycorrhizal or endophytic fungi to colonize *A. halleri*.

Some studies have shown that the co-cultivation of several accumulator species can also improve biomass yields (Wu et al. 2007; Wei et al. 2011). In field conditions, Grignet et al. (2020) demonstrated the synergetic effect of *A. halleri* and the accumulator *Salix viminalis* on Zn and Cd phytoextraction, the co-culture increasing the Zn and Cd extraction in comparison to a monoculture. Willows are fast growing TE-accumulating trees (Van Slycken et al. 2013; Kidd et al. 2015) that can be mycorrhized by both AMF and ectomycorrhizal fungi (ECM) (Aliferis et al. 2015). Very few studies, of which only one was carried out in field conditions, have reported the impact of mycorrhizal fungi on the phytoextraction performance of willows (Lingua et al. 2008; Labrecque et al. 2006; Bissonnette et al. 2010).

Thus, focusing on agronomic practices (N fertilization; AMF, ECM and endophytic fungi inoculation), the present study was conducted in both controlled and field conditions on TE-contaminated soil with the aim of (i) optimizing phytoextraction performances of the hyperaccumulator *A. halleri* by improving biomass production and/or shoot Cd/Zn concentration, and (ii) determining the optimum stage of plant development to maximize Zn concentration for ecocatalysis. To meet these objectives, the Zn and Cd concentrations in the plant at different stages of its life cycle (rosette, flowering and fruiting) and its biomass in the presence or absence of N-fertilizer were measured. This is the first time that the natural mycorrhizal status of *A. halleri* has been investigated in the field over an entire life cycle. In addition, mycorrhization was induced in controlled conditions in *A. halleri* and *S. viminalis* (as a co-actor of a phytoextraction strategy), to investigate the possibility of using such a biological amendment in a pre-inoculation step before scaling up at field scale. The efficiency of the pre-inoculation on willows was assessed by measuring the growth parameters.

Materials and Methods

Site description

The field trials were conducted in the urban area of Montataire (Oise, France, 49°15’12’’N, 2°26’48’’E). Details of the overall physicochemical characteristics of the site have been provided previously (Grignet et al. 2020). Briefly, the topsoil was contaminated mostly by Zn (616 ± 248 mg kg^{-1}) and Cd (1.7 ± 0.2 mg kg^{-1}) and presented an alkaline pH (> 8). Nitrogen and phosphorus concentrations were respectively about 2.08 ± 0.28 and 0.80 ± 0.1 $g.kg^{-1}$.

In situ experimental design

The experimental design consisted of several 1m² plots of *A. halleri* set up between the rows of a willow plantation (650 m²). The study was carried out on four plots of 1m², set up in the summer 2018 (P14, P15, P16 and P17) (Fig 1). In these plots, *A. halleri* seedlings were transplanted at a density of 50 plants per m² after 2 months of growth on organic compost (Terreau potager bio, Gamm vert) in a culture chamber (20°C, 70% moisture, 12 h day cycles). The *A. halleri* seeds came from a metallicolous population (Parc Péru, Auby, Nord, France). Before transplantation, 100 g per m^2 of nitrogen (N-) fertilizer (Biogine® NPK 4-6-10) was incorporated into the top-soil (10 cm depth) on the plots P14 and P15.

In situ experiments: plant and soil samplings and analyses

On the *A. halleri* plots (Fig. 1), 500g of contaminated soil was collected (March 2018) randomly to determine the number of AMF spores. The AMF spores were isolated by wet sieving (Gerdeman and Nicolson 1963). 100g of fresh soil was deposited in a series of sieves with decreasing meshes (250, 125, 40 µm) under a trickle of water. The particles from each sieve were collected, placed in a beaker and then observed with a binocular magnifying glass. Spores from each fraction were counted and collected for preservation.

The aerial parts and roots were sampled in spring and summer 2019 to allow collection at various developmental stages (rosette, flowering and fruiting) on the four plots (Fig. 1). The aerial materials were carefully washed with tap and deionized water and then dried at 40° C until they reached a constant weight. The Zn and Cd in the plant samples (0.5 g dry weight) were quantified by ICP-OES (Agilent 5110) after digestion at 180 °C for 20min in 10 ml of nitric acid (67% AnalaR Normapur) and 3 ml of ultra-pure water using a microwave digester (Mars Xpress CEM). One plant of standard reference material was used for analytical quality control (cabbage “NCSZC 73012”, NACIS).

The roots of three colonizer plants (*Mercurialis annua*, *Lamium purpureum*, *Potentilla reptans*) were collected at the same time as the *A. halleri* roots to check their natural mycorrhizal status.

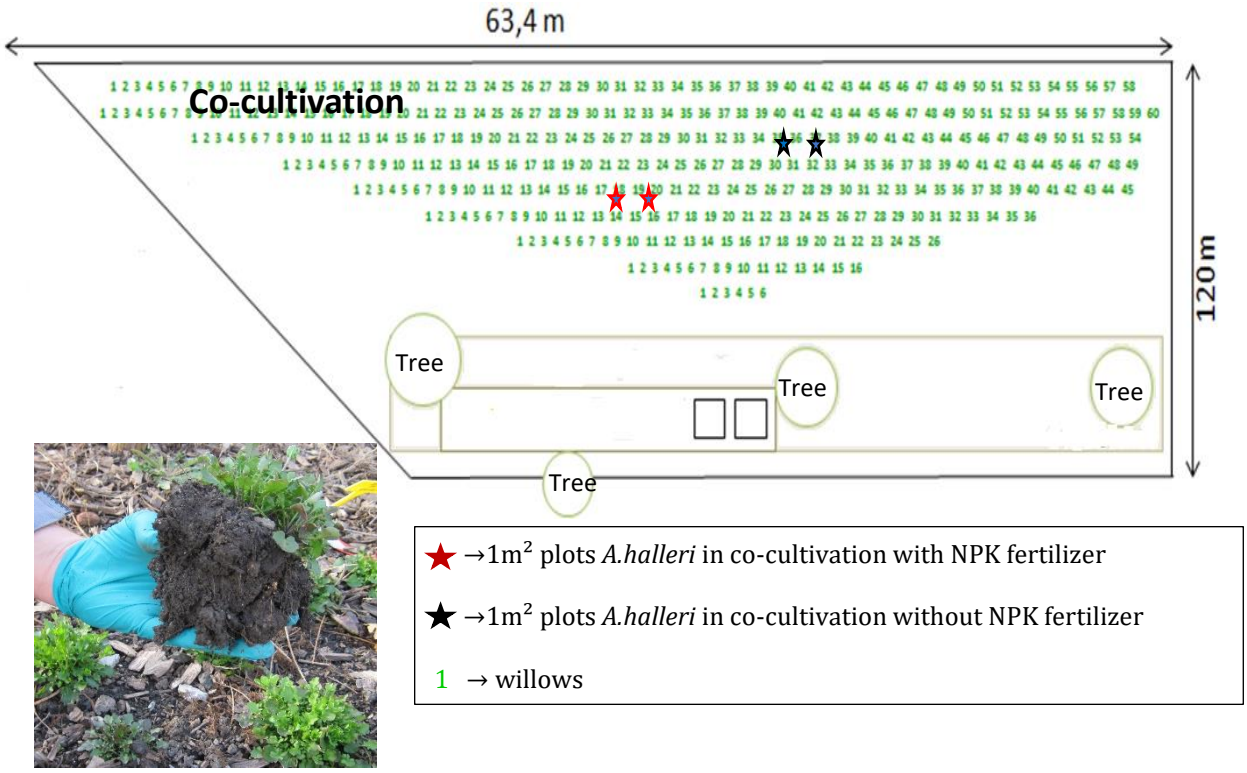

Fig. 1 Experimental design of the field trial.

Biological amendments

The commercial mycorrhizal inoculums used in the present study were the AMF inoculum FR140® (*Funneliformis mosseae*, MycAgro Ltd., France) and the ectomycorrhizal inoculum Ectovit® (Symbiom, Lanskroun, Czech Republic) containing the mycelium of four ECM (*Cenococcumgeophilum*, *Hebeloma velutipes*, *Laccariaproxima* and *Paxillus involutus*). The endophytic fungus *Piriformospora indica* (strain DSM 11827) was kindly provided by Professor P. Franken from the University of Lausanne. The *P. indica* was cultivated in liquid culture Kaefer Medium (KM; Hill and Käfer 2001) and incubated for ten days at 28 °C at 150 rpm in a rotary shaker. After 10 days, the liquid *P. indica* culture was harvested by centrifugation and the mycelium was washed three times with sterilized water. One gram of fresh mycelium was added to 100 ml of sterilized water and fragmented by blending for one minute to obtain the inoculum.

Pot experimental set up to induce mycorrhizal inoculation of *A. halleri*

The *A. halleri* was cultivated on composite soil (sand, perlite, vermiculite 2:2:1, v/v/v) either alone in pots (1L) with 100g of AMF inoculum (FR140®) or together with a host species (*Trifolium repens*, known to be well mycorrhized with AMF, was used to pre-establish the arbuscular mycorrhizal network) in 1.5L pots. These latter were divided into two equal compartments by a 30 µm nylon mesh to separate roots while allowing the passage of AMF hyphae (Fig 2). The nylon mesh was used to reduce the effects of direct root competition. Each half received 0.75 L of soil. The host plant *T. repens* seedlings were transplanted in half of the pot with 75g of AMF inoculum (FR140®) and the *A. halleri* seedlings were transplanted in the other half.

A. halleri was also cultivated in pots (1L) then inoculated with 2 ml of mycelium of *P. indica* suspension directly injected to the root rhizosphere 2 weeks after seed germination.

The plants (5 replicates per condition) were irrigated three times a week with tap water and maintained in a growth chamber (20°C, 70% moisture, 12 h day cycles) for 8 weeks.

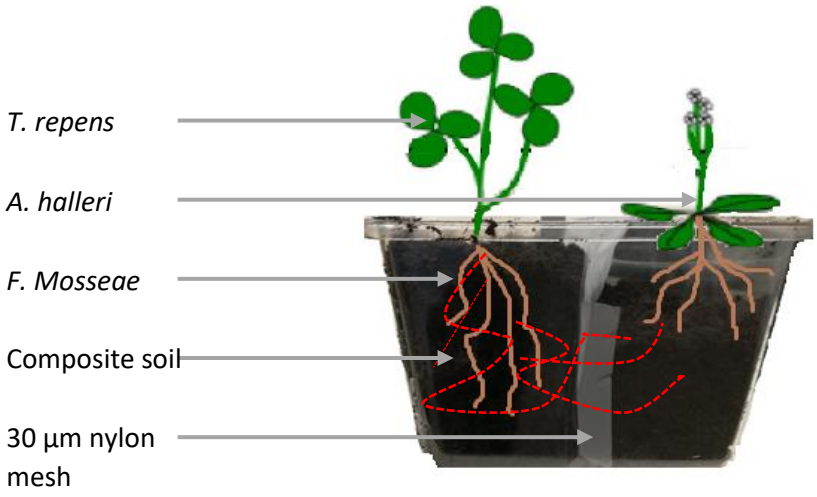

Fig. 2 Representation of a dual-compartment microcosm containing on the left, the host plant (*T. repens*), used to pre-establish the arbuscular mycorrhizal network and on the right, *A. halleri*. The two root systems were separated by a 30 mm nylon mesh (permeable to hyphae) to reduce the effects of direct root competition.

Pot experimental set up for induced mycorrhizal inoculation of *S. viminalis*

Unrooted cuttings of *S. viminalis* of 20 cm length were bought at a wood nursery (De Vos “Salix”, Belgium, Lokeren). The experiment was carried out on composite substrate (sand, perlite, vermiculite, 2:2:/1, v/v/v). 4

conditions were tested (none, AMF, ECM, AMF+ ECM), with 10 replicates (4L pots) for each condition. 400g of AMF (FR140®) and/or 20g of ECM (*Ectovit®)* were added per pot, according to the suppliers' instructions.

The morphometric parameters of the willows (height, diameter, number of stems and leaves) were measured at the start and at 6 weeks of culture in a growth chamber (20°C, 70% moisture, 12 h day cycles). This non-destructive method allowed the further willow transplantation on the field site.

In situ transplantation of pre-inoculated willows

In July 2019, after 6 weeks of culture in a growth chamber, 30 pre-inoculated willows and 10 non-inoculated willows were transplanted on the TE-polluted site (Fig. 1). The willows were set up in two rows with an inter-row distance of 1.5 m and 0.5 m spacing between plants. The survival rate was measured in March 2020.

P. indica metal tolerance tests

The sensitivity of *P. indica* to Cd and Zn was evaluated by investigating the minimum inhibitory concentration (MIC) on fungal growth. MIC is defined as the minimum inhibitory concentration of metals required to completely inhibit fungal growth. The plugs (5 mm) were cut from the edge of actively growing two-week-old colonies and placed on solid Kaefer Medium (KM; Hill and Käfer 2001) amended with metals at the following concentrations: 0, 2, 4, 6 and 8 mM for $ZnCl_2$ and 0, 10 and 50 µM for $CdCl_2$. The concentrations tested were selected based on previous studies (Berthelot et al. 2016; Lacercat-Didier et al. 2016). Radial growth was measured after two weeks of incubation at 24°C.

Determining fungal root colonization of *A. halleri*, willows and colonizer plants

To quantify the AMF root colonization, fresh roots of *A. halleri*, willows and colonizer plants were collected from the contaminated soil and/or the pots, then carefully washed with demineralized water and cleared in KOH (10%) for 10 min at 90 °C. The roots were rinsed in water and stained with trypan blue (0.05%), as described by Philips and Hayman (1970) and modified by Koske and Gemma (1989). The stained root fragments were stored in a water/glycerol/lactic acid (1:1:1, v/v/v) solution until observation. The mycorrhizal rate was determined by microscopic examination of the stained root samples, using McGonigle et al. (1990) method. One hundred and thirty-five root 1cm fragments from each different pot were observed under an optical microscope (× 100 magnification) in order to count the mycorrhizal structures (arbuscules, vesicles and hyphae). Three grid-line intersections per root fragment were examined. In total, more than 400 observations per condition were analysed.

To quantify the ectomycorrhizal root colonization, the fresh willow roots were collected from the pots and carefully washed with demineralized water. 100 root points were then observed with binocular magnifiers to determine the mycorrhizal rate.

The endophytic fungi (*P. indica*) was quantified using the *A. halleri* root sample measurements resulting from the induced inoculation experiment previously described. The same staining protocol as that used for the AMF observation was applied. The mycorrhizal rate was determined by microscopic examination. *A. halleri* root fragments of 1 cm in length were extracted from each pot and observed under an optical microscope (× 100 magnification) in order to count the chlamydospores (Johnson et al. 2011).

Statistical analyses

All data were pre-analysed by normality tests (Shapiro-Wilk test) and homogeneity of variances (test F). The *A. halleri* metal concentrations and dry weight (n=6) and the willow morphometric parameters (n=10) were analysed with the Kruskal-Wallis H test. All statistics were performed with the R statistical software 3.2.2 (R Core Team).

Results and discussion

What effect did the N-fertilizer have on *A. halleri* biomass and Cd/Zn concentrations in function to the developmental stage?

Without N-fertilizer, the highest biomasses were obtained at the flowering and fruiting stages (Fig. 3 A). While a significant increase in biomass was observed without N-fertilizer (0.32 ± 0.05 g at rosette stage to 1.00 ± 0.41 g at flowering stage), no significant difference was observed in the biomass obtained with N-fertilizer due to a large individual heterogeneity (2.33 ± 0.7 g at rosette stage to 5.28 ± 1.5 g at flowering stage and 6.02 ± 2.9 g at fruiting stage). This finding confirms previously obtained results showing that N-fertilizer has a long-term effect on biomass (Grignet et al. 2020). However, it is noteworthy that the quantity of biomass obtained on the plot with N-fertilizer was significantly higher (+50 %) than that on the plot without N-fertilizer for each developmental stage (Fig 3 A). These results demonstrate for the first time that N-fertilizer increases the biomass of *A. halleri*, whatever its growth stage. They are in line with previous studies conducted on *N. caerulescens* showing that N-fertilizer additions can lead to an increase in biomass of up to 50% (Bennette et al. 1998; Xie et al. 2009; Jacobs et al. 2018). In Jacobs et al. (2018), the dose of N-fertilizer was the same as that used in this study.

Without N-fertilizer, the concentrations of Cd and Zn in aerial parts of *A. halleri* did not change in function to life cycle stages (Fig. 3 B and C). However, during the flowering stage, a large individual heterogeneity was observed in Zn accumulation. At this stage, some individuals exceeded the threshold of hyperaccumulation for Zn (>10,000 mg. kg^{-1}), as previously reported on the same site (Grignet et al. 2020). Contrary to Zn (Fig. 3B), the Cd concentrations increased according to life cycle in the presence of N-fertilizer (Fig. 3C), the highest Cd concentration being observed at the fruiting stage (10.2 ± 1.48 $mg.kg^{-1}$ DW). The increase in Cd concentrations can be explained by the fact that Cd follows paths designed for essential ions such as calcium channels (Cataldo et al. 1983). N-fertilizer could have decreased the soil pH, facilitating Cd mobility and thus its absorption, although this was not observed in Grignet et al. (2020). N-fertilizer did not significantly increase the mean concentration of Zn between stages. Although, a tendency in Zn was noted in presence of the fertilizer compared to the conditions without fertilizer. Contrary to the previous study, a homogenization in Zn concentrations between stages and within a single stage (decrease of the inter-individual variability) was observed.

Our findings are in line with many previous studies showing that N-fertilizer increases phytoextraction potential by enhancing biomass and/or TE concentration in hyperaccumulators (Bennett et al. 1998; Xie et al. 2009, Jacobs et al. 2018a; Grignet et al. 2020).

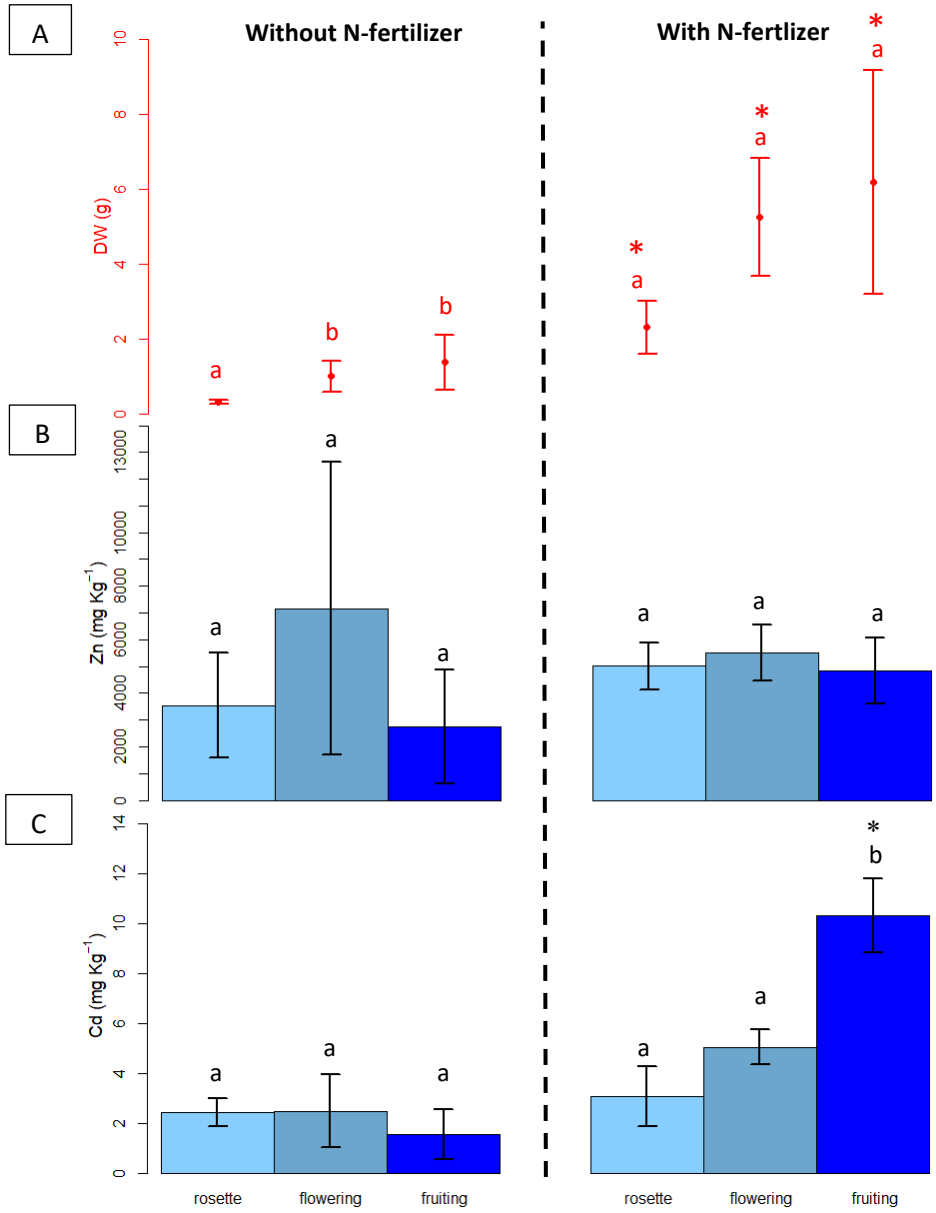

Fig. 3 Dry weight (A), Zn (B) and Cd (C) concentrations of *A. halleri* shoots (mean ± SD) according to different developmental stages (rosette (light blue), flowering (blue grey) and fruiting (dark blue). Significant differences between stages are indicated by different letters at $\alpha = 0.05$, significant differences between conditions at the same stage are indicated by ∗ at $\alpha = 0.05$ (n=5).

Grignet et al. (2020) found that the most suitable growth stages for use in Zn-ecocatalysis production were the flowering and fruiting stages when *A. halleri* was not exposed to N-fertilizer, and the rosette stage after 1 month of exposure to N-fertilizer. This study confirmed that the highest Zn concentrations were found at the flowering stage when the plant was not exposed to the fertilizer. Although the Zn concentrations did not differ in the presence and absence of N-fertilizer, the average concentrations were roughly doubled which suggests that all stages are suitable for ecocatalysis production. Nevertheless, it would be advisable to select the rosette or flowering stages

to decrease the Cd concentration, which could represent a limitation for this purpose (Grignet et al. 2020). The estimated biomass yield was between 1.3 and 3.7 t. ha^{-1} for the N-fertilizer plots, and between 0.4 and 1.5 t. ha^{-1} for the plot without N-fertilizer (Supplemental Table 1). These results confirm those previously obtained in Grignet et al. (2020). The quantity of Zn exported was higher at the flowering stage for plots without N-fertilizer and at the flowering and fruiting stages for plots with N-fertilizer (respectively 11 and 17 kg. ha^{-1}. $Year^{-1}$) (Supplemental Table 1). To obtain the maximum Zn concentrations for ecocatalysis and to maximize Zn export while limiting Cd, the flowering stage is the most suitable in both cases.

Does *A. halleri* naturally mycorrhize on a contaminated site?

Before testing the relevance of a mycorrhizal inoculum amendment in controlled experimental conditions, the natural mycorrhizal status of *A. halleri* was checked in the field site. The AMF colonization of *A. halleri* roots was investigated at different stages (rosette, flowering and fruiting) in the plots with and without N-fertilizer (Fig. 1). In these soil conditions, no specific AMF structures (arbuscules, vesicles, hyphae) were observed in *A. halleri* roots whatever the growth stage and regardless of the presence of N-fertilizer. Although the members of the Brassicaceae family are widely known to be non-mycorrhizal, natural mycorrhization of *A. halleri* could still be expected. Indeed, Hildebrandt et al. (1999) found a low colonization rate in *A. halleri* ranging from 1 to 7%, although no arbuscules, the main sites for nutrient exchange between the two partners of the mycorrhizal symbiosis, were observed. As reported for some Brassicaceae such as *Noccaea* species and *Biscutella laevigata,* it is possible that *A. halleri* could be mycorrhized during the reproductive period (flowering and fruiting stages) (Reagvar et al. 2003; Orlowska et al. 2002). Several hypotheses could explain the absence of mycorrhization in *A. halleri*. Firstly, TE may have a deleterious effect on AMF growth (reducing fungal biomass and mycorrhizal colonization) (Karlinski et al. 2009; Christie et al. 2004). Pawlowska and Charvat (2004) showed a decrease in hyphae density, spore germination rate, and presymbiotic hyphal extension in the presence of metallic stress on two AMF species (*Claroideoglomus etunicatum* and *Rhizophagus irregularis*). Secondly, TE could affect AMF spore formation and thus their availability in soil (Cabello 1997; Gattai et al., 2011). Indeed, several studies have shown that the number of AMF spores in polluted soil is low, ranging from 200 to 350 spores / 100 g dry soil (Cabello 2006; Mozafar et al. 2002), which agrees with the low quantification in the polluted soil studied in this work (220 spores / 100g of dry soil). For comparison, in unpolluted soil, the number of spores can reach 700 - 3000 spores / 100 g dry soil (Cabello 2006; Oehl et al. 2003; Gosling et al. 2010). Thirdly, it is known that high N

and phosphorus (P) concentrations are unfavourable to the establishment of mycorrhizal symbiosis and reduce spore germination (Christie et al. 2004). In our experimental conditions, the P concentration was 0.8 $g.kg^{-1}$ dry soil, demonstrating a P content to 8 times higher than the average P content measured in French soil (0.014 - 0.172 $g.kg^{-1}$ (Delmas et al. 2015)). Many works on the use of N-fertilizer have shown that the nature and the quantity of fertilizer used affect soil microbial communities (Bardgett et al. 1999; Lundquist et al. 1999), particularly AMF (Egerton-Warburton and Allen 2000). Indeed, the addition of N-fertilizer has been shown to reduce AMF species diversity and abundance, spore abundance, as well as hyphal and vesicular root colonization (Bardgett et al. 1999; Lundquist et al. 1999; Egerton-Warburton and Allen 2000). The initial amount of N in our soil was high (about 2.8 $g.kg^{-1}$ dry soil) compared to local soil concentrations (1 to 2.5 $g.kg^{-1}$ (Gis Sol, BDAT, 2011)), and so the addition of N-fertilizer is assumed to not favoured the spore germination, colonization and mycorrhization of *A. halleri*.

However, despite the high P and N content of the contaminated soil, the mycorrhizal rate of the natural colonizer plants collected on the site ranged between 6 and 15% (Table 1).The results may be low but they do suggest that mycorrhization of *A. halleri* could have occurred. Altogether, these findings tend towards the hypothesis that TE contamination and, probably the P and N contents, of the soil could have reduced the AMF colonization of *A. halleri* roots, although these factors did not completely inhibit it. This strongly suggests that *A. halleri* is a non-mycorrhizal species.

Table 1. Mycorrhizal status of the colonizer plant species grown on the contaminated experimental site (Mean ± SD).

Family	**Species**	**Total colonization rate %**	**Arbuscules %**	**Vesicules %**
Euphorbiaceae	***Mercurialis annua***	**6 ± 3**	**3 ±2**	**4 ±1**
Lamiaceae	***Lamium purpureum***	**12± 8**	**6 ±2**	**8 ±4**
Rosaceae	***Potentilla reptans***	**15± 11**	**8 ±4**	**9 ± 3**

Can mycorrhization be induced in *A. halleri* under controlled conditions?

To confirm the previous data obtained *in situ* conditions, an induced mycorrhization of *A. halleri* was carried out in controlled conditions in an uncontaminated substrate, as described in *A. thaliana* (Veiga et al. 2013). The controlled conditions made it possible to overcome the potential limitations described previously (limited number of spores, high levels of N, P and TE in soil). As a result, *T. repens* and/or *A. halleri* were cultivated on a neutral soil, which did not contain TE, in 1.5L pots, in the presence or absence of an AMF inoculum.

The total mycorrhizal rate of *T. repens* was estimated at 38% (Table 2) and arbuscules were observed (5%). Contrary to *T. repens*, the *A. halleri* roots did not show any colonization either when grown alone or in the presence of the host plant (Table 2). Microscopic observations of stained *A. halleri* roots showed extra-radical hyphae (Fig. 4), which proved that the experimental device worked well. The extra-radical fungal hyphae, produced by *T. repens* colonization, was able to cross the nylon membrane and reach part of the *A. halleri*. In spite of this, no colonization of *A. halleri* was observed. The same device was used in a study by Veiga et al. (2013) on *A. thaliana*, which was found to be colonized by the AMF *R. irregularis* (12%) when grown with the host plant *T. pretense.* However, no arbuscules were observed. Several hypotheses could be proposed to explain the non-mycorrhization of *A. halleri* in these controlled experimental conditions. The commercial AMF inoculum used may not be well-adapted to *A. halleri*. Indeed, in some studies, AMF have been found to be non-host specific (Gianinazzi-Pearson et al. 1985; Zhu et al. 2000) whereas others suggest a host plant-AMF relationship specificity (Sanders and Fitter 1992; Van der Heijden et al. 1998; Sanders 2002; Bever et al. 2003). Some plant species are susceptible to being colonized by one or more AMF species. The preference for one AMF species, known as ecological specificity (McGonigle and Fitter 1990), could be due to different root exudates (Steinkellner et al. 2007) or different types of arbuscular structure (Arum or Paris) (Smith and Read 2008).

Table 2. Root colonization of *Trifolium repens* and *Arabidopsis halleri* inoculated with AMF inoculum (FR140®) and *P. indica;* (Mean ± SD).

Inoculums	Culture conditions	Plant	Total colonization rate %	Arbuscules %	Vesicules %
AMF FR140®	**Monoculture**	***A. halleri***	**0**	**0**	**0**
	Culture combination	***T. repens***	**38.3 ± 7.4**	**5.3 ± 3.1**	**15.6 ± 4.2**
		A. halleri	**0**	**0**	**0**
Endophyte fungus P. indica	**Monoculture**	***T. repens***	**18 ± 4**	/	/
		A. halleri	**8 ± 2**	/	/

Taken together, our results demonstrate that *A. halleri* does not appear to be a mycorrhizal plant species or have an ecological specificity. To validate the last hypothesis, it would be interesting to test other mycorrhizal inoculums composed of other AMF species.

The most widespread hypothesis to explain why Brassicaceae plant species are not mycorrhizal is the deletion of symbiotic genes grouped under the name "symbiotic toolkit" (Delaux et al. 2013). They affect several colonization steps in pre-symbiotic, fungal entry, intraradical hyphal colonization and arbuscular formation (Cosme et al. 2018). In *A. thaliana,* these genes are not present. In *A. halleri,* no information is available. It would be interesting to assess these gene expressions in *A. halleri* in the future to reach a conclusion on the mycorrhizal potential of this species.

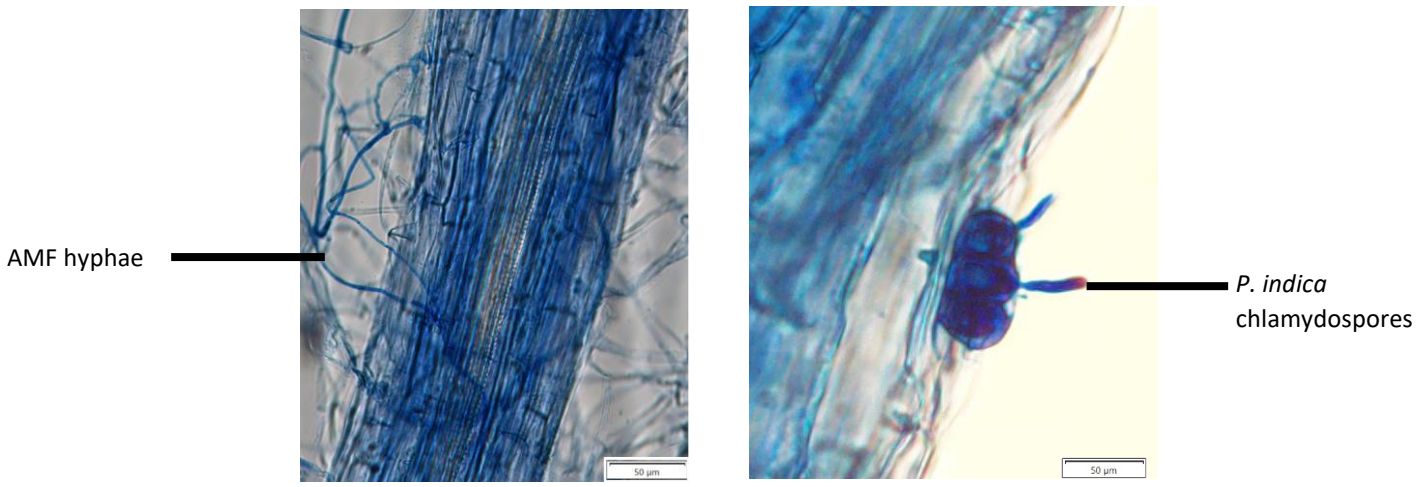

Fig. 4 Morphological characteristics of fungal structures in *A. halleri* roots (pot experiment). **a** extraradical hyphae, **b** colonization by fungal endophyte (*P. indica*, chlamydospores).

Can *A. halleri* be colonized by the endophytic fungus *P. indica*?

If *P. indica* was to be TE-tolerant, it could be an alternative to AMF in *A. halleri* to boost the plant's growth and/or TE accumulation. Recent studies have shown that *A. thaliana* can be colonized by *P. indica* (Thürich et al. 2018; Abdelaziz et al. 2017), and so a similar colonization of *A. halleri* was expected. Indeed, beyond the success of the experiment indicated by the colonization rate of *T. repens* (18%), *A. halleri* was colonized by *P. indica* at a rate of 8% (Table 2, Figure 4). Apart from the fact that this endophytic fungus has an exceptionally large plant host range (Qiang et al. 2012), several studies have shown that it has a beneficial effect on the growth of plants exposed to TE soil pollution (Padash et al. 2016; Hui et al. 2015; Shahabivand et al. 2017; Nanda and Agrawal 2018).

