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Biodisponibilité du cuivre et du zinc pour les plantes et les vers de terre : interactions entre les effets de fertilisants organiques sur le long-terme et des organismes sur l'évolution des propriétés physico-chimiques du sol

Céline Laurent

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INSTITUT DES SCIENCES ET INDUSTRIES DU VIVANT ET DE L'ENVIRONNEMENT
PARIS INSTITUTE OF TECHNOLOGY FOR LIFE, FOOD AND ENVIRONMENTAL SCIENCES

Biodisponibilité du cuivre et du zinc pour les plantes et les vers de terre : interactions entre les effets de fertilisants organiques sur le long-terme et des organismes sur l'évolution des propriétés physico-chimiques du sol

Thèse de doctorat de l'Université Paris-Saclay
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Thèse présentée et soutenue à Paris, le 17/12/2019, par

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Thèse de doctorat

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Introduction générale

Le sol est une ressource naturelle assurant de multiples fonctions qui interviennent dans des processus écologiques comme la production végétale, la dynamique de la matière organique et les cycles des éléments nutritifs, le stockage de carbone et de l'eau, ou encore l'émission d'oxyde d'azote, de gaz carbonique et de méthane (Lavelle, 1996). Si le sol est un habitat pour les organismes et stocke des ressources naturelles, il est aussi le récepteur de contaminants. Le sol représente alors une voie de transfert de ces contaminants vers les eaux de surfaces, les nappes phréatiques et les réseaux trophiques (Adriano, 2001). Les sites répertoriés comme pollués en France sont décrits dans la base de données BASOL (2019) à partir de données majoritairement sur les éléments traces et les hydrocarbures aromatiques polycycliques.

L'accroissement de la population mondiale et du style de vie, comme une consommation de viande en augmentation, ont entrainé une augmentation des déchets d'origine organique. La gestion de ces déchets est devenue une problématique majeure à laquelle doivent désormais faire face les pays développés et en développement. La gestion de ces déchets par épandage sur les sols agricoles apparait être une forme de valorisation intéressante car l'apport de ces déchets organiques sur les sols est une pratique culturale fertilisante, amendante pour le sol, fertilisante pour les plantes et d'intérêt économique (Wassenaar et al., 2014). De plus, l'utilisation de ces fertilisants organiques est un bon substitut aux engrais minéraux. Ces derniers sont encore majoritairement appliqués mais leur utilisation durable est compromise par le fait que leur fabrication se fait à partir de minerais naturels dont les ressources sont épuisables et inégalement réparties sur la planète.

Si la valorisation des déchets organiques en agriculture apparait intéressante, on sait néanmoins que l'apport de fertilisants organiques contribue à la contamination des sols agricoles en éléments traces, en particulier en cuivre (Cu) et en zinc (Zn) qui se retrouvent dans les fertilisants organiques car apportés à la base en complémentation dans l'alimentation des animaux d'élevage (Jondreville et al., 2003). Or le temps nécessaire estimé pour que les concentrations de Cu et Zn dans le sol atteignent les limites réglementaires est plus court que pour d'autres éléments traces. Si l'on considère qu'au-delà d'un certain seuil Cu et Zn peuvent devenir toxiques pour les organismes, cela pourrait remettre en cause la durabilité de l'apport de fertilisants organiques sur les sols agricoles. Des études récentes utilisant l'approche d'analyse de cycle de vie (ACV) pour évaluer le risque environnemental lié à l'apport de fertilisants organiques à long-terme ont montré en effet que Cu et Zn étaient les principaux contributeurs à l'écotoxicité terrestre de cette pratique (Leclerc et Laurent, 2017 ; Sydow et al., 2018). Ces études ont révélé que l'impact écotoxicologique de ces éléments traces est corrélé à la masse

totale d'élément trace apporté avec l'application de fertilisants organiques aux sols. Le risque environnemental lié à cette pratique agricole serait donc relié au niveau de contamination en Cu et Zn sans forcément prendre en compte leur biodisponibilité au sens large, c'est-à-dire leur disponibilité dans le sol et leur biodisponibilité pour les organismes.

Même si les méthodes ACV se basent sur des approches écotoxicologiques, celles-ci restent classiques dans le sens où la toxicité de Cu et Zn pour les organismes est étudiée à partir d'un ou plusieurs organismes cibles modèles mais sans forcément de lien avec la réalité du terrain (Pelosi et al., 2013), exposés à des sols souvent artificiels comme décrits dans les normes et également artificiellement contaminés au laboratoire avec des sels solubles des éléments trace en question, souvent dans des conditions de contamination létale. Les mesures d'effets toxiques (par exemple l'élongation racinaire chez les plantes, la reproduction chez les vers de terre) permettent ensuite de construire des courbes doses-réponses dans un objectif de prédiction, mais qui demande toujours à être validé sur le terrain ce qui est pour le moment encore peu fait.

Une des problématiques liée aux fertilisants organiques est qu'ils apportent au sol des niveaux modérés de contamination à des doses sublétales. Il est alors difficile voire inutile de viser à détecter des effets toxiques sur les organismes cibles en suivant ce type d'approche expérimentale, et il convient plutôt de changer d'approche pour comprendre les impacts de ces fertilisants organiques sur les sols. Les apports de fertilisants organiques n'introduisant pas uniquement Cu et Zn dans le sol, de multiples facteurs confondants peuvent influencer la biodisponibilité de Cu et Zn et ne sont actuellement pas pris en compte dans l'approche écotoxicologique classique. Par exemple, il a été observé que les apports de fertilisants organiques sur le long-terme modifient les propriétés physico-chimiques du sol, en particulier le pH et la concentration en matière organique (Vanden Nest et al., 2016), qui sont des facteurs influençant la biodisponibilité de Cu et Zn. Les modifications des propriétés chimiques du sol peuvent donc agir comme des facteurs confondants, susceptibles d'influencer les résultats obtenus avec des tests écotoxicologiques classiques, sans que cela soit pris en compte. Une autre limite à la réalisation des approches classiques est que les organismes sont considérés uniquement comme des cibles du/des contaminants considérés. Cependant, les organismes peuvent également être considérés comme des acteurs de la contamination, car ils modifient aussi les propriétés physico-chimiques du sol dans le volume de sol qu'ils bio-influencent. Il a été montré que ces modifications induites par la bio-influence des organismes modifient la disponibilité des éléments traces dans la zone de sol qu'ils bio-influencent (Bravin et al., 2012 ; Sizmur et Hodson 2009). Ainsi, l'activité des organismes sur les modifications de biodisponibilité des éléments traces peut être considéré comme un facteur confondant supplémentaire dont il s'agirait de connaitre l'importance pour savoir si il doit être pris en compte dans l'évaluation des risques écotoxicologiques.

En contexte d'apport de fertilisants organiques, les modifications induites par l'évolution temporelle du pH et de la matière organique de différents types de sol, combinées aux modifications de ces paramètres par les organismes du sol ont des effets sur la disponibilité et la biodisponibilité de Cu et Zn qui restent encore mal renseignés. Dans ce cadre, l'objectif de mon travail de thèse a été de distinguer les effets respectifs de la contamination du sol et des évolutions temporelles de pH et de la matière organique dues à des apports de fertilisants ou dues à l'activité d'organismes du sol dans le volume de sol bio-influencé, sur la biodisponibilité de Cu et Zn pour ces organismes en contexte d'apports de fertilisants sur le long-terme dans des types de sols différents. Ce travail est fondé sur l'étude de sols provenant d'une collection d'échantillons issus de trois essais de terrain conduits à La Réunion et fertilisés pendant une décennie soit avec des engrais minéraux, soit des fertilisants organiques ou jamais fertilisés. Des mesures chimiques sur les sols ont été réalisées pour évaluer les évolutions de pH, de la matière organique et de la disponibilité de Cu et Zn dans les différents sols. La biodisponibilité de Cu et Zn pour les organismes a été évaluée par la mise en œuvre de tests biologiques permettant de prendre en compte la bio-influence des organismes sur le sol et les modifications qu'ils sont susceptibles d'induire sur la (bio)disponibilité de Cu et Zn. Nous avons choisi d'étudier l'action de plantes ou de vers de terre afin d'englober deux niveaux d'organismes (végétal et invertébré) qui ont des rôles clés dans les sols et dans le transfert des contaminants dans les réseaux trophiques.

La question de la valorisation agronomique des déchets organiques est particulièrement importante dans les territoires à fortes contraintes comme les espaces insulaires et d'actualité. En effet à La Réunion, plus de 270 000 t de matières organiques brutes par an sont produites par les élevages (MVAD, Mission de Valorisation Agricole des Déchets, Ile de la Réunion) et la DAAF (Direction de l'Alimentation, de l'Agriculture et de la Forêt) a mis en place localement le projet DEFI (Développement de l'Elevage et des Filières des Interprofessions) avec pour objectif d'augmenter le nombre d'éleveurs sur l'île. La quantité de matières organiques à épandre est donc amenée à augmenter, nécessitant une gestion durable des déchets organiques. Le territoire de La Réunion représente donc un bon cas d'étude pour évaluer le risque environnemental de l'apport de fertilisants organiques sur le long-terme et la durabilité de cette pratique.

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Chapitre I : Synthèse bibliographique

1. Concept de biodisponibilité des éléments traces dans les sols

D'après Peijnenburg et al. (1997), le concept de biodisponibilité se décompose en trois sous-concepts : la disponibilité environnementale, la biodisponibilité environnementale et la biodisponibilité toxicologique. L'évaluation des effets sur les organismes de la présence de contaminants dans les sols tels que les éléments traces nécessite donc d'évaluer la disponibilité des éléments dans les sols et la biodisponibilité pour les organismes.