The results of the tolerance test showed a MIC of 2 mM for Zn and of 0.01 mM for Cd but rather different results were found with the same *P. indica* strain in a different study (MIC for Zn = 9mM and for Cd = 0.3mM in Berthelot et al. (2016)). However, since the culture medium and the TE bioavailability differed between the two studies, it is not possible to compare the MIC. In our experimental conditions, *P. indica* showed a low tolerance to Zn and no tolerance to Cd. Future work is required to confirm the TE tolerance of *P. indica* in soil conditions.

Did the pre-inoculation of willows by AMF and ECM improve their growth?

Induced mycorrhization was also performed on willows as a pre-requisite before scaling up to field scale, since it was assumed that this practice would reduce competition with indigenous fungi (Niwa et al. 2018) and favour the survival and growth of the willows (Maltz and Treseder 2015).

Table 3. Fungal colonization of *Salix viminalis* pre-inoculated (pot experiment) with AMF inoculum (FR140®) and Ectovit® (ECM). (Mean ± SD)

Inoculums	**Total colonization rate %**	**Arbuscules %**	**Vesicules %**
None	0	0	0
AMF FR140®+ *Ectovit®*	6 ± 1.3	2 ± 0.8	3 ± 1.2
AMF FR140®	10 ± 2.5	4 ± 1.8	3 ± 2.1
Ectovit®	0	0	0

In the presence of AMF alone, the mycorrhizal rate of *S. viminalis* was 10% (Table 3). When *S. viminalis* was co-inoculated with AFM and ECM, a colonization rate of 6% was observed. Arbuscular and vesicular structures of 2-4% and 3% respectively were observed in both cases (ECM+AMF and AMF) (Fig. 5). ECM were not observed, although the willows are able to make associations with both AMF and ECM (van der Heijden 2001; Puttsepp et al. 2004). These low mycorrhizal rates are consistent with those reported for *S. repens* inoculated with *F. mosseae*, where the mycorrhizal rates ranged between 2.5 and 4.2% (van der Heijden 2001). Another study, carried out in field conditions, reported that the AMF mycorrhizal rate of *S. viminalis* on a TE-polluted site ranged from 1 to 14% after 2 years of culture (Bissonnette et al. 2010).

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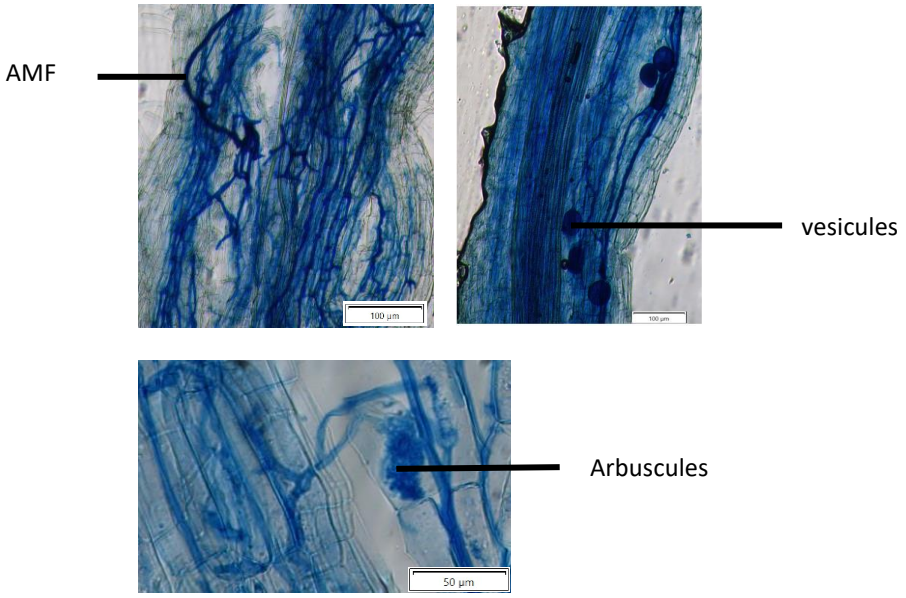

374 **Fig. 5** Fungal structures in *Salix viminalis* roots pre-inoculated (microcosm experiment) with AMF. **a**

375 colonization with AMF hyphae, **b** vesicules, **c** arbuscules.

376 We found no difference in morphometric parameters between non pre-inoculated and pre-inoculated willows (Fig.

377 6). For example, the willows' height increments were irrespective of the mycorrhization (218 ± 82 % versus 242

378 ± 52 %). To the best of our knowledge, there is no literature data available reporting the potential growth

379 improvement in willows after mycorrhizal pre-inoculation.

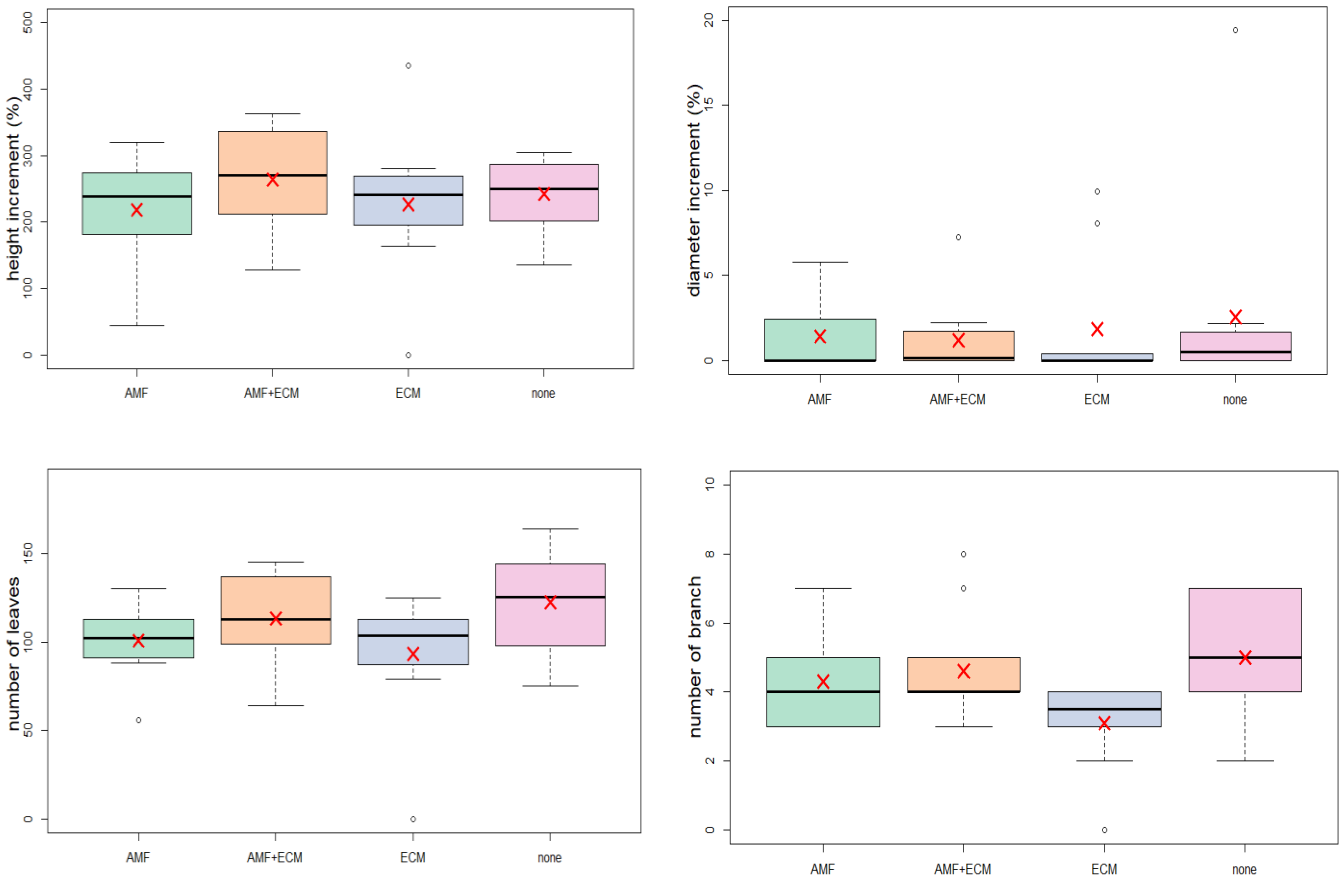

Fig. 6 Effect of different inoculums on morphometric parameters of *Salix viminalis* grown on composite soil. AMF inoculum (AMF); Ectovit® (ECM); AMF inoculum and Ectovit (AMF + ECM). The data indicate the median and the 25 and 75 percentiles; ×= mean, n=10. S

After the pre-inoculation, the willows were planted *in situ* and left to grow for one year. In March 2020, the survival rate of the pre-inoculated willows was between 10 and 20 %, regardless of the mycorrhizal modality. These low survival rates were probably due to late transplantation on the site (July 2019) and competition for light and water with colonizer plants. This result does not make it possible to draw a conclusion on the potential positive effect of pre-inoculating willows on this parameter.

AMF are dominant in the early years of willows and are gradually replaced by ECM (van der Heijden 2001; Puttsepp et al. 2004). Future work should focus on the long-term effect of AMF and ECM on survival rate and growth parameters, while comparing pre-inoculation and direct inoculation to the foot of the willow to ensure the efficiency of these practices on these parameters. Indeed, direct inoculation is the current practice for fast-growing trees (Bissonnette et al. 2010; Ciadamidaro et al. 2017). In addition, effect of AMF and ECM on TE willow shoot transfer should be studied.

Conclusion:

In this study, N-fertilizer and fungal inoculation were studied as a means of increasing the biomass of the hyperaccumulator *A. halleri*, since the plant's low biomass yield has been identified as a blocking point for an optimal Zn and Cd phytoextraction. For the first time, based on an *in situ* field study over one life cycle (rosette, flowering and fruiting), we have shown that N-fertilization increases both the biomass and the Cd concentration of *A. halleri*. These results confirm, on one hand, the benefit of N-fertilization as an agronomic practice to reduce the biomass limitation and, on the other hand, the usefulness of *A. halleri* in removing Zn and Cd from the soil. Further studies will focus on upscaling to produce practical advice for workers on polluted soils. This study confirms the benefit of *A. halleri* at all developmental stages for ecocatalysis production, based on the Zn concentrations obtained in the presence of N-fertilizer. In addition, this is the first time that the natural mycorrhizal status of *A. halleri* has been studied on a TE-polluted soil over one life cycle. and that induced mycorrhization on the species was performed. The results suggest that, like most members of the Brassicaceae family, *A. halleri* is not mycorrhizal. To definitively state on the mycorrhizal status of the species, other AMF inoculums should be tested. As an alternative to AMF, the effect of endophytic fungi such as *P. indica*, which successfully colonized *A. halleri*, on biomass and TE improvement could be investigated in future work. In a phytoextraction strategy, the use of several (hyper)accumulators should be considered in order to maximize the metal removal from the site, while focusing on other benefits such as aesthetics, biomass production for bioenergy and raw matter. For these purposes, our research will focus in the future on further investigating aspects of *A. halleri* and willows co-cultivation.

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Supplemental Table 1 : Biomass yield (based on an extrapolation to 1ha from the biomass of the plants collected on the $1m^2$, Cd and Zn concentration in *A. halleri* (mean ± SD, n = 6) and Cd and Zn removal per hectare and per year for each stage without and with N-fertilizer.

	Without N-fertilizer			With N-fertilizer		
	rosette	flowering	fruiting	rosette	flowering	fruiting
Biomass yield ($Kg\ ha^{-1}$)	466	1538	1303	1398	3168	3720
Zn concentration ($mg\ Kg^{-1}$ DW)	3549 ± 1969	7168 ± 5454	2755 ± 2134	5011 ± 885	5519 ± 1052	4832 ± 1233
Zn removal ($Kg\ ha^{-1}\ year^{-1}$)	1.6	11	3.5	7	17.4	17.9
Cd concentration ($mg\ Kg^{-1}$ DW)	2.42 ± 0.56	2.48 ± 1.47	1.56 ± 1.00	3.07 ± 1.19	5.04 ± 0.71	10.3 ± 1.48
Cd removal ($Kg\ ha^{-1}\ year^{-1}$)	0.001	0.003	0.002	0.004	0.015	0.038

Chapitre 4 : Evaluation du fonctionnement du sol pollué à l'aide d'indicateurs biologiques dans un contexte de phytomanagement

Ce chapitre est présenté sous forme d'un article qui sera prochainement soumis dans un journal scientifique à comité de lecture.

Les saules sont de bons candidats pour la phytoextraction en raison de leurs caractéristiques physiologiques, notamment leur tolérance élevée aux ET, leur capacité d'accumulation des ET (Delplanque et al. 2013; Van Slycken et al. 2013; Kidd et al. 2015), ainsi que leur aptitude à produire une quantité importante de biomasse sur plusieurs saisons (Pitre et al. 2010). Dans le premier chapitre, nous avons démontré que la phytoextraction du Cd et du Zn par *S. viminalis* a été efficace dans nos conditions de site sur les trois premières années de culture (Grignet et al. 2020). Dans le chapitre 3, nous avons pu mettre en évidence un éventuel effet négatif de la pollution aux ET sur les CMA (nombre de spores de CMA faible par rapport à des sols non pollués, faible taux de mycorhization des racines des espèces végétales se développant spontanément sut le site et des saules). Ces impacts négatifs des ET sur les CMA, microorganismes telluriques symbiotiques bénéfiques des plantes peuvent perturber les fonctionnalités du sol et par conséquent ses services écosystémiques.

Les bénéfices environnementaux du traitement des sols pollués par phytoextraction sont peu renseignés notamment par manque d'application *in situ* et à grande échelle et de recul opérationnel (Kumpiene et al. 2014). Outre l'évaluation des transferts des ET dans les différentes parties de la plante, l'efficacité de la phytoextraction est généralement évaluée par la mesure de la réduction des concentrations en ET totales ou extractibles du sol. La phytoextraction peut améliorer la « santé » du sol, définie comme la capacité du sol à remplir ses fonctions (Pardo et al. 2014). L'évaluation de la santé du sol peut être mesurée, grâce à des paramètres agronomique, physique, chimique et biologique (Breure et al. 2005; Pardo et al. 2014; Le Guédard et al. 2017). Il a été montré dans plusieurs études que les phytotechnologies (phytoextraction ou phytostabilisation) pouvaient avoir un impact positif sur les paramètres biologiques du sol (biomasse microbienne, activités enzymatiques, biomasse lombricienne) (Nahmani and Rossi 2003; Breure et al. 2005; Bouzaiane et al. 2007; Epelde et al. 2009a; Al Souki et al. 2017).

L'objectif de ce chapitre était de déterminer le potentiel bénéfice de l'utilisation du saule des vanniers ou d'un couvert végétal formé par la coculture du saule des vanniers et de l'arabette de Haller sur la santé d'un sol pollué par rapport à un sol pollué non végétalisé à l'aide de paramètres agro-physico-chimiques, de biomarqueurs (activités enzymatiques et biomasse microbienne) et d'un bioindicateur (vers de terre). Le deuxième objectif était d'évaluer le potentiel de phytoextraction du saule des vanniers sur une nouvelle période de 3 ans. Les analyses agro-physico-chimiques et le suivi du bioindicateur ont été réalisé sur la saulaie et sur le sol non végétalisé. Les biomarqueurs ont été suivis sur les 4 conditions présentes sur notre dispositif expérimental (Saulaie, coculture saule/arabette, parcelle d'arabette, sol non végétalisé). Les biomarqueurs utilisés dans cette études étaient 2 activités enzymatiques [la déshydrogénase (DHA) et la fluoresceine diacetate hydrolase (FDA)] et la biomasse microbienne mesurée via les acides gras associés aux phospholipides (AGPL) spécifiques de chaque groupe microbien (bactérie Gram + et Gram - ; champignons mycorhiziens à arbuscules, champignons ectomycorhiziens et saprotrophes).

L'évaluation du fonctionnement du sol a montré que l'utilisation du saule peut améliorer certains paramètres biologiques. En effet, la DHA et la biomasse de vers de terre sur le sol couvert par les saules est accrue par rapport au sol non végétalisé. En ce qui concerne la FDA et les AGPL, aucune différence n'est observée entre le sol végétalisé par le saule et le sol non végétalisé. Des différences saisonnières ont été observées pour la biomasse des bactéries Gram - et pour les CMA. L'utilisation du saule seul ou en coculture avec l'arabette ne change pas les paramètres biologiques du sol pollué. Les paramètres agro-physiques du sol, à savoir le taux de MO et le rapport C/N, augmentent et pourraient être à l'origine d'un dysfonctionnement du sol à l'avenir. Ces augmentations pourraient être limitées par l'exportation de la MO du site si une valorisation de la biomasse est identifiée.

Dans cette étude, nous avons démontré le potentiel de phytoextraction du Zn et du Cd par *S. viminalis* sur une nouvelle période de 3 ans (soit 6 années de culture sur milieu pollué), malgré des concentrations foliaires en Zn et en Cd qui diminuent. En 2020, les concentrations foliaires en Zn et en Cd sont cependant 2,7 et 6,2 fois supérieures aux concentrations moyennes retrouvées chez les végétaux poussant sur milieux non pollués (Kabatas-Pendias, 2010). Les saules ont une bonne croissance et un bon taux de survie sur le sol pollué.

International Journal of phytoremediation

Title: Evaluating TE-polluted soil function with biological indicators in a phytomanagement context

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Abstract

Plant cover can be used to extract trace elements (TE) from polluted soil and improve the overall health of such soil. The objectives of this study, carried out at field scale, were to evaluate the Cd and Zn phytoextraction potential of willows and to determine the potential impact of various plant covers (*Salix viminalis*, *Arabidopsis halleri*, tested alone or in co-cultivation) and seasonality on the functioning of a TE-polluted soil. This was assessed using agro-physicochemical and biological parameters, including biomarkers of soil microbial biomass (through specific phospholipid fatty acid analysis) and activity (by assessing dehydrogenase (DHA) and fluorescein diacetate hydrolase (FDA) enzyme activities), and bioindicators (earthworms). A very short rotation coppice of willows was set up in a field site in 2013. The agro-physicochemical soil parameters were monitored every 3 years and the biological soil parameters were evaluated in 2019 and 2020. The potential of willows for Cd and Zn phytoextraction was confirmed. Moreover, they increased the OM and C/N ratio of the TE-polluted soil. However, they did not have any effect on FDA microbial activity nor on the total amount of microbial biomass in the soil. A beneficial effect on DHA microbial activity was observed in the willow plantation compared to the unvegetated soil. Seasonality did not affect FDA activity nor the total amount of microbial biomass, but it did influence DHA activity and the soil biomasses of Gram negative bacteria and arbuscular mycorrhizal fungi. In addition, the willows increased the biomass and modified the structure of the earthworm community. *A. halleri* alone or in co-

cultivation with *S. viminalis* did not change the soil biological parameters (enzyme activity and microbial biomass) compared to the unvegetated soil.

Keywords: phytoextraction, Dehydrogenase, Fluorescein diacetate hydrolase, earthworms, microbial biomass, Cd, Zn, willow

Acknowledgments

This work was supported by national funds through the PHYTOAGGLO and EXTRA-Zn projects (ADEME - Agence De l'Envrionnement et de la Maîtrise de l'Énergie, conventions n° 1072C0045, 2013-2017 and 1972C0007, 2019-2022). It was also carried out in the framework of the Alibiotech project financed by the European Union, the French State and the French Region of Hauts-de-France. A. Grignet received grants from ADEME (n° TEZ17-21) and the Hauts de France Region. The authors would like to thank the Creil conurbation, and particularly L Raphael Pardon (ACSO – Agglomération Creil Sud Oise) and JL Deremy (Mairie de Montataire), for providing access to the field site and technical support during the entire duration of the project. The authors are also grateful to Fabrice Richez, and Hacène Meglouli for their technical contribution during the sampling campaigns and biochemical analyses. We also thank the Solten company (https://solten.co.uk/en/) for improving the quality of the English text.

Introduction

Phytoextraction is a new green technique to manage polluted soils. Phytotechnology in urban areas could represent an advantageous low-cost method for the rehabilitation and management of polluted land compared to physical and chemical techniques. Phytoextraction uses the capacity of accumulator plants to extract trace elements (TE) from polluted soil (McGrath et al., 2006; Hooper et al., 2016) and export them to the biomass produced (Vangronsveld et al. 2009). This technique could provide ecosystem services such as producing non-food biomass, increasing biodiversity in urban areas for instance, and increasing the well-being of the population through the creation of aesthetic green recreational areas.

Willows are suitable candidates for phytoextraction due to their physiological characteristics: high TE-tolerance and TE-accumulating capacity, in particular Cd and Zn (Van Slycken et al. 2013; Delplanque et al. 2013; Kidd et al. 2015), ability to resprout after coppicing (Labrecque and Teodorescu 2005) and production of high biomass over several growing seasons (Pitre et al. 2010). The Cd and Zn phytoextraction with *S. viminalis* was effective in the field trials and provided significant metal extraction and biomass yields (Hammer et al. 2003, Grignet et al. 2020).

Other than measuring TEs in the plant, the effectiveness of phytoextraction is generally evaluated by measuring the reduction of total or extractable TE fractions in the soil. Therefore, it is shown that phytoextraction can increase soil health, defined as the soil's ability to perform its functions (Pardo et al. 2014). The effectiveness of this technique can also be ascertained by assessing the recovery of soil health after a remediation procedure. Agro-physicochemical and biological soil parameters are relevant indicators for the evaluation of soil health (Breure et al. 2005; Pardo et al. 2014). Bioindicators such as earthworms, and biomarkers such as soil microbial biomass and enzymatic activities, can be used to evaluate the effect of phytoextraction on soil function (Le Guédard et al., 2017). Indeed, earthworms are sensitive organisms that provide information on the overall transfers and the impacts of pollutants such as TEs on the ecosystem and ecological status of the soils (Le Guédard et al., 2017). Biomarkers include all the biochemical, cellular and physiological parameters that can be measured in an organism (enzymatic activities, microbial biomass, etc.). They provide information on the initial response to environmental disturbances and contamination (Fontanetti et al. 2011). Therefore, the bioindicators and biomarkers evaluated in polluted soils generally give lower values than in unpolluted soils (Li et al. 2009, Nahmani and Rossi, 2003). For example, Li et al. (2009) showed that in TE-polluted soil, soil enzyme activities such as fluorescein diacetate

hydrolase (FDA), β-glucosidase and invertase were decreased compared to unpolluted soil. Nahmani and Rossi (2003) also demonstrated that the number of earthworms in polluted soil was much lower than in unpolluted soil. Plant cover on TE-polluted soil may have positive impact on the biological functioning of the soil compared to an unvegetated soil (Epelde et al., 2009b; Al Souki et al., 2017; Nahmani and Rossi 2003). Indeed, in a TE-polluted soil, planting poplars was shown to increase the number of earthworm species from 3 to 5, and the number of individuals from 12 to 57/m² (Nahmani and Rossi 2003). Epelde et al. (2009b) showed in a phytoextraction experiment with *Sorghum* that the soil biological parameters such as the glucose-induced respiration (used as a measure of potentially active microbial biomass) were improved and reached the levels of the unpolluted control soil also vegetated with *Sorghum*.

To our knowledge, no studies on improving agro-physicochemical and biological parameters by using different plants in polluted environments have been conducted.

The main objective of this field-scale study was to evaluate the potential contribution of different plant covers (*Salix viminalis*, *Arabidopsis halleri*, *S.viminalis* and *A. halleri* in co-cultivation) and seasonality in the functioning of a TE-polluted soil. This was done by assessing the agro-physicochemical and biological parameters, including biomarkers (such as soil microbial biomass end enzyme activities) and bioindicators (earthworms). The introduction of a plant cover was assumed to improve these parameters in comparison with an unvegetated condition. The soil function results for the TE-polluted soil were also compared to unpolluted conditions. The second objective of this study was to evaluate the phytoextraction potential of willows in terms of survival rate, biomass, and Zn/Cd foliar concentrations.

This study was a part of the ExtraZn project set up in 2013 in an urban area polluted by Zn and Cd due to diverse past industrial activities. The project aims not only to study the phytoextraction potential of willows but also the potential for landscaping and the production of biomass from a polluted site in an urban environment.

Material and methods

Site description

The field trial was set up in 2013 at the Carrefour des Forges site, located in Montataire, France (49°15’12’’N, 2°26’48’’E). The topsoil was polluted with TEs, mostly Zn (616 mg kg^{-1}) and Cd (1.7 mg kg^{-1}) and presented an alkaline pH (pH > 8) (Grignet et al. 2020). The main agro-physicochemical properties of the soil are summarised in Table 1.

Table 1: Agronomic soil parameters of the willow soil in 2013, 2016 and 2019. The values are expressed as mean ± SD (n = 9 for willow, n=1 for non-vegetated). Differences between years are indicated by different letters, at $\alpha = 0.05$.

	Willow			**Unvegetated**
Years	2013	2016	2019	2019
CEC (meq/100g)	12.4 ± 0.564 (ab)	12.0 ± 0.65 (a)	13.2 ± 0.75 (b)	10.5
C/N	8.7 ± 0.045 (a)	18.5 ± 2.53 (b)	22.2 ± 2.10 (c)	24.5
OM %	4.0 ± 0.447 (a)	6.3 ± 0.64 (b)	7.9 ± 0.79 (c)	6.5
pH	8.12 ± 0.045 (a)	8.32 ± 0.06 (a)	8.39 ± 0.10 (a)	8.29
Soil calcium saturation %	254 ± 10 (a)	315 ± 17 (b)	283 ± 16 (b)	349
Clay (%)	13 ± 0.76	14.8± 0.73	12.75 ± 0.94	10.29
Silt (%)	31± 3.57	32.2 ± 1.89	30.53 ± 4.64	32.17
Sand (%)	56 ± 5.66	53 ± 4.55	56.72 ± 4.42	57.54
$Ca(NO_3)_2$ Extractable Zn	1,32 ± 0,98 (a)	0,96 ± 0,85 (a)	0,79 ± 0,38 (a)	0.27
% mobile	0.21	0.16	0.12	
$Ca(NO_3)_2$ Extractable Cd	0,0007 ± 0,0001 (a)	0,0007 ± 0,0004 (a)	0,00061 ± 0,00019(a)	0.005
% mobile	0.04	0.04	0.04	

In situ experimental design

The site was divided into four field trials as shown in Fig. 1: (i) willow plantation, (ii) *Arabidopsis halleri* and willows in co-cultivation, i.e. the *A. halleri* was cultivated all around willows, (iii) *A. halleri*, and (iv) unvegetated. These four field trials were considered as conditions in which various assays were performed. *A. halleri* was used in this study as a plant cover, its phytoextraction potential having been previously assessed (Grignet et al. 2020). In May 2013, 350 unrooted cuttings of *S. viminalis* of 1.5 m length were purchased from a wood nursery (Cheille, France, Hardouin Tressage) and planted in a very short rotation coppice (VSRC). The design consisted of 9 rows with an inter-row distance of 1.5 m and a spacing of 0.6 m between cuttings. *A. halleri* was cultivated in a $1m^2$ plot set up in September 2015.

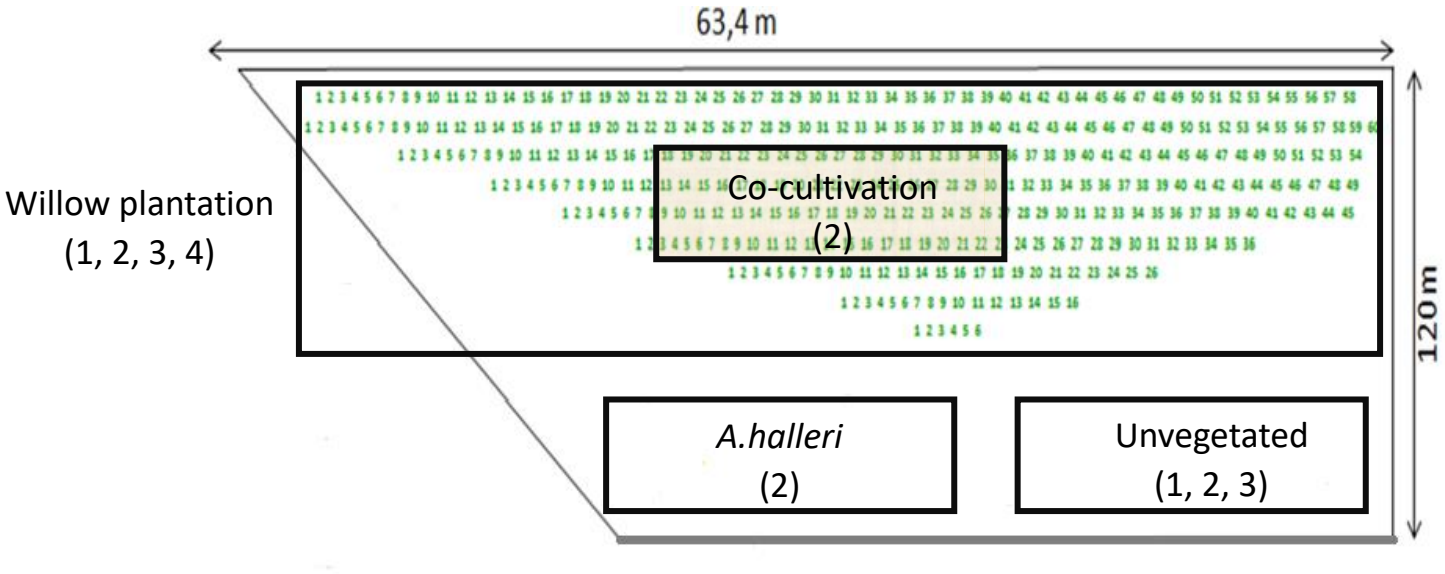


Fig 1: Experimental device with the different conditions studied. Each number corresponds to the different tests performed on each condition: 1) Agro-physicochemical analyses, 2) Enzyme and microbial biomass assays, 3) Earthworm assays, 4) Morphometric monitoring, survival rate and Zn and Cd concentrations.

Agro-physicochemical soil analyses

In 2013, 2016 and 2019, the organic matter (OM), C/N, cation exchange capacity (CEC) and soil texture were measured in 9 composites made from 99 topsoil samples collected with a hand auger on the whole site of the willow plantation (Fig. 1 (1)). In addition, in 2019, one composite was made from 5 topsoil samples collected from the unvegetated condition (Fig. 1 (1)). All topsoil samples were collected with a hand auger in June and were sent to the La Mayenne laboratory (laboratoire departemental d'analyse, Laval, France) for analysis. In addition, the

soil pH was measured in all the collected samples following the NF ISO 10390 (2005) standard. 5g of 2 mm sieved soils were mixed with 25 mL of distilled water and shaken for 2 hours. Water pH was measured after 1 h rest.

To estimate the plant available fractions of Zn and Cd, a selective extraction was performed with 0.01M $Ca(NO_3)_2$ solution on soils collected in 2018, 2019 and 2020 in the willow plantation (Fig. 1 (1)). 60g of dried soil was shaken with 120 mL $Ca(NO_3)_2$ for 48 h in an agitation table. The Zn and Cd in eluates were filtrated through a Millipore filter (Ø 0.45µm) and acidified to a pH of 2 for preservation. They were then analysed either by ICP-OES (Agilent 5110) or by ICP-MS (Agilent 7500).

Enzyme and phospholipid fatty acid (PLFA) soil analyses

For the enzymatic activity and PLFA assays, the soil was sampled in March 2018 and October 2019 on the four conditions of the site (Fig. 1 (2)). Five topsoil samples per condition were taken and stored at 4°C until analysis.

Soil enzyme assays

Analysing fluorescein diacetate hydrolase (FDA) and dehydrogenase (DHA) is widely accepted to be an accurate and simple method for measuring total microbial activity in a range of environmental samples, including soils (Adam and Duncan 2001).

The DHA was measured using a method modified from Tabatabai et al. (1982). The DHA measurement was based on determining the triphenyl formazan formed from the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) in the soil. Five 6g samples of sieved soil per condition were mixed with 0.06g of $CaCO_3$, 1 ml of 3% 2.3.5-triphenyltetrazolium chloride, and 2.5 ml of distilled water. The samples were then incubated for 24 h at 37 °C. After incubation, 10 ml of methanol was added to each tube and the samples were vortexed. The tubes were then incubated 3 times for 1h under horizontal agitation (200 rpm) at room temperature. After centrifugation at 2000 rpm for 5 min, the absorbance of the supernatant was read spectrophotometrically at 485 nm (Cary 100, Agilent Technologies). The DHA activities in the samples were calculated by using calibration graphs. The results are expressed as µg triphenyl formazan (TPF) g^{-1} dry soil released from a pre-given TTC solution within 24 h^{-1}.

The FDA was assayed using a method modified from Adam and Duncan (2001). One gram of fresh soil (sieved < 2mm) was added to 7.5 ml of 60 mM potassium phosphate buffer pH 7.6 and 100 µl of 0.1M fluorescein diacetate substrate (Sigma-Aldrich). The mixture was incubated at 30 °C in a rotary shaker at 50 rpm for 60 min. The soil was extracted from the incubation solution by centrifugation for 5 min at 10000 rpm. To stop the reaction, 1 ml of acetone was then added immediately to 1 ml of supernatant. The amount of fluorescein diacetate hydrolysed was measured in using a spectrofluorimeter (Fluoroskan Ascent, ThermoScientific) (λemission = 523 nm, λexcitation

= 494 nm). The FDA activities were calculated using calibration standard graphs. The results are presented as micrograms of fluorescein per gram of soil.

Phospholipid fatty acid (PLFA) analyses

After removing the plant debris from the soil samples, two replicates of 3g of freeze-dried soil (from each soil sample) were analysed to measure their PLFA contents. The amounts of PLFA specific to arbuscular mycorrhiza fungi (AMF) (C16:1ω5), saprotrophic/ectomycorrhizal fungi (C18:2 ω 6.9), Gram-positive bacteria (GP) (i15:0, a15:0, i16:0, i17:0 and a17:0) and Gram-negative bacteria (GN) (cy17:0, C18:1v7 and cy19:0) were measured. The total PLFA concentration was used as a measure of soil microbial biomass.

The total lipid extraction procedure followed that described by Frostegard et al. (1991), which is based on the method by Bligh and Dyer (1959) as modified by White et al. (1979). Briefly, 3g (dry wt) portions of soil were extracted in a one-phase mixture consisting of chloroform, acetone and methanol (1: 2:2 v/v/v). After splitting the extracts into two phases by adding chloroform and a buffer, the lipid-containing phase was dried under a stream of N, and stored at -20°C. The lipid material was fractionated with Solid Phase Extraction (SPE) - columns containing silica (500mg/6ml) into neutral lipids, glycolipids and polar lipids containing phospholipid (Frostegard et al., 1991). The chloroform fraction (containing the neutral lipids) and the methanol fraction (containing the phospholipids) were subjected to a transesterification using a base solution (KOH 0.2 M) prepared in methanol to transform the PLFA into free fatty acid methyl esters. The resulting fatty acid methyl esters were separated on a Gas Chromatography–Mass Spectrometry (GC-MS) SHIMADZU QP2010 ULTRA equipped with a single quadrupole mass detector simultaneously coupled with a flame ionization detector (FID).

The samples were analysed on a (10m length x 0.1mm inner diameter x 0.1µm phase thickness) ZB-1MS (100 % dimethylpolysiloxane, Zebron, Phenomenex, Torrance Calif, U.S.A.) fast capillary column using helium as a carrier gas at a constant linear velocity (40.0 cm/sec), and the injections were made in a split mode (ratio 80:1). The injector temperature was 28O°C, and the detector temperatures were respectively 330°C for FID and 280°C for the ion source. The temperature program was: initial temp 175°C, increase 25°C. min-^{1}, final temp 275°C for 0.5 min. The relative retention times of the supposed fatty acid methyl esters were compared with those of commercial standards. Identifications were made using GC-MS. The ionization mode was electronic impact at 70 eV and the mass range between 50.0 and 400.0 was scanned. The SIM mode was used simultaneously. Identifications of fatty acid methyl esters were based on comparison with spectra that were either obtained from

commercial standards and/or reported in the literature (NIST Standard Reference Database). Quantification using FID Fames quantities were calculated from the GC/FID peak areas compared to the known amount in the internal standard.

Earthworm biomass, abundance and identification

Earthworms were collected in April 2019 from 6 quadrats (50 cm × 50 cm) set up in two of the field conditions (Fig. 1 (3)), i.e. three in the willow plantation and three in the unvegetated condition. The earthworm extraction was carried out according to the ISO 23611-1 standard. Over each quadrat, the first 30 centimetres of soil were removed and the earthworms were manually sorted. 10 L of allyl isothiocyanate (AITC 94 %, Acros Organics) and isopropanol diluted in water (1:100:10,000 L) were then added to the quadrat. We collected all the earthworms coming out of the soil until no more individuals emerged (we waited up to 30 min for each quadrat). These were first transferred to a solution of 70% dilution alcohol and water for 24h, and then stored in 90% alcohol before being taxonomically identified in the laboratory. The earthworms were then rinsed with water, dried at room temperature and weighed to determine their biomass. The mass lost during fixation varies between 10 and 20% according to ISO 23611-1. The abundance per condition was calculated as the number of individuals per m^2. The identification of earthworms was carried out with dichotomous determination keys under a binocular magnifying glass (Edwards and Lofty, 1977 and Bouché, 1972).

Morphometric monitoring, survival rate and foliar Zn and Cd concentration of the willows

Over three years (2018, 2019 and 2020), the willow leaves were collected annually in June at the time of the soil sampling (Fig 1 (4)). The leaves were carefully washed with tap and deionized water before drying at 40 °C until they reached a constant weight. Zn and Cd were analysed in the plant samples (0.5 g) after digestion at 180 °C for 20 min in 10 mL of nitric acid (67% AnalaR Normapur) and 3 mL of ultra-pure water using a microwave digester (Mars Xpress CEM) by ICP-OES (Agilent 5110) or by ICP-MS (Agilent 7500). One standard reference material was used for analytical quality control (cabbage “NCS ZC 73012”, NACIS). In addition, willow leaves were also collected using a leaf blower after leaf fall in autumn 2019. They were then dried and weighed.

The height and diameter (at 1.30m) of each living willow were measured during the 3 years of the monitoring (2018 to 2020) (Fig 1 (4)). Height and diameter increments were calculated as follows:

((Height or diameter – initial height or diameter) / (initial height or diameter)) *100.

The willow survival rate was determined in 2018 and 2019 during the monitoring by counting each living willow (Fig 1 (4)).

The Zn and Cd extraction was calculated using the maximal, minimal and mean foliar concentrations measured in June of each monitored year and the amount of biomass measured in autumn 2019. These calculations are only indicative as it was not possible to collect the leaves on the trees in June.

Statistical analyses

All data were pre-analysed using normality tests (Shapiro-Wilk test) and homogeneity of variances (test F). ANOVA and post hoc comparisons were then made with HSD tests for height, diameter and metal concentrations for the willows. The Kruskall-Wallis test and pairwise comparisons were used for the soil enzyme assays, PLFA and earthworms assay. All the statistical analyses were performed using R statistical software 3.2.2 (R Core team).

Results and discussion

The impact of willow plantation on the agro-physicochemical parameters of the TE-polluted soil

The agro-physicochemical parameters of the polluted soils were measured three times in the willow plantation, which made it possible to study the evolution of these parameters in this condition. In 2019, these parameters were compared to those obtained in the unvegetated condition (Fig. 1 (1)). In addition to modifying soil pollution, the establishment of a plant cover on a site is assumed to modify the agro-physicochemical parameters of the soil such as the pH, OM, soil structure and stability (Yan et al. 1996; Quideau et al. 2001; Pérez-Bejarano et al. 2010). It is also a source of nutrients for all soil organisms once mineralized (Pérez-Bejarano et al. 2010).

Table 1 gives the results of the agro-physicochemical parameter monitoring. The CEC was stable over time, but it was high in both the willow-vegetated soil and the unvegetated soil. These results show that the plant cover did not influence the CEC in the soil conditions studied. Since the cationic exchanges between OM and soil solution can be characterized by CEC, the high CEC can be directly related to the high amount of OM found and suggests

a malfunctioning of the soil (Kabata-Pendias, 2004). The CEC in the unvegetated condition was lower than in the willow plantation, which might suggest an influence of the plant cover.

The pH of the soil in the willow plantation was alkaline and remained stable between 2013 and 2019 (Table 1). This result could be explained by the large calcium reserve in the soil. Indeed, the soil calcium saturation rate in the willow plantation was much higher than that normally found (>200% vs 65%, respectively) (Gis Sol, BDAT, 2011). The willow plantation pH did not differ from that measured in the unvegetated condition, which shows that the willows did not influence this parameter in our soil conditions (Yan et al. 1996).

The texture of the willow plantation soil did not change over time and did not differ from the unvegetated condition. As in the case of pH, this result suggests that willows did not modify this parameter. This result could be explained by the fact that the willow plantation is young, i.e. set up in 2013, and did not have time to change the soil structure (Pérez-Bejarano et al. 2010).

In the willow plantation, we found a significant increase in the amount of OM over time, from 4 to 7.9% (Table 1), which is higher than that found in unpolluted soil (between 1 and 5% in Le Villio et al. 2001). Indeed, in an *in situ* phytoextraction experiment with *Celosia argentea*, Yu et al. (2020) also showed an increase in OM levels after planting. Similarly, the C/N ratio in our plantation increased from 8.7 to 22.2. Contrary to unpolluted soils where the C/N ratio is less than 12 (Agro-transfert, 2007) reflecting a well-functioning OM mineralization (Sorensen, 1981; Schmidt et al., 2000), the C/N ratio evolution in the willow plantation reflected a poor OM humification and mineralization by microorganisms. Contrary to expectations, the comparison with the unvegetated condition showed similar results, suggesting that the malfunctioning could be due to the polluted soil rather than the plant cover. Indeed, several studies have shown a negative effect of TE on soil microorganisms (Epelde et al., 2009 a,b; Leita et al., 1995; Liao and Xie, 2007; Papa et al., 2010; Renella et al., 2003, 2005; Wang et al., 2007; Zhang Y. et al., 2008; Zhang N. et al. 2010) as well as a decrease in microbial biomass compared to unpolluted soils (Leita et al. 1995; Liao and Xie 2007).

To assess whether the soil pollution affected the soil microbial status, we tested the soil microbial enzyme activities (DHA, FDA) and biomass (PLFA) in the four experimental conditions - willow plantation, *A. halleri* alone, *A. halleri* and willow co-cultivation, unvegetated (Fig. 1 (2)) - and compared these to unpolluted soil values. In addition, the comparison between the polluted soil conditions and between two seasons (winter, autumn) made it possible to evidence, on one hand, the potential effect of cover by a plant or a combination of plants and, on the other hand, the seasonal effect on soil microbial activities.

Plant cover and seasonality effects on microbial biomass and enzymatic activities in TE-polluted soil

Fig. 2 presents the soil DHA and FDA activities assessed in the four conditions (willow plantation, co-cultivation, *A. halleri* and unvegetated). In 2018, no significant differences between the tested conditions were observed for the two enzyme activities (Fig 2). In 2019, in the willow plantation, the DHA activity was between 0.3 and 0.8 µg TPF.g of soil $^{-1}$.24 H^{-1}, and was also at its lowest in the other conditions. These values were much lower than in results from unpolluted soil. Indeed, Baum et al. (2020) showed that in unpolluted soil vegetated with willow, the DHA activity was between 0.6 and 1.8 µg TPF.g of soil $^{-1}$.24 H^{-1}. These results confirm the negative impact of TE on DHA activity. Compared to winter, the DHA activity increased significantly in autumn in both the willow-vegetated and unvegetated soil, which suggests a seasonal effect on DHA activity. To our knowledge, no literature has reported the effect of seasonality on DHA activity. However, Luo et al. (2020) observed the effect of seasonality on other soil enzyme activities (urease and phosphatase). They found that the activities of these two enzymes were reduced in winter compared to spring.

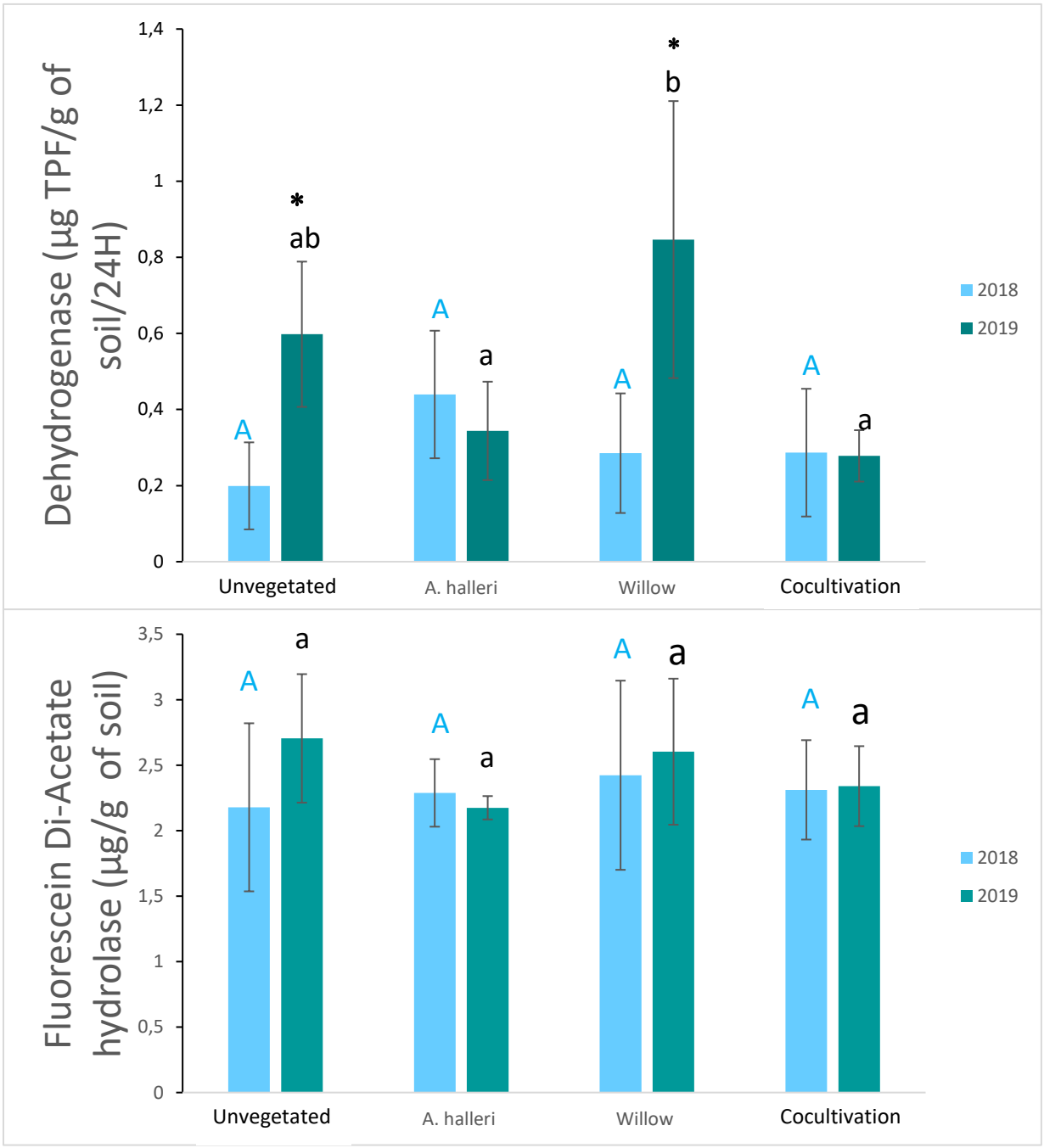


Fig 2: Soil dehydrogenases (a) and fluorescein diacetate hydrolases (b) activities in the soil in March 2018 and October 2019. The values are expressed as mean ± SD. Differences between conditions at the same year are indicated by different letters (blue for 2018 and black for 2019) and differences between the same condition over the two years are indicated by ∗.

We found no variation in FDA activity between our conditions, showing that neither cover by a single plant or a combination of plants nor seasonality had an effect on this activity (Fig. 2). Unlike our results for DHA, no seasonality effect on FDA activity was reported. In unpolluted soil, the amount of FDA varies between 70 and 250

296 µg. g^{-1} of soil (Tripathy et al. 2014; Elbl et al. 2019), but the concentrations in our soil were much lower in
297 comparison. TE in soil appears therefore to significantly reduce FDA activity.

298 Vegetated soils allow an increase in microbial populations through the increase of OM (Cui and Holden 2015) and
299 root exudates which stimulate the rhizosphere microbial growth (Qin et al. 2015; Ridl et al.2016). As a result, an
300 increase in the total microbial biomass in the vegetated soil conditions was expected. In 2018 and 2019, the
301 amounts of microbial biomass, measured in the same year with PLFA biomarkers, ranged from 7.56 to 13.23 µg
302 g^{-1} of dry soil and did not differ between the four study conditions (Fig. 1 (2); Table 2). These findings suggest
303 that the plant cover did not affect the soil microbial biomass. This result does not concord with studies reporting
304 that the presence of a plant cover significantly increased soil PLFA (Hortal et al. 2013; Qin et al. 2015; Yang et
305 al. 2017). The studies by Moore-Kucera and Dick (2008) and Shi et al. (2013) were carried out on polluted soil
306 and found that the total amount of PLFA was greater in the presence of a plant cover during the growing season
307 (April) than in autumn (October). However, to the contrary, we observed no significant differences in the total
308 microbial biomass between seasons in our experimental conditions. This lack of difference between the seasons
309 could be due to the sample timing, since they were in fact taken in two different years. In addition, the unvegetated
310 part of the site is not far from the willow grove and is bordered by a vegetative barrier, thus willow roots and
311 border plants may be present in the unvegetated part and influence microbial biomass and enzyme activity.

312 In 2018, a predominance of Gram-positive bacteria and AMF biomass was observed in the soil (Table 2), whereas
313 in 2019, a predominance of Gram-negative bacteria and a balance of AMF and other fungi (saprotrophic +
314 ectomycorrhizal fungi) was detected. In October 2019 (autumn), the amount of AMF was significantly lower in
315 the willow, *A. halleri* plantation and co-cultivation condition soils than in March 2018 (winter). These differences
316 may be due to seasonal differences, as reported in Bardgett et al. (1999) and Yokobe et al. (2018). Various
317 researchers have used the bacteria/fungi ratio to indicate environmental change and the functioning of the soil
318 microbial communities (Hinojosa et al. 2005; Strickland and Rousk 2010; Yang et al. 2017). In our study, the
319 bacteria/fungi ratio was significantly higher in October 2019 than in March 2018 in all the different conditions,
320 which is in accordance with the previous result suggesting a seasonal effect.

321 Studies comparing polluted and unpolluted soils have shown that TE in soils can reduce the bacteria/fungi ratio
322 and the microbial biomass (Hinojosa et al. 2005; Leita et al., 1995; Liao and Xie, 2007). Soil enzymatic activities
323 are closely related to microbial biomass, as reported in Burns (1982) and Yuan and Yue (2012). The low amount
324 of microbial biomass and DHA and FDA enzyme activities found in our soil seems to be consistent with these

reports. Soil enzyme activities indicate the mineralization rates of soil OM (Burns, 1982; Frankenberger and Dick, 1983) and are directly involved in the soil C, N and P cycles (Sinsabaugh et al., 2008, Xu et al., 2017). The low enzymatic activity found in our soil could therefore explain the high OM and C/N in the four conditions.

328 Table 2: Bacterial and fungal biomass estimated by PLFA (µg/g dry soil) analysis in the soil in March 2018 and October 2019. The value is expressed as mean ± SD (n=5).

329 Differences between the different conditions in the same year are indicated by different letters and the differences between the same condition over the two years are indicated

330 by ∗ at α= 0.05.

	Conditions	Bacterial			Fungi			Bacteria/ Fungi	Total microbial biomass
		Gram negative (GN)	Gram positive (GP)	GN/GP	Arbuscular Mycorrhizal fungi (AMF)	Saprotrophic (S) + Ectomycorrhizal fungi (EMF)	Total fungi (AMF+S+EMF)		
March 2018	Unvegetated	1.95 ± 0.84 **a**	2.49 ± 0.91 **a**	0.76 ± 0.14 **a**	2.39 ± 0.49 **a**	0.73 ± 0.16 **a**	3.12 ± 0.65 **a**	1.40 ± 0.18 **a**	7.56 ± 1.97 **a**
	A. halleri	2..47 ± 1.01 **a**	4.2 ± 1.46 **a**	0.61 ± 0.14 **a**	3.20 ± 0.39 **a**	1.09 ± 0.56 **a**	4.29 ± 0.95 **a**	1.44 ± 0.10 **a**	10.46 ± 2.23 **a**
	Willow	2.41 ± 0.83 **a**	2.87 ± 1.14 **a**	0.86 ± 0.26 **a**	2.95 ± 0.97 **a**	0.93 ± 0.39 **a**	3.88 ± 1.35 **a**	1.37 ± 0.18 **a**	9.16 ± 3.07 **a**
	Co-cultivation	3..28 ± 1.74 **a**	3.92 ± 1.59 **a**	0.91 ± 0.36 **a**	3.27 ± 0.06 **a**	0.90 ± 0.41 **a**	4.17 ± 0.47 **a**	1.75 ± 0.51 **a**	11.37 ± 1.40 **a**
October 2019	Unvegetated	2.78 ± 1.69 **a, ∗**	1.67 ± 0.98 **a**	1.39 ± 0.63 **a**	0.79 ± 0.53 **a**	0.85 ± 0.69 **a**	2.44 ± 1.18 **a**	3.85 ± 2.26 **a, ∗**	13.23 ± 12.80 **a**
	A. halleri	3.31 ± 1.30 **a**	2.66 ± 1.67 **a**	1.49 ± 0.82 **a, ∗**	1.11 ± 0.92 **a, ∗**	0.99 ± 0.68 **a**	2.10 ± 1.52 **a**	3.27 ± 0.79 **a, ∗**	8.09 ± 4.07 **a**
	Willow	4.69 ± 1.19 **a, ∗**	3.11 ± 0.94 **a**	1.71 ± 1.03 **a**	1.63 ± 0.38 **a, ∗**	1.04 ± 0.55 **a**	2.68 ± 0.71 **a**	3.05 ± 0.84 **a. ∗**	8.89 ± 2.79 **a**
	Co-cultivation	4.65 ± 2.03 **a**	4.2 ± 2.41 **a**	1.24 ± 0.60 **a**	1.12 ± 0.6 **a, ∗**	1.86 ± 0.90 **a**	2.98 ± 1.01 **a**	2.88 ± 0.55 **a, ∗**	11.84 ± 5.06 **a**

331

Influence of a willow plantation on earthworm biomass, abundance and ecological categories in a TE-polluted soil

We studied the potential impact of the willow plantation on the earthworm biomass, abundance, species richness and ecological categories, in comparison with the unvegetated condition (Fig. 1 (3)). The abundance between the two soil conditions did not differ (Table 3) which suggests that the willows did not influence earthworm abundance. The numbers of earthworms in our soil, both in the willow plantation and in the unvegetated condition, are very low compared to those found in a poplar plantation (468 indiv/m²) or meadow (342 indiv/m²) on unpolluted soil (Nahami and Rossi, 2003; Spurgeon and Hopkins, 1999), but they are comparable to those measured in polluted industrial wastelands (0 to 200 indiv/m² in Pérès et al. 2011). This result suggests that earthworm abundance was affected by TE pollution (Grumiaux et al. 2015).

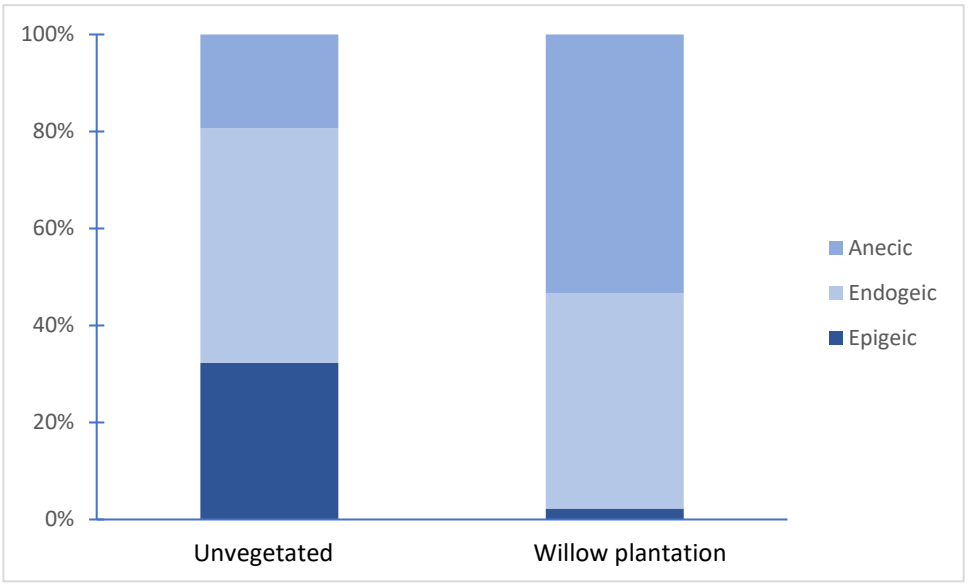
The earthworm biomass was significantly higher in the willow plantation than in the unvegetated soil (Table 3). This result is consistent with the literature on polluted sites (0 to 75 g/m^2 in Cluzeau et al. 2012) and could be due to an increase in the willow plantation of available OM, which is an important part of the diet of earthworms (Sims and Gerard, 1999).

The number of species was higher in the unvegetated condition than in the willow plantation (6 and 5 species, respectively, Table 3). *Lumbricus terrestris* and *L. castaneus* were present in both conditions; they are known to be tolerant to metal pollution (Spurgeon and Hopkins, 1999; Grumiaux et al. 2015). *Allolobophora rosea* was only present in the unvegetated condition. This species is known to be sensitive to TE, however Grumiaux et al. (2015) did find it in a TE-polluted site, which concords with our results.

Table 3: Biomass, abundance and species of earthworms found in willow and unvegetated conditions. The values are expressed as mean ± SD (n=3). Differences between the different conditions are indicated by different letters, at $\alpha = 0.05$.

	Willow plantation	**Unvegetated**
Biomass (g / m²)	86.71 ± 35.95 a	16.41 ± 14.90 b
Abundance (ind/ m²)	51.33 ± 14.18 a	37.33 ± 9.01 a
Richness	5	6
Species	*Aporrectodea caliginosa*, *Lumbricus terrestris*, *Lumbricus castaneus*, *Octolasium cyaneum*, *Aporrectodea giardi*	*Aporrectodea caliginosa*, *Lumbricus terrestris*, *Lumbricus Castaneus*, *Dendrobaena octaedra*, *Allolobophora rosea* , *Dendrodrilus rubidus*

Fig. 3 presents the distribution of individuals in terms of the three ecological categories of earthworms. The three categories were represented in both conditions, but a majority of endogeic earthworms was found in the unvegetated condition (Fig 3). In the willow plantation, a significant decrease of epigeic earthworms and a dominance of anecic earthworms was observed. Epigeic earthworms are surface species. As such, the high Ca concentration in the soil and the tillage operation between willow rows before the test might have affected their presence in the willow plantation, as already reported (Grumiaux et al. 2015, Fragoso and Lavelle 1992). The increase in anecic earthworms could be explained by their dietary specificity. Indeed, they are saprophagous and are highly affected by the presence of OM (Pérès et al. 2011), which concords with our results on OM.



363 Fig 3: Percentage distribution of the number of earthworms per ecological category (anecic, endogeic and
364 epigeic).

365 Willow growth on a TE-polluted soil and Cd and Zn phytoextraction efficiency

366 Since willow growth can be altered by TEs (Morris et al. 200), the willows' height and diameter and survival rate
367 were measured throughout the three years of the monitoring to record their development. We observed a stagnation
368 of the height and diameter increments during the monitoring (2018 to 2020) (Fig 4) whereas, during the three
369 previous years, we observed an increase of these two parameters (Grignet et al. 2020). This stagnation seems to
370 be because the willows have reached their maximum height and diameter. In this study, for aesthetic reasons, one
371 half of the willow plantation was cut following the current practice performed on unpolluted soil, i.e. harvest every
372 3 or 4 years (Larsen et al. 2014; Wang and MacFarlane 2012), whereas the other half was used to study growth
373 development. As a result, to the best of our knowledge, this study is the first to report the growth of willows on a
374 polluted soil after 6 years of culture in VSRC. In 2018, the trees were 1.22 times higher than previously reported
375 on polluted soils (Mleczek et al. 2010). The willows showed a high survival rate during the two years of monitoring
376 (respectively 85% for 2018 and 84% for 2019). After 6 years of cultivation, the willow growth in our soil
377 conditions was a success with no visible alteration, ensuring the aesthetics of the landscaping.

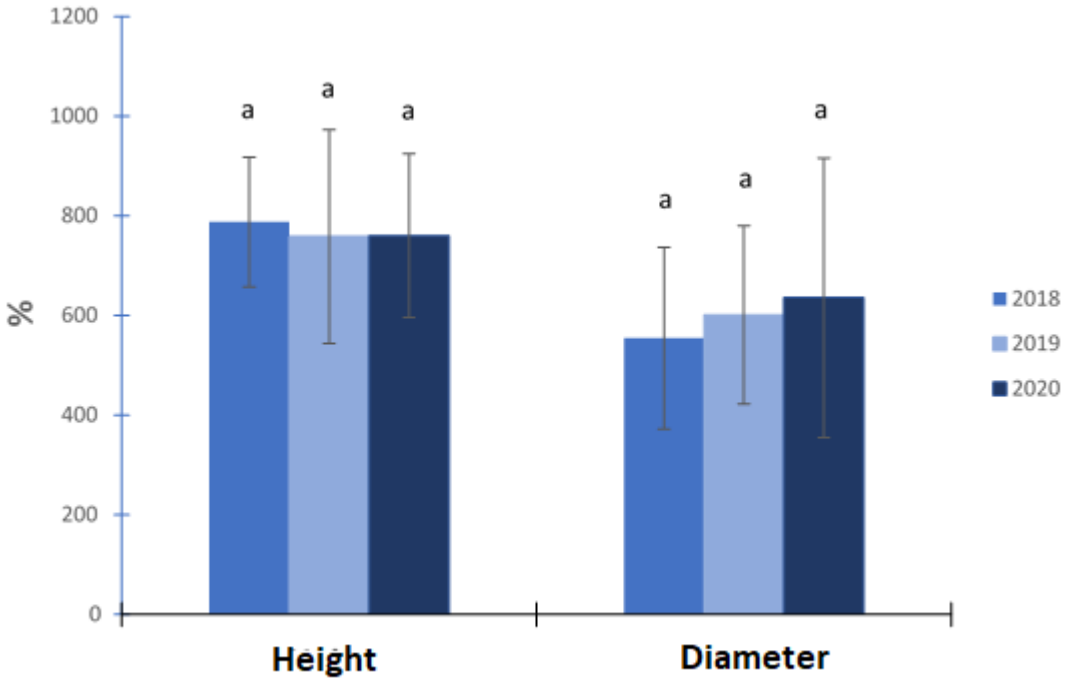


Fig 4: *Salix viminalis* annual height and diameter increments (mean ± SD). Different letters indicate significantly different values, at $\alpha = 0.05$

The foliar concentrations in Zn and Cd (Fig 5) were higher in our study (respectively 2.7 and 6.2 times more) than the basal values measured in plants on unpolluted soils (Kabata-Pendias, 2004). These results agreed with many studies that report willows accumulating high concentrations of Zn and Cd in their leaves even in moderately polluted environments (Van Slycken et al. 2013; Grignet et al. 2020). Over the three years, we observed a significant decrease in the foliar Cd and Zn concentrations. This decrease and the stagnation of growth parameters after 6 years of cultivation could limit the potential for phytoextraction. This assumption is supported by the constant biomass measured for the two indirect parameters of biomass (height and diameter) and the decrease in the TE concentration. As a result, the quantity of metal exported (leaf TE concentration * leaf biomass) decreases each year (Table 4). This hypothesis should be confirmed by a measurement of foliar biomass, although direct leaf biomass measurement is difficult to perform due to the need to collect the leaves on living trees in June.

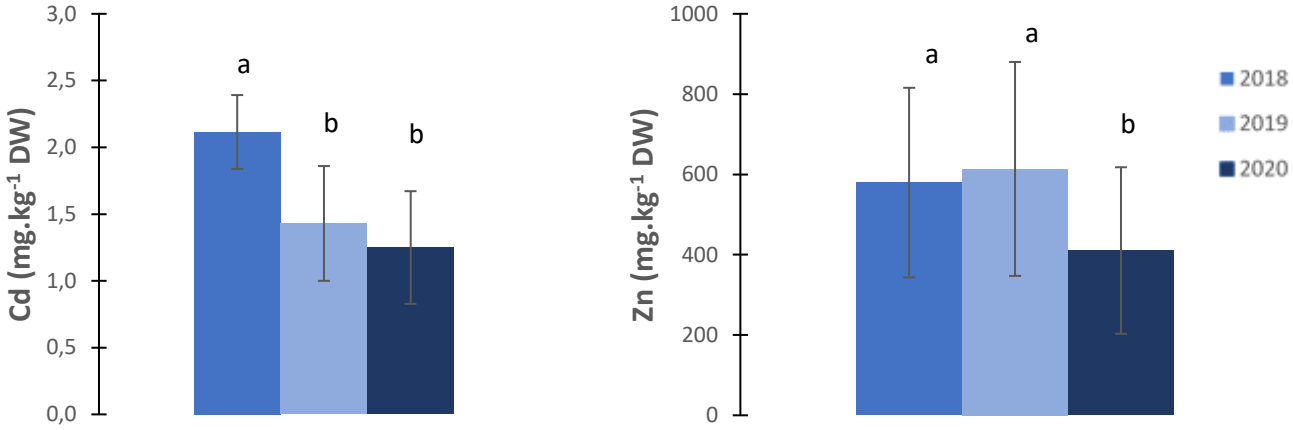


Fig 5: Cd and Zn *Salix viminalis* foliar concentrations over the 3 years of growth (mean ± SD). Significant differences between years are indicated by different letters, at $\alpha = 0.05$

The phytoavailability of Zn and Cd was assessed by measuring the extractable concentrations of these two metals in the soil. Extractable Zn represented 0.1% of the total Zn and extractable Cd represented 0.04% of the total Cd (Table 1). This low metal mobility might be due to the alkaline pH of the soil (pH > 8), this parameter being the most important factor influencing the phytoavailability of Zn and Cd (Eriksson 1989). Contrary to what was expected, the extractable concentrations of Cd during the monitoring did not change. For Zn, a decreasing trend was observed (Table 1). In agreement with these results, no correlation was observed between the Zn and Cd foliar concentrations and the extractable concentrations ($p > 0.05$). This concords with the fact that the willows did not decrease the metal extractability of the soil as expected in a phytoextraction scenario. Indeed, the phytoavailability of Cd and Zn must decrease by the extraction of these two elements and/or by modification of the speciation by root exudates (Meers et al. 2007; Antoniadis et al. 2017).