1.1 Disponibilité environnementale

La disponibilité environnementale est définie comme « la fraction du contaminant potentiellement disponible pour des organismes et qui résulte de processus physico-chimiques de désorption » (norme ISO 17402, 2008). La disponibilité environnementale peut donc être évaluée au travers d'approches chimiques opérationnelles d'extraction avec des extractants de différentes forces. Peijnenburg et Jager (2003) ont par ailleurs proposé d'étudier la disponibilité environnementale au travers de la spéciation chimique des métaux dans les sols, i.e. la détermination de leurs différentes espèces chimiques en interaction avec les différentes phases du sol (Fig.1). L'étude de la spéciation chimique des métaux dans les sols permet de mettre en avant la solution du sol dans les mesures de disponibilité environnementale des métaux. En effet, parmi les différentes formes chimiques des éléments traces dans les sols, les ions libres (Cu^{2+}, Zn^{2+}…) sont connus comme les principales espèces chimiques prélevables par les organismes du sol et qui contrôlent la toxicité de ces éléments pour les organismes (Hough et al., 2005; Sauvé et al., 1996). Dans les sols, évaluer la spéciation chimique des éléments traces peut donc permettre d'avoir accès à l'exposition des organismes vivants. Cependant, la spéciation des métaux en solution se fait par l'étude des équilibres chimiques et ne permet pas de prendre en compte les contraintes cinétiques qui peuvent être en jeu dans le réapprovisionnement en métaux de la solution du sol par la phase solide. Pour cela des techniques comme la DGT (diffusive gradient in thin film), permettant de prendre en compte (i) les processus à l'équilibre qui régissent le partitionnement phase solide-solution des métaux disponibles et (ii) des processus cinétiques qui régissent la labilité des métaux (Degryse et al., 2009).

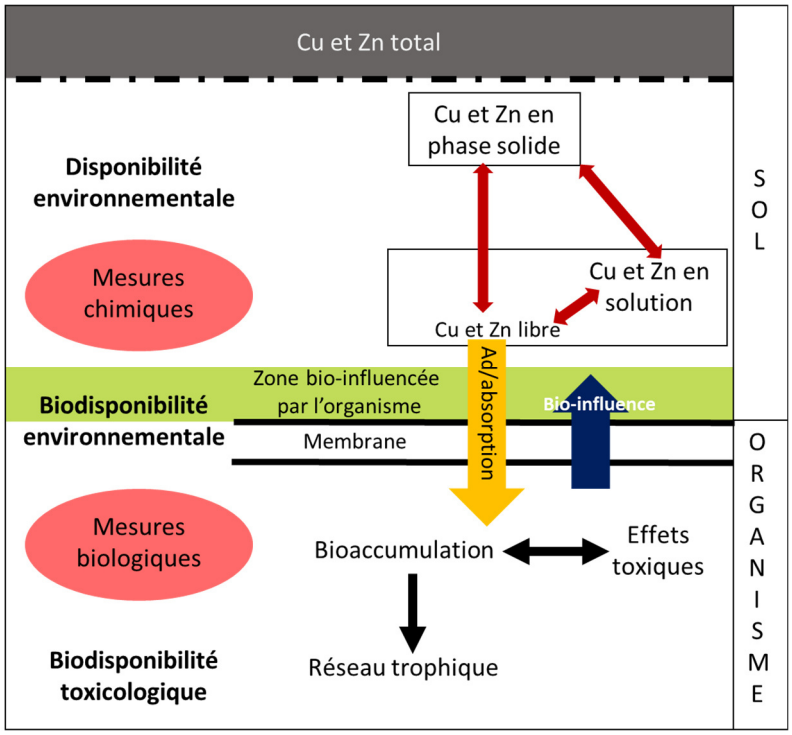

Figure 1 : Schéma conceptuel des trois étapes clés de la biodisponibilité des éléments traces (adaptés à Cu et Zn) pour les organismes du sol : disponibilité environnementale, biodisponibilité environnementale et biodisponibilité toxicologique (modifié d'après Harmsen, 2007).

Si les éléments traces peuvent interagir avec les organismes, en retour les organismes du sol peuvent modifier la disponibilité environnementale des éléments traces dans la zone dite bio-influencée du sol. Par exemple il est connu que les organismes peuvent modifier certaines conditions chimiques (e.g. pH, concentration en matière organique dissoute) dans le sol, par exemple dans le volume de sol bio-influencé par les vers de terres ou la rhizosphère soumise aux activités racinaires des plantes, ce qui en conséquence peut modifier la disponibilité environnementale des éléments traces (Hinsinger et al., 2003, 2006 ; Sizmur et Hodson, 2009). La zone de sol bio-influencée par les organismes est intégrée dans le concept plus général de domaines fonctionnels défini par Lavelle, (2002). Ces domaines fonctionnels sont hiérarchisés dans l'espace, le temps et en fonction de l'hétérogénéité des ressources pour les organismes. Les domaines fonctionnels sont constitués de zones de sol influencées par des régulateurs biotiques et abiotiques et opèrent à des échelles spatiales et temporelles qui peuvent différer. La notion de domaine fonctionnel prend en compte des échelles de temps et d'espace que nous n'avons pas considéré dans ce travail, nous parlerons de zone/sol bio-influencé par les organismes. Cette zone bio-influencée du sol est en général moins bien caractérisée et encore peu prise en compte dans la compréhension et la modélisation de la spéciation chimique des éléments traces dans les sols.

1.2. Biodisponibilité environnementale

La biodisponibilité environnementale est « la fraction du composé disponible dans le sol qu'un organisme absorbe par des processus physiologiques » (ISO 17402, 2008). Harmsen et al. (2005) ont proposé une définition plus opérationnelle de la biodisponibilité en intégrant la durée d'exposition des organismes à la mesure de biodisponibilité environnementale. La biodisponibilité environnementale peut donc également être définie comme le flux d'un élément trace donné vers un organisme cible pour un temps d'exposition donné (Harmsen et al., 2005) (Fig. 1). Le flux de contaminant vers un organisme peut être estimé via la mesure de l'accumulation du contaminant dans un organisme cible, rapporté au temps d'exposition de l'organisme au sol contaminé. La norme ISO 17402 suggère que la biodisponibilité environnementale puisse être approchée via des mesures chimiques ou biologiques, les mesures chimiques n'étant alors qu'un indicateur de la biodisponibilité pour les organismes et non une mesure directe de la biodisponibilité environnementale (Harmsen, 2007; Harmsen et al., 2005). L'évaluation de la biodisponibilité des éléments traces au travers de mesures chimiques de disponibilité de ces éléments dans le sol ne peut donc pas être envisageable car ne tient pas compte de la spécificité de l'organisme, à moins de faire ces mesures dans la zone bio-influencée par l'organisme-cible. D'après Harmsen (2007) il est difficile de définir la biodisponibilité d'une manière générale, puisque la mesure de biodisponibilité est susceptible de varier d'un organisme à un autre. Il faudrait donc définir et mesurer des biodisponibilités spécifiques à chaque organisme cible. Cela a été rendu opérationnel avec la mise en place de bioessais standardisés pour évaluer la biodisponibilité pour les organismes. Cependant souvent peu d'espèces-cibles sont conseillées d'utiliser pour un type d'organisme, la/les espèces ne sont pas nécessairement représentatives des conditions hors laboratoires, et le plus souvent dans ces bioessais les sols sont contaminés artificiellement. Récemment, pour les plantes un bioessai a été standardisé (ISO-16198, 2015) pour évaluer la phytodisponibilité environnementale des métaux et préconise l'utilisation d'espèces végétales agricoles communes qui sont capables de maximiser la phytodisponibilité. Il est également possible avec ce type de bioessai de prendre en compte la bio-influence de la plante sur la disponibilité.

La biodisponibilité environnementale des métaux pour les organismes peut être reliée à la théorie de l'ion libre (Free Ionic Activity Model, FIAM) et, pour le sol, au modèle du ligand biotique terrestre (Terrestrial Biotic Ligand Model, TBLM). Ces deux modèles se basent sur la détermination de la spéciation des éléments traces en solution. Le modèle FIAM établit que la forme libre du métal en solution est la seule à interagir physico-chimiquement avec les surfaces des organismes exposés et que l'intensité de cette interaction est proportionnelle à la bioaccumulation et in fine à l'effet toxique sur l'organisme (Slaveykova et Wilkinson, 2005). Le modèle du ligand biotique terrestre se base également sur cette théorie en la complétant par la prise en compte des mécanismes de compétitions pour

l'adsorption sur les surfaces de l'organisme-cible entre les formes libres des éléments traces, des protons et des éléments majeurs (e.g. Ca^{2+}, Mg^{2+}). In fine ces modèles permettent d'évaluer la biodisponibilité toxicologique des métaux pour les organismes par la mesure d'indicateurs d'effets toxicologiques (Thakali et al., 2006; Thakali et Herbert, 2006) comme par exemple l'élongation racinaire chez les plantes. Cependant depuis la mise en place de ces modèles, de nombreuses exceptions ont été mis en évidence pour les sols (e.g. Mourier et al., 2011). Les modèles FIAM et TBLM ont été établis sur une base physico-chimique et appliqués à des cas évaluant des expositions à de fortes concentrations. A de plus faibles niveaux d'expositions, des phénomènes de régulations biologiques par les organismes peuvent être observés (e.g. Spurgeon and Hopkins, 1999), ce qui pourrait expliquer que les relations entre disponibilité et biodisponibilité ne sont pas toujours évidente.

1.3 Biodisponibilité toxicologique

La biodisponibilité toxicologique concerne les effets toxiques que peuvent avoir les éléments traces accumulés au sein des organismes (Fig. 1). Les effets toxiques des éléments traces sont en général observés à partir d'un seuil d'accumulation dans les organismes Harmsen (2007), mais avant d'atteindre de tels seuils, des indicateurs d'effet plus précoces peuvent être mis en évidence au niveau biochimique ou moléculaire à l'échelle infra-individuelle (e.g. Marchand et al., 2017; Pérès et al., 2011).

La biodisponibilité toxicologique d'un élément trace dépend ainsi de son interaction et plus encore sa pénétration dans un organisme, c'est-à-dire de sa biodisponibilité environnementale qui est donc un prérequis à l'évaluation de la biodisponibilité toxicologique. Cette biodisponibilité environnementale est elle-même dépendante de « l'offre du sol », donc de la disponibilité environnementale de l'élément trace dans le sol. Cette dernière est principalement déterminée par les conditions physico-chimiques dans un sol donné ainsi que par la bio-influence des organismes vivant dans ce milieu (voir 1.1) et en revanche généralement peu par le niveau total de contamination du sol. Ainsi, la concentration totale des éléments traces dans les sols explique peu la biodisponibilité de ces éléments pour les organismes vivants et n'est en général pas considérée comme un bon indicateur de la biodisponibilité. Considérant que la biodisponibilité d'un élément trace dans le sol est dépendante de sa disponibilité, la détermination des deux composantes est nécessairement complémentaire pour comprendre et évaluer le transfert vers les organismes et la toxicité à partir de données de disponibilité de cet élément dans le sol. Les preuves empiriques et les relations de cause à effet du concept de biodisponibilité restent cependant encore partielles dans la littérature, notamment pour les organismes du sol autres que la plante dans la littérature (Beaumelle et al., 2016).