Table 4: Biomass yield of willow leaves collected using a leaf blower after leaf fall in autumn 2019, and extrapolation of metal removal based on the concentrations measured in the leaves in June of each year and on the average.

		Biomass yield *(Kg ha^{-1}) S. viminalis collected in autumn*	Metal removal (kg. ha^{-1}. years^{-1})
Zn	2018	532	0.30 ± 0.12
	2019		*0.32* ± 0.14
	2020		*0.21* ± 0.11
	Mean		*0.28* ± 0.12
Cd	*2018*		*0.0011* ±0.0001
	2019		*0.00076* ±0.0002
	2020		*0.00066* ±0.0002
	Mean		*0.00085* ±0.0002

Conclusion:

In this study performed on a metal-polluted soil, we demonstrated that introducing a willow plantation can improve some soil biological parameters. Indeed, DHA microbial activity and earthworm biomass were higher compared to the unvegetated soil, which confirms the usefulness of a plant cover on a TE-polluted soil. The use of *A. halleri* alone or in co-cultivation with *S. viminalis* did not change the soil biological parameters (enzyme activity and microbial biomass) compared to the unvegetated soil. Since the OM and C/N ratio are increasing over time, serious soil dysfunction could occur in the future. To avoid this, these parameters should be monitored. These increases could be limited by exporting the OM. In addition, this study confirms the potential of willows for Zn and Cd phytoextraction despite the non-optimal agro-physicochemical and biological parameters. After 6 years of culture, the willows showed sufficient Zn concentrations for use in green chemistry (ecocatalysts), an ongoing option for reusing the biomass of this species.

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Chapitre 5 : Influence de la fertilisation NPK+S et du temps de culture sur les concentrations en Zn, Cdt en glucosinolates (GLS) chez *Arabidopsis halleri.* Rôles du Zn, du Cd et des GLS dans la défense de la plante face à un herbivore (*Cantareus aspersus*).

Ce chapitre est présenté sous forme d'article et sera prochainement soumis dans un journal scientifique à comité de lecture.

Dans le chapitre 2, une diminution de la consommation des feuilles d'arabette a été observée lorsque celles-ci étaient fortement chargées en Zn et Cd. Les observations de terrain tout au long du projet ont également montré que les jeunes plantules d'arabette étaient les plus consommées.

Les GLS sont des composés secondaires riches en soufre qui sont majoritairement retrouvés chez les Brassicaceae (Fahey et al. 2001). Constitutives dans cette famille, les GLS représentent plus de 20% de la fraction du soufre total de la plante (Fahey et al. 2001; Castro 2004; Halkier and Gershenzon 2006). Les GLS sont impliqués dans la défense des plantes contre les herbivores et les agents pathogènes, lors d'attaques de champignons et de bactéries. Ils ont aussi un rôle toxique ou dissuasif pour de nombreux herbivores (oiseaux, mammifères, mollusques) (Brader et al. 2001; van Dam et al. 2009; Buxdorf et al. 2013). L'hyperaccumualtion de métaux démontrée chez certaines espèces de Brassicaceae pourrait constituer pour ces espèces un mécanisme supplémentaire de défense contre les herbivores et les pathogènes (Boyd and Martens 1998).

Les objectifs de cette étude ont été tout d'abord d'étudier l'effet d'un fertilisant azoté soufré (NPK+S) sur les concentrations en Zn, Cd et S des parties aériennes d'*A. halleri*, sur sa biomasse et sa survie en condition *in situ* sur le site de Montataire. Pour cela 6 nouvelles parcelles avec et sans fertilisant ont été mises en place en avril 2019. Un mois après la plantation, le taux de survie a été calculé, les parcelles ont été fauchées afin de mesurer la biomasse produite et les concentrations en ET ont été mesurées dans les plantes.

Les résultats ont montré que l'utilisation de fertilisant NPK+S sur des parcelles *in situ* permettait d'augmenter les concentrations en Cd dans la plante de 1,2 fois comme cela a déjà observé dans les chapitres 2 et 3. Une augmentation significative (x 1.6) de la biomasse est également notée en présence de fertilisant. Ce résultat est novateur chez *A. halleri.* Dans les chapitres 2 et 3, seule une tendance à l'augmentation de la biomasse était observée. Un doublement de la concentration en S foliaire a également été observé due à la présence de S assimilable dans le fertilisant. Les plantes sur les parcelles avec fertilisant présentent également une meilleure survie. La quantité de métal exportée (Zn et Cd) sur ces parcelles est également augmentée (respectivement par 1,3 et 1,5 fois).

Pour connaître la cinétique de l'accumulation du Zn et du Cd sur sol pollué chez *A. halleri*, un test en condition contrôlée a été réalisé. Les résultats ont montré que l'accumulation du Zn au cours du temps suivait une courbe exponentielle avec un décrochage rapide après la 4ème semaine de culture. Cette courbe ressemble à la courbe de l'hyperaccumulation proposée par (Baker 1981). Concernant le Cd, la cinétique a montré que les plantes accumulaient rapidement le Cd jusqu'à un plateau. L'existence d'un plateau dans l'accumulation du Cd a été proposée par (Hamon et al. 1999).

De plus, une expérimentation en condition contrôlée avec de la terre polluée du site expérimental a été réalisée dont l'objectif était d'évaluer l'effet du fertilisant NPK+S et du

temps de culture sur les concentrations en Zn, Cd et GLS dans les feuilles d'*A. halleri* avant et après consommation par un herbivore (*Cantareus aspersus*). Les résultats ont montré que l'utilisation de fertilisant et le temps de culture diminuaient les concentrations en GLS. L'herbivorie n'a pas eu d'effet sur ces concentrations. La consommation des feuilles d'*A. halleri* par l'escargot, *Cantareus aspersus,* est influencée par les concentrations en Zn et Cd pour les plantes cultivées sans fertilisant. Les plantes présentant les concentrations en Zn et Cd les plus importantes sont les moins consommées. Les GLS dans les plantes cultivées avec et sans fertilisant n'influencent pas la consommation par l'herbivore .Plus les plantes ont de Zn et Cd foliaire et moins elles sont consommées présentant un moindre risque de dispersion des ET dans l'environnement moindre.

Title : Fertilization to improve Zn and Cd phytoextraction of *A. halleri* and potential role of TE and glucosinolates against herbivore attack

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Abstract

The use of fertilizer can increase the phytoextraction potential of metal hyperaccumulators. The effect of adding NPK+S fertilizer on the phytoextraction potential of *Arabidopsis halleri* (L.) O'Kane & Al Shehbaz to partially remove Zn and Cd in soil was investigated in field plots. Zn and Cd concentrations in *A. halleri* increase over time. The accumulation kinetics of Zn and Cd in *A. halleri* was characterized in a semi-controlled experiment. In addition, the effect of this fertilizer and the growing duration on a TE polluted soil during an herbivory attack were investigated on Zn, Cd, S and glucosinolate (GLS) concentrations in *A. halleri*. Field results showed that plots with NPK+S showed an increase in In presence of the fertilizer, the biomass of the plants as well as Cd and S concentrations in the plants were increased by 1.6), 1.2 and 2, respectively. Similarly, the quantity of Zn and Cd exported following plot harvest was increased by1.3 and 1.5, respectively. The accumulation kinetics of Zn showed that Zn concentrations exponentially increased over time while for Cd no increase over time was observed. In the laboratory, the results showed that fertilizer use and growing time decreased GLS concentrations. Herbivory had no effect on these concentrations. Consumption of *A. halleri* leaves by the snail, Cantareus aspersus, is influenced by Zn and Cd concentrations for plants grown without fertilizer. Plants with the highest Zn and Cd concentrations are the least consumed. GLS in plants grown with and without fertilizer did not influence herbivore consumption; the more foliar Zn and Cd the plants had, the less they were consumed, thus presenting a lower risk of dispersal of ETs in the environment.

Keywords : Cd, Zn, phytoextraction, glucosinolate, herbivory, snail

Acknowledgments

This work was supported by national funds through PHYTOAGGLO and EXTRA-Zn projects (ADEME - Agence De l'Envrionnement et de la Maîtrise de l'Énergie, conventions n° 1072C0045, 2013-2017 and 1972C0007, 2019-2022). A. Grignet received grants from ADEME (n° TEZ17-21) and the Hauts-de-France Region. The authors would like to thank the Creil conurbation, and particularly L Raphael Pardon (ACSO – Agglomération Creil Sud Oise) and JL Deremy (Mairie de Montataire), for providing access to the field site and technical support during the entire duration of the project. The authors are also grateful to Fabrice Richez for the technical contribution during the sampling campaigns and biochemical analyses. The authors thank A. de Vaufleury who kindly provided by the snails for the study.

Introduction

Phytoextraction is an emerging technique for partial soil remediation (Vangronsveld et al. 2009; Li et al. 2009; Li et al. 2012). This technique uses the ability of certain plant species to transfer significant amounts of trace elements (TE) from the soil to their above-ground parts. Several types of plant species can be used for phytoextraction: TE accumulators that rapidly produce large amounts of above-ground biomass, such as willow and poplar, and hyper-accumulators that generally produce less above-ground biomass but have the capacity to accumulate larger amounts of TE (Mench et al. 2010). *Arabidopsis halleri* belongs to the Brassicaceae family and is known to be a hyper-accumulator of Zn and Cd. In fact, this species accumulates more than 10,000 $mg.kg^{-1}$ of Zn and 100 $mg.kg^{-1}$ of Cd in its above-ground parts (Dahmani-Muller et al. 2000; Bert et al. 2002). To our knowledge, only two field studies have been undertaken with *A. halleri* (McGrath et al. 2006; Grignet et al. 2020). Only the study by Grignet et al. (2020) revealed the potential of this species for phytoextraction of Zn and Cd. Compared to the results obtained by Mcgrath et al. (2006), in this study the biomass produced per hectare is 14 times higher and the zinc concentrations are 3.6 times higher. This study also demonstrated that using agricultural practices such as the application of nitrogen fertiliser increased the phytoextraction potential (biomass and/or Cd and Zn concentrations) in this species.

Sulphur plays a role in several mechanisms in Brassicaceae, including the synthesis of defence molecules (glucosinolate (GLS)), of molecules related to TE absorption and transport (glutathione, cysteine, nicotianamine) and of proteins (Kramer et al., 2000; Verbruggen et al., 2009; Ueno et al., 2008; Huguet et al., 2012, Takahashi et al. 2001; Blažević et al. 2020). GLS are sulphur-rich secondary compounds that are predominantly found in Brassicaceae (Blažević et al. 2020). As constituent elements in this family, GLS represent more than 20% of the total sulphur fraction of the plant (Castro et al., 2004). More than 120 GLS have been identified, classified into three main groups: indole, aliphatic and aromatic (Blažević et al. 2020; Halkier and Gershenzon 2006). *A. halleri* is known to produce at least ten GLS (Windsor et al. 2006). At least two GLS are herbivory-induced in *A. halleri* (6MSOH and 7MSOH) and therefore play a role in defending against herbivores (Stolpe et al. 2017). The dominant GLS in *A. halleri* are aliphatic GLS (Kazemi-Dinan et al. 2015; Stolpe et al. 2015; Stolpe et al. 2017).

GLS play a role in defending the plants against herbivores and pathogens (fungi and bacteria). They also act as a toxin or deterrent for many herbivores (birds, mammals, molluscs) (Brader et al. 2001; Buxdorf et al. 2013; van Dam et al. 2009). However, GLS are not effective against specialist herbivores (Buxdorf et al. 2013; Renwick et al. 1992). Indeed, some herbivores have co-evolved with their host plant and have developed defence mechanisms

against GLS. For example, the *Pieris rapae* caterpillar produces an intestinal protein that prevents the formation of isothiocyanates by redirecting the hydrolysis of the glucosinolate towards the formation of nitrile which is less toxic than isothiocyanates (Wittstock et al. 2004). Damage from herbivores and pathogens leads to the induction of GLS in many Brassicaceae species (Textor and Gershenzon 2009). This induction is dependent on many factors, the plant species and population and the nature of the herbivore (Textor and Gershenzon 2009). Furthermore, not all GLS are induced. GLS with indole groups are more induced by herbivory than GLS with aliphatic groups (the increase in indole is 1.2 to 20 times the initial concentration while that of aliphatic is 1.2 to 3 times the initial concentration) (Textor and Gershenzon 2009). GLS induction is quick. The induction peak is observed between 24 and 48 hours after the passage of the herbivores or pathogens (Textor and Gershenzon 2009; Chen et al. 2020). The use of sulphur fertiliser can also have an impact on the quantity of GLS produced. Indeed, in most of the *Brassica sp.* species tested, the use of sulphur fertiliser increases the amount of GLS by more than 20%. This increase is dependent on the dose of sulphur fertiliser tested, the species and the age of the plant (Falk et al. 2007). *Cantareus aspersus* (also known as *Helix aspersa aspersa*) is a snail used as a bioindicator. This snail is known to accumulate metals in its viscera and for its ability to not excrete them. Cantareus aspersus has a great capacity to accumulate and sequester heavy metals in its tissues. However, they suffer sublethal toxic effects when the level of absorption exceeds the metabolic storage rates and detoxification processes (Laskowski and Hopkin, 1996). Several studies have shown that the toxic effect of Zn in herbivores was only manifested when the concentration in hyper-accumulating plants was greater than 5000 mg kg^{-1} (Gomot-de Vaufeury 2000; Noret et al. 2007). Falk et al. (2014) showed in Arabidopsis thaliana that slug attacks produced a day/night response in the production of GLS. The production of aliphatic GLS peaked at night in order to respond to nocturnal slug attacks.

The hyper-accumulation of metals demonstrated in some Brassicaceae species could constitute a defence mechanism against herbivores and pathogens (Boyd and Martens 1992). This hypothesis has been studied in several Zn and Cd hyper-accumulators such as *N. caerulescens* and *A. halleri* (Assunção et al. 2003; Noret et al. 2007; Stolpe et al. 2017). Indeed, high concentrations of TE in leaf tissue could be toxic to herbivores and pathogens suggesting a direct effect of TE (Martos et al. 2016). TEs may be a stress signal and play a mediating role in other defence mechanisms, thus suggesting an indirect effect of TEs (Poschenrieder et al., 2006; Fones and Preston, 2013). Metals stored in the leaves are released by the damage caused by herbivores, leading to a domino effect in responses such as the production of jasmonic acid and its derivatives (Poschenrieder et al., 2006).

The effects of TE accumulation on GLS concentrations are complex and very wide-ranging. Indeed, metal accumulation can lead to a decrease in GLS production (Tolra et al., 2001; Asad et al., 2015; Stolpes et al., 2017),

or an increase (Foroughi et al., 2014), or have no effect either way (Noret et al., 2005). Under the hypothesis of the existence of a trade-off, the plant would produce less GLS as the metal concentrations in the plant increase. The production of secondary compounds for defence against herbivores would be considered too costly for the plant (Bekaert et al. 2012). This trade-off hypothesis has been tested, in *A. halleri*, in studies by Kazemi-Dinan et al. (2015) and Stolpe et al. (2017). These studies did not show a trade-off between total GLS concentrations and TE concentrations.

Our study was carried out on a metal-polluted site. The primary endpoint was to study the effect of a nitrogen-sulphur fertiliser (NPK+S) on the concentrations of Zn, Cd and major elements in the above-ground parts of *A. halleri,* and on its biomass and survival. UsingNPK+S was expected to increase its phytoextraction potential by increasing its Zn/Cd concentrations and/or biomass. In addition, the amount of sulphur was expected to increase in the above-ground parts of the plants (since sulphur is found in assimilable form in the fertiliser). The plants' survival rates were also expected to increase due to a better mineral nutrition and/or a higher production of sulphur-containing defence molecules in *A. halleri*. This was the first time that the effect of NPK+S fertiliser on the phytoextraction potential, mineral nutrition and survival of *A. halleri* was tested in field conditions. The secondary endpoint of this study was to investigate the effects of NPK+S fertiliser and cultivation time in a polluted environment on the leaf concentrations of Zn/Cd and GLS when the leaves are consumed by a herbivore in *A. halleri*. The use of NPK+S fertiliser was expected to increase the foliar Zn, Cd and S concentrations. In this study, the hypothesis was that over time, the GLS are replaced by metals in the plant to protect against herbivory. In order to test this hypothesis, an experiment under controlled conditions was conducted, during which 3 variables were tested: herbivory, the presence of fertiliser and the cultivation time. For this purpose, we measured the concentrations of GLS, trace and major elements, and leaf consumption.

Materials and methods

Field experiment

Site description

A field trial was conducted in the urban area of Montataire (Oise, France, 49°15'12''N, 2°26'48''E) on the experimental site of the PHYTOAGGLO project. Details of the overall physicochemical characteristics of the site can be found in Grignet et al. (2020). Briefly, the topsoil was contaminated mostly by Zn (total concentration: 616 ± 248 mg kg^{-1}, mean ± SD) and Cd (1.7 ± 0.2 mg kg^{-1}), it presented an alkaline pH (> 8) and OM of 7.9 ± 0.79 %. The percentage of mobile Zn was 0.12 % and of mobile Cd 0.04%.

Arabidopsis halleri cultivation

The *Arabidopsis halleri* seeds came from a metallicolous population (Parc Péru, Auby, Nord, France). The *A. halleri* seedlings were grown in organic compost (Terreau potager bio, Gamm vert) in a culture chamber (20 °C, 70% moisture, 12 h day cycles) for 2 months. In April 2018, six plots of 1m² were set up (P20 to P25) on the experimental site of the PHYTOAGGLO project (Fig. 1). Before transplantation, 100 g of nitrogen fertiliser (Biogine® "NPK+S" 4-6-10 and 9.4% SO_3) was incorporated into the topsoil (10 cm depth) of plots P20, P23 and P25. 60 individuals were transplanted in each plot.

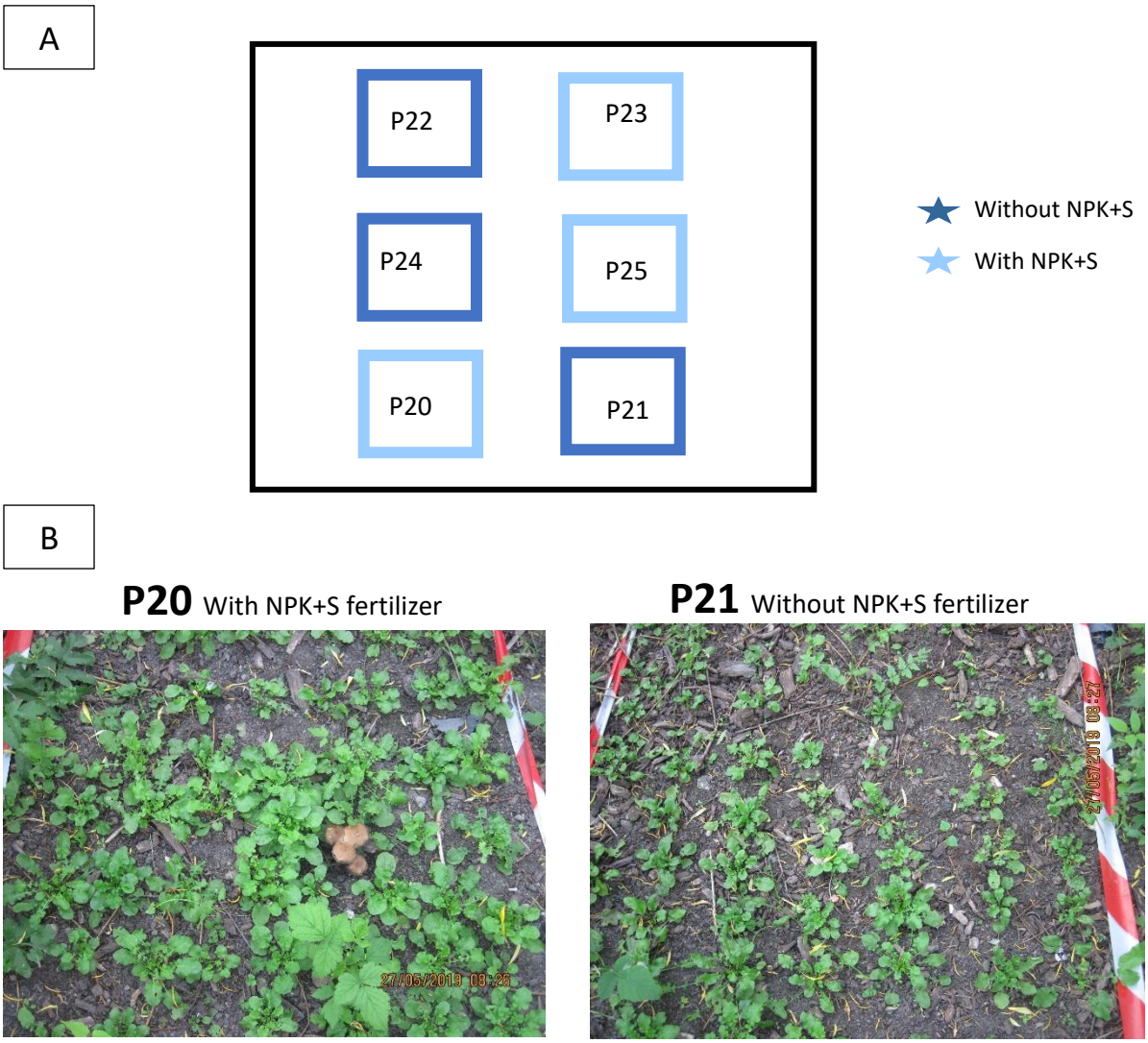


Figure 1: Experimental design of the 6 1m² *A. halleri* plots on the PHYTOAGGLO experimental site and photographs of plants at the rosette stage before harvest (B) (1 month after transplantation).

A. halleri, soil samples and analyses

One month after transplantation on site, all plants at the rosette stage were harvested and the survival rate was calculated by counting the living plants. The plant materials were carefully washed with tap and deionized water before drying at 40 °C until constant weight. The dry biomass was measured per plot. The plant material from each plot was ground to make one composite per plot and 5 samples of each of the composite plant powders were taken for mineral analysis.

The trace and major elements (Ca, Cd, Cu, Cr, K, Pb S and Zn) in the plant samples (0.5 g) were analysed by ICP-OES (Agilent 5110) or ICP-MS (Agilent 7500) after digestion (Mars Xpress CEM) at 180°C for 20 min in 10 mL of nitric acid (67% AnalaR Normapur) and 3 mL of ultrapure water. One standard reference material was used for analytical quality control (cabbage "NCS ZC 73012", NACIS).

Before the *A. halleri* was transplanted, 3 soil samples were taken in each plot with a hand auger (0–20 cm depth) and mixed to form one composite. The 6 soil composites were dried at 40 °C in a forced air oven to constant weight, then ground with a grinder (agate mortar, Retsch RM100) and sieved to < 20 mm (Retsch AS 200 digit). The soil pH was measured following the NF ISO 10390 (2005) standard. 5 grammes of 20 mm-sieved soils were mixed with 25 mL of distilled water and shaken for 2 hours. The water pH was measured after 1 h rest. Selective extraction was performed with 0.01 M Ca(NO3)2 to estimate the plant-available fractions of Zn, Cd, Cu and S. 60 grammes of dried soil were shaken with 120 mL 0.01 M $Ca(NO_3)_2$ for 48 h in an agitation table. After filtration (Millipore filter; Ø 0.45 µm) and acidification to a pH of 2 for preservation, the Zn, Cd, Cu and S in eluates were analysed either by ICP-OES (Agilent 5110) or ICP-MS (Agilent 7500) depending on the eluates' sample concentrations.

Experiments in controlled conditions

Short-term kinetics experiment

In order to determine the changes in Zn and Cd concentrations in *A. halleri* during 6 weeks of growth on a contaminated soil, a weekly monitoring of the leaf concentrations was carried out using topsoil (0-30 cm) collected on the contaminated experimental field site (Extra-Zn). The *A. halleri* seeds (Parc Péru, Auby, Nord, France) were germinated on organic compost (Terreau potager bio, Gamm vert) in 2 boxes (mini greenhouse 38 * 24 * 5 cm) in a culture chamber (20 °C, 70% moisture, 12 h day cycles). After 1 month, all the *A. halleri* seedlings from one of the two boxes were collected in order to obtain sufficient plant material to measure the initial Zn and Cd concentrations. 40 *A. halleri* seedlings from the other box were then transferred into one mini greenhouse (38 * 24

* 5 cm) filled with the contaminated soil to the monitor the leaf concentrations. Every week until week 6, 5 plants were randomly harvested, and their leaf Zn and Cd concentrations were measured by ICP-OES or ICP-MS, as previously described.

TE and GLS concentrations in the snail herbivory experiment

To ascertain whether there is a link between TE and GLS accumulation during a snail attack, an herbivory experiment was carried out in controlled conditions. In this experiment we tested the effects of soil fertilisation and weeks of growth on a contaminated soil on the concentrations of TE and GLS before and after snail damage.

Plant cultivation

The *A. halleri* seeds (Parc Péru, Auby, Nord, France) were germinated on blotting paper and cultivated for 1 month on a composite medium (perlite and vermiculite). After 1 month, the seedlings were transferred to 1L pots filled with contaminated soil (Extra-Zn soil), with and without NPK+S fertiliser. The seedlings were grown for 4 to 6 weeks. All the conditions are reported in Table 1. One gramme of NPK+S was added to the soil and hand-mixed to ensure soil homogeneity. The fertiliser dose was calculated based on that added to the $1m^2$ field plot. The length of the cultivation period on the contaminated soil was determined by the results of the short-term kinetic experiment, leading to 4, 5 and 6 weeks of cultivation. In addition to the pots with the contaminated soil, 5 pots were filled with organic compost, with and without fertiliser, with a cultivation period of 4 weeks (Table 1).

Herbivory experiment

One quarter of the plant rosette was collected from each pot (Table 1) to measure the initial concentration of GLS before snail damage. Before introducing the herbivore, a plastic tarp was added to the top of each pot to avoid any contact between the contaminated soil and the snails. One seven-week-old sub-adult *Cantareus aspersus* snail (previously *Helix aspersa aspersa*) (5.0 g ± 0.30 g) was added to each pot and caged with a netting. The snail was then left in contact with the plant for two days. After this period, each snail was put in a plastic box (plastic box with ventilation 24 * 10.5* 8 cm) and fed a non-contaminated diet for 2 days (the faeces were removed every 24h). The snails were then frozen at -80°C for further analysis (TE in viscera). In addition, 5 snails were raised in a box (plastic box with ventilation 24 * 10.5* 8 cm) and fed with uncontaminated commercial snail meal (Helixal) to serve as a control when calculating the accumulation quotient (AQ).

After contact with the snails, the *A. halleri* plants were left to grow for 3 more days in the culture chamber. The remainder of the rosette was then harvested, washed with demineralized water to remove any residues of snail faeces and frozen at -80°C for further analysis (TE and GLS in *A. halleri*).

Table 1. Design of the herbivory experiment.

Fertilizer	Soil	Weeks	Number of replicates per condition	Measurements
With	uncontaminated	4	5	Foliar trace and major concentrations, GLS concentrations (before and after herbivory), trace and major concentrations in snails viscera
	contaminated	4	5	
		5	5	
		6	5	
Without	uncontaminated	4	5	
	contaminated	4	5	
		5	5	
		6	5	

TE analysis in snail viscera and Arabidopsis Halleri leaves

The Zinc, Cd and S in the plant samples were analysed at the end of the experiment (after snail contact) by ICP-OES or ICP-MS, as previously described.

The trace and major elements in the snail viscera were measured according to the protocol in Grignet et al. (2020). For each TE, the mean snail viscera concentrations were compared to those of the control snails. The transfer of metals was calculated using the accumulation quotient (AQ) as follows: AQ = Concentration of the metal in the snail viscera after the end of exposure / Concentration of the metal in the control snail viscera. AQ > 1 represents an abnormal transfer of a metal. An overall metal transfer (SET) was also calculated from the sum of the excess of transfer, where SET represents the overall excess of metal transfer calculated from the transfer excess of each metal (AQmetal-1). For this index, the AQ < 1 was adjusted to 1 and considered as a normal transfer (AQmetal – 1 = 0). SET = $\sum$AQmetal-1.

Leaf consumption was determined by the difference in leaf area using photos taken before and after the snail consumption. Leaf area was determined using the software ImageJ.

Glucosinolate and metabolites analysis in A. halleri.

GLS were extracted from powdered freeze-dried leaf material (25 mg) following the protocol in Salem et al. (2016), adding 2-propenyl GLS (Sigma-Aldrich) as an internal standard to each sample at first extraction. The solvent M1 (lipophilic phase) was prepared with 75% methyl tert-butyl ether and 25% of methanol (3:1, v/v) and stored at -20°C. The solvent mixture 2 (M2; hydrophilic phase) was prepared with 75% water and 25% methanol (3:1, v/v). The ultrapure water was prepared by filtration using a Milli-Q system from Millipore (Bedford, MA, USA). The MS quality reagents were purchased from Sigma-Aldrich (Steinheim, Germany). For each 25 mg sample, 1 mL of extraction solvent M1 was added to the homogenized leaf powder in a 2 mL tube. After adding the extraction solvent, the tubes were thoroughly vortexed for 1 min and then placed in an orbital shaker (100 rpm) for 45 min at 4 °C, followed by a 15 min sonication step. Solvent M2 (650 µl) was then added to each tube and the samples were again thoroughly vortexed for 1 min. After that, the samples were centrifuged at a speed of 20,000g for 5 min at 4 °C. The two lipophilic and hydrophilic phases were then pipetted into separate tubes. Several extraction replicates were performed to check the extraction repeatability. The tubes were then stored at -80°C until analysis.

The analyses were performed using a 1200 series rapid resolution LC (RRLC) system coupled to a 6520 series electrospray ionization (ESI)-quadrupole time-of-flight (QTOF) high-resolution mass spectrometer (Agilent Technologies, Waldbronn, Germany). The column used was a Poroshell 120 EC-C18 (2.7 µm; 100 mm × 2.1 mm; Agilent). The column temperature was set at 40 °C. The mobile phases were composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient applied was as follows: 0–1 min, 0–1% B; 1–13 min, 1–45% B; 13–14.5 min, 45–30% B; 14.5–15.5 min, 30–99% B; 15.5–17 min, 99% B; 17–17.1 min, 99–1% B; post-run 3.9 min at 0.5 mL/min. The ESI-QTOF parameters were as follows: negative mode, 2 GHz mode for resolution, mass range 100–1700 m/z, drying gas temperature and flow of 350 °C and 9 L/min respectively, nebulizer pressure 50 psig, and capillary voltage 4500 V.

Nitrogen was used as the nebulizer gas. The data acquisition and LC-MS data analysis were carried out using MassHunter Acquisition® software for QTOF (Version B.04 SP3), MassHunter Qualitative Analysis® (Version B.06) software and MassHunter Quantitative Analysis® (Version B.04) software (Agilent Technologies). All samples were analysed in one batch; a quality control (QC) sample (mix of all samples) was regularly injected

(every ten samples approximately). GLS were identified by comparing retention times and exact m/z values to in-house library and online databases (MassBank; www.massbank.jp) and the scientific literature (Stolpe et al. 2017).

Statistical analysis

All analyses were performed with the R statistical software 3.2.2 (R Core Team) (except Friedman ANOVA). The data were first checked for normality (Shapiro-Wilk test) and homogeneity of variances (F-test). The trace metal and major metal concentrations and the dry weight (n=3) of the plants grown in the field were analysed with the Kruskal-Wallis H test. A PCA was carried on dry weight, trace and major elements measured in the soils of the *A. halleri* plots and the samples were distributed according to the presence or absence of NPK + S. For GLS concentrations, the data were ln+1-transformed to meet the requirements of the ANOVA. In order to test the difference in GLS concentrations before and after snail damage, a Friedman ANOVA for dependent data was performed with STATISTICA software. All the other analyses on GLS and metal and major element concentrations were performed on the post-herbivory data (ANOVA, Post Hoc test (Tukey HSD) and linear correlations).

Results and discussion

The effect of NPK+S fertiliser on biomass, leaf concentrations of Zn, Cd and S and phytoextraction potential of *A. halleri in situ*

The NPK+S fertiliser is used here in order to maximise Zn and Cd leaf concentrations and/or increase the biomass as reported in several studies in Brassicaceae (Ji et al. 2011, Jacobs et al. 2018a; Grignet et al. 2020). The *A. halleri* produced a significantly higher biomass (x 1.7) on the plots with NPK+S than on the plots without fertiliser (Figure 2). Several studies have already reported an increase in biomass in *N. caerulescen*s with the use of nitrogen fertiliser (Schwartz et al. 2002; Jacobs et al 2018a). In *A. halleri*, Grignet et al. (2020) observed a trend towards increased biomass at the rosette stage with the use of the same NPK+S fertiliser at the same dose.

The Zn leaf concentration did not change in function to the plots with and without NPK+S (Figure 2). Despite a pH that is not optimal for phytoextraction (values), the Zn concentrations were 15 times higher than the average ordinary concentration found in plants growing in an unpolluted environment (Kabata-Pendias, 2010). These results confirm the Zn-accumulative character of *A. halleri*. On the other hand, the foliar Cd concentration was significantly higher on plots with NPK+S (+ 18%) compared to the unfertilised plots, confirming the results previously found (Grignet et al. 2020). On the contrary, Sirguey et al. (2006) showed in *N. caerulescens* that the combined use of nitrogen and sulphur fertiliser did not increase Cd leaf concentrations nor biomass. Similarly,

Kayser et al. (2000) also showed that the use of elemental sulphur in *N. caerulescens* did not increase biomass nor leaf concentrations of Zn and Cd. The increase in Cd concentration could be due to a decrease in the soil pH leading to an increase in Cd mobility, as a result of the sulphur fertiliser application (Kabata-Pendias, 2010). Contrary to what was expected, no decrease in pH was observed on the plots with NPK+S,(8.25 ± 0.08 for the plots with NPK+S and 8.23 ± 0.07 for the plots without NPK+S). Similarly, no increase in Cd mobility was found (0.0016 ± 0.006 for the plots with NPK+S and 0.0016 ± 0.0008 for the plots without NPK+S). In the presence of NPK+S, the leaf concentration of sulphur doubled (Figure 2). This increase in foliar S was also found in other Brassicaceae (*Brassica napus* and *Sinapsis alba*) when sulphur fertiliser was applied (Podleśna 2004, Barczak et al. 2011).

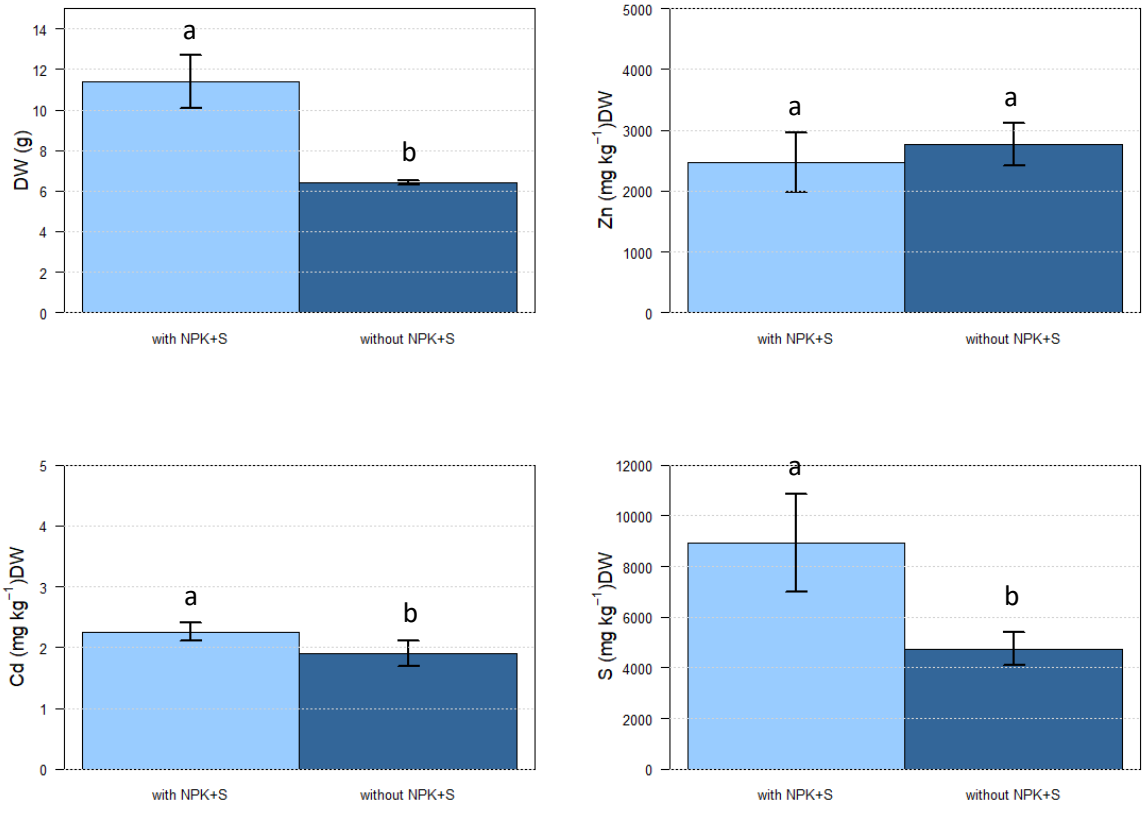


Figure 2. Dry weight per 1 m^2 plot (mean ± SD, n=3 plots) and foliar Zn, Cd and S concentrations in *A. halleri* depending on the plots with (light blue) or without (dark blue) NPK + S (mean ± SD, n=15). Different letters indicate significantly different values at α = 0.05

The Principal Component Analysis (PCA) and sample projection confirmed that the soils of the plots with and without NPK+S were different (Figure 3). The first 2 principal components F1 and F2, accounted for 58.8% of the variability in the data. We also observed that the foliar concentrations of Cd, S, Cu, K and Ca of plants growing with or without fertilisation varied in the same direction. The Zn concentrations are not correlated with those of

the other elements (orthogonal position). The most discriminating factors between plots with and without NPK+S were the foliar concentrations of Cd and S. In line with the previous results, these results suggest that foliar S was assimilated from the NPK+S fertiliser and was involved in the Cd accumulation mechanisms. However, in *A. halleri*, it is organic acids rather than sulphur molecules that transport and sequestrate Cd and Zn (Sarret et al. 2002; Ueno et al. 2008; Huguet et al. 2012). The increase in foliar S under our study conditions was therefore not be related to the transport and sequestration of Cd but rather to defence mechanisms against biotic and abiotic stresses through the production of sulphur molecules such as glutathione, defensins and GLS (Na and Salt 2011; Mirouze et al. 2006).

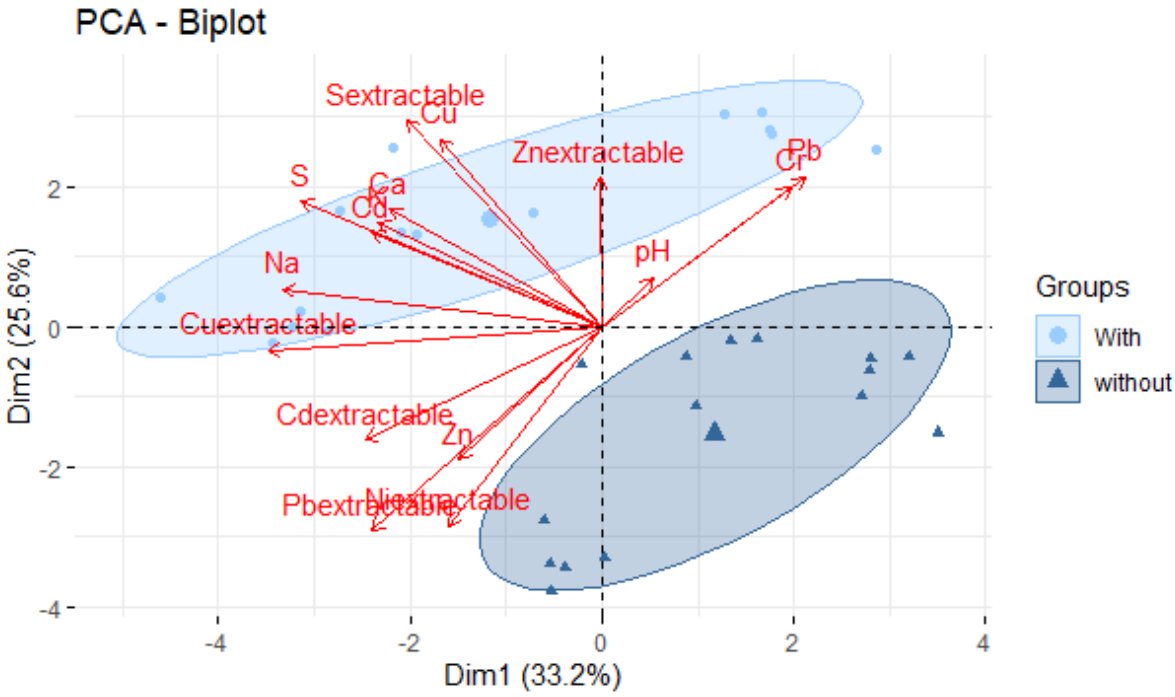


Figure 3. PCA on dry weight, trace and major elements measured in *A. halleri* and distribution of samples labelled according to the presence or absence of NPK + S, with projection on F1 and F2 axes (the ellipse represents the dispersion of the individuals in relation to the average of all the individuals (centroid)).

The effect of the fertiliser on the phytoextraction potential of *A. halleri* was studied by calculating the quantity of metals exported (Table 2). An extrapolation of the biomass produced per hectare showed a significantly higher biomass production in the presence of NPK+S after cutting at the rosette stage (0.11 $t.ha^{-1}$ compared to 0.08 $t.ha^{-1}$ without NPK+S). These results are lower than those reported in Grignet et al. (2020) where the biomass was estimated at 3.5 $t.ha^{-1}$. This difference in biomass can be explained by a difference in the cultivation time (1 month compared to 2 months in Grignet el al. (2020)) and in the calculation itself. In the study by Grignet et al. (2020), the biomass per hectare was estimated based on the biomass estimate of the $1m^2$ plots extrapolated from the

biomass of 5 plants. Whereas in this study, the biomass per hectare estimate was extrapolated from the average biomass measured for all the plants in the plots. This would suggest a significant heterogeneity of plant biomass within the same plot, which was not measured (Table 2 and Chapter 3). Other factors could also explain this result (e.g. climatic factors). The export of Zn and Cd was significantly higher on the plots with NPK+S than on the plots without NPK+S due to the increase in biomass for Zn and the combined increase in biomass and Cd concentrations for Cd. As before, these results are poorer than those reported in Grignet et al. (2020), which can be explained by a lower biomass production and lower Zn and Cd concentrations under our experimental conditions. The presence of the NPK+S fertiliser also influenced the survival of *A. halleri* (Table 2). Indeed, the plant survival rate was higher on the plots with fertiliser than on the plots without fertiliser (92% vs. 78%). This increase in survival rate may be due to the S contained in the fertiliser. Indeed, sulphur can allow a better mineral nutrition of the plants in line with the results of the PCA in which the major elements (K, Ca) vary in the same way as S. The survival rate may also be due to an increased production of the sulphur molecules involved in plant defence such as GLS.

Table 2 : Biomass yield based on an extrapolation to 1 ha from the average biomass harvested on $1m^2$ plot after 1-month at the rosette stage. Cd and Zn removal per hectare were calculated as average Cd and Zn concentrations X biomass yield. The survival rate was as assessed after one month on plots with and without NPK + S (mean ± SD, n=3 plots).

	With NPK + S	**Without NPK + S**
Biomass yield (t ha^{-1})	0.11 ± 0.01 (a)	0.08 ± 0.02 (b)
Zn removal (kg ha^{-1})	0.28 ± 0.06 (a)	0.21 ± 0.05 (b)
Cd removal (g ha^{-1})	0.25 ± 0.03 (a)	0.16 ± 0.07 (b)
Survival rate (%)	91.61 ± 5.13 (a)	78 ± 10.44 (b)

Increased GLS concentrations in the presence of sulphur fertiliser have been demonstrated in several Brassicaceae species in unpolluted soil conditions (Rosen et al., 2005; Falk et al. 2007). This increase in GLS concentration can be up to +20%, depending on the dose of sulphur fertiliser tested, the species and the age of the plant. GLS are also induced by herbivore and pathogen damage in many species (Textor and Gershenzon 2009). The nature of the GLS synthesised depends on the species and plant population studied, but also on the herbivore or pathogen provoking the induction (Newton et al. 2010).

Characterisation of Zn and Cd accumulation in *A. halleri* as a function of the cultivation time on polluted soil

In order to characterise the accumulation of Zn and Cd in *A. halleri* over time, we carried out an experiment in semi-controlled conditions on polluted soil. The highest Zn concentrations were measured in the plants grown for the longest time on the polluted soil (Figure 4). To our knowledge, this is the first time that a curve of this type has been established for *A. halleri*. We found an exponential increase in Zn concentrations, with a sharp increase after the 4th week; this curve resembles the theoretical hyper-accumulation curve put forward by Baker in 1981. However, for the Cd concentrations, we observed no significant difference between growth weeks. The leaf concentrations of Cd in the polluted conditions were identical to those measured in the *Arabidopsis halleri* plants grown for 1 month in potting compost. The compost contained 0.85 mg kg^{-1} of total Cd whereas the polluted soil contained 1.7 mg kg^{-1} total Cd. This result confirms the Cd hyper-accumulative character of the species (BCF >>1). The Cd accumulation curve stagnates around 2.5 mg kg^{-1}. It could be that the accumulation of Cd is very rapid and that it had already reached a plateau. Indeed the existence of a plateau in the accumulation of Zn and Cd has been suggested by Hamon et al (1999). The cultivation time has an effect on the Zn concentrations and GLS concentrations in *A. halleri* plants (Stolpe et al. 2017). If a trade-off exists between Zn concentration and GLS concentration, *A. halleri* plants that have spent less time on polluted soils should have less foliar Zn and therefore more GLS, and vice versa.

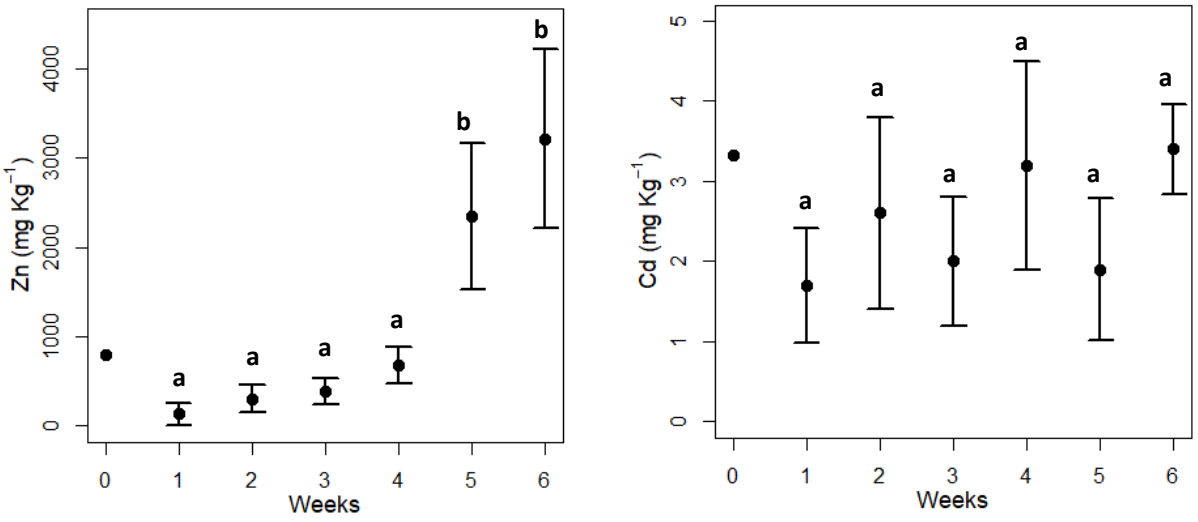


Figure 4. Foliar Zn and Cd concentrations in *A. halleri* plants grown for 6 weeks in controlled conditions. 0 = after 1 month in composite unpolluted soil (n=1 composite plant); 1-6 = weeks of growth in a polluted soil (n=5 plants, mean ± SD).

Effect of NPK+S fertiliser, cultivation time on polluted soil and herbivory on leaf GLS concentrations in *A. halleri*

In order to find out whether the use of NPK+S fertiliser and the cultivation time on polluted soil increased leaf concentrations of Zn, Cd and GLS, and to support the hypothesis of the role of Zn and GLS in defence against herbivores, a two-factor cross-over experiment with a herbivore (snail) was carried out under controlled conditions. The GLS concentrations were measured before and after herbivory, and the major and trace element concentrations were measured after herbivory. A total of 8 GLS were detected in the leaves of the *Arabidopsis halleri* plants, 6 with an aliphatic chain (6-methylthiohexyl GLS (6MTH), 7-methylthio-heptyl GLS (7MTH), 6-methylsulfinylhexyl GLS (6MSOH), 7-methylsulfinylheptyl GLS (7MSOH), 8-methylthiooctyl GLS (8MTO), 8-methylsulfinyloctyl GLS (8MSOO)) and 2 with an indole chain (4-methoxy-indol-3-yl-methyl GLS (4MOI3), indol-3-ylmethyl GLS (I3M)). Aliphatic GLSs were in the majority compared to indolics (Figure 5A). 7MTH and 6MTH accounted for more than 80% of the total GLS. This dominance of aliphatics in *A. halleri* was also demonstrated in the studies by Kazemi-Dinan et al. (2015) and Stolpe et al. (2017). However, in these two studies, the majority GLSs were 6MSOH and 7MSOH. This widely documented difference in the composition of GLS is probably due to the nature of the populations studied (Kazemi-Dinan et al. 2015; Mosleh et al. 2008; Katz et al. 2020). Katz et al. (2020) showed a very marked difference in the composition of GLS in *A. thaliana* between populations in Eastern and Western Europe. GLS composition also varies within a population, as Kazemi-Dinan et al (2015) found in *A. halleri*.

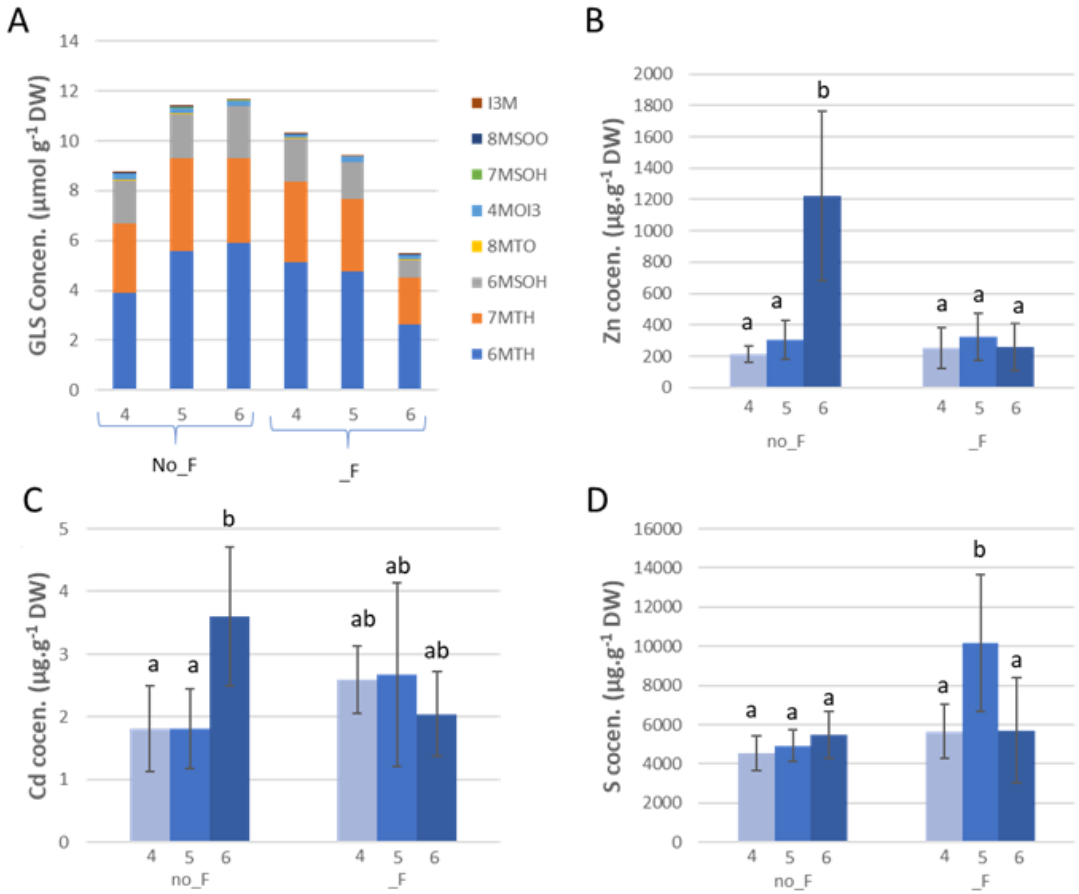


Figure 5. Composition of leaf glucosinolates (GLS) of Arabidopsis halleri *plants grown with (_F) or without (No_F) NPK+S fertilisation at different times (4,5 and 6 weeks) (A), and effect of NPK+S fertilisation and time on foliar Zn (B), Cd (C) and S (D) concentrations (Different letters indicate significantly different values at α = 0.05, two-way ANOVA with fixed factors; n=5 for each week * fertilisation combination).*

Without the NPK+S fertiliser, the Zn concentrations in the leaves of the *Arabidopsis halleri* plants were significantly higher in the 6th week of growth (Figure 5B). This significant increase in Zn concentrations was expected based on the Zn accumulation characterisation study described earlier (Figure 3). In the presence of fertiliser, no significant difference was found. This homogenisation of Zn concentrations over time was observed in the field at different stages of development (Chapter 3, Grignet et al. submitted). For the Cd concentrations, the Arabidopsis plants without NPK+S fertiliser accumulated significantly more Cd after 6 weeks in the polluted environment than after 4 and 5 weeks in the polluted environment, which differs from the Cd accumulation result obtained previously (Figure 4). This can be explained by significant inter-individual heterogeneity. Contrary to the *in situ* experiment (Figure 2), no significant difference in leaf Cd accumulation between fertilised and

unfertilised polluted soils was observed, thus the NPK+S had no effect. In agreement with the *in situ* experiment, in the presence of fertiliser, the leaf concentration in S was significantly higher (1.6 times) but only at the 5th week.

In order to characterize the link between Zn and Cd accumulation and the foliar GLS and S concentrations, Arabidopsis plants were grown for 4 weeks on compost and on polluted soil with and without NPK+S. We observed no significant difference in the Zn, Cd and GLS concentrations (Supplementary data Table 1) between the two growing conditions. On the other hand, there was a significant difference in the concentration of leaf S, which was higher in the presence of fertiliser in both the polluted soil and in the compost (Figure 6). It should be noted that the compost seems to contain more assimilable S than the polluted soil. The addition of the NPK+S fertiliser did not have the expected effect on Cd and GLS concentrations. The S present in the fertiliser therefore does not seem to increase the leaf GLS concentrations.

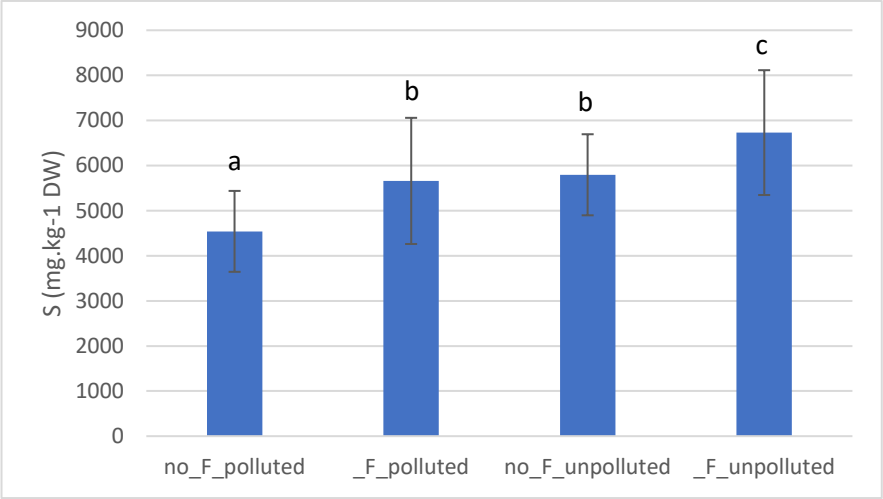


Figure 6 Effect of soil type (growth for 4 weeks on contaminated versus uncontaminated [compost]soil) and NPKS fertilization on S concentration (Different letters indicate significantly different values at the level of α = 0.05, Posthoc Tukey HSD test, n=5)

In order to test the effect of herbivory on GLS concentrations, we performed Friedman's ANOVAs in function to fertilisation and cultivation time (Supplementary data Table 2). Contrary to the study by Stolpe et al. (2017), we found no significant difference before and after herbivory on GLS concentrations in *A. halleri* suggesting that no induction occurred. In the majority of studies, a significant increase in indole GLS concentrations was observed after herbivory. Aliphatic GLS concentrations also increase, but to a lesser extent (Textor and Gershenzon, 2009). The lack of induction of GLS by herbivory in our study conditions may be due to the fact that the plants come from a metallicolous population.

Table 3. Effect of NPK+S fertilisation and time (after 4, 5 or 6 weeks of growth on a polluted soil) on *A. halleri* foliar concentrations of several glucosinolate (6MTH, 7MTH, 6MSOH, 7MSOH, 8MTO, 8MSOO, 4MOI3, I3M; see text for full names of molecules) concentrations (two-way ANOVA with fixed factors; n=5 for each week * fertilisation combination). Significant differences (P < 0.05) are highlighted in bold.

		6MTH		**7MTH**		**6MSOH**		**7MSOH**		**8MTO**		**8MSOO**		**4MOI3**		**I3M**	
	df	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F.	P
fertilization	**1**	**5.68**	**0.02**	**4.06**	**0.05**	**15.47**	**0.01**	1.25	0.27	**4.98**	**0.03**	**12.56**	**0.01**	1.12	0.3	2.87	0.10
weeks	2	2.77	0.08	2.95	0.07	1.38	0.27	1.25	0.30	**5.89**	**0.01**	1.97	0.15	1.08	0.35	0.47	0.63
fertization*weeks	2	0.97	0.39	0.57	0.57	1.53	0.23	0.49	0.61	2.26	0.12	0.25	0.77	0.54	0.58	0.03	0.96

Indeed, it has been shown that herbivore pressure was lower on metallicolous sites (Noret et al. 2007; Notten et al. 2006). Given that the maintenance of primary defences via the production of organic molecules is an energy-intensive mechanism, it has been suggested that metallicolous populations of *N. caerulescens* produce less GLS in favour of accumulating metals for their defence (Fones and Preston 2013). However, as GLS induction is rapid (24 h and 48 h after the start of consumption by herbivores), the induction peak may not have been observed under our experimental conditions since the dosage was measured 72 h after leaf consumption (Textor and Gershenzon 2009).

Studying the effects of fertilisation and cultivation time on GLS concentrations showed that fertilisation had a depressing effect on 6MTH, 7MTH, 6MSOH and 8MSOO concentrations and that cultivation time had this same effect on 8MTO (Table 3). Post-hoc tests (Tukey HSD) showed that the Arabidopsis plants with NPK+S fertiliser had less 6MTH, 7MTH, 6MSOH, 8MSOO and 8MTO than the Arabidopsis plants without NPK+S (Figure 7 A and B). In *Brassica sp*. a decrease in total GLS was found when a nitrogen fertiliser was used (Chen et al. 2004; Aires et al. 2006; Schonhof et al. 2007). To the contrary, the use of sulphur fertiliser increased the concentration of GLS, especially aliphatic GLS (Withers and O'Donnell, 1994; Zhao et al., 1994, Textor and Gershenzon 2009). When the nitrogen/sulphur ratio in the plant increases, the GLS content decreases, probably because the plant allocates its resources to its growth rather than to GLS biosynthesis, which would dilute these metabolites in the plant (Hugentobler and Renwick, 1995; Kim et al., 2002; Rosen et al., 2005). NPK+S fertiliser use is therefore not relevant for increasing GLS concentrations in *A. halleri*.

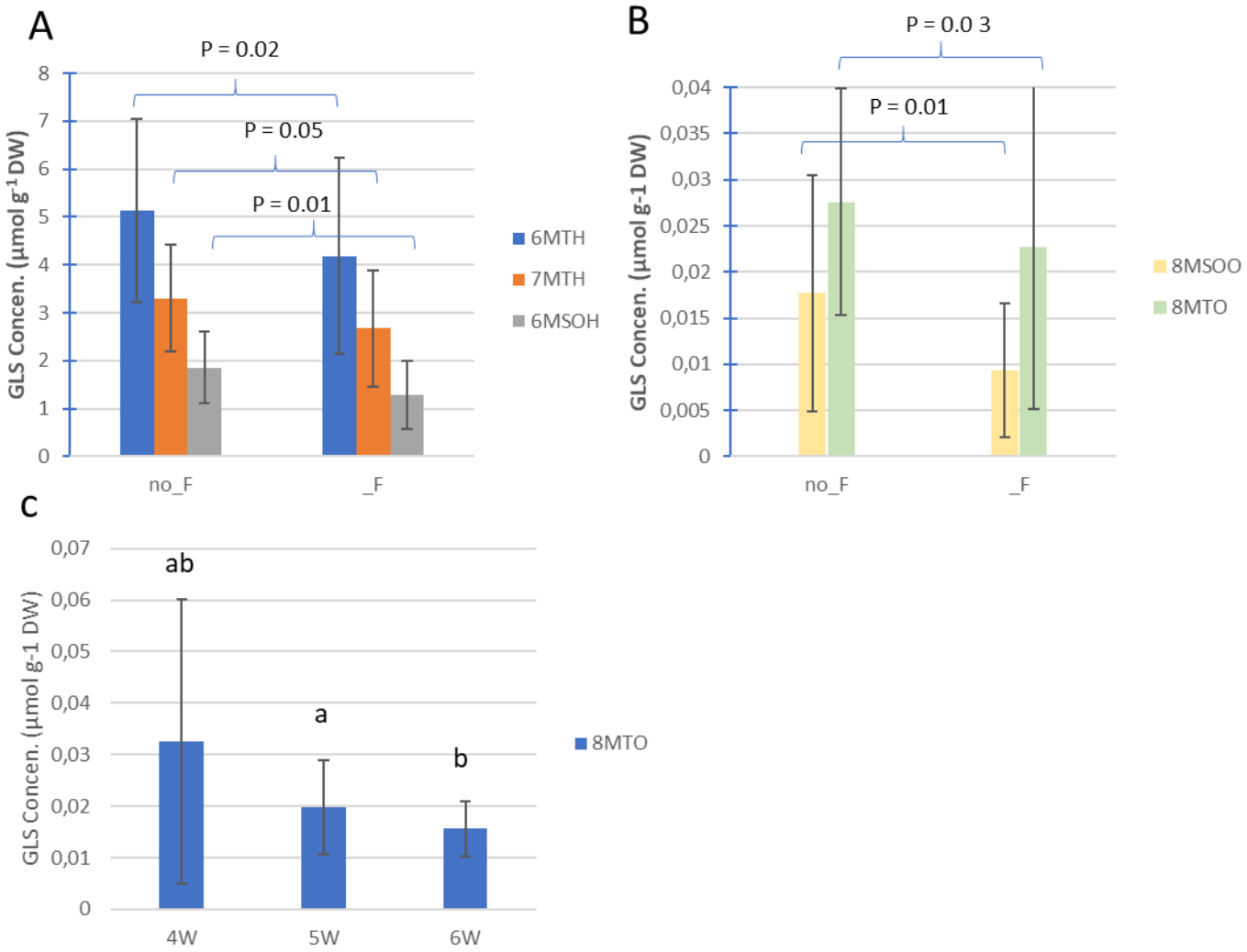
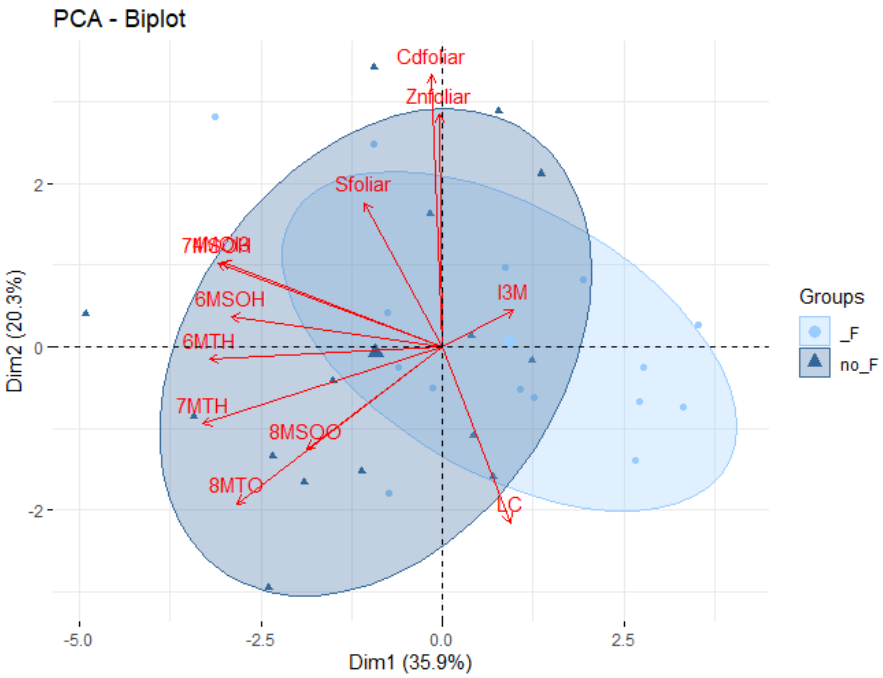
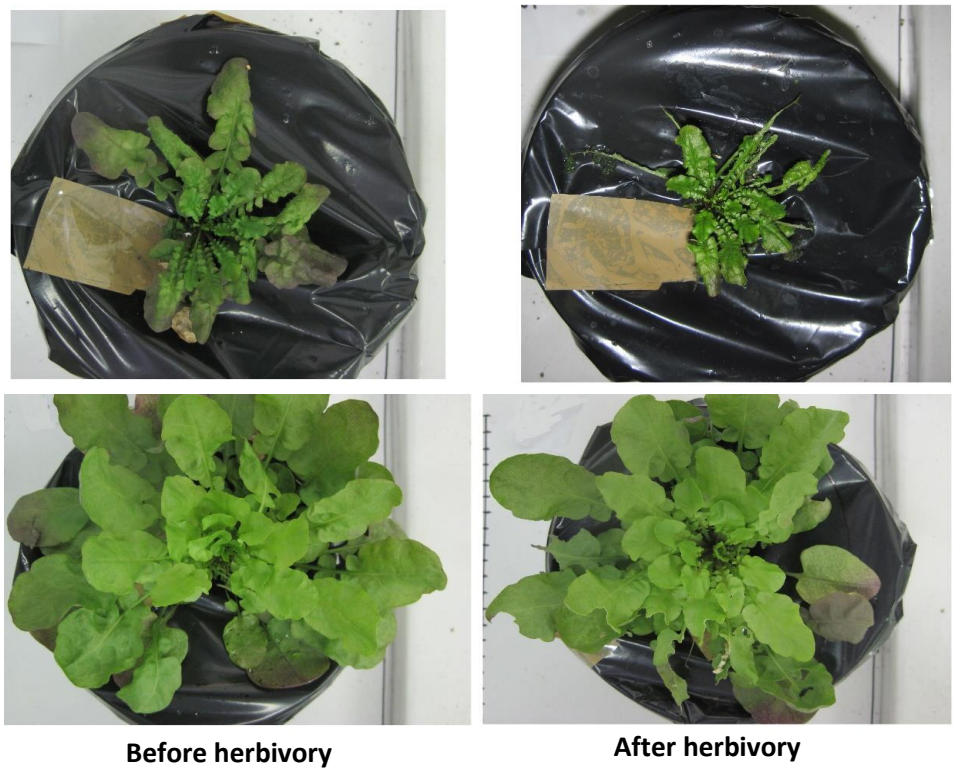


Figure 7 Effect of NPK+S fertilization on 6MTH, 7MTH and 6MSOH GLS concentrations (A), effect of NPK+S fertilization on 8MSOO and 8MTO GLS concentration (B), effect of time cultivation on 8MTO concentration (4,5 and 6 weeks)(c). (Different letters indicate significantly different values at the level of α = 0.05 Posthoc Tukey HSD test; n=5).