Dans ce manuscrit nous étudierons spécifiquement les deux premières composantes de la biodisponibilité et leurs interactions (i.e. entre disponibilité et biodisponibilité environnementales), en considérant deux éléments traces, le cuivre et le zinc, et deux types d'organismes cibles, un modèle plante et un modèle ver de terre.

2. Présence et devenir du cuivre et du zinc dans les sols

2.1. Sources de cuivre et zinc dans les sols agricoles

Le fond pédo-géochimique des sols et les apports anthropiques sont les principales sources d'éléments traces dans les sols. Le cuivre (Cu) et le zinc (Zn) sont des oligo-éléments pour les organismes, que l'on retrouve naturellement dans la croute terrestre et donc dans le fond pédo-géochimique de tous les sols à des concentrations comprises environ entre 10 et 30 mg Cu kg^{-1} et 10 et 300 mg Zn kg^{-1}, respectivement (Adriano, 2001). Ces concentrations peuvent être augmentées du fait de contaminations. Dans les sols agricoles plusieurs sources anthropiques d'éléments traces existent : les pesticides, les engrais minéraux, les fertilisants organiques (FO) (e.g. effluents d'élevages bruts et transformés, compost de déchets verts, boues d'épuration…), les amendements chaulant ou encore les dépôts atmosphériques. Parmi les éléments traces apportés dans les sols agricoles, Cu et Zn sont ceux qui sont apportés en plus grande quantité avec respectivement en moyenne 4869 $t.an^{-1}$ et 15190 $t.an^{-1}$ (Belon et al., 2012). Notamment, Cu et Zn sont apportés en complément dans l'alimentation des animaux d'élevage pour améliorer l'efficacité des antibiotiques et comme facteurs de croissance. Le Cu et Zn sont rejetés par le corps de l'animal et se retrouvent ainsi dans les déjections animales (Jondreville et al., 2003), qui peuvent être ensuite épandues sur les sols agricoles. Ainsi, l'application de FO constitue la source anthropique principale de Cu et Zn. Le Cu et Zn sont donc les deux éléments traces pour lesquels le temps nécessaire estimé pour que la concentration dans le sol atteigne les limites réglementaires est le plus court (Luo et al., 2009; Nicholson et al., 2003). Cela est problématique si l'on considère qu'au-delà d'un certain seuil, Cu et Zn sont toxiques pour les organismes, en particulier pour les plantes (Kopittke et al., 2011).

2.2 Déterminants physico-chimiques de la disponibilité environnementale de cuivre et zinc dans les sols

Le sol peut être décomposé en deux phases : une phase solide et une phase liquide (appelée solution du sol) dans lesquelles on trouve les différentes formes de Cu et Zn (Fig. 2). A l'interface de la phase solide et de la solution du sol, Cu et Zn peuvent être liés à des oxyhydroxydes de fer, d'aluminium et

de manganèse, aux argiles, aux carbonates et à la matière organique du sol (McBride, 1989). Le Cu et Zn à l'interface de la phase solide-solution du sol peuvent alimenter le pool de Cu et Zn de la solution du sol. Dans la solution du sol, le Cu lié à la matière organique dissoute (MOD) est la forme majoritaire (par rapport à la forme ionique libre en général très minoritaire ou au Cu lié à des ligands inorganiques) du fait de sa forte affinité pour la MOD (Ma et al., 2006; Sauvé et al., 1997). Le Zn, qui présente une plus faible affinité pour la MOD, se retrouve plus facilement sous sa forme ionique libre (Zn^{2+}) (Bonten et al., 2008) mais également sous ses formes liées aux ligands inorganiques dans la solution du sol.

Le Cu et Zn liés à des ligands inorganiques ne présentent qu'une faible partie de Cu et Zn dans la solution (Bonten et al., 2008), et comme illustré sur la figure 2, le pool de Cu et Zn disponibles correspond aux formes de Cu et Zn présentes dans la solution du sol ainsi qu'aux formes de Cu et Zn présentes à l'interface entre la phase solide et la solution du sol et capables de réalimenter le pool de Cu et Zn en solution sous l'effet d'un prélèvement.

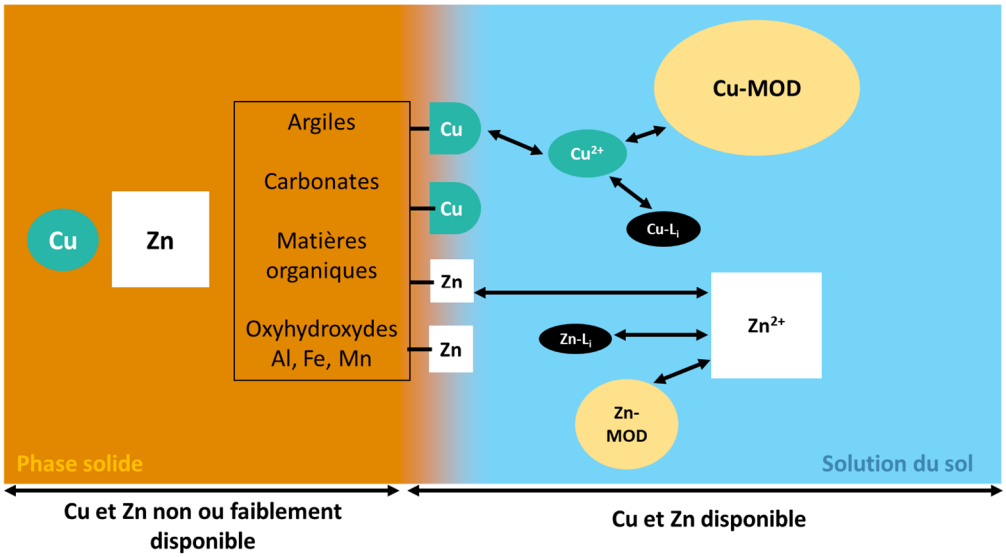

Figure 2 : Répartition des différentes formes de cuivre (Cu) et de zinc (Zn) dans le sol : ions libres (Cu^{2+} et Zn^{2+}), Cu et Zn liés à la matière organique dissoute (Cu-MOD et Zn-MOD) et Cu et Zn liés à des ligands inorganiques ($Cu\text{-}L_i$ et $Zn\text{-}L_i$).

2.2.1 Rôle du pH

Il a été montré que le partitionnement entre la phase solide et la solution du sol (Kd) est fortement dépendant du pH de la solution du sol pour Zn, contrairement au Cu (Sauvé et al., 2000). En effet, en compilant plusieurs centaines de données expérimentales, Sauvé et al. (2000) ont montré que le pH expliquait à lui seul 60% de la variabilité du Kd pour Zn, contre 29% pour Cu. Les auteurs ont également montré que la prise en compte de la concentration en matière organique du sol en plus du pH

expliquait 42% de la variabilité de Kd pour Cu alors que l'amélioration de cette relation pour Zn restait marginale. Le flux de Cu mesuré par la technique DGT appliquée à un sol acide a été plus important que dans un sol calcaire, du fait d'une corrélation négative avec le pH (Bravin et al., 2010). Cela a également été rapporté dans le cas de Zn (Zhang et al., 2004).

Concernant la spéciation dans la solution du sol, il a été montré que la concentration totale de Zn ainsi que l'activité de Zn^{2+} et de Cu^{2+} sont fortement dépendantes du pH du sol. Pour un sol donné, la concentration totale de Zn, l'activité de Zn^{2+} et de Cu^{2+} diminuent en général linéairement avec l'augmentation du pH du sol ou dans la solution de sol (Meers et al., 2006; Stephan et al., 2008; McBride et Blasiak, 1979; Salam et Helmke, 1998). Par contre, la relation entre la concentration totale de Cu en solution et le pH n'est pas linéaire (McBride et Blasiak, 1979). Ces auteurs ont montré que la concentration totale de Cu dans la solution du sol est minimale pour un pH de 6,5 et augmente pour des pH plus élevés ou plus faibles (Fig. 3). Pour des pH élevés (> 6.5), cette augmentation de Cu dans la solution peut être expliquée par la solubilisation de la matière organique et une MOD chargée de plus en plus négativement, favorisant ainsi la désorption de Cu depuis la phase solide par la formation de complexe Cu-MOD en solution (Tyler and Olsson, 2001). Par contre, en milieu acide (pH < 5-5,5), la matière organique n'est pas ou peu complexante, la compétition pour la complexation sur les sites portés par la MOD étant en faveur du proton et la MO étant également peu désorbée, Cu reste en phase solide peu échangeable.

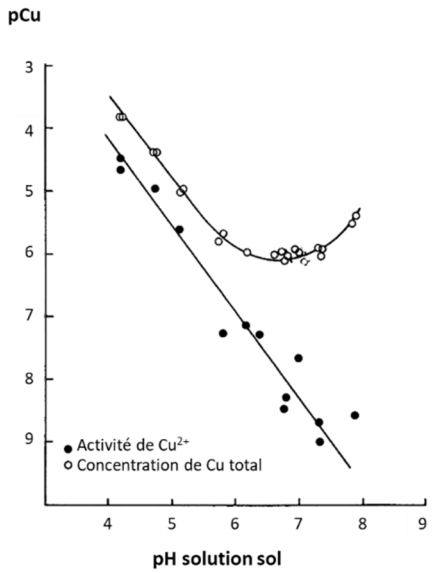

Figure 3 : Concentration totale de Cu et activité de Cu libre (Cu^{2+}) en fonction du pH dans la solution d'un sol amendé avec 40 mg Cu kg^{-1} (d'après McBride et Blasiak, 1979).

2.2.2 Rôle de la MOD

Le rôle de la MOD dans la détermination de la spéciation de Zn dans la solution du sol est généralement peu important du fait de la faible affinité de Zn pour la MOD. Par contre, la concentration totale de Cu dans la solution de sol a été trouvée positivement liée à la concentration en MOD (Vulkan et al., 2000; Weng et al., 2002). Cependant dans ces deux études, les auteurs ont montré une faible relation de la concentration en Cu^{2+} avec la concentration en MOD alors que Bravin et al. (2012) et Djae (2017) ont montré une corrélation négative entre la concentration en MOD et l'activité de Cu^{2+} en solution. En ce qui concerne l'activité de Cu^{2+}, Djae et al. (2017) ont mis en évidence que l'utilisation de modèles géochimiques avec un paramétrage par défaut (WHAM VII dans le cas de leur étude) biaise la prédiction de l'activité de Cu^{2+} en solution (étude sur 55 sols présentant des caractéristiques physico-chimiques variées). L'optimisation des propriétés de complexation de la MOD (densité de sites et affinité de la MOD) a permis une prédiction adéquate de l'activité de Cu^{2+} et a ainsi montré l'importance de la prise en compte de l'hétérogénéité des propriétés de complexation de la MOD pour la prédiction de l'activité de Cu^{2+} (Fig. 4).