We carried out a PCA to characterise the interactions between GLS concentrations and Zn, Cd and S concentrations in function to the presence or absence of the fertiliser (Figure 8). Most of the GLS were positively correlated with each other, except for the I3M GLS which was negatively correlated with the others. The leaf concentrations of Zn, Cd and S were not correlated with the GLS concentrations, which disproves the hypothesis of a trade-off between metal and GLS accumulation in *A. halleri* under our study conditions.



28 Figure 8 PCA on GLS (6MTH,7MTH, 6MSOH, 7MSOH, 8MTO, 8MSOO, 4MOI3, I3M), trace and major
29 elements and leaf consumption measured in *A. halleri* and distribution of samples labelled according to the
30 presence or absence of NPK + S with projection on F1 and F2 axes.



31 Figure 9 : Photos of 2 *A. halleri* plants before and after 48 hours with 1 herbivore (top photos at 4 weeks and
32 bottom photos at 6 weeks without NPK+S)

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In addition, leaf consumption by snails (LC) was not influenced by the GLS concentrations (orthogonal vector; Figure 8). To the contrary, independently of the presence of fertiliser, it was influenced by leaf concentrations of S, Zn and Cd (Figure 9).

We carried out linear correlation tests between Zn, Cd concentrations and leaf consumption for each fertilisation condition. Leaf consumption by herbivores was negatively correlated with the leaf Cd and Zn concentrations for the Arabidopsis plants growing on polluted soil without NPK+S (pvalue < 0.05) (Figure 10).

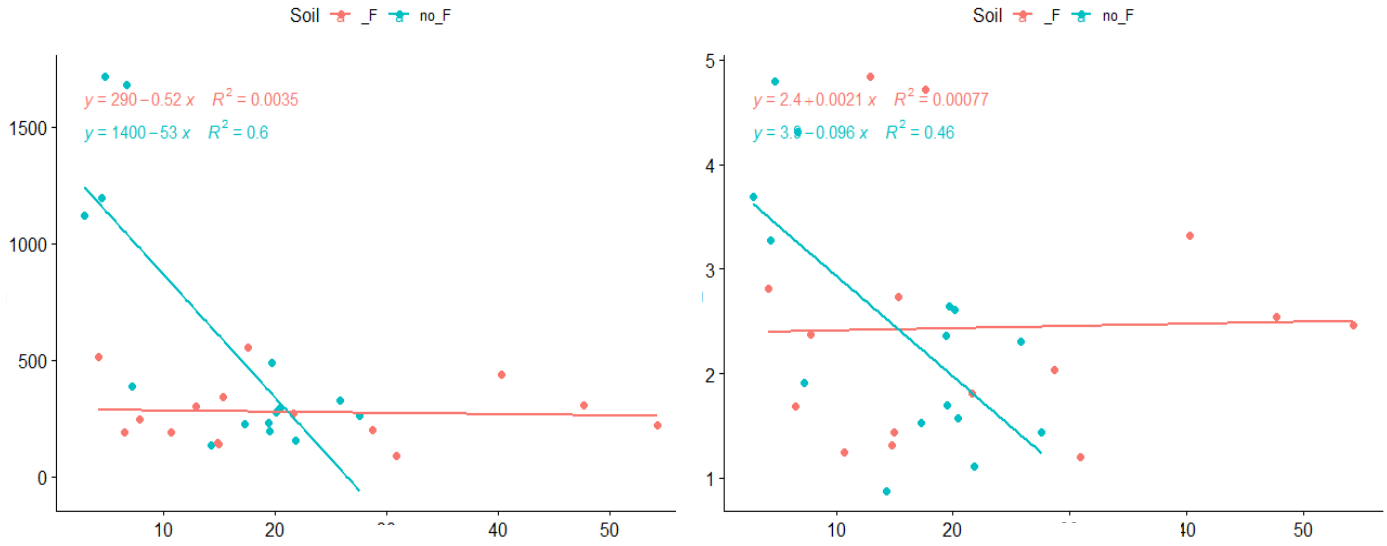

Figure 10. Linear correlation between leaf consumption and Zn, Cd concentrations according to the presence or absence of NPK + S.

The snails consumed less Arabidopsis plants with more Zn and Cd, which corroborates the results obtained by Grignet et al. (2020). Similarly, in *Noccaea praecox*, the leaves with high Cd concentrations were found to be the least consumed (Llugany et al. 2019). These results therefore suggest an involvement of Zn and Cd in the defence of the plant against herbivores. On the contrary, with the NPK+S fertiliser, we found no linear correlation (pvalue > 0.05) between LC and the Cd, Zn and S in the leaves, which confirms the fact that the fertiliser did not have the expected effect. In this case, the snails consumed leaves with similar Zn and Cd concentrations differently (figure 5 B and C). Several studies have shown that the toxic effect of Zn in herbivores only occurred when the concentration in the hyper-accumulating plants was above 5000 mg kg^{-1} (Gomot-de Vaufeury 2000; Noret et al. 2007). Similarly, Huitson and Macnair (2000) showed that there was no difference in leaf consumption by snails when *A. halleri* had Zn concentrations below 5000 mg kg^{-1}, which is the case in our study conditions without

NPK+S. On the other hand, the differences in consumption in this case could be attributed to factors other than the metals. In *Noccaea praecox*, the leaves containing the most sugar were found to be the most consumed (Llugany et al. 2019).

The Zn accumulation in snail viscera was not correlated with leaf Zn concentrations for both conditions, with and without NPK (pvalue < 0.05) (Figure 9). Cd accumulation in snail viscera was however correlated with Cd concentrations in the leaves of the Arabidopsis plants without NPK+S (Figure 11).

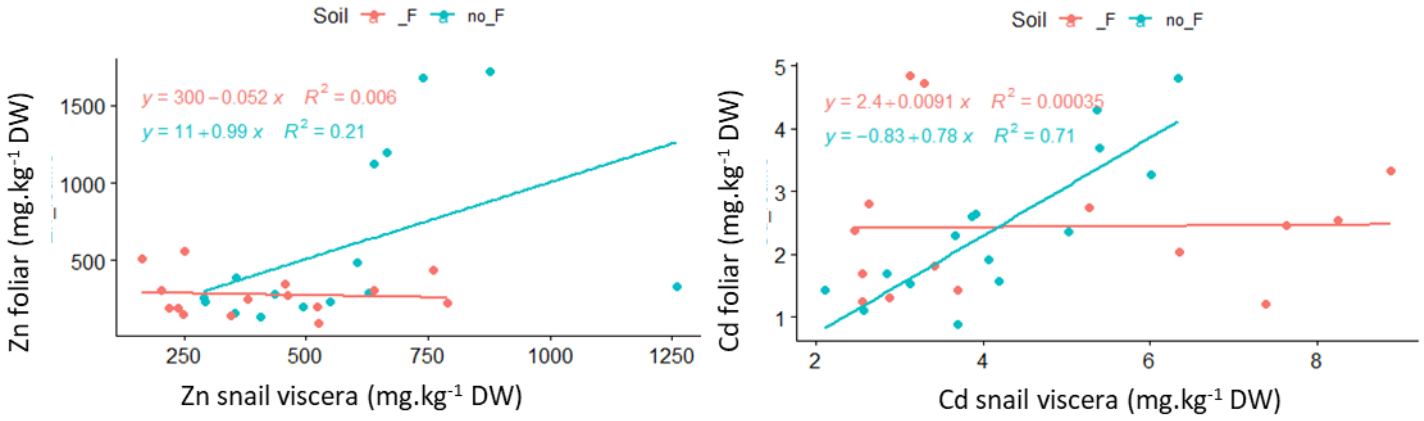

Figure 11 Linear correlation between Zn concentration in snail viscera and Zn concentration in A. halleri (left) and Cd concentration in snail viscera and Cd in A. halleri (right).

The snails bioaccumulated the Cd, and to a lesser extent the Zn, found in the *A. halleri* leaves (Supplementary Table 3). The amount of leaves consumed was lower in the condition without NPK+S compared to the condition with NPK because of the higher Cd concentrations, but this consumption is sufficient to induce a higher bioaccumulation of Cd in this case (figure 12). These results agree with those reported in Grignet et al. (2020) and suggest that there may be a risk of Cd dispersing in the environment. Concerning Zn, which is an essential element for biological functioning, these results can be explained by the fact that the snails already presented Zn concentrations before consuming the leaves of *A. halleri*, or by the fact that part of the Zn allocated to the metabolism is not accumulated in the viscera.

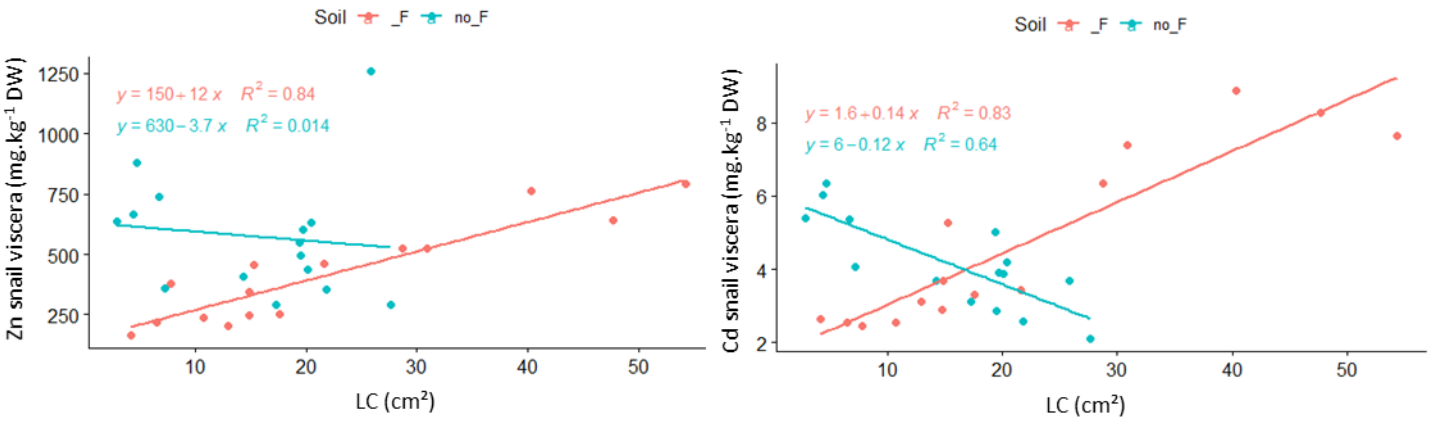

Figure 12. Linear correlation between Zn concentration in snail viscera and leaf consumption (left) and Cd concentration in snail viscera and leaf consumption (right).

Conclusions

In this study, we showed that the use of NPK+S fertiliser *in situ* increased the biomass of *A. halleri* at the rosette stage and thus its phytoextraction potential. To our knowledge, this study is the first to report a very significant increase (1.7 times) in biomass in this species in the presence of fertiliser without a decrease in Zn and Cd leaf concentrations. For the first time, this study made it possible to characterise the accumulation of Zn and Cd in *A. halleri* over time, which confirms the plant's hyper-accumulative behaviour for these two elements in polluted soil conditions. This study also revealed that Zn and Cd played a role in defence against a herbivore (snail) in this species in the absence of fertilisation, the plants with the highest concentrations of these elements being the least consumed. We found no evidence of the existence of a trade-off between Zn and Cd concentrations and GLS. The involvement of GLS in the defence against herbivory could therefore not be established. One of the reasons for this could be that we were working with a metallicolous population. In order to confirm this hypothesis, we would need to make a comparison between metallicolous and non-metallicolous populations. Finally, we cannot rule out the hypothesis that there was in fact an induction peak, but it was not identified in our experimental conditions. We have also demonstrated the risk of Cd dispersing in the environment if the plants are consumed by herbivores. This potential transfer will need to be controlled if *A. halleri* is used for *in situ* phytoextraction.

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Supplemental data

*Table 1. Effect of soil type (growth for 4 weeks on contaminated versus uncontaminated [compost] soil) and NPKS fertilisation on Trace Elements (Cd. Zn), S and glucosinolate (6MTH. 7MTH. 6MSOH. 7MSOH. 8MTO. 8MSOO. 4MOI3. I3M) concentrations in A. halleri. (two-way ANOVA with fixed factors; n=5 for each soil type * fertilisation combination). Significant differences (P < 0.05) are highlighted in bold.*

		Cd		Zn		S		6MTH		7MTH		6MSOH		8MTO		4MOI3		7MSOH		8MSOO		I3M	
	df	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Soil type		0.37	0.55	0.04	0.83	**4.93**	**0.04**	2.15	0.16	1.61	0.22	1.75	0.21	1.75	0.20	0.02	0.87	0.03	0.85	0.22	0.63	0.05	0.82
Fertilization		0.08	0.77	0.19	0.66	3.84	0.06	1.55	0.23	1.05	0.31	1.45	0.24	0.21	0.64	1.29	0.27	1.19	0.29	0.28	0.60	0.05	0.81
Soil type * Fertilization		0.99	0.33	1.4	0.24	0.03	0.86	0.43	0.51	0.49	0.49	3.65	0.07	0.004	0.95	1.97	0.17	2.13	0.16	2.21	0.15	3.24	0.08

Table 2. Effect of herbivore damage on foliar concentrations of several glucosinolates (6MTH, 7MTH, 6MSOH, 7MSOH, 8MTO, 8MSOO, 4MOI3, I3M; see text for full names of molecules) in *A. halleri* as a function of fertilisation (with or without NPKS fertilisation) or time (after 4, 5 or 6 weeks of growth on a polluted soil) (n=5). Data were ln-transformed and analysed by a Friedman ANOVA for dependent data collected on the same individual before *vs* after herbivory. No significant differences were found ($p < 0.05$).

Before/after herbivory		**6MTH**		**7MTH**		**6MSOH**		**7MSOH**		**8MTO**		**8MSOO**		**4MOI3**		**I3M**	
	df	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P
With NPK+S	1	0.6	0.43	0.6	0.43	0.6	0.43	0.06	0.79	0.06	0.79	0.06	0.79	0.06	0.79	3.26	0.07
Without NPK+S	1	0.06	0.79	0.06	0.79	0.06	0.79	0.6	0.43	0.06	0.79	0.06	0.79	0.06	0.79	0.06	0.79
Before/after herbivory		**6MTH**		**7MTH**		**6MSOH**		**7MSOH**		**8MTO**		**8MSOO**		**4MOI3**		**I3M**	
	df	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P	Chi Sqr.	P
4 Weeks	1	0.4	0.52	0.4	0.52	0	1	0.4	0.52	0.4	0.52	0	1	0.4	0.52	0	1
5 Weeks	1	0.4	0.52	0.4	0.52	0.4	0.52	0.4	0.52	0.4	0.52	0	1	0.4	0.52	0	1
6 Weeks	1	0.4	0.52	0.4	0.52	0.4	0.52	0.4	0.52	0.4	0.52	0.4	0.52	0	1	0	1

Table 3. Accumulation quotient (AQ) and sum of the excess of transfer in snail viscera for each condition. Abnormal transfer (AQ>1) is indicated in red

Weeks	Fertilizer	AQ							SET
		Cd	Cu	Fe	Ni	S	Zn	Pb	
4	without	1.26	0.85	0.99	1.05	0.94	1.80	1.03	1.13
5	without	1.12	1.28	1.48	1.26	0.92	2.59	1.04	2.76
6	without	1.84	1.09	1.51	1.21	0.96	2.71	1.25	3.61
4	with	2.60	2.57	4.22	2.19	0.99	2.67	2.73	10.98
5	with	1.28	1.49	1.28	1.33	1.06	1.42	1.15	2.01
6	with	0.89	1.25	1.03	1.22	0.93	1.03	1.01	0.54

Chapitre 6 : Options de valorisation de la biomasse (*A. halleri* et *S. viminalis*) produite sur un site phytomanagé

Ce chapitre est présenté sous forme d'article et sera prochainement soumis dans un journal scientifique à comité de lecture.

Nous avons dans les précédents chapitres étudié plusieurs options pour augmenter la biomasse d'*A. halleri* et de *S. viminalis*. L'un des enjeux majeurs pour permettre le déploiement de la phytoextraction est de trouver des débouchés pour la biomasse produite sur sol pollué. Au contraire de la phytostabilisation, la biomasse est enrichie en métaux ce qui peut être un atout ou une limite à sa valorisation.

En 2010, le nombre de sites gérés par les phytotechnologies en France était de 0,3% (Ernst and Young 2012). Plusieurs voies de valorisation sont évoquées pour la biomasse issue des phytotechnologies. Les arbres à croissance rapide de la famille des salicacées sont souvent implantés en TTCR pour une production de bois à usage énergétique. Quelques études ont démontré que le bois de salicacée issu des phytotechnologies pouvait être utilisé en combustion (Chalot et al. 2012; Delplanque et al. 2013) ou en pyrolyse (Lievens et al. 2008b, a; Bert et al. 2017) à condition d'utiliser des filtres spéciaux et de surveiller les concentrations en Cd et, dans une moindre mesure, en Zn tout au long du process. Une étude a démontré que le Zn contenu dans les feuilles de saule pouvaient être utilisées pour produire des écocatalyseurs de Zn (Deyris et al. 2018). Cette nouvelle voie de valorisation pourrait permettre un double usage des saules (feuilles + bois). À notre connaissance, une seule étude s'est intéressée à la valorisation des adventices présentes sur un site phytomanagé (Jeannin et al. 2020) alors que ces plantes peuvent représenter une biomasse importante.

Dans notre étude, 101 arbres présents sur le site ont été coupés en février 2018. Des échantillons ont été prélevés à différentes hauteurs sur 30 arbres afin de caractériser la distribution du Zn et du Cd le long du tronc. Un inventaire floristique a été réalisé en mars 2019 ce qui a permis d'identifier 13 espèces végétales sur lesquelles des mesures de Zn et de Cd ont été réalisées. Ces concentrations ont ensuite été comparées aux concentrations retrouvées dans la législation française pour chaque voie de valorisation identifiée (combustion, pyrolyse et paillage horticole pour le bois ; compostage et méthanisation pour les adventices).

Au contraire du Cd, le zinc présentait une distribution verticale dans le bois de saule, les concentrations les plus importantes étant retrouvées dans les parties terminales du tronc. La concentration en Cd dans le bois était largement inférieure à la concentration d'entrée pour les combustibles dans certaines chaudières alors que celle du Zn était supérieure (250 vs 200 mg.kg-1). En vertu du principe de non dilution, les résultats suggèrent que la combustion n'est pas une option pour valoriser le bois de saule issu du site. Une autre voie de valorisation envisagée était le paillage. Cependant les résultats ont montré que les concentrations en Zn et en Cd étaient supérieurs à la concentration d'entrée en paillage (150 et 0.5 $mg.kg^{-1}$ pour le Zn et le Cd respectivement). Cette option n'était pas non plus une option pour valoriser le bois. Les adventices présentes sur le site ont des concentrations en Zn et en Cd comprises dans la gamme de valeurs ordinairement retrouvées pour des plantes poussant sur milieu non pollué. Aucune gestion particulière n'est donc nécessaire pour ces plantes qui peuvent donc intégrer les filières courantes des déchets verts (compostage et méthanisation).

Title :Valorization options of the biomass produced on a TE phytomanaged site: what to do with *Salix viminalis* and plant colonizers?

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Introduction

The economic valorization of biomass is a major stake in the deployment of phytomanagement. In fact, without a viable and perennial recycling process for the biomass produced, phytoextraction is not a technique considered by the managers of polluted sites and soils as a relevant alternative. From an economic point of view, plant technologies are often considered less expensive than conventional methods (Conesa et al., 2012; Cundy et al., 2016). The use of phytotechnologies is often evoked during rehabilitation operations of industrial or urban site, but rarely implemented due to a lack of feedback. In 2010, only 0.3% of land volumes were treated by phytotechnologies in France according to Ernst and Young (2012).

For site dedicated to woody biomass production, fast-growing trees (e.g. willows and poplars) are generally planted in short rotation coppice (SRC) or verry short rotation coppice (VSRC) (Bert et al., 2012a; Ciadamidaro et al., 2017; Kidd et al., 2015; Phanthavongsa et al., 2017). *Salix spp*. (Salicaceae) are well-known fast-growing trees with Zn- and Cd-accumulating behavior and consequently are studied for their phytoextraction potential (Van Slycken et al. 2013; Delplanque et al. 2013; Kidd et al. 2015). To date, several studies have been conducted under real conditions assessing the time needed to reduce the TE soil pollution to acceptable regulated values (Kidd et al. 2015). Although generally developing well on metal-contaminated soils, metal extraction and biomass yield greatly depend on *Salix* clones (Maxted et al. 2007). The common practice for *Salix* cultivation is the SRC which allows wood production for around 24 years. The first harvest of willows generally occurs after 3 or 4 years then every 3 years. Calculations performed hypothesizing the collection of leaves, stems, or both, during the time frame

of the plantation, pointed out the remediating potential of willows as well as their potential for bioenergy (Delplanque et al. 2013).

For several years the use of Salicaceae wood from phytomanagment has been studied for energy purposes. Different techniques have been used such as combustion (Chalot et al. 2012; Delplanque et al. 2013) and pyrolysis (Bert al. 2017; Lievens et al. 2008a,b). Also, willow wood could be used for mulching. Willow leaves from phytoextraction can be used as an ecocatalyst (Deyris et al. 2018). To our knowledge, only one study considers the fate of colonizing plants on phytomanaged sites (Jeanin et al. 2020). However, these plants can represent an important source of biomass and therefore call for management. Colonizing plants can be used as a source of plant fiber for the textile industry such as *Urtica dioica* (Jeanin et al. 2020). These colonizing plants could also be used in composting and anaerobic digestion if trace element (TE) concentrations comply with the legislation. Colonizing plants may compete with plants used in the phytoextraction project for resources and decrease their biomass and TE extraction. Moreover, among these plants, accumulators could be found. Management of these plants could thus be set up.

The objectives of this *in situ* study were to evaluate the possibility of using willow wood in an energy chain (combustion or pyrolysis) or mulching and to determine the best way of valorization for the colonizing species. To estimate the potential for willow wood fuel use, TE concentration measurements were carried out in the wood as well as height and diameter measurements. After a floristic inventory, measurements of TE concentrations were made on each species found to determine the relevant valorization option for the colonizing species and. The TE contents of willow woods and colonizing plants were compared to the French regulatory values for each valorization option considered (combustion; pyrolysis; mulching; composting and anaerobic digestion). Zn ecocatalyst production was also considered. This study was a part of the Extra-Zn project set up in 2013 in an urban area contaminated by Zn and Cd due to diverse past industrial activities.

Material and methods

Site description

Field trials were conducted in the urban area of Montataire (Oise, France, 49°15’12’’N, 2°26’48’’E). The overall physicochemical characteristics of the site have been provided previously (Grignet et al. 2020). Briefly, the topsoil was contaminated mostly by Zn (616 mg kg^{-1}) and Cd (1.7 mg kg^{-1}) and presented an alkaline pH (pH > 8).

Willow harvest and weight and Cd and Zn concentrations in woods

In February 2018, the end of the first cycle of the very short rotation coppice (VSRC) of willows was reached. A VSRC is generally cut every 4 years (AILE, 2007). One third of the willow plantation was cut, i.e. 101 trees, in order to measure the TE concentrations in wood and to verify their possible valorization in wood energy (combustion, pyrolysis). The number of trees harvested was decided in cooperation with the green space services of Montataire in order to preserve the landscaping objective of the Extra-Zn project. Over the 101 trees, 30 trees were annually monitored since 2015 (morphometric parameters and TE foliar concentrations) (Grignet et al. 2020) (Fig. 1). After the harvest, the 30 trees belonging to the monitoring were measured (height and diameter at 1.3m). On these trees, sections of wood ranging from 5 cm to 10 cm and twigs were sampled to cover three different heights (from the bottom to the top of the tree: 1m50; 3m and terminal parts, respectively) to see the distribution of TE concentrations along trees (Fig. 2A).

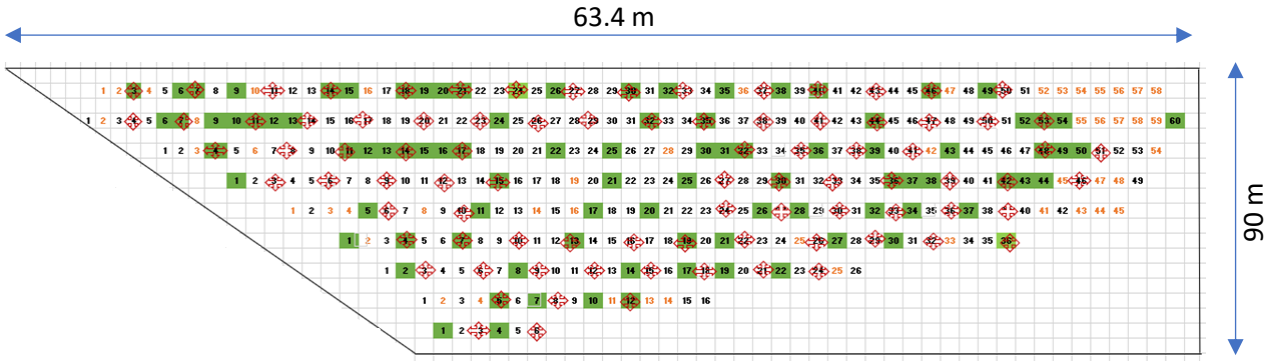

Figure 1 Cutting plan of the willow. The red cross indicates the cut trees (n=101). The willows which showed both green and red colors were monitored in this study (n=30).

The section samples were then washed with tap water, then with demineralized water, dried in an oven (40° C, until constant weight) and finally crushed to obtain a sample of about 2mm (RETSCH SM2000). The rest of the monitored trees were crushed separately on site and packed in bags by the green services of Montataire (Fig. 2B), leading to 30 bags. After hand mixing, one representative sample of crushed wood was taken from each monitored bag. The non-monitored trees were also crushed on site and stored as a mixture in 6 big bags of which two samples of crushed wood from each mixture bag were taken. The average Cd and Zn concentrations were further measured on all the crushed wood samples (n=42). After transportation to Ineris, each section of wood and bags of wood from the monitoring were weighed using a field weight scale in the laboratory. Each big bag was transported to Ineris and weighed with a lift truck. From the average biomass measured on the 101 trees cut, an extrapolation per

hectare was made. TE mineralomass for each tree in the follow-up was determined by calculating the average concentration * biomass.

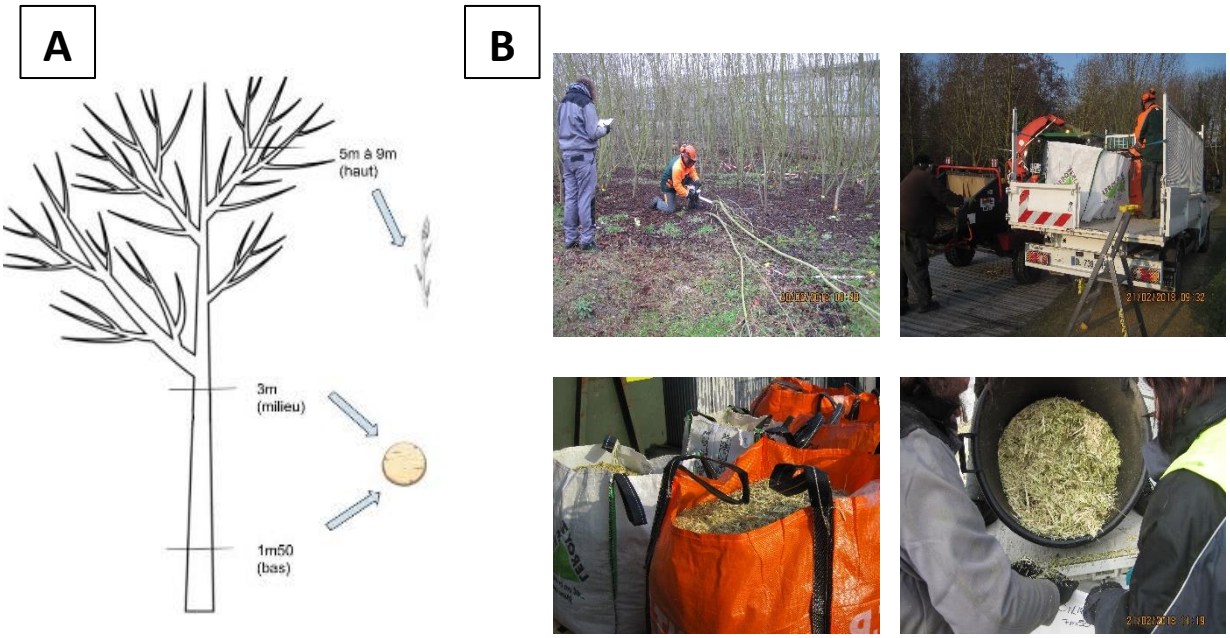

Figure 2 Harvest of willows in March 2018; A) schema of a willow tree with the cutting of the different sections. B) photo of the cutting and crushing of the willows.

In June 2018 and 2019, the stump recovery rate of the trees was calculated by counting the number of regrowths over the number of trees cut.

For correlation calculations between wood and willow leaves, the concentrations in willow leaves used are those harvested in June 2017 and reported in Grignet et al. (2020).

Floristic inventory and plant colonizer sampling and preparation

A floristic inventory was carried out in March 2019 on the willow part. 3 to 5 individuals per species were collected in order to identify them and measure the concentrations of Cd and Zn in their aerial parts. The identification was carried out with the help of a reference book for plant taxonomy (Streeter, 2011). After their identification the plants were washed with tap and demineralized water then dried in an oven (40° C, until constant weight), crushed (2 mm) and preserved until the TE concentration was measured.

TE analysis in willow wood and aerial parts of plant colonizers

Zn and Cd were analyzed in wood and plant samples (0.5 g) after digestion at 180 °C for 20 min in 10 mL of nitric acid (67% AnalaR Normapur) and 3 mL of ultra-pure water using a microwave digester (Mars Xpress CEM) by ICP-OES (Agilent 5100) or ICP-MS (Agilent 7500). One standard reference material was used for analytical quality control (cabbage "NCS ZC 73012", NACIS).

Statistical analyses

Data were analyzed using ANOVA and post hoc comparisons with HSD tests for metal concentrations for willows and Kruskall-Wallis test and pairwise comparison for metal concentration for colonizing plant. All statistics were performed with R statistical software 3.2.2 (R Core team).

Results and discussion

Concentration of Zn and Cd in willow wood

The stump recovery rate of the trees calculated in June 2018 was 80% (Table 1) and approximated the one reported on unpolluted soil. Indeed, the stump recovery rate after the first rotation of a *Salix sp.* VSRC was estimated at 90% (AILE, 2017). In June 2019, the stump recovery rate dropped to 31% (Table 1) and showed heterogeneity. The cut trees on the site border showed a higher stump recovery than those inside the site (60% versus 10%, respectively). This large difference could be explained by a significant competition for the light within willows. These results showed that the harvesting plan was not appropriate for reaching the objectives of the study, i. e. biomass production and landscaping. To our best knowledge, there are no data in the literature on the stump recovery rate of such a cutting design plan. Indeed, VSRC of willows is entirely cut in a single cut for the biomass valorization whereas the landscape aspect is not considered. It is necessary to carry out a consultation with the different actors of the project to know if the landscape aspect is more important than the biomass valorization aspect, to be able to re-evaluate the cutting plan. An estimate of the biomass produced per hectare was also made from the biomass produced on the cut samples. The total estimated biomass was 58T/ha, which is equivalent to unpolluted on-site production (AILE, 2017) which suggested that willows used in this study were not impacted by the TE pollution and were adapted to the site conditions. This result also suggested that willows could be produced on TE polluted sites without biomass yield decrease and valorized in different sectors.

Table 1: Stump recovery rate on polluted site in 2018 and 2019 (n= 30)

Years	Stump recovery rate
2018	80%
2019	31%

Analyses carried out on the 101 willow wood samples sectioned at different sections showed significantly higher concentrations (p-value < 0.05) of Zn and Cd in the terminal parts (Figure 3). Zn concentrations showed a stepped pattern along the trunk, the parts that accumulate the most being the terminal ones (Figure 3). The gradation of Zn concentrations in the wood was observed in several studies (Laureysens et al. 2004;2005; Maxted et al. 2007) and is thought to be due to a gradient effect of the xylem. TE circulates in the xylem vessels from the roots to their storage sites (leaves) (Laureysens et al. 2005). A decrease in diameter and dry weight from the bottom of the trunks to the terminal parts was observed (Table 2). On the contrary, Zn and Cd concentrations in the sections increased from the bottom to the terminal parts. This may be due to a dilution or concentration effect of the metals in the bottom and terminal parts, respectively. To our knowledge, the distribution of metals along the willow trunk, from its bottom to its top was never reported. Nevertheless, several studies have shown that the part that accumulates most in willow is the bark (Laureysens et al. 2004; Maxted et al. 2007, Bissonnette et al. 2010). Zn and Cd concentrations can be three to six times higher in bark than in wood (Laureysens et al. 2004).

Table 2 Mean diameter (cm) and Zn and Cd concentrations (µg/g) as well as the correlation coefficient between diameter and concentrations. Significant differences are represented by letters at the threshold α<0.05; n=34

trunk section	*Average diameter (cm)*	*Zn concentration (µg/g)*	correlation coefficient	*Cd concentration (µg/g)*	correlation coefficient
Bottom	*45.5 (a)*	*146.8 ± 53.6 (a)*	-0,70 (*p-value<0,01*)	*0.89 ± 0.19 (a)*	-0,42 (*p-value<0,01*)
Middle	*31.6 (b)*	*162.3 ± 60.3 (a)*		*0.90 ± 0.22 (a)*	
Top	*5.7 (c)*	*439.6 ± 169.1 (b)*		*1.24 ± 0.32 (b)*	

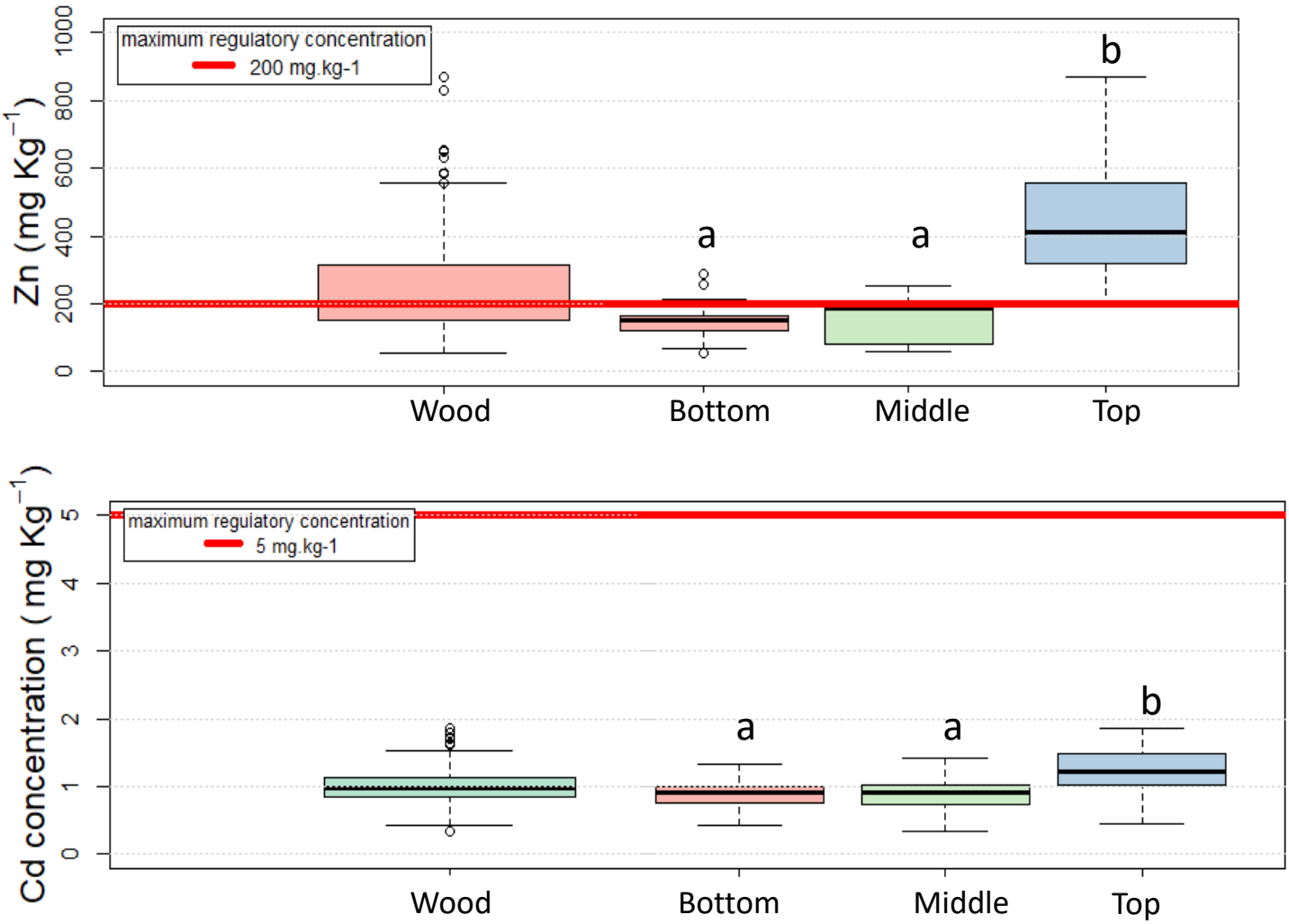

Figure 3 :Zn and Cd concentrations measured (mg.Kg-1 DW) in each section of the wood of the trees with respect to the concentrations allowed for wood energy use in some boilers (French Decree of September 24, 2013, article 8) (red line). Significant differences are represented by letters at the threshold $\alpha<0.05$; n=101.

The average TE concentrations of willow wood harvested in the VSRC were compared with French TE limit values for fuel entry in registration combustion plants (French Decree of August, 3, 2018, article 8). The average concentration of Cd in willow wood was 1 $mg.kg^{-1}$ which was well below the maximum regulatory concentration of 5 $mg.kg^{-1}$ for this element. The Zn average concentration in willow wood was 240 $mg.kg^{-1}$. This concentration is slightly higher than the maximum Zn regulatory concentration of 200 $mg.kg^{-1}$. On unpolluted soil, the average concentration of Zn and Cd in willow wood ranges between 25.2 to 161 $mg.kg^{-1}$ and 0.5 to 4.5 $mg.kg^{-1}$, respectively (Adegbidi et al. 2001, Adler et al. 2005, Labrecque & Teodorescu 2003, Maxted et al.200).The Zn concentration is higher than the average found on unpolluted sites confirming the accumulating behaviour of the willow for this element. The Cd concentration is in the average found on unpolluted sites. The use of willow wood resulting from

phytoextraction in the wood energy sector has been studied to our knowledge in only one study (Delplanque et al. 2013) where Zn and Cd concentrations in willows were respectively 7.3 $mg.kg^{-1}$ of Cd and 285 $mg.kg^{-1}$ of Zn. In our study, Zn could be the limiting element for combustion, as it exceeds the maximum Zn regulatory concentration of 200 $mg.kg^{-1}$. As the values observed in our case study are relatively close to the physiological values, Zn is therefore a parameter to be monitored to ensure the feasibility of this valorization option.

To overcome this constraint, another option could be to consider debarking trees to decrease Zn and Cd concentrations as performed for eucalyptus cultivated in VRC and VSRC (Meulun and NGuyen, 2012). Nevertheless, this is not a common practice in the valorization of willow cultivated in VSRC because this will lead to a decrease in biomass yield. In addition, in our field conditions, it could be difficult to access to the site with the machines used in silviculture such as a debarked one.

More specifically, on the 30 monitored trees cut in March 2018, we looked at the relationships between the average Zn and Cd concentrations and the average diameter of the trees, on one hand, and, on the other hand, the quantities of metals exported. We observed a negative linear correlation (-0.48, Pvalue = 0.005) (Figure 4) between the average Zn concentration and the average diameter of trees. The larger the diameter of the trees, the lower the Zn concentrations. Concerning Cd, no correlation is observed. The average Cd concentrations are identical whatever the average diameter of the tree (Figure 4). We also observe a positive correlation between the mineralomass in Zn and Cd with the diameter of our trees (respectively R^2=0.66, Pvalue<0.01 for Zn and R^2=0.80, Pvalue<0.01 for Cd) (Figure 4). The larger the diameter of the truncks, the greater the quantity of metal exported. We have compared our data with the threshold values for use in combustion (French Decree of August, 3, 2018, article 8). For Cd, whatever the diameter of willows, the average concentration is lower than the threshold value. For Zn, we can recommend using trees with an average diameter > 30 mm in order to comply with the combustion regulation. If the cultural itinerary allows to get a majority of willows with this diameter, debarking could not be needed. Cutting trees with a diameter >30 mm thus makes it possible to maximize the partial depollution of the site (higher mineralomass) but also to be able to valorize the biomass. Indeed, in this way, the average concentration in the wood (190 rather than 240 $mg.kg^{-1}$) might be below the threshold value for entry in combustion

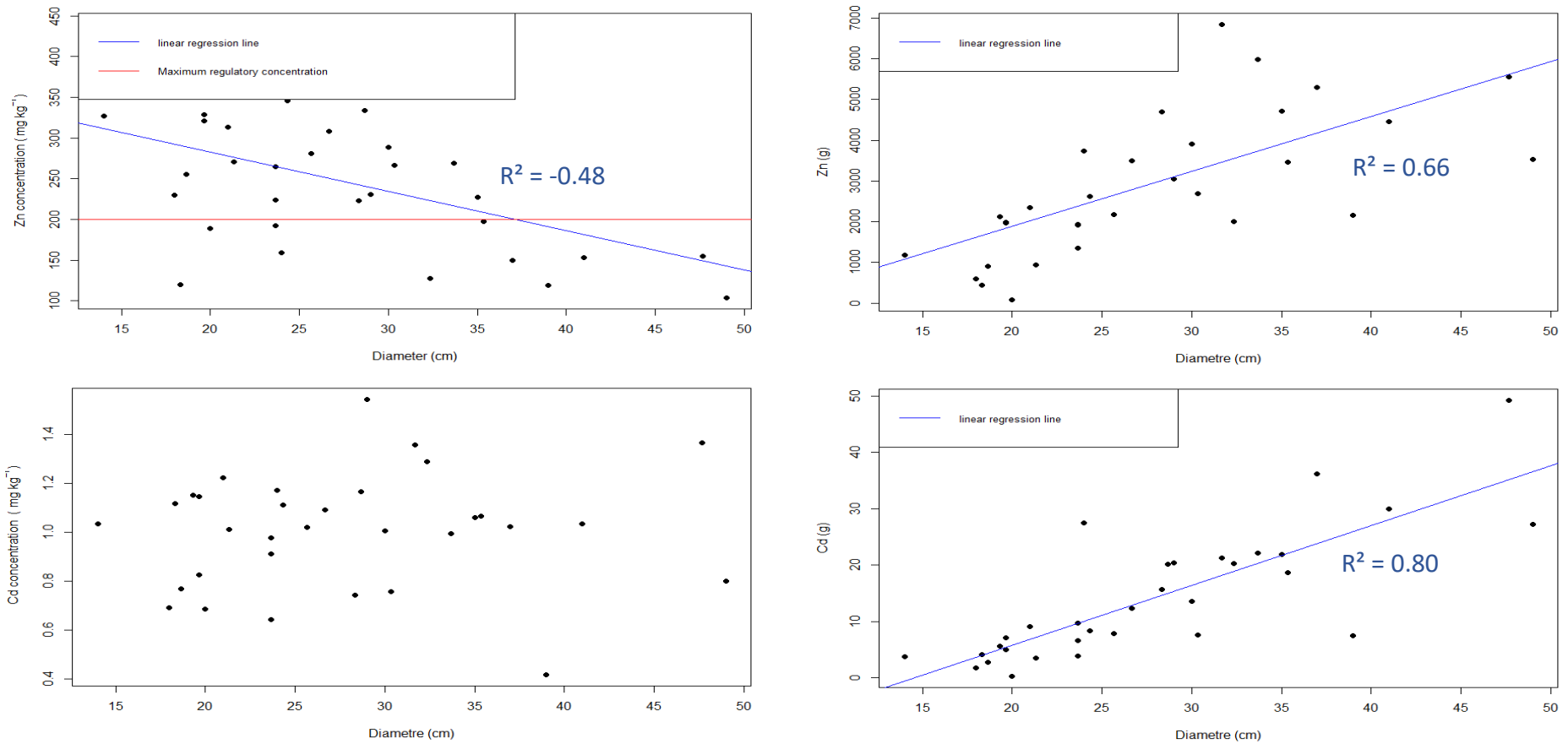

Figure 4 Correlation between Zn concentration, Zn mineralomass, Cd concentration, Cd mineralomass and average willow diameter. The linear correlation lines and the associated correlation coefficient are displayed in blue, the maximum Zn concentration allowed for wood energy use in some boilers is displayed by the red line (French Decree of September 24, 2013, article 8).

Other ways of valorization could be envisaged, such as the use of wood in mulching or pyrolysis. Several studies have shown that the pyrolysis of biomass from phytotechnology is possible if the concentrations of TE and especially Cd in gases and in bio-oil and biochar are monitored (Bert et al. 2017, Lieven et al 2008a, b). Concerning mulching, the return to the ground of TE in the biomass must not exceed the values of the French standard NF U52-001 (Février 2005). Zn and Cd concentrations in the biomass should not exceed 150 and 0.5 $mg.kg^{-1}$ respectively. The concentration of Zn and Cd in willows being higher than these limit concentrations, it is therefore not possible to use willow wood from phytoextraction in mulch.

Correlations between the concentration in the wood harvested in March 2018 and the concentration in the leaves in June 2017 were carried out. A positive correlation between wood concentration and leaf concentration was found for Zn (R^2= 0.50, Pvalue= 0.003), while no correlation for Cd was demonstrated (R^2= 0.006, Pvalue= 0.97). The higher the Zn concentrations in the wood, the higher the concentrations in the leaves (the average concentration in the leaves was 590 ± 200 $mg.kg^{-1}$). Trees with smaller diameters have higher wood and leaf Zn concentrations. However, no direct relationship between diameter and leaf concentrations is observed. For an optimal valorization of willow in phytomanagement, trees with a diameter greater than 30 mm should be cut down in winter in order to valorize the wood biomass in combustion. In addition, trees with diameters less than 30 mm in spring showed leaf concentrations relevant for ecocatalysis (Deyris et al 2018). These results suggest two ways of recovery: one for the leaves in ecocatalysis harvested each autumn and one for the wood when the majority of the trees reached an average diameter of 30 mm for use in combustion. These two ways of valorization would make it possible to maximize the valorization of the biomass and the land while controlling the risks.

Floristic inventory

Twelve species were identified (Table 3). We can observe that the species encountered are mainly characteristic of open, rich and basic environments. 70% of the plants found are perennial plants (Table3).

Table 3 Species inventoried in March 2019 with associated life cycle (A: annual, P: perennial, B: biennial),environment and TE behavior.

Family	Species	Life cycle	Environment	TE behaviour
Euphorbiaceae	*Mercurialis annua*	A	open, rich and basic pH	Not known
Asteraceae	*Senecio vulgaris*	A	open, rich and basic pH	Indicator [1]
Lamiaceae	*Lamium purpureum*	A	open, rich and basic pH	Not known
Rubiaceae	*Galium aparine*	A	shaded, rich, neutral pH	Cd accumulator [2]
Papaveraceae	*Chelidonium majus*	P	open, rich and basic pH	Not known
Asteraceae	*Taraxacum officinale*	P	open, balanced	Indicator [3]
Lamiaceae	*Glechoma hederacea*	P	undergrowth, rich, pH neutral to basic	Not known
Urticaceae	*Urtica dioica*	P	open and undergrowth, rich, neutral to basic	Tolerant [4]
Rosaceae	*Rubus fruticosus*	P	woodland clearings, roadsides, poor, acid pH	Tolerant [5]
Rosaceae	*Potentilla reptans*	P	ditches, balanced, basic pH	Not known
Apiaceae	*Pastinica sativa*	B	open, rich and basic pH	Tolerant [6]
Poaceae	*Anthoxanthum odoratum* *Bromus erectus*	P	undergrowth, balanced, neutral pH open, poor, basic pH	Tolerant [7]

[1] Salinitro et al. 2019 ; [2] Liao et al. 2015;[3] Gómez-Arroyo et al. 2018 ; [4] Jeanin et al. 2020 ; [5] Lassalle et al. 2021 ; [6] Ciura et al. 2004 ; [7] Qureshi et al. 1985

Zn and Cd concentrations were also measured (Figure 5). Cd concentrations (Figure 5) are all lower than the concentrations ordinary found in plants growing on uncontaminated soils (2 $mg.kg^{-1}$ DW) (kabata-Pendias, 2010). Zn concentrations (Figure 5) are all lower than physiological concentrations (150 mg.kg-1) except in one species, *Mercurialis* (male and female) and in one family, *Poaceae*, which are slightly higher than physiological concentrations. These values are within the ranges of excessive or toxic concentrations (100-400 mg.kg-1) (Kabadias-Pendias, 2010), but individuals do not show signs of toxicity. These results may reflect the tolerance of plant colonizers to high concentrations of pollutants at this site and probably an ability to exclude contaminants. In addition, half of the colonizing plants found on the site are plants known to be metal tolerant and found on polluted sites (Salinitro et al. 2019 ; Liao et al. 2015; Jeanin et al. 2020 ; Lassalle et al. 2021 ; Ciura et al. 2004 ;

Qureshi et al. 1985). Bioconcentration factors (BCF), calculated as the ratio of shoots element concentration in the plant to the total element concentration in the soil (Mench et al., 2010), were all below 0.3, confirming that the plant limited Cd and Zn transfers to the aboveground parts.

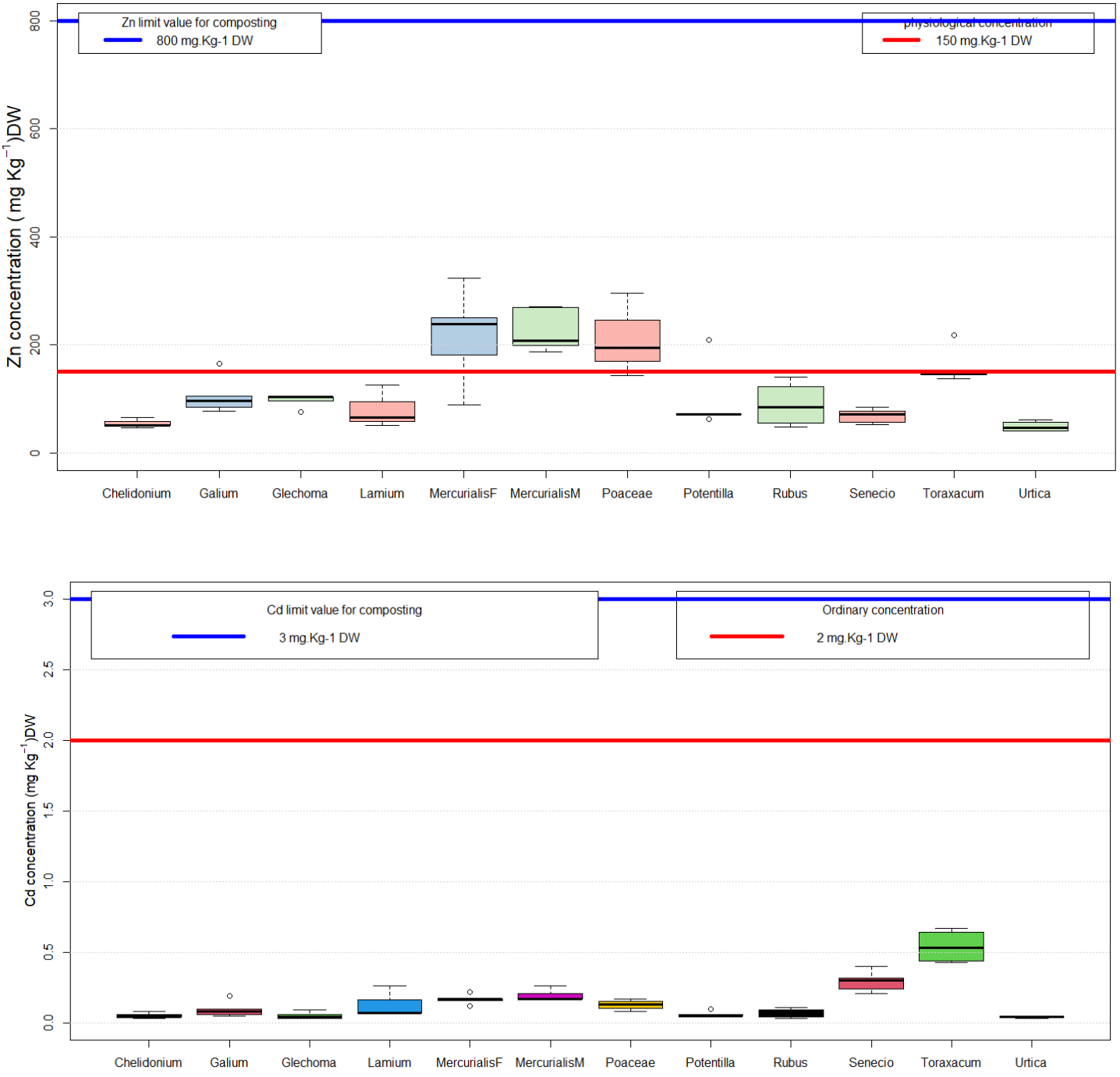

Figure 5 : Concentrations of Zn (low) and Cd (high) measured (mg.kg-1) in colonizers with respect to physiological concentrations for Zn and ordinaryconcentrations for Cd (red line) and at the threshold values of the composting standard (standard NF U44-051/A3, 2019) (blue line), (n=3 to 5 per species).

During composting a loss of about 50 to 60% of OM is observed (Alburquerque et al; 2009). To account for the loss of OM during the composting process, the concentrations measured in the colonizing plants were multiplied by 2 to be compared with the limit values applicable to composts (standard NF U44-051/A3, 2019). Cd and Zn concentrations in the plant colonizers after the application of the factor 2 ranged between 96 and 454 $mg.kg^{-1}$ for Zn and 0.1 and 1.08 $mg.kg^{-1}$ for Cd. These concentrations are well below the limit values for these elements (3 and 800 $mg.kg^{-1}$ DW for Cd and Zn, respectively). Limit values for metals may change in the next version of the French Standard NF U 44-051, which is currently under revision. They could become close to those of the European Regulation EU 2019/1009 for which Cd and Zn concentrations in organic fertilizers and soil improvers must not exceed, respectively, 1,5 and 800 $mg.kg^{-1}$ DM. Plant colonizers are therefore not subject to any particular management, and the harvested biomass can be used in compost by the city's green space services. Colonizing plants can also be valorized in anaerobic digestion. Indeed, according to the French legislation (Arrêté du 22 octobre 2020), the vegetable waste resulting from the maintenance of gardens and green spaces (mowing, pruning, leaves) can enter in methanization. However, digestates should comply with the DigAgri 3 specifications (Arrêté du 8 août 2019) in order to be valorized (Zn and Cd values should not exceed 1000 and 1.5 $mg.kg^{-1}$ MS, respectively).

Conclusions

In the present study, we reported the results of Zn and Cd concentrations in willow wood and colonizing plants by comparing them with the limit values provided by the French legislation for the different valorization options. Zn concentrations in willow wood showed a gradation. The highest concentrations were found in the terminal parts. To comply with the current French legislation on combustion, it could be advised to use trunks with a diameter greater than 30mm. Based on Zn and Cd concentrations, no particular management of colonizing plants is required. Thus, a valorization in compost or in anaerobic digestion can be envisaged.

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DISCUSSION GENERALE

/CONCLUSIONS ET PERSPECTIVES

Discussion Générale

Pour réhabiliter les sols pollués et valoriser économiquement le foncier, le phytomanagement et en particulier la phytoextraction apparaît comme une stratégie pertinente pour certains sites pollués (Robinson et al. 2006; Mench et al. 2010; Bert V et al. 2012; Chalot et al. 2012; Kidd et al. 2015). A ce jour, la plupart des travaux de phytoextraction ont été réalisés en pot ou en petite parcelle sur du court terme (Kidd et al. 2015). Depuis quelques années et suite au développement d'options de valorisation de la biomasse produite sur sols pollués, notamment via la voie de la chimie verte, les études sur les (hyper)accumulateurs en conditions de terrain commencent à se développer (Jacobs et al. 2017, 2018b; Grignet et al. 2020). Mes travaux de thèse s'inscrivent dans ce cadre et contribuent à alimenter les connaissances liées au déploiement de la phytoextraction *in situ.* Ils ont permis, en particulier, de mieux comprendre les mécanismes de tolérance et d'accumulation du Zn et du Cd chez deux espèces (hyper)accumulatrices de ces métaux à travers l'étude (i) des transferts sol- plantes- herbivores, (ii) des risques environnementaux potentiels associés et (iii) de l'impact sur le fonctionnement biologique du sol. Ils ont également contribué à l'identification des conditions optimales pour la valorisation des biomasses produites par l'étude des effets de plusieurs pratiques culturales.

I) Potentiel de phytoextraction de *Salix viminalis* et d'*Arabidopsis halleri*

Un des verrous à lever pour optimiser la phytoextraction du Zn et du Cd par les (hyper)accumulateurs comme *A. halleri* et *S. viminalis* est l'augmentation de la biomasse et des concentrations foliaires en Zn et en Cd.

Mes travaux de thèse ont permis de démontrer que l'utilisation de *S. viminalis* dans un contexte de phytoextraction en milieu urbain était une option pertinente (chapitres 2 et 4, publications 1 et 3). En effet, *S. viminalis* présente une croissance en hauteur et en diamètre sur le sol pollué étudié meilleure que celle rapportée dans (Mleczek et al. 2010) après 3 ans de culture (2015, 2016 et 2017) et une croissance identique à celle de saule sur sol non pollué (Aile 2007). La

croissance des saules stagne les trois dernières années du suivi (2018, 2019 et 2020). Ceci est sans doute dû au mode de plantation choisi, le TTCR. En effet, dans les TTCR de saule sur sol non pollué, la hauteur des saules ne dépasse pas les 9 mètres après 4 ans de culture (Aile 2007). Or dans nos conditions expérimentales, les saules atteignent en moyenne 9.5m de hauteur en 2020. Sur la durée de l'étude (6 ans), les concentrations en Zn et en Cd dans les feuilles de saule sont en diminution, probablement en lien avec l'augmentation des paramètres de croissance, ce qui pourrait révéler un effet de dilution déjà décrit dans d'autres études (Hammer et al. 2003; Wieshammer et al. 2007). Les concentrations en Zn et Cd restent tout de même 6 et 7 fois supérieures à la concentration moyenne retrouvée pour des plantes poussant sur milieu non pollué (Kabata-Pendias 2010) et ce malgré un pH non-optimal (> 8) pour la phytoextraction. Les feuilles de saule présentent des concentrations en Zn de 410 ± 120 $mg.kg^{-1}$ très intéressantes pour une utilisation dans une filière combinant phytoextraction et valorisation de la biomasse enrichie en Zn en catalyseur pour la chimie verte (Deyris et al. 2018). Bien que prometteurs par rapport à des catalyseurs traditionnels, issus des mines, grâce à leur plus grandes efficacité et sélectivité et capacité de réutilisation, ces nouveaux écocalyseurs ne sont pour le moment étudiés qu'à l'échelle du laboratoire (Grison 2015). Un changement d'échelle est nécessaire afin de valider leur utilisation dans divers secteurs de la chimie verte.

Bien qu'*A. halleri* soit un hyperaccumulateur de Zn et de Cd reconnu, cette espèce, contrairement à *N. caerulescens* n'a bénéficié que de peu d'études pour la qualifier en phytoextraction de ces 2 métaux. Seules trois études menées *in situ* en parcelles ont été rapportées dans la littérature (Baker et al. 1994; McGrath et al. 2006; Tlustoš et al. 2016). Celles-ci ont classé l'arabette de Haller comme moins intéressante pour la phytoextraction comparée à *N. caerulescens*. Dans ces études *A. halleri* produit moins de biomasse et accumule moins de Zn et de Cd que *N. caerulescens*. Mes travaux de thèse sur l'arabette de Haller ont démontré que malgré un pH du sol basique entraînant une faible mobilité du Zn, *A. halleri*

présentait des concentrations en Zn proches du seuil définissant l'hyperaccumulation du Zn (> 10000 mg kg^{-1}, (Baker and Brooks 1989)). Pour la première fois, les concentrations en Zn et en Cd ont été observées sur un cycle complet de cette espèce végétale, du stade rosette au stade sénescence. Alors que les concentrations en Zn ne changent pas tout au long du cycle de la plante, les concentrations en Cd sont significativement plus importantes au stade rosette et diminuent aux stades floraison et fructification, probablement pour protéger les graines (Dahmani-Muller et al. 2000). Une remobilisation du Cd est observée au stade sénescence.

Le potentiel de phytoextraction (augmentation de la biomasse et/ou des concentrations en ET foliaire) des deux espèces étudiés pourrait être augmenté avec l'utilisation de pratique agronomique comme cela a déjà été démontré dans plusieurs études sur des (hyper)accumulateurs (Wieshammer et al. 2007; Vangronsveld et al. 2009; Jiang et al. 2010; Ji et al. 2011; Kidd et al. 2015; Li et al. 2016; Jacobs et al. 2018a, b).

1) Est-ce que l'utilisation de pratiques agronomiques permet d'augmenter le potentiel de phytoextraction (augmentation de la biomasse et/ou des concentrations foliaires en ET) ?

   - L'ajout de NPK+S

Pour la première fois, chez *A. halleri*, un fertilisant NPK+S a été ajouté au stade rosette (Chapitres 2, 3 et 5, publications 1, 2 et 4). Les résultats ont montré que la concentration foliaire en Cd en présence de NPK+S a été augmenté (de 1,2 à 2 fois). Cette augmentation n'est pas due à une mobilité plus importante du Cd dans le sol. Une augmentation significative de la biomasse (de 1,7 fois) en présence du fertilisant NPK+S a également été mise en évidence. Ceci a déjà été observé chez *N. caerulescens* (Schwartz et al. 2002; Jacobs et al. 2018a) mais jamais chez *A. halleri*. De plus, dans ces études une diminution des concentrations en Zn et en Cd a été montrée, due probablement à un effet de dilution. De plus, l'export en Zn et en Cd par fauche, c'est à dire le potentiel de phytoextraction de *A. halleri,* est augmenté en présence de fertilisant. L'utilisation du fertilisant NPK+S montre un effet positif et qui persiste sur le long

terme sur la production de biomasse. En effet, dans le chapitre 3 (publication 2) le doublement de la biomasse par rapport aux parcelles sans NPK+S était observée jusqu'au stade floraison (6 mois après l'application du fertilisant).

Une très bonne production de biomasse (1.3 à 3.7 $t.ha^{-1}$) a été calculée sur la base de l'extrapolation à l'hectare de la biomasse de 5 plantes échantillonnées aléatoirement sur les parcelles de 1m² (chapitres 2 et 3 et publications 1 et 2). Ces résultats étaient largement supérieurs à l'étude de McGrath et al. (2006) (0.002 to 0.25 t ha^{-1}). Cependant, une forte variabilité individuelle dans la biomasse a été observée. Dans le chapitre 5 (publication 4), afin de réduire la variabilité entre les individus, nous avons fait les calculs de biomasse à l'hectare à partir de la récolte de l'ensemble des plantes issues des parcelles de 1m². Dans ce cas, la biomasse est proche de celle obtenue dans les études de Baker et al. (1994) et McGrath et al. (2006). *A. halleri* produit donc peu de biomasse même si celle-ci est augmentée par l'utilisation du fertilisant NPK+S.

- La fauche :

Mes travaux de thèse ont également montré que la fauche d'*A. halleri* était possible. Celle-ci permet d'augmenter les concentrations foliaires en Zn et Cd sans augmenter la biomasse. Bien que peu nombreux, les essais réalisés sur les hyperaccumulateurs montrent l'effet positif d'une double fauche sur l'augmentation de leur biomasse et des concentrations foliaires (Ji et al. 2011; Li et al. 2016). Faucher plusieurs fois *A. halleri* permet de maximiser la phytoextraction et les concentrations en ET en vue de leur valorisation en écocatalyse.

- La co-culture

La co-culture d'espèces (hyper)accumulatrices a très rarement été testée. Dans le cadre de mes travaux de thèse, *A. halleri* a été cultivée selon deux modalités : (1) entre les rangées de *S. viminalis* et (2) au pied de *S. viminalis*. La co-culture de *S. viminalis* et d'*A. halleri* en inter-

rangée est une stratégie qui peut donc permettre d'augmenter l'extraction du Zn du sol (chapitre 2, publication 1). En revanche, nos résultats montrent que la seconde modalité n'est pas favorable à la phytoextraction du Zn. Un effet de compétition pour cet élément a été mis en évidence comme cela avait été rapporté dans l'étude en pot de (Wieshammer et al. 2007).