En conclusion, le pH du sol et dans une moindre mesure la matière organique sont des paramètres d'influence pour la disponibilité de Zn dans les sols tandis que ces deux paramètres influencent fortement la disponibilité de Cu dans les sols.

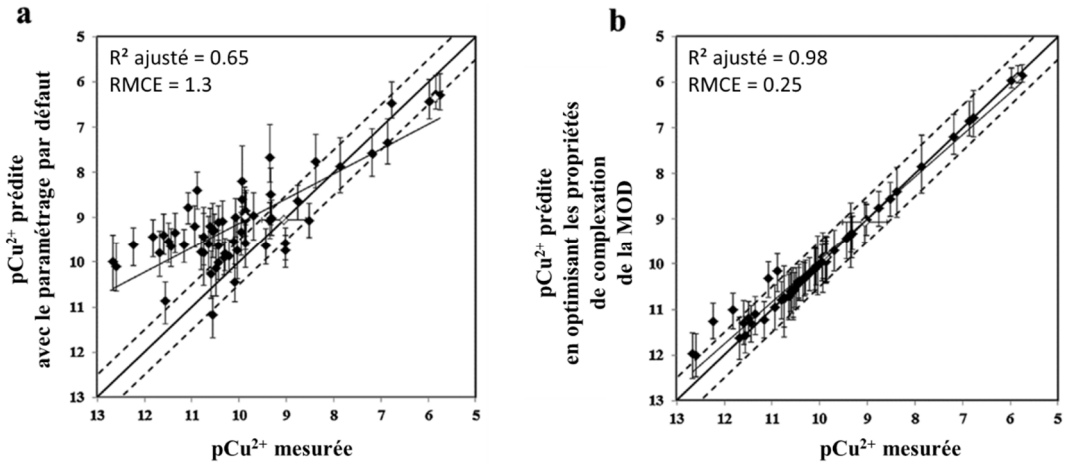

Figure 4 : Activité de Cu^{2+} (pCu^{2+} = -log{Cu^{2+}}) prédite avec le modèle WHAM (Windermere humic aqueous model) en fonction de l'activité de Cu^{2+} mesurée dans 55 échantillons de sols (a) avec un paramétrage par défaut du modèle et (b) en optimisant les propriétés de complexation de la MOD vis-à-vis de Cu. RMCE signifie racine carré de la moyenne du carré des erreurs (d'après Djae et al., 2017).

2.3 Déterminants de la biodisponibilité environnementale du cuivre et du zinc pour les plantes et les vers de terre

2.3.1 Phytodisponibilité environnementale du cuivre et zinc

L'évaluation de la biodisponibilité des métaux pour les plantes (encore appelée phytodisponibilité), peut être utilisée pour prévenir (i) de leur accumulation possible dans les organes consommés des plantes pouvant induire des risques pour la santé humaine et (ii) d'une éventuelle toxicité de ces éléments, cela pouvant entrainer une baisse de rendements agricoles (Adriano, 2001).

La phytodisponibilité de Cu et Zn a souvent été reliée au pH de la solution du sol non bio-influencé (i.e. qui n'est pas influencé par l'activité des racines des plantes) et à la spéciation de Cu et Zn dans la solution du sol. Ainsi, Cornu and Denaix, (2006) ont montré que la concentration de Zn dans les parties aériennes de la laitue (*Lactuva sativa*) augmentait avec la diminution du pH de la solution du sol. Lexmond (1980) a rapporté une diminution de la concentration de Cu dans les parties aériennes du maïs (*Zea mays*) suite à une augmentation du pH du sol. Néanmoins, Hough et al. (2005) ont montré que le pH n'expliquait que 51 à 58% de la variabilité de la concentration de Zn dans le ray grass (*Lolium perenne*) en étudiant 40 sols présentant un niveau de contamination en Zn contrasté et une large gamme de variation de pH. En ce qui concerne le Cu, sa concentration dans les parties aériennes et racinaires de la tomate (*Lycopersicon esculentum*) a été faiblement expliquée par la concentration totale de Cu dans la solution (entre 39 et 54%) et à l'activité de Cu^{2+} dans la solution de sol (entre 21 et 41%) (Zhao et al., 2006). L'explication de la biodisponibilité de Cu pour la tomate a été améliorée par la prise en compte du pH du sol mais restait faible, entre 40 et 50% (Zhao et al., 2006). Les observations des études présentées ici suggèrent que le pH dans le bulk soil (sol non bio-influencé) n'est pas le seul facteur qui permet d'expliquer la phytodisponibilité. D'autres facteurs à prendre en compte, en particulier dans la rhizosphère (i.e. le volume de sol influencé par l'activité des racines des plantes), peuvent influencer le pH et la MOD (Hinsinger et al., 2009). Le pH et/ou la MOD étant les principaux déterminants mis en avant dans les processus de disponibilité de Cu et Zn dans les sols (voir 2.2), les modifications de ces facteurs dans la rhizosphère pourraient participer à la détermination de la phytodisponibilité de Cu et Zn. Il parait ainsi évident que la prise en compte de ces modifications dans la rhizosphère soit importante dans l'évaluation de la phytodisponibilité de Cu et Zn.

La modification du pH dans la rhizosphère est principalement due au prélèvement de cations (Ca^{2+}, Mg^{2+}, K^{+}, NH_4^{+}) ou d'anions (Cl^{-}, $H_2PO_4^{-}$, NO_3^{-}, SO_4^{2-}) par les racines et à la libération concomitante d'hydroxydes ou de protons pour maintenir l'électroneutralité dans le milieu intracellulaire (Hinsinger et al., 2003). Loosemore et al. (2004) ont montré pour le tabac (*Nicotiana tabacum*) que l'accumulation de Zn était expliquée par l'acidification du pH de la rhizosphère. En étudiant deux cultivars de blés

tolérants à la déficience en Zn, Khoshgoftarmanesh et al. (2018) ont montré un prélèvement de Zn plus important dans la rhizosphère d'un des deux cultivars, en lien avec une diminution plus importante du pH et une augmentation du carbone organique dissous (COD) dans la solution de sol de la rhizosphère de ce cultivar par rapport au bulk soil. En ce qui concerne Cu, Bravin et al. (2009) ont montré une alcalinisation dans la rhizosphère du blé dur (*Triticum turgidum durum*) induisant une diminution de la disponibilité de Cu dans la rhizosphère et de la phytodisponibilité de Cu.

Les racines des plantes peuvent exsuder des composés organiques comme des sucres, des vitamines, des enzymes, des acides aminés et des anions organiques dans la rhizosphère induisant une augmentation de l'activité microbienne considérée et généralement une augmentation de la MOD (Jones et al., 2009). Il a été montré par ailleurs que la quantité d'anions organiques dans la rhizosphère est liée à l'état nutritionnel de la plante dans des cas de déficience en Zn ou des cas de toxicité au Cu, induisant une modification de la biodisponibilité de Cu et Zn (Hoffland et al., 2006; Nian et al., 2002). L'exsudation de ces composés organiques ne modifie pas seulement la quantité de la MOD dans la rhizosphère mais en modifie également les propriétés de complexation vis-à-vis des métaux. Le malate, le citrate et l'oxalate souvent retrouvés dans la rhizosphère peuvent former des complexes stables avec Cu^{2+} (Jones, 1998). Les plantes peuvent produire des phytosidérophores qui sont des ligands organiques (Parker et al., 2005) qui peuvent former des complexes avec les métaux (Dell'Mour et al., 2010). Cu semble présenter une affinité élevée pour les phytosidérophores produits par des graminées par rapport à Zn (Murakami et al., 1989). Dans le cas de Zn, Degryse et al. (2008) ont montré que des exsudats racinaires produits par de l'épinard (*Spinacia oleracea*) et de la tomate (*Lycopersicon esculentum*) ont une plus grande affinité vis-à-vis de Zn dans un cas de déficience en Zn dans le sol. Ainsi les plantes peuvent avoir un effet non négligeable dans la modification du pH et de la MOD dans la rhizosphère induisant des changements de la disponibilité de Cu et Zn dans la rhizosphère par rapport au bulk soil et de la biodisponibilité de Cu et Zn.

2.3.2 Biodisponibilité environnementale du cuivre et du zinc pour les vers de terre

Parmi les organismes du sol, les vers de terre (Annélides Oligochètes) représentent 80% de la biomasse vivante (Yasmin and D'Souza, 2010) dans les écosystèmes terrestres. Ils participent au phénomène de bioturbation en mélangeant les horizons de sols. Par leur activité dans les sols, les vers de terre forment des galeries et favorisent l'infiltration de l'eau (Bertrand et al., 2015) et l'aération des sols (Jégou et al., 2002). Par leur alimentation, ils agissent également sur la dégradation de la matière organique, et sont des acteurs essentiels de la dynamique de la matière organique dans le sol (Bertrand et al., 2015). Les déjections des vers de terre sont riches en nutriments qui sont potentiellement disponibles pour

les autres organismes du sol (e.g. plantes, microorganismes) (Blouin et al., 2013; Chaoui et al., 2003). Les vers de terre modifient ainsi la disponibilité des ressources pour les autres organismes dans un écosystème et sont donc qualifiés d'ingénieurs des écosystèmes (Jones et al., 1994). L'ingestion de sol et le contact épidermique permanent avec celui-ci les rendent susceptibles d'être affectés par les éléments traces et de les accumuler (Lanno et al., 2004). Les vers de terre sont ainsi reconnus pour être des bioindicateurs de la qualité des sols et de stress chimiques (Paoletti, 1999; Suthar et al., 2008).