- L'inoculation fongique : CMA et champignon endophyte

Les espèces de Brassicaceae sont réputées non mycorhizables (Demars and Boerner 1996). Pourtant, quelques travaux ont montré qu'à certaines périodes de leur cycle de vie et/ou dans certaines conditions, certaines espèces végétales appartenant à la famille des Brassicaceae [*Biscutella laevigata* (Orłowska et al. 2002) et des hyperaccumulateurs de cette famille (*Noccaea* (précédemment *Thlaspi*) *caerulescens, T. goesingense, T. calaminare, T. cepaeifolium* (Regvar et al. 2003)] pouvaient être mycorhizées (Hildebrandt et al. 2007). Ainsi, dans le cadre de mes travaux de thèse, j'ai voulu vérifier l'éventuel aptitude à la mycorhization de l'arabette de Haller dans une perspective d'augmentation de sa biomasse et/ou ses concentrations foliaires en ET. En effet, les CMA permettent d'augmenter le volume de sol prospecté par les racines en développant un réseau mycélien dense et ramifié. Ainsi, ils améliorent les nutritions hydriques et minérales de la plante hôte et par conséquent sa croissance et sa tolérance aux stress environnementaux (St-Arnaud and Vujanovic 2007; Beauregard et al. 2008). Nos résultats ont montré qu'*A. halleri* n'était pas mycorhizable par les inoculums mycorhiziens testés dans le cadre de notre étude et, ce, quelles que soient les conditions de culture (en présence ou en absence de fertilisant NPK, *in situ* ou en pot en présence d'une plante nurse) et quelles que soient les stades de développement (rosette, floraison et fructification) (Chapitre 3, publication 2). Toutefois, nous avons montré que la colonisation racinaire de *A. halleri* était possible en utilisant un champignon endophyte *P. indica.* En effet, cette colonisation s'est faite avec succès en pot.

Les saules sont capables de s'associer avec des CMA et des ECM (Aliferis et al. 2015). Cependant, le rôle des champignons mycorhiziens dans la croissance, la tolérance et l'accumulation d'ET chez les saules ont très peu été étudiés *in situ* (Lingua et al. 2008; Bissonnette et al. 2010). Plusieurs études sur l'utilisation de champignons mycorhiziens en bioaugmentation ont montré des effets contradictoires sur la croissance et/ou sur le transfert des ET. En effet, alors que certaines études montrent des effets bénéfiques pour le potentiel de phytoextaction (augmentation de la biomasse ou des concentrations en ET foliaires, et/ou une tolérance accrue aux ET)(Toler et al. 2005; Sell et al. 2005; Marques et al. 2007a), d'autres études montrent des effets inverses (diminution des concentrations en ET foliaires) (Chen et al. 2004; Mrnka et al. 2012), ou aucun effet (Lingua et al. 2008). Mes travaux de thèse ont montré que la pré-inoculation des saules par un inoculum mycorhizien commercial à base de CMA était possible. Des taux de mycorhization à hauteur de 10% ont été mis en évidence dans des conditions contrôlées. Ces résultats sont cohérents avec ce que l'on retrouve dans la littérature. En effet, les saules sont faiblement mycorhizés à l'état naturel que ce soit sur milieux pollués ou non (Van der Heijden 2001; Bissonnette et al. 2010). Le taux de mycorhization des saules était compris entre 2 et 14% dans ces deux études. L'absence d'ECM est tout à fait cohérent. En effet dans les premières années de vie des arbres, les CMA sont dominants et sont progressivement remplacés par les ECM (Van der Heijden 2001; Püttsepp et al. 2004).

Dans les sols pollués, les paramètres biologiques (activités enzymatiques, biomasse microbienne …) présentent en général des valeurs plus faibles que dans des sols non pollués. Plusieurs études ont montré un effet négatif des ET sur le fonctionnement du sol affectant la croissance, la morphologie (altération des protéines et de l'intégrité membranaire des cellules) et l'activité microbienne du sol (Leita et al. 1995; Renella et al. 2003, 2005; Liao and Xie 2007; Wang et al. 2007; Zhang et al. 2008, 2010; Epelde et al. 2009b; Papa et al. 2010). Cependant l'utilisation d'un couvert végétal sur des sols pollués permet d'augmenter ces paramètres par

rapport au sol non végétalisé (Nahmani and Rossi 2003; Epelde et al. 2009b; Al Souki et al. 2017).

2) Est-ce que l'utilisation d'un couvert végétal sur un sol pollué améliore le fonctionnement biologique du sol ?

L'évaluation de l'efficacité de la phytoextraction est généralement abordée en utilisant des mesures physico-chimiques (mesure de la réduction des concentrations totales ou extractibles en ET du sol). Or, il est admis que l'utilisation d'un couvert végétal pour la phytoextraction comparé au même sol non végétalisé peut améliorer le fonctionnement biologique ou « la santé » des sols, définie comme la capacité du sol à remplir ses fonctions (Pardo et al. 2014). Cette capacité, à accroître la santé du sol peut être mesurée grâce à plusieurs indicateurs (physique, chimique et biologique) (Breure et al. 2005; Pardo et al. 2014). Mes travaux de thèse ont permis de démontrer que l'utilisation du saule dans nos conditions d'étude permettait d'augmenter certains paramètres biologiques du sol pollué par rapport au sol pollué non végétalisé (Chapitre 4, publication 3). Nous avons notamment mis en évidence une augmentation de la biomasse des vers de terre ainsi que de l'activité microbienne du sol (DHA) en présence de saule. Contrairement aux études de Hortal et al. (2013; Qin et al. (2015); Yang et al. (2017) où la présence d'un couvert végétal augmente la biomasse microbienne par rapport au sol sans végétation , aucune différence entre les conditions n'a été observée concernant la biomasse microbienne ,ce qui suggère que l'utilisation d'un couvert végétal dans nos conditions d'étude ne contrebalance pas l'effet négatif des ET sur ce paramètre..

3) Est-ce que l'hyperaccumulation du Zn et du Cd chez *A. halleri* lui permet de résister aux herbivores ?

Mes travaux de thèse ont montré que la dispersion des ET dans l'environnement à partir *d'A. halleri* était faible mais non négligeable (chapitre 2, publication 1). Comme dans les études de Kazemi-Dinan et al. (2015) et Stolpe et al. (2017), mes résultats n'ont pas permis de discriminer un trade off entre concentrations en ET et les glucosinolates (GLS) chez *A. halleri*. Cependant, mes résultats ont montré que le Zn et le Cd étaient directement impliqués dans la défense d'*A. halleri* face à l'herbivorie (Chapitre 5, publication 4). Plus les plantes sont chargées en Zn et en Cd et moins elles sont consommées par les escargots. Il y a donc moins de risque de dispersion des ET via l'herbivorie.

Noret et al. (2007) ont montré chez *T. caerulescens* que les herbivores préfèrent les plantes provenant de sites métallicoles aux plantes provenant de sites non métallicoles. Les plantes métallicoles présentent des concentrations en GLS plus faibles que les plantes non métallicoles. Différentes études en conditions contrôlées démontrent que les populations non métallicoles ont des capacités d'absorption des ET plus importantes que les populations métallicoles (Bert et al. 2000). Les populations non métallicoles présentant des concentrations en GLS plus importantes et des capacités d'accumulation en ET plus grandes, leur utilisation en phytoextraction pourrait présenter des avantages pour extraire plus d'ET et pour limiter la dispersion dans l'environnement des ET par l'herbivorie.

II) Recommandations sur les itinéraires culturaux et la gestion d'un site phytomanagé

1) Arabidospis halleri

Les itinéraires culturaux d'*A. halleri* n'étant pas connus, mes résultats de thèse ont permis d'apporter une grande quantité d'informations pratiques. Pour l'installation des plantes, le repiquage est nettement supérieur au semis direct pour la mise en place de la phytoextraction avec *A. halleri* (Tableau 3) et devrait être favorisé pour les petites surfaces. En 2017, les graines récoltées sur les parcelles du site (P8 et P9) présentaient un taux de germination sur

terreau de 35%. En 2019 et en 2020, les graines récoltées sur milieu pollué (parc Péru) présentaient un taux de germination de 86 et de 80 % sur terreau, respectivement. Aucun test sur milieu pollué n'a été effectué avec ces graines. A ma connaissance, aucune étude ne rapporte le taux de germination des graines d'*A. halleri* sur milieu pollué. L'origine des graines pourrait jouer un rôle dans le pouvoir de germination des graines. Le taux de germination des graines de 2017 était de seulement 25% comparé à celui de 74% en 2018. Le test de germination des graines a été réalisé au même moment en 2018, les graines de 2017 avait donc plus d'un an. Soit les graines ont été mal conservées soit le taux de germination des graines diminue rapidement au cours du temps.

Tableau 3Taux de germination des graines d'arabette de Haller (population parc Péru, nord, France) selon différentes modalités (semi en conditions contrôlées en terreau et in situ sans NPK+S).

Condition	Taux de germination (%)
Terreau graine 2017	25
Semi direct graine 2017 (100 graines)	0
Terreau graine 2018	74
Semi direct graine 2018 (440 graines)	10
Semi direct 2018 (880 graines)	5

Entre les parcelles d'*A. halleri* nous avons observé un développement de rosette sans doute dû à la germination des graines *in situ* ou la propagation clonale (figure 10). En effet *A. halleri* est une espèce pouvant se propager par la reproduction sexuée et asexuée par formation de rosettes clonales (Kudoh et al. 2018).

Figure 10Plantule d'A. halleri au stade rosette entre deux parcelles d'A. halleri

La densité de repiquage peut être maximisée (30 au début du projet et 60 plantes m-² après 2016). Une forte densité permet à l'arabette de Haller de se développer en tapis dense de façon à limiter la dispersion des polluants par envol de poussières.

De plus, le taux de survie des arabettes implantées dépend de la date d'installation des parcelles. Les plantes des parcelles mises en place en automne (septembre à novembre) ont un meilleur taux de survie que celles des parcelles installées au printemps (mars à juin) (76% en automne vs 58% au printemps). Les observations de terrain montrent que les arabettes plantées aux printemps subissent plus d'herbivorie que celle plantées en automne. La lutte contre les herbivores au printemps est cruciale pour la survie des jeunes plantules. Mise en place dès le début du projet, la lutte chimique (granulé de phosphate de fer utilisable en agriculture biologique) contre les herbivores permet de limiter la mortalité des plantules. Bien qu'utilisé qu'une seule fois l'utilisation des granules de phosphate de fer peut poser des questions sur l'utilisation de produits phytosanitaires sur un site pollué. L'apport de phosphate de fer pourrait modifier les paramètres physico-chimiques des parcelles d'*A. halleri* et modifier les capacités d'accumulation de celles-ci.

Le suivi des arabettes peut se faire la première année de culture. Par la suite, il devient difficile de suivre un individu, car l'arabette peut se propager de manière clonale par la formation de rosettes sur les méristèmes apicaux et latéraux (Honjo and Kudoh,2019).

Par ailleurs, la faible compétitivité d'*A. halleri,* observée pour la première fois *in situ* dans le cadre de mes travaux de thèse, face aux adventices demande beaucoup de temps et de main d'œuvre afin de désherber les parcelles. Par ailleurs, une fois transplantées, les rosettes d'*A. halleri* mettent plus d'un an à couvrir complètement le sol. Pour des grandes parcelles, la lutte contre les adventices devrait être mécanisée. L'utilisation de mulch les premières années du projet avait permis de limiter l'apparition d'adventices sur la saulaie et sur les parcelles d'arabette. Le mulch peut être une solution afin de limiter les adventices et de réduire les coûts en main d'œuvre. Cependant dans le chapitre 4 (publication 3) une augmentation de la MO a été observée, peut-être en partie dû à l'apport de mulch les premières années. L'apport de mulch peut perturber le fonctionnement du sol. En effet, une faible minéralisation de la MO a été observée sur le site. Une forte densité de plantules au moment des repiquages peut également être une solution pour limiter la compétition avec les adventices.

Les résultats pratiques obtenus lors de ma thèse, concernant la mise en place d'un projet de phytoextraction avec *A. halleri* sont synthétisés dans le tableau 4. En résumé, afin de maximiser la phytoextraction avec *A. halleri,* il est conseillé de réaliser la plantation en automne avec un nombre de 70 plantules par mètre carré. La parcelle doit être fertilisée avec un fertilisant NPK+S à une dose de 100g. m² et en inter-rangée, si elle est utilisée en co-culture avec le saule. La récolte de la biomasse peut être réalisée 2 fois par an au stade rosette.

Tableau 4 : Recommandations pour la mise en place de la phytoextraction avec A. halleri (++ bonne pratique, + pratique pouvant être mise en place avec restriction, - pratique non recommandée).

	Pratiques Agronomiques				Culture		
	Fertilisant NPK+S (100g m²)	Fauche (1 et2 fauches)	Co-culture (Inter-rangée ou autour des saules)	Fertilisant biologique (CMA)	Semis direct (hiver)	Transplantation de plantules *in situ*	Période de repiquage
A. halleri	++	++ (2 fauches)	+ Inter-rangé uniquement	-	-+	++ 60 à 70 Nombre de plantules/m²	Septembre /novembre >>> Mars/avril

2) Salix Viminalis

Mes travaux de thèse sur le saule ont également démontré que les feuilles de saule ne présentaient pas de risque de dispersion des métaux dans l'environnement (Chapitre 2, publication 1). Cependant, depuis 2015, nous avons observé à plusieurs reprises l'attaque des bois de *S. viminalis* par le puceron géant du saule *Tuberolachnus salignus.* Collins et al. (2001) ont montré que les attaques du saule par *T. salignus* se produisent en été pendant une longue période de sècheresse. Chez la plupart des plantes, une hydrolyse des protéines foliaires est observée en période de sècheresse. Cette hydrolyse provoque une augmentation du taux d'azote soluble dans le phloème, ce qui rend les arbres plus sensibles aux pucerons (Wearing and Van Emden 1967). Les pucerons ont un effet négatif sur la croissance des arbres. En effet, Collins et al. (2001) ont montré que les croissances racinaire et foliaire étaient réduites après l'attaque des pucerons. *T. salignus* a également un impact sur la structure du bois. Une réduction de la masse des tissus ligneux est en effet observée (Collins et al. 2001).

Stolpe et al. (2017a) ont montré que l'attaque d'*A. halleri* par des insectes suceurs de sève avait pour conséquence l'augmentation des concentrations de Cd et de Zn dans le phloème. La question se pose de savoir si dans ce cas, l'attaque d'insectes suceurs de sève tels que le *T.*

salignus entraîne chez le saule des vanniers une augmentation du Cd et du Zn dans la sève. *T. salignus* est une espèce de puceron produisant du miellat qui attire les abeilles, les guêpes et les fourmis (Tun et al. 2020). La mesure du Cd et du Zn dans la sève des arbres et dans les pucerons devrait être effectuée pour vérifier cette hypothèse. Ces résultats pourront alimenter la question de l'évaluation des risques si le transfert, en particulier, de Cd est avéré chez le puceron. Dans ce cas, une réflexion sera à mener par rapport à l'éventuel passage de cet élément à un niveau trophique supérieur. De plus, nous avons observé que le miellat sécrété pas les pucerons et retombant au sol avait un effet négatif sur les plantes du couvert végétal et notamment sur les parcelles d'arabette de Haller se trouvant en co-culture en inter-rangée avec le saule. En effet, les plantes se retrouvant couvertes de sucre brulent (observations personnelles, figure 11). De plus, les attaques de pucerons probablement plus fréquentes, en lien notamment avec une fréquence accrue en période de sécheresse due au changement climatique, des modifications de l'itinéraire cultural de *S. viminalis* sont à prévoir en lien notamment avec la santé et la croissance des arbres. La recherche de clones moins sensibles ou résistants a *T. salignus* pourrait être effectuée afin d'anticiper les effets du changement climatique. L'utilisation de produit phytosanitaire (insecticide) pourrait être envisagée, mais n'est pas recommandée dans un site urbain. L'utilisation de traitement écoresponsable existe, comme l'utilisation de larve de coccinelle au début de l'apparition des pucerons (attention au risque de dispersion des ET). Le traitement à base d'insecticide naturel (décoction d'ail, savon noir, etc.) peut être réalisé lorsque les pucerons sont installés.

Figure 11Exemples de Tuberolachnus salignus infestant le tronc de Salix viminalis à gauche, état du couvert végétal suite au miellat de Tuberolachnus salignus (la zone rouge montre la zone affectée par le miellat) à droite.

3) Gestion du site

La valorisation de la biomasse et des ET extraits est un enjeu important dans le déploiement de la phytoextraction. La chimie verte permettrait de valoriser les métaux contenus dans les feuilles des plantes. Pour cela, des procédés d'extraction des ET (Ni, Zn et Mn) ont été mis au point pour produire de nouveaux catalyseurs appelés « écocatalyseurs » (Grison 2015). Au laboratoire, des tests encourageants ont été réalisés sur des feuilles de saules et d'arabette de Haller (Deyris et al. 2018). La valorisation du Ni est à ce jour, la plus aboutie pour produire des sels de Ni (Simonnot et al. 2018). Dans le projet Extra-Zn, différentes biomasses issues de mon travail de thèse sont à l'étude pour une utilisation en écocatalyse (feuilles de saule, d'arabette et du bois de saule [parties terminales les plus chargées et biomasse totale]) (Grison et al. en cours). L'utilisation des feuilles de saules en écocatalyse de Zn a été confirmée dans le chapitre 2 (publication 1) et le chapitre 4 (publication 3) sur la base des concentrations en Zn dans les feuilles de saule mesurées en automne et en juin 2016 et 2019 (tableau 5). Afin de

maximiser les concentrations en Zn pour la production d'écocatalyseurs, il est donc recommandé de collecter les feuilles de saule tombées en automne.

Tableau 5 Comparaison des concentrations en Zn dans les feuilles de saule récoltés en juin ou en octobre 2016 et 2019.

Concentration en Zn dans les feuilles de saule ($mg.kg^{-1}$)	Juin	Automne
2016	856 ± 409	1309 ± 490
2019	554 ± 268	950 ± 124

Mes travaux de thèse ont apporté des réponses pour la gestion des adventices sur un site phytomanagé et les possibilités de valorisation des bois de saule. Les adventices peuvent être utilisées en compostage ou en méthanisation (Chapitre 6, publication 5) car elles présentent des concentrations en Zn et en Cd toutes inférieures aux limites réglementaires.

En absence de cadre règlementaire pour les biomasses issues de sol pollué, les concentrations en Zn et en Cd du bois de saule des vanniers ont été comparées avec les valeurs d'entrée en Zn et en Cd dans les combustibles pour des chaudières soumises à enregistrement. (Arrêté du 3 août 2018). La valeur du Zn (200 mg kg^{-1}) correspond à une valeur physiologique moyenne observée sur toutes les essences de bois issus de sites non pollués. Sur sites non pollués, les valeurs moyennes en Zn mesurées chez les saules sont comprises entre 25,2 et 161 $mg.kg^{-1}$ (Adegbidi et al. 2001; Labrecque and Teodorescu 2005; Adler et al. 2005; Maxted et al. 2007). Le saule étant une espèce accumulatrice d'ET (Delplanque et al. 2013; Van Slycken et al. 2013), il était attendu des valeurs potentiellement supérieures à 200 mg kg^{-1}, ce que nos résultats ont montré. Contrairement au Cd qui n'est pas limitant, le Zn pourrait constituer une limite à la valorisation du bois de saule en combustion s'il était la seule ressource pour la chaudière. La pyrolyse pourrait constituer une autre option de valorisation pour le bois de saule (Lievens et al. 2008b, a; Bert et al. 2017). Une attention particulière devra néanmoins être portée

au Cd qui pourrait être limitant pour une valorisation des cendres et du biochar (Delplanque et al. 2013; Bert et al. 2017). De même, le principe de non dilution (mélange de biomasse de plusieurs origines pour constituer des lots) est à considérer pour ces options. Pour pallier à ces potentielles limitations, l'utilisation d'arbres ayant un diamètre supérieur à 30mm pourrait être une solution. En effet, comme démontré par la corrélation négative entre le diamètre des troncs et la concentration en Zn, pour diminuer la concentration moyenne en Zn, le gestionnaire du site devrait attendre que la majorité des arbres ait atteint un diamètre supérieur à 30 mm afin de ne pas dépasser la concentration limite en entrée de combustion (chapitre 6, publication 5).

Le projet Extra-Zn avait un double objectif, paysager et de dépollution partielle du site. Lors de la coupe de la saulaie, l'aspect paysager a été conservé et seulement 1/3 des arbres a été coupé. Pour une meilleure gestion du site, les saules devraient être coupés en une seule fois afin de favoriser la reprise des souches. Les saules étant une espèce à croissance rapide, l'aspect paysager ne sera impacté que pendant une faible période (quelques mois à 1 an après la coupe). Cette option de gestion est à l'étude depuis février 2021.

Conclusions et Perspectives

Cette étude sur le suivi de la phytoextraction du Zn et du Cd par *S. viminalis* et *A. halleri* sur un site urbain est la seule étude conduite en condition *in situ* et sur le long terme (6 ans) avec ces deux espèces. Mes travaux de thèse ont permis de démontrer que *A. halleri* et *S. viminalis* pouvaient être utilisés en synergie dans une stratégie de phytoextraction et que les résultats étaient comparables à ceux obtenus avec d'autres (hyper)accumulateurs au regard des conditions de site non optimales . La co-culture d'une espèce accumulatrice (le saule) avec en couvert végétal une espèce hyperaccumulatrice (l'arabette) permet de maximiser la dépollution partielle du site ainsi que la production de biomasse à des fins économiques. Les feuilles de saule ainsi que les parties aériennes d'*A. halleri* peuvent être utilisées en écocatalyse et le bois de saule peut être valorisé en combustion à condition de suivre la concentration en Zn. Nous avons pu identifier plusieurs pratiques culturales (ajout de fertilisant NPK+S, fauche, co-culture) efficaces pour une amélioration du potentiel de phytoextraction d'*A. halleri* et *S. viminalis*. Ces pratiques ont été utilisées séparément. L'une des perspectives qui découle de mes travaux de thèse serait d'étudier l'effet combiné des différentes pratiques pour optimiser le potentiel de phytoextraction de la co-culture saule/arabette.

La coupe entière de la saulaie en février 2021, va permettre d'approfondir les résultats obtenus en février 2018. Des mesures de concentrations en Zn et Cd vont être réalisées sur les arbres présentant un diamètre supérieur à 30mm. Ces mesures vont permettre de compléter les recommandations pour une utilisation du bois en combustion. La poursuite de l'étude des saules va également permettre d'étudier les concentrations foliaires en Zn et Cd et les paramètres de croissance sur une nouvelle rotation.

Les connaissances acquises sur l'itinéraire cultural d'*A. halleri* dans le cadre de ma thèse vont servir de base à des études plus approfondies. En effet, dans le cadre du projet Extra-Zn, l'optimisation des conditions de germination des graines et de culture d'*A. halleri* va être

poursuivie en collaboration avec les services espaces verts de la ville de Creil afin d'assurer une production locale et à grande échelle. Afin de maximiser les taux de germination un enrobage des graines avec un fertilisant pourrait être envisagé. Sur le terrain des tests a différentes saisons pourront également être réalisés.

Mes travaux ont également montré que *A. halleri* était une espèce non mycorhizable par les CMA. Afin de compléter ces résultats et de comprendre l'origine de l'inaptitude de *A. halleri* à établir une association symbiotique avec les CMA, la recherche des gènes « symbiotic Toolkit » pourrait être intéressant (Delaux et al. 2013). Par ailleurs, *A. halleri* étant colonisable par un champignon endophyte (*P. indica)*, l'étude de l'influence de cette association sur les performances de croissance et de phytoextraction de *A. halleri* pourrait être développée. Une autre approche serait d'isoler les microorganismes (bactéries et champignons) de la rhizosphère de l'arabette de la population mère et de l'injecter au moment de la transplantation des plantules. Plusieurs études ont montré l'importance de la communauté microbienne dans l'accumulation du Zn et du Cd chez *A. halleri* (Farinati et al. 2011; Muehe et al. 2015; Borymski et al. 2018). De plus, une caractérisation moléculaire des communautés microbiennes du sol présentes sur le site pourrait apporter des informations supplémentaires sur l'impact de la végétalisation et de la phytoextraction sur la structure et l'identité des populations microbiennes du sol pollué. D'autre part, lors de notre étude, nous avons utilisé des inoculums mycorhiziens commerciaux (MycAgro et Symbivit) à base de souches de CMA non sélectionnées spécifiquement pour leur caractère de tolérance aux ET. Un isolement des souches de CMA présentes sur le site (au niveau de la rhizosphère des saules et des adventices), suivi d'une identification moléculaire et d'une caractérisation de leurs capacités de tolérance aux ET permettrait de produire un inoculum local bien adapté aux conditions du site (concentration en ET, conditions pédo-agronomiques du sol).

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Annexes

Annexe 1 :Liste des communications

Communications orales :

GRIGNET A., LOUNES-HADJ SAHRAOUI A., BERT V. Phytoextraction with *Arabidopsis halleri* intercropped with *Salix viminalis* to redevelop metal contaminated urban area. 15th International Phytotechnology Conference (2-4 October 2018, Novi Sad, Serbie)

GRIGNET A., LOUNES-HADJ SAHRAOUI A., FONTAINE J., BERT V. Phytoextraction in situ du Zn et du Cd par *Arabidopsis halleri* et *Salix viminalis* (PHYTOEXCO). 4es rencontres nationales de la recherche sur les sites et sols pollués (26 et 27 novembre 2019, Le Beffroi de Montrouge, Paris, France).

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Phytoextraction du Zn et du Cd par l'arabette de Haller en co-culture avec le saule en milieu urbain : projet PHYTOEXCO. INTERSOIL 2020 (2 et 3 Mars 2020, Bruxelles, Belgique)

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Phytoextraction du Zn et du Cd par l'arabette de Haller en co-culture avec le saule en milieu urbain : projet PHYTOEXCO. Journée doctorante INERIS 18 juin 2020..

Posters :

GRIGNET.A, BERT.V, FREJAFON. E, LOUNES-HADJ SAHRAOUI A. Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec le Saule (PHYTOEXCO). Journée doctorant ADEME (14-16 Mars 2018, Nice, France)

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Mycorhization spontanée et induite de l'arabette de Haller par des champignons mycorhiziens à arbuscules dans un contexte de pollution aux éléments potentiellement toxiques. SFR journées Condorcet (14-15 Juin 2018, Calais, France)

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Essai d'inoculation de l'arabette de Haller (*Arabidopsis halleri*) par des champignons mycorhiziens à arbuscules dans un contexte de pollution aux éléments potentiellement toxiques. 5éme journées francophone des mycorhizes (27-29 Juin 2018, Dunkerque, France)

GRIGNET.A, BERT.V, FREJAFON. E, LOUNES-HADJ SAHRAOUI A. Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec le Saule (PHYTOEXCO). Journée doctorant INERIS (19 septembre 2018, INERIS, Verneuil-en-Halatte, France)

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Phytoextraction in situ du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec le saule des vanniers (PHYTOEXCO). Journées Jeunes Chercheurs Transfrontalières (les 2 et 3 avril 2019, Reims, France)

GRIGNET.A, FREJAFON. E, LOUNES-HADJ SAHRAOUI A, BERT.V. Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec le Saule (PHYTOEXCO). Journée doctorant INERIS (12 Juin 2019, INERIS, Verneuil-en-Halatte, France)

GRIGNET A., BERT V., FONTAINE J., LOUNES-HADJ SAHRAOUI A. Phytoextraction in situ du Zn et du Cd par l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec le saule des vanniers (PHYTOEXCO). Journées Condorcet 2019 (25 et 26 juin 2019, Reims, France)

Etude des performances de phytoextraction du Zn et du Cd de l'hyperaccumulateur *Arabidopsis halleri* en co-culture avec *Salix viminalis*.

Mots clés : phytoextraction, *Arabidopsis halleri*, *Salix viminalis*, pratiques agronomiques, valorisation biomasse

La phytoextraction a pour objectif de réduire partiellement la pollution des sols. Ce mode de gestion de terre polluée *in situ* évite leur excavation, transport et mise en site de stockage. Pour alimenter les retours d'expérience en conditions réelles et sur le long terme, une expérimentation de phytoextraction du Zn et du Cd par une co-culture d'*Arabidopsis halleri* et de *Salix viminalis* sur un site urbain contaminé avec des teneurs moyennes en Cd et en Zn de 1,7 et 616 mg kg^{-1}, respectivement, a été mise en place en 2013 à Montataire (Oise, France). Mes travaux de thèse s'inscrivent dans ce contexte et contribuent à alimenter les connaissances liées au déploiement de la phytoextraction *in situ.* Ils avaient pour objectifs d'étudier (i) les transferts de métaux sol- plantes- herbivores, (ii) les risques environnementaux potentiels associés (iii) l'impact de la pollution métallique et d'un couvert végétal sur le fonctionnement biologique du sol et (iv) d'identifier les conditions optimales pour la valorisation des biomasses produites par l'étude des effets de plusieurs pratiques culturales.

L'efficacité de phytoextraction de *A. halleri* et *S. viminalis* a été démontrée. Les deux espèces se sont bien adaptées aux conditions du site (pH basique). Les concentrations foliaires en Zn des deux espèces sont largement supérieures aux valeurs physiologiques et en Cd supérieures aux valeurs ordinaires confirmant les caractères tolérants et accumulateurs de ces espèces pour ces deux éléments. Les pratiques agronomiques testées (fertilisant NPK+S, fauche, co-culture) ont permis d'augmenter le potentiel de phytoextraction d'*A. halleri* (biomasse et/ou accumulation d'ET (élément trace)). Nos résultats ont également montré que la pré-inoculation des saules par un inoculum mycorhizien commercial à base de CMA était possible. Cependant, *A. halleri* n'était pas mycorhizable par les inoculums mycorhiziens testés dans les conditions expérimentales de notre étude et, ce, quelles que soient les conditions de culture (en présence ou en absence de fertilisant NPK, *in situ* ou en pot en présence d'une plante nurse) et quels que soient les stades de développement (rosette, floraison et fructification). Toutefois, nous avons montré que la colonisation racinaire d'*A. halleri* était possible en utilisant un champignon endophyte *Piriformospora indica*.

Les ET ont un effet négatif sur le fonctionnement biologique du sol (biomasse microbienne, activité enzymatique, biomasse et abondance des vers de terre). L'utilisation du saule en couvert végétal à un effet bénéfique sur certains paramètres biologiques du sol (biomasse des vers de terre, activité enzymatique microbienne déhydrogénase) par rapport au sol non végétalisé.

Plus les parties aériennes d'*A. halleri* sont enrichies en Zn et en Cd et moins elles sont consommées ce qui suggère un lien direct entre les concentrations en Zn et Cd et la défense face aux herbivores chez cette espèce. Au contraire, aucun lien n'a été établi avec les concentrations et la nature des glucosinolates.

Sur la base des concentrations en ET dans la biomasse produite (*S. viminalis* ; *A. halleri* ; adventices) sur le site d'expérimentation, plusieurs voies de valorisation semblent émerger : la production d'écocatalyseur de Zn (*A. halleri*, feuilles de *S. viminalis*), combustion (*S. viuminalis* tronc) et le compostage et la méthanisation pour les adventices du site.

Zn and Cd phytoextraction performances of the hyperaccumulator *Arabidopsis halleri* in co-cultivation with *Salix viminalis*.

Key words: phytoextraction, *Arabidopsis halleri*, *Salix viminalis*, agronomic practices, biomass valorization

Phytoextraction aims at partially reducing soil pollution. This *in situ* polluted soil management method avoids its excavation, transport and disposal. In order to provide feedback in real conditions and on the long term, an experiment of phytoextraction of Zn and Cd was set up in 2013 in Montataire (Oise, France) by co-cultivating *Arabidopsis halleri* and *Salix viminalis* on a contaminated urban site (average Cd and Zn contents of 1.7 and 616 mg kg-1, respectively). My thesis work is part of this experiment and contributes to the knowledge related to the deployment of *in situ* phytoextraction. The objectives were to study (i) soil-plant-herbivore metal transfers, (ii) potential associated environmental risks, (iii) the impact of metal pollution and a plant cover on the biological functioning of the soil and (iv) to identify optimal conditions for the valorization of the biomass produced by studying the effects of several cultural practices.

The phytoextraction efficiency of *A. halleri* and *S. viminalis* was demonstrated. Both species adapted well to the site conditions (basic pH). Zn leaf concentrations in both species were well above physiological values and Cd was above normal values confirming the tolerant and accumulating characteristics of these species for this two elements. The agronomic practices tested (NPK+S fertilizer, mowing, co-cultivation) increased the phytoextraction potential of *A. halleri* (biomass and/or TE accumulation). Our results also showed that pre-inoculation of willows with a commercial mycorrhizal inoculum was possible. However, *A. halleri* was found to be not mycorrhizable in our exprimental conditions, regardless of the growing conditions (in the presence or absence of NPK fertilizer, *in situ* or in pots in the presence of a nurse plant) and regardless of the developmental stages (rosette, flowering and fruiting). However, we showed that root colonization of *A. halleri* was possible using an endophytic fungus *Piriformospora indica*.

Trace elements have a negative effect on soil biological functioning (microbial biomass, enzymatic activity, earthworm biomass and abundance). The use of willow as a vegetation cover has a beneficial effect on several soil biological parameters (earthworm biomass, the microbial enzyme activity dehydrogenase) compared to the non-vegetated soil.

The more the aerial parts of *A. halleri* are enriched in Zn and Cd, the less they are consumed, suggesting a direct link between Zn and Cd concentrations and defense mechanisms against herbivores in this species. Conversely, no link was established with the concentrations and nature of glucosinolates.

Based on the TE concentrations in the biomass produced (*S. viminalis*; *A. halleri*; colonizer plant) on the experimental site, several valorization pathways seem to emerge with Zn ecocatalyst production (*A. halleri*, *S. viminalis* leaves), combustion (S; viminalis trunk) and composting as well as methanization for colonizer plant on the site.

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