Les vers de terre se répartissent en trois grandes catégories écologiques, les épigés, les anéciques et les endogés (Bouché, 1977), qui occupent différentes niches écologiques dans le sol et vont influencer différemment la disponibilité de Cu et Zn dans les sols. Dans une review de Sizmur et Hodson, (2009), il est précisé que l'espèce de vers de terre la plus souvent utilisée pour évaluer l'effet des vers de terre sur la disponibilité des métaux est *Eisenia fetida* (épigé). Cette espèce peut augmenter la disponibilité des métaux dans le sol. Comme pour les épigés, les études réalisées pour évaluer l'effet des anéciques sur la disponibilité des éléments traces ont montré que la présence de cette catégorie écologique dans des sols contaminés peut augmenter la disponibilité de ces éléments (e.g. Kızılkaya, 2004; Zorn et al., 2005a). En ce qui concerne les endogés, Coeurdassier et al. (2007) et Fritsch (2008) ont montré que la présence d'*Aporrectodea tuberculata* dans un sol contaminé augmentait la disponibilité des métaux tandis que Lukkari et al. (2006) et Zorn et al. (2005b) ont montré que *Aporrectodea caliginosa* et *Allobophora chlorotica* pouvaient diminuer la disponibilité des métaux.

La capacité des vers de terre à prélever et accumuler des métaux est bien documentée, en particulier dans des contextes de forte contamination en métaux. Dans une review de Nahmani et al. (2007), il est expliqué que certaines études montrent que la concentration en métaux dans les vers de terre augmente principalement avec la concentration totale du métal dans le sol (également observé par Leveque et al., 2013). Dans d'autres études, la teneur en métal dans les vers de terre a été reliée à la concentration totale de Cu et Zn dans le sol mais également à des propriétés du sol telles que le pH du sol, le pourcentage de matière organique, la capacité d'échange cationique. Néanmoins, ces paramètres du sol n'expliquent pas toujours complètement la concentration en Cu et Zn dans les vers de terre (e.g. Beyer et Cromartie, 1987; Van Vliet et al., 2005).

Crommentuijn et al. (1997) estiment qu'il est difficile de corréler la teneur en Cu et Zn chez les invertébrés au pH de la solution du sol, à la concentration de COD ou à la disponibilité des métaux dans la solution de sol du fait de différentes voies d'exposition des vers de terre aux métaux (i.e. voie tégumentaire et ingestion orale). Vijver et al. (2003) ont étudié, dans des sols contaminés en métaux, l'exposition par la voie tégumentaire des vers de terre (*Lumbricus rubellus*) en sellant la bouche des vers, et comparée à des vers dont la bouche n'avait pas été scellée. Les auteurs ont montré que la

concentration de Cu dans les vers avec la bouche scellée n'était pas significativement différente de celle dans les vers exposés à la bouche non scellée, tandis que la concentration de Zn dans les vers à la bouche scellée était significativement inférieure à celle des vers à la bouche non scellée. Les auteurs ont attribué la concentration interne de Cu dans les vers à la voie d'exposition tégumentaire alors que pour Zn, ils ont estimé qu'entre 20 et 30% de la concentration interne était due à l'ingestion du sol. Néanmoins, des études ont montré que les vers de terre influençaient la disponibilité des métaux via des modifications de pH et COD induites par leur activité. Cela entraîne alors des modifications en termes de biodisponibilité environnementale pour les vers de terre ou pour d'autres organismes comme les plantes (Sizmur et Hodson, 2009).

En particulier, les vers de terre peuvent augmenter le pH du sol du fait d'une sécrétion cutanée de mucus (Sizmur et Hodson, 2009). Dans un sol contaminé, Udovic et Lestan, (2007) ont montré une augmentation de pH du fait de la présence d'*Eisenia fetida* dans le milieu. Les auteurs n'ont pas rapporté d'augmentation de la concentration de Zn en solution dans les turricules d'*E. fetida* par rapport à la concentration de Zn dans la solution du sol. L'augmentation de pH pourrait avoir été contrebalancée par un autre phénomène susceptible de modifier la disponibilité. D'autres auteurs ont montré une augmentation de la disponibilité des métaux malgré l'augmentation du pH du sol du fait de la présence de vers de terre (Ma et al., 2002; Udovic et al., 2007; Wen et al., 2006). Cela suggère qu'en présence de vers, la modification du pH n'est pas le seul facteur impliqué dans l'évolution de la disponibilité des métaux.

L'activité des vers de terre dans le sol peut également augmenter le COD (Dandan et al., 2007; Wen et al., 2004). Dans leur étude, Wen et al. (2004) ont montré une augmentation de la concentration totale de Cu et Zn dans la solution de sol en présence de vers de terre (*Eisenia fetida*), corrélée à l'augmentation de COD. Sizmur et al. (2011) ont montré des concentrations en solution des éléments traces plus importantes dans les turricules de vers de terre mettant en évidence que le passage du sol dans le tube digestif des vers de terre pouvait être à l'origine de modification de la mobilité des éléments traces et de leur spéciation. Les auteurs ont suggéré que cela pourrait conduire à des modifications de la disponibilité des éléments traces dans le sol et de leur phytodisponibilité. De leur côté, Leveque et al. (2014) ont montré que *Lumbricus terrestris* et *A. caliginosa* peuvent augmenter la phytodisponibilité de Cu et Zn sans corrélation directe avec l'augmentation de la disponibilité des métaux dans la solution du sol mais en relation avec leur activité de bioturbation qui a entrainé une augmentation de la macroporosité du sol. Les mécanismes impliqués dans ces modifications de disponibilité et de biodisponibilité de Cu et Zn restent encore peu clairs. Il est pourtant important de comprendre les déterminants de l'influence de la présence de vers de terre, qui sont des organismes

indispensables au bon fonctionnement des sols, sur la (bio)disponibilité de Cu et Zn pour les vers de terre.

L'évaluation de la biodisponibilité de Cu et Zn pour les plantes et les vers de terre est ainsi décrite dans la littérature comme dépendante de la/des espèces et du/des sols étudiés et des modifications induites par les organismes eux-mêmes. De plus, la plupart des études présentées dans la partie 2.3 ont été réalisées sur un ou plusieurs sol(s) fortement contaminé(s) prélevé(s) sur le terrain ou contaminé(s) au laboratoire à des doses croissantes de contaminants (plusieurs fois à plusieurs dizaines de fois le fond géochimique). Ces résultats semblent peu généralisables à des sols étudiés en contexte de contamination en Cu et Zn plus modérée, comme c'est le cas avec l'apport de fertilisants organiques, et d'une façon plus générale pouvant induire des effets sublétaux pour les organismes vivants.

3. Influence de l'apport de fertilisants organiques sur la disponibilité du cuivre et du zinc

L'apport de FO (effluents d'élevage, résidus végétaux et boues de station d'épuration) sur les sols agricoles améliore les propriétés physico-chimiques des sols, contribue à la fertilisation en apportant les nutriments essentiels aux cultures et présente des intérêts économiques et aussi environnementaux en termes de gestion des déchets (Wassenaar et al., 2014). Cependant les FO font partie des sources de contamination en Cu et Zn dans sols agricoles (voir 2.1). L'apport répété de FO au cours du temps modifie les propriétés chimiques des sols tels que le pH et la quantité et les propriétés de complexation des matières organiques (voir 3.1.2 et 3.1.3), principaux déterminants de la disponibilité de Cu et Zn (voir 2.2). Evaluer a minima les modifications de ces deux paramètres doit ainsi permettre d'évaluer le risque environnemental lié à cette pratique en termes de disponibilité et de biodisponibilité de Cu et Zn pour différents organismes (i.e. plantes et vers de terre).

3.1 Modifications induites par l'apport de fertilisants organiques

3.1.1 Contamination du sol en cuivre et zinc

Si Cu et Zn sont majoritairement apportés par l'épandage de fertilisants organiques dans les sols agricoles (voir 2.1), l'apport continue de FO au cours du temps induira une augmentation des teneurs en Cu et Zn dans le sol (Guan et al., 2018). Certaines études suggèrent que l'écotoxicité de Cu et Zn pour les organismes liée aux apports de FO serait reliée au niveau de contamination en Cu et Zn induit par ces apports plutôt qu'aux modifications de la disponibilité de Cu et Zn via les modifications de pH et de MOD dans le sol qui ne sont pas prises en compte dans ces méthodes (Leclerc et Laurent, 2017;

Sydow et al., 2018). Cependant, Smolders et al. (2012) ont montré dans une étude portant sur des échantillons de sols provenant de 22 essais de terrain ayant reçu des apports de FO sur le long-terme, que la disponibilité de Cu était en moyenne diminuée d'un facteur 6 dans les sols ayant reçu des FO par rapport aux mêmes sols n'ayant pas reçu de FO mais contaminés artificiellement avec Cu^{2+} pour corriger le niveau de contamination. Les auteurs ont suggéré que la modification de la disponibilité de Cu dans les sols fertilisés avec des FO était due à une plus faible disponibilité initiale de Cu dans les FO. Néanmoins, cette étude n'a pas pris en compte les modifications de pH suite à l'apport de FO, pourtant observées, car les différences de pH entre les sols ayant reçu ou non des FO ont été corrigées au laboratoire préalablement aux mesures. D'autres études ont montré une modification de la disponibilité de Cu et Zn en lien avec les modifications de pH et de matière organique suite à l'apport de FO (e.g. Minnich et McBride, 1987; Pérez-Esteban et al., 2014). Il reste donc encore à éclaircir l'effet de la contamination en Cu et Zn sur la disponibilité de Cu et Zn, en lien avec les modifications de pH et de la matière organique du sol, afin de s'assurer de la durabilité de cette pratique agricole.

3.1.2 Modifications de pH

Suite à l'apport répété de FO dans le temps, des études ont montré une augmentation du pH (Haynes et Mokolobate, 2001; Vanden Nest et al., 2016). L'augmentation du pH induite par l'apport de fertilisants est attribuée à différents processus, et notamment à la décarboxylation des anions organiques lors de la minéralisation du carbone qui libère des ions OH^- et/ou à la protonation de molécules organiques qui consomment des ions H^+ (Mkhabela et Warman, 2005; Shi et al., 2019; Yan et al., 1996).

Dans la solution de sol, il a été montré que l'apport de fertilisants organiques (à court- ou long-terme) pouvait augmenter ou diminuer la concentration totale de Cu et Zn en solution (Cambier et al., 2014; Pérez-Esteban et al., 2014, 2012). Bien que ces études ne mettent pas en évidence une tendance générale de l'évolution de la concentration totale de Cu et Zn dans la solution du sol, la concentration totale de Zn dans la solution de sol était principalement déterminée par le pH de la solution du sol (De Conti et al., 2016; Pérez-Esteban et al., 2014) et il en était de même pour l'activité de Zn^{2+} (De Conti et al., 2016). En ce qui concerne la concentration totale de Cu en solution, Pérez-Esteban et al. (2014) ont montré que celle-ci était déterminée par le pH et la concentration de COD, eux-mêmes modifiés par l'apport de FO. La modification du pH, par l'apport de FO, est donc un facteur d'influence de la disponibilité de Cu et Zn mais n'est pas le seul à prendre en compte, notamment pour étudier la spéciation de Cu dans la solution du sol.

3.1.3 Modification des concentrations et de la nature des matières organiques

Dans le sol, l'apport de FO à long-terme augmente la quantité de carbone organique (Smolders et al., 2012; Vanden Nest et al., 2016) et la concentration de COD (Cambier et al., 2014; Seo et al., 2019). L'augmentation de COD dans la solution du sol est due en particulier à l'augmentation de pH qui favorise la solubilisation de la matière organique du sol (Bolan et al., 2011). L'aromaticité de la MOD, qui est un indicateur de la qualité de la MOD (évaluée par la mesure de l'absorbance spécifique à 254 nm) augmente également avec l'apport de FO. Araújo et al. (2019) ont montré pour des sols amendés pendant trois ans avec des FO que l'augmentation de la concentration totale de Cu dans la solution de sol est expliquée par l'augmentation de la concentration en COD et de l'aromaticité, ce qui a amené ces auteurs à suggérer que les groupements fonctionnels associés aux groupements aromatiques étaient plus complexants. Amery et al. (2008) ont en effet montré que la proportion de Cu lié à la MOD était corrélée positivement à l'aromaticité de la MOD indiquant ainsi que plus la matière organique est aromatique plus elle aura d'affinité pour Cu. Dans une étude où le lien entre la concentration totale de Cu dans la solution de sol et l'activité de Cu^{2+} a été évalué dans un sol amendé avec des boues, il a été montré une augmentation de la concentration totale de Cu en solution et une diminution de l'activité de Cu^{2+} avec l'augmentation de concentration de carbone organique du sol (Minnich et McBride, 1987). Les auteurs ont expliqué ces variations par la formation de complexes en solution entre Cu et MOD concomitante à l'augmentation du pH.

Les propriétés de complexation de la matière organique du sol vis-à-vis des métaux sont également modifiées par l'apport de FO sur le long-terme (i.e. densité de site et affinité) (Sebastia et al., 2007). Les auteurs ont montré dans un sol fertilisé pendant 15 ans avec de la paille ou un compost de résineux, une diminution de la densité de site mais une augmentation de l'affinité des acides fulviques (i.e. font partie des substances humiques de la matière organique du sol) vis-à-vis de Cu, comparé au sol non amendé. Au contraire, dans des sols amendés avec du lisier de porc et des boues de station d'épuration Plaza et al. (2006, 2005) ont montré une diminution à la fois de la densité de site et de l'affinité des substances humiques de la matière organique du sol (i.e. acides fulviques et humiques) vis-à-vis de Cu et Zn par rapport à des sols non-amendés. Plaza et al. (2006, 2005) ont donc suggéré que la disponibilité de Cu et Zn serait plus importante dans des sols amendés avec ce type de FO que dans des sols non-amendés. Cependant, dans ces études, l'évolution de pH et de la matière organique au cours du temps du fait de l'apport de FO n'a pas été prise en compte.

Les études présentées en 3.1 sur l'effet de l'apport de FO sur le court-(quelques mois), moyen-(quelques années) ou plus long-terme (une ou plusieurs décennies) montrent que le pH et la concentration et les propriétés de complexation de la MOD sont des facteurs d'influence de la

disponibilité de Cu et Zn. De plus, l'apport de FO modifie également la contamination de Cu et Zn. Néanmoins, l'absence de prise en compte simultanée du niveau de contamination et de l'évolution de pH et de la matière organique pose question quant aux effets respectifs de ces paramètres sur la disponibilité de Cu et Zn dans le contexte d'apport de FO. En effet, dépendant du/des facteur(s) déterminant la disponibilité de Cu et Zn, la durabilité de la pratique agricole d'épandage de FO sur les sols agricoles pourrait être remise en question. Il parait ainsi nécessaire de mener des études en tenant compte à la fois des modifications du niveau de contamination en Cu et Zn, et de pH et MOD.

3.2 Effet des fertilisants organiques sur la biodisponibilité du cuivre et du zinc pour les plantes et les vers de terre

3.2.1 Phytodisponibilité du cuivre et du zinc

Dans la partie 2.3.1 nous avons vu que la biodisponibilité de Cu et Zn pour les plantes pouvait augmenter ou diminuer selon le type de plante étudié mais surtout que les plantes pouvaient induire des modifications de pH et de MOD dans la rhizosphère modifiant ainsi la disponibilité et la biodisponibilité de Cu et Zn. En contexte d'apport de FO, Singh et al. (2010) ont montré une diminution de Cu et Zn dans des bettes (*Beta vulgaris* var All green) cultivées sur un sol amendé avec du fumier. Mais d'autres expériences ont montré une diminution, une augmentation ou pas de changement de la concentration de Cu et Zn dans des plantes cultivées sur des sols amendés avec des FO en comparaison avec des sols sans apports (Benedet et al., 2016; Wang et al., 2016; Zheljazkov et Warman, 2004). Cependant, dans ces études, il n'a pas été distingué l'importance de l'effet de l'apport de FO par rapport à l'effet de la plante sur les facteurs déterminants la disponibilité de Cu et Zn dans la rhizosphère. Les modifications de la teneur en Cu et Zn dans les plantes ont été attribuées uniquement à l'apport de FO et/ou à l'augmentation de la concentration totale de Cu et Zn dans les sols avec l'apport de FO, sans tenir compte des possibles facteurs confondants comme le pH ou la MOD. Dans une expérience en pot utilisant un sol collecté sur un essai expérimental et amendé pendant 11 ans avec des FO, Benedet et al. (2019) ont étudié la disponibilité de Cu et Zn (via la mesure de Cu et Zn totale dans la solution de sol) dans la rhizosphère et le bulk soil et la phytodisponibilité de Cu et Zn (via les mesures de concentration et teneur de Cu et Zn) pour l'avoine (*Avena sativa*). Les auteurs ont montré une augmentation de la disponibilité de Cu et Zn dans le sol, une augmentation de la concentration et de Cu et Zn dans les plantes (biodisponibilité environnementale) mais pas d'effet toxique sur les plantes (biodisponibilité toxicologique) suggérant une régulation de ces éléments dans la plante. Smolders et al. (2012) ont montré que, à même niveau de contamination, la toxicité de Cu pour l'orge (rhizotoxicité) était plus faible lorsque Cu est apporté sur le long-terme par l'apport de FO

que lorsque Cu est apporté artificiellement au laboratoire sous forme de sel soluble. Cependant, dans cette étude, l'évolution du pH n'a pas été prise en compte bien qu'elle puisse avoir un effet sur la disponibilité de Cu (cf. partie 3.1.2) et in fine sur sa biodisponibilité.

Dans les étude présentées ici, les relations entre les modifications de la (phyto)disponibilité de Cu et Zn et les modifications de pH et matière organique n'ont été que suggérées. De plus, l'importance de l'effet de l'apport de FO n'a pas toujours été distinguée de la bio-influence de la plante sur la disponibilité de Cu et Zn.

3.2.2 Biodisponibilité de Cu et Zn pour les vers de terre

En ce qui concerne la biodisponibilité de Cu et Zn pour les vers de terre en contexte d'apport de FO, Centofanti et al. (2016) ont montré que la concentration de Cu chez *Lumbricus terrestris* n'a pas augmenté dans un sol contaminé et fertilisé avec des FO par rapport au sol non fertilisé, après une exposition des vers au sol pendant 45 jours. La concentration en Zn dans les vers de terre exposé au sol fertilisé avec des FO était comparable aux teneurs mesurées chez *L. terrestris* exposé à des sols non contaminés ou forestier. Dans cette étude, les auteurs n'ont pas déterminé les facteurs expliquant la biodisponibilité de Cu et Zn pour les vers de terre. Kızılkaya, (2004) a montré que l'augmentation de la teneur en Cu et Zn chez *L. terrestris* est corrélée positivement à l'apport de boues d'épuration à des doses croissantes (de 0 à 800 t de boues ha^{-1} sol) après 30 jours d'exposition des vers en microcosmes (Fig. 5). Les auteurs ont montré que la biodisponibilité de Cu et Zn avait augmenté avec l'apport de FO du fait d'une augmentation de la concentration de Cu et Zn dans le sol avec l'apport de FO. Sizmur et al. (2011a) ont évalué l'effet de *L.terrestris* sur la disponibilité de Cu et Zn dans deux sols contaminés et fertilisés avec des FO, en conditions de laboratoire. Les auteurs ont montré qu'en l'absence de vers de terre, l'apport de FO a diminué les concentrations de Cu et Zn en solution. La présence de vers de terre a entraîné une augmentation de la concentration de Zn en solution et une diminution de la concentration de Cu en solution. Ces modifications de concentration de Cu et Zn en solution ont été reliées à l'impact des vers de terre sur le pH et le carbone organique soluble. Les auteurs ont également montré que bien que les vers de terre ont un effet sur la disponibilité de Cu et Zn en solution, l'effet des vers de terre diminue quand la dose de FO apportés au sol augmente.

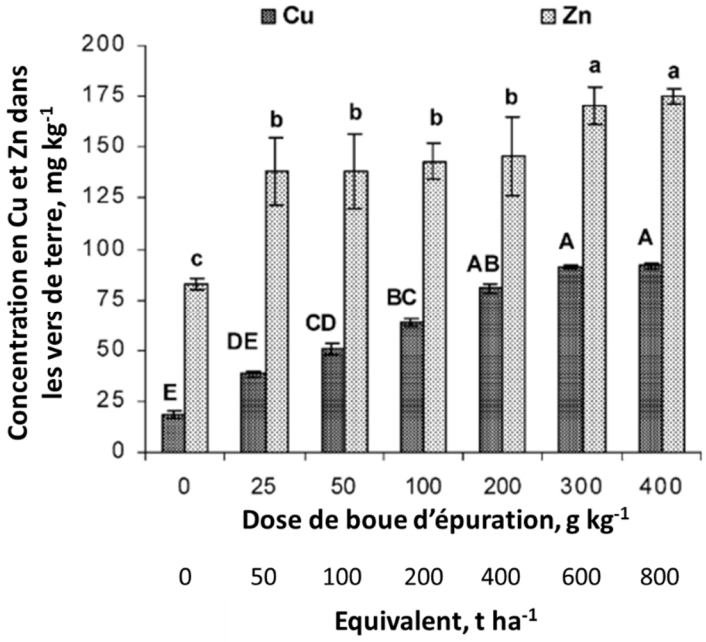

Figure 5: Teneurs en Cu et Zn dans *Lumbricus terrestris* en fonction de la dose de boue d'épuration appliquée. Des lettres majuscules et minuscules différentes indiquent une différence significative pour Cu et Zn, respectivement ($p < 0.01$) (d'après Kızılkaya, 2004).

Les vers de terre peuvent présenter des différences en termes de biodisponibilité de Cu et Zn en fonction de la catégorie écologique à laquelle ils appartiennent. Suthar et al. (2008) ont montré que *Metaphire posthuma* (endogé) accumulait plus les métaux que *Lampito mauritii* (anécique) dans des sols amendés avec des FO par rapport à des sols non amendés. Les auteurs suggèrent que la solution du sol serait plus retenue dans les galeries créées par les endogés (qui font des galeries d'orientation quelconque dans le sol), ce qui favoriserait la disponibilité des métaux pour cette catégorie écologique. Certaines espèces de vers de terre comme *L. terrestris* (anécique) possèdent une glande calcifère active, qui affecte les teneurs en métaux dans les tissus, comparé à des espèces endogées chez qui cette glande est non sécrétoire. Centofanti et al. (2016) ont suggéré que cela pouvait expliquer des teneurs en métaux plus faibles mesurées dans les tissus d'espèces anéciques par rapport à des espèces endogées. Cependant, moins d'études sur la biodisponibilité des métaux ont été réalisées en utilisant des espèces de vers endogés (Sizmur and Hodson, 2009 ; Blouin et al., 2013). Contrairement aux épigés et aux anéciques que l'on retrouve dans la litière à la surface du sol, car cela correspond à leur habitat et/ ou leur base alimentaire, les endogés vivent en permanence dans le sol (Sizmur and Hodson, 2009; Suthar et al., 2008). D'un point de vue écologique cette catégorie semble être la plus adaptée pour l'étude de la biodisponibilité pour les vers de terre en cas de contamination par les FO incorporés sur

les premiers centimètres du sol. De plus, les endogés sont couramment observés près des racines des plantes, ce qui renforce l'intérêt écologique des espèces de cette catégorie pour évaluer l'influence des vers de terre sur la disponibilité et la biodisponibilité des métaux pour d'autres organismes comme les plantes.

Dans la plupart de ces études, les FO ont été apportés sur les sols au laboratoire, les évolutions à long-terme de pH et MOD n'ont donc pas été prises en compte. De plus les effets observés sur la biodisponibilité de Cu et Zn pour sont en général aux doses les plus importantes qui ne sont pas toujours représentatives des doses appliquées au champ (e.g. Fig. 5). En effet, les doses généralement appliquées au champ se situent entre le témoin non fertilisé et la première dose apportée (50 t ha^{-1}) pour laquelle aucune augmentation significative de concentration de Cu dans les vers de terre n'est observée par rapport au sol non fertilisé. Enfin, dans ce contexte d'apport de FO, les relations entre modifications de la (bio)disponibilité de Cu et Zn et les modifications de pH et MOD qui peuvent être dues à l'apport de FO et/ou à l'influence des vers de terre n'ont été que suggérées, mais jamais vraiment démontrées.

4. Objectif et stratégie de recherche

L'apport de FO à long-terme modifie la contamination en Cu et Zn mais aussi les facteurs déterminants principalement la disponibilité de Cu et Zn dans les sols (i.e. pH et MO). Cependant, certaines études relient la disponibilité de Cu et Zn à la contamination du sol et peu d'études montrent un lien explicite entre modifications de pH et MOD et disponibilité de Cu et Zn lors de l'application de FO à long-terme. Si la contamination du sol en Cu et Zn est un facteur majeur de la détermination de la disponibilité de Cu et Zn, cela pourrait remettre en cause la durabilité de la valorisation des déchets d'origine organique par épandage sur les sols agricoles. Une autre hypothèse pourrait être que ce sont les modifications de pH et de MO induites par l'apport de FO qui vont être les principaux facteurs influençant la disponibilité de Cu et Zn. L'importance du niveau de contamination en Cu et Zn dans les sols fertilisés sur le long-terme avec des FO par rapport à l'effet des modifications de pH et MO au cours du temps sur la disponibilité de Cu et Zn reste encore à distinguer.

Les organismes comme les plantes et les vers de terre peuvent aussi influencer de façon substantielle les propriétés physico-chimiques du volume de sol avec lequel ils interagissent, induisant une modification de la (bio)disponibilité de Cu et Zn. De plus, ces modifications induites par l'apport de FO sont généralement observées sans faire de distinction entre le sol non bio-influencé et la zone bio-influencée par les vers de terre ou les plantes (la rhizosphère), c'est-à-dire deux zones bio-influencées au sein desquelles une modification substantielle de la disponibilité est susceptible d'être observée.

L'effet de l'organisme dans la zone bio-influencée pourrait ainsi s'ajouter ou s'opposer à l'effet de l'apport de FO à long-terme sur le pH et la MOD, résultant en une modification encore plus importante de la disponibilité et in fine de la biodisponibilité de Cu et Zn pour les organismes. Il apparait donc important dans le contexte d'apport de FO de distinguer les effets des FO sur la contamination du sol en Cu et Zn, sur la disponibilité et la biodisponibilité de Cu et Zn pour les plantes et les vers de terre, de celle que ces organismes peuvent avoir en retour, afin de s'assurer de la durabilité de cette pratique.

4.1 Objectif scientifique de la thèse

L'objectif de ma thèse est donc d'évaluer les effets respectifs (i) de la contamination du sol, (ii) de l'évolution du pH et de la MO du sol et (iii) des vers de terre et des plantes dans le volume de sol qu'ils sont susceptibles de bio-influencer, sur la disponibilité de Cu et Zn dans le sol et in fine leur biodisponibilité pour ces organismes en contexte d'apports de FO sur le long-terme.

Dans ce travail, nous avons choisi de travailler en deux temps :

Dans un premier temps, j'ai choisi de cibler le compartiment sol sans bio-influence, afin d'évaluer d'une part la disponibilité de Cu et Zn en solution suite à l'apport sur le long-terme de FO, et d'autre part de distinguer les effets dus à l'augmentation de la contamination de Cu et Zn (due aux apports) de ceux dus à des modifications des propriétés physico-chimiques du sol (pH et MOD) dans le processus de disponibilité.

Dans un second temps, j'ai choisi d'évaluer les effets des interactions sol-organismes, sur :

(i) la disponibilité de Cu et Zn dans la zone bio-influencée par les plantes (i.e. rhizosphère) et la biodisponibilité de Cu et Zn pour les plantes (i.e. phytodisponibilité) et ses déterminants ;

(ii) la disponibilité de Cu et Zn dans la zone bio-influencée par les vers de terre et la biodisponibilité de Cu et Zn pour les vers de terre et ses déterminants.

4.2 Stratégie expérimentale

Afin de répondre à l'objectif scientifique, trois études correspondant aux chapitres II et III du manuscrit ont été menées, portant sur la disponibilité (étude 1) et la biodisponibilité de Cu et Zn pour les vers de terre (étude 2) et les plantes (étude 3) en contexte d'apport de FO à long-terme (Fig. 6).

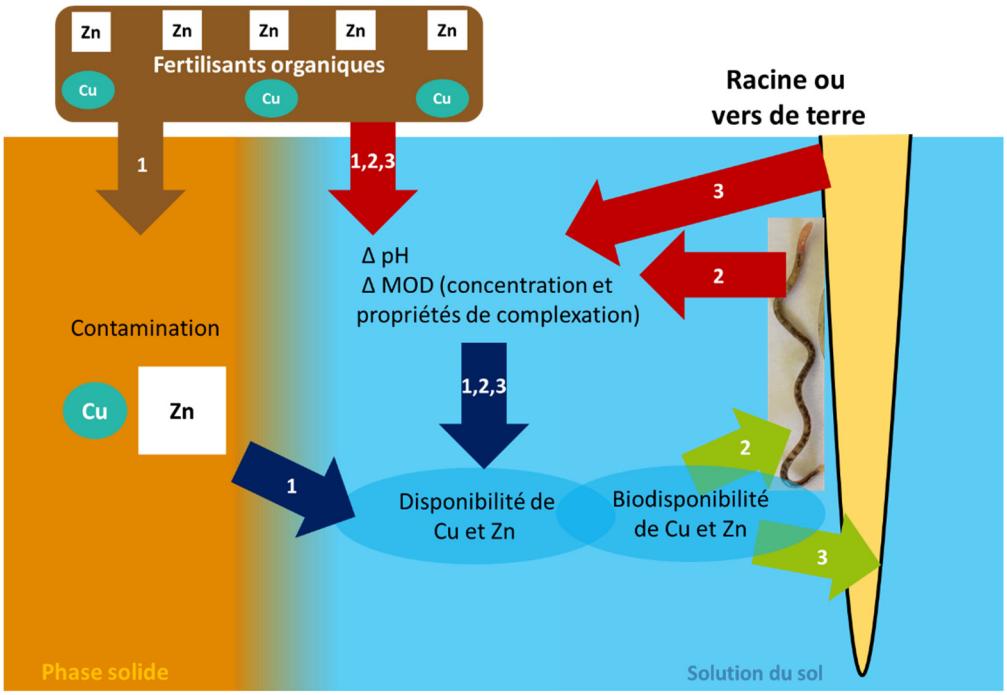

Figure 6 : Stratégie expérimentale de recherche. Les flèches représentent les modifications des phénomènes ciblés (variation de pH et MOD, (bio)disponibilité environnementale de Cu et Zn) qui seront étudiées dans les trois études et induites par différents agents (apport de FO, racines des plantes, vers de terre). Les chiffres dans les flèches indiquent les études (1 : disponibilité de Cu et Zn dans le sol non bio-influencé par les organismes ; 2 : biodisponibilité de Cu et Zn pour les vers de terre ; 3 : biodisponibilité de Cu et Zn pour les plantes) présentées dans la stratégie expérimentale.

Etude 1 : chapitre II

La première étude a pour but de distinguer les effets respectifs du niveau de contamination du sol et des modifications de pH et de la concentration et des propriétés de complexation de la MO sur la détermination de la disponibilité de Cu et Zn dans des sols ayant reçu des FO pendant une décennie. Pour explorer l'effet de l'apport à long-terme de FO sur les facteurs déterminant la disponibilité de Cu et Zn dans le sol, nous avons choisi de travailler sur des échantillons de sols issus d'une pédothèque de plus de 300 échantillons, et correspondant à trois essais agronomiques localisés sur l'île de La Réunion. Ces trois essais ont été établis sur trois types de sol qui sont représentatifs des sols cultivés sur l'île : un andosol, un cambisol et un arénosol. L'étude de différents types de sol permettra de voir si les effets des apports de FO sont dépendants du type de sol ou présentent des effets similaires entre les types de sol induisant une généricité des effets des FO. Ces essais ont reçus des historiques de fertilisations variés et ont été étudiés après un à huit ans d'apports. Afin d'avoir une gamme relativement étendue de propriétés physico-chimiques des sols, nous avons sélectionné un total de 74

échantillons de sols issus d'une base de données d'environ 320 échantillons de sols ayant reçu des fertilisants organiques, minéraux ou n'ayant pas été fertilisés pendant la durée des essais. La base de données regroupait entre autre les données de pH-H_20 des sols, les teneurs de C organique et les concentrations totales de Cu et Zn dans les sols. Sur la base des régressions empiriques de McBride et al. (1997) et Stephan et al. (2008) nous avons prédit pour chaque échantillon de sol l'activité de Cu^{2+} et de Zn^{2+}. Nous avons ensuite sélectionné les 74 échantillons en fonction du gradient de pH et de C organique pour représenter tous les types de fertilisations et différentes années de collecte depuis le début de l'essai pour un échantillon donné. Pour chaque échantillon, le pH, la concentration et l'aromaticité de la MOD ont été mesurées dans la solution du sol. Les propriétés de complexation de la MOD ont été évaluées avec un modèle géochimique (i.e. Windermere Humic Aqueous Model – WHAM-). La contamination des sols en Cu et Zn a été évaluée par la mesure de la concentration totale de Cu et Zn du sol. Le fond pédo-géochimique naturel des sols de La Réunion étant naturellement élevé (Dœlsch et al., 2006a, 2006b) nous avons aussi évalué le niveau de contamination en mesurant les concentrations dans le sol de Cu et Zn extractible au DTPA, attendant de cette méthode qu'elle soit plus sensible que la mesure de la teneur totale de Cu et Zn dans le sol. La disponibilité de Cu et Zn a été évaluée via trois proxys :

(i) la concentration totale de Cu et Zn dans la solution du sol ;

(ii) l'activité ionique libre de Cu et Zn dans la solution du sol ;

(iii) la mesure de Cu et Zn via la méthode DGT (diffusive gradient in thin film) directement sur les échantillons de sol.

L'utilisation de ces trois indicateurs permet d'avoir une vision plus globale de la disponibilité de Cu et Zn, ne prenant pas uniquement en compte l'équilibre chimique dans la solution du sol, mais tenant compte aussi du réapprovisionnement de la solution du sol en Cu et Zn par la phase solide.

Afin d'évaluer les facteurs déterminant la disponibilité de Cu et Zn nous avons expliqué chacun des proxys de la disponibilité avec les variables de la solution du sol (i.e. pH, concentration, aromaticité et propriétés de complexation de la MOD).

Etude 2 : chapitre III.1

La deuxième étude a pour but d'évaluer l'effet de l'apport à long-terme de FO sur (i) la disponibilité de Cu et Zn dans le volume de sol bio-influencé par les vers de terre et (ii) sur la biodisponibilité de Cu et Zn pour les vers de terre. Cette étude est basée sur un nombre plus restreint d'échantillons de sol par

rapport à l'étude 1 car à la période de l'année à laquelle nous les avons collectés, nous avons trouvé moins d'individus qu'attendu. De plus les échantillons provenant d'une pédothèque et utilisés par ailleurs pour d'autres projets, ne nous permettaient pas d'utiliser la totalité des quantités de sol à disposition. Cependant, nous nous sommes assurés que l'effet attendu des types de fertilisation sur les propriétés physico-chimiques du sol et la disponibilité de Cu et Zn était conservés. Pour cette expérience, nous avons utilisé une espèce de vers de terre endogée (*Dichogaster saliens*). Nous avons choisi cette espèce car elle est présente en milieu tropical et nous avons pu la trouver facilement dans deux des essais expérimentaux, dont l'essai conduit sur le cambisol. De plus la catégorie écologique de cette espèce parait intéressante pour l'évaluation de la biodisponibilité de Cu et Zn (voir 3.2.2). Dans une expérience en pot, nous avons exposé les vers de terre aux échantillons de sols présentant des modalités de fertilisations différentes. Nous avons mesuré le pH, la concentration et l'aromaticité de la MOD, les concentrations totales de Cu et Zn dans la solution du sol et l'activité ionique libre, après exposition des vers de terre aux sols. La biodisponibilité de Cu et Zn pour les vers de terre a été mesurée par la concentration totale de Cu et Zn dans les vers et exprimée en quantité de Cu et Zn dans les vers de terre, pour s'affranchir de l'effet de la biomasse des vers de terre. Nous avons expliqué la disponibilité de Cu et Zn par les variables de la solution du sol et les modifications de la détermination de la disponibilité de Cu et Zn pour isoler les paramètres les plus explicatifs. Nous avons également déterminé les facteurs de la solution du sol contrôlant la biodisponibilité de Cu et Zn pour *D.saliens*.

Etude 3 : chapitre III.2

La troisième étude a pour but d'évaluer l'effet de l'apport à long-terme de FO (i) sur la disponibilité de Cu et Zn dans la rhizosphère et (ii) sur la phytodisponibilité de Cu et Zn. Parmi les échantillons de l'étude 1, nous avons sélectionné un nombre restreint d'échantillons tout en s'assurant que l'effet attendu des types de fertilisation sur les propriétés physico-chimiques du sol et la disponibilité de Cu et Zn était conservé. Pour cette étude, le dispositif RHIZOtest a été utilisé afin d'évaluer la (phyto)disponibilité dans la rhizosphère. Ce dispositif expérimental normalisé pour la mesure de la phytodisponibilité des éléments traces (norme ISO-16198, 2015) permet un accès facilité à la rhizosphère par la séparation physique des racines et du sol, pour mesurer le pH, la concentration et l'aromaticité de la MOD, les concentrations totales de Cu et Zn dans la solution de sol et l'activité ionique libre. La phytodisponibilité environnementale de Cu et Zn a été déterminée par la mesure de la concentration totale de Cu et Zn dans les plantes entières (i.e. parties aériennes et racinaires) puis l'estimation du flux de Cu et Zn du sol vers la plante. La plante utilisée pour cette expérience est la fétuque (*Festuca arundinacea* cv Callina) qui est une espèce standard recommandée pour

l'expérimentation en RHIZOtest. Cette espèce est particulièrement intéressante pour l'étude de la bio-influence de la plante dans la rhizosphère car elle a montré des modifications de pH et de concentration et de propriétés de complexation de la MOD sur 55 sols (dont l'essai conduit sur le cambisol) (Djae, 2017).

Nous avons ensuite relié les deux indicateurs de la disponibilité de Cu et Zn aux variables de la solution de sol et évalué les modifications de la détermination de leur disponibilité. Nous avons également établi le lien entre la phytodisponibilité de Cu et Zn et les variables de la solution du sol contrôlant leur disponibilité.

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Chapitre II : Disponibilité du cuivre et du zinc dans le sol

L'article *Increased soil pH and dissolved organic matterafter a decade of organic fertilizer application mitigates copper and zinc availability despite contamination* (DOI : 10.1016/j.scitotenv.2019.135927), constitue ce chapitre II et a pour objectif de distinguer les effets respectifs du niveau de contamination du sol et des modifications de pH et de la concentration et des propriétés de complexation de la MO sur la détermination de la disponibilité de Cu et Zn dans des sols ayant reçu des FO pendant une décennie. Cet article a été publié dans le journal *Science of the Total Environment*.

Increased soil pH and dissolved organic matter after a decade of organic fertilizer application mitigates copper and zinc availability despite contamination

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Abstract

Long-term organic fertilizer (OF) application on agricultural soils is known to induce soil Cu and Zn contamination, along with pH and organic matter changes, which in turn alter the soil Cu and Zn availability. Our study was aimed at assessing Cu and Zn availability in long-term OF-amended soils by distinguishing the importance of increased contamination levels versus pH and organic matter changes in soil. Seventy-four soil samples were collected over time from fields corresponding to three soil types upon which no, mineral, or organic fertilization had been applied over a decade, and thus exhibited a gradient of Cu and Zn contamination, pH, and organic matter concentration. Soil Cu and Zn contamination (i.e. total and DTPA-extractable Cu and Zn concentration), soil solution chemistry (i.e. pH and dissolved organic matter concentration and aromaticity) and Cu and Zn availability (i.e. total concentration and free ionic activity in solution and DGT-available concentration in soil) levels were measured. The Windermere humic aqueous model (WHAM) was used to estimate Zn^{2+} activity and dissolved organic matter (DOM) binding properties in soil solution. Regardless of the soil type, organic fertilization increased Cu and Zn contamination in soil, in addition to the pH and the DOM concentration, aromaticity and binding properties in soil solution. The pH increase prompted a decrease in the total Zn concentration and Zn^{2+} activity in soil solution. The concomitant pH increase and DOM concentration, aromaticity and binding properties boosted the total Cu concentration but decreased the Cu^{2+} activity in soil solution. DGT-available Cu and Zn varied very little between the three fertilization modalities. Our results suggest that pH and DOM changes were able to regulate Cu and Zn availability in long-term OF amended soils by exerting a protective effect that offset the concomitant increase in soil Cu and Zn contamination.

Keywords: Ecotoxicity; Livestock residues; Sorption; Speciation; Trace elements

1. Introduction

The recycling of organic fertilizers (OF) in crop fields is known to improve the soil physical-chemical properties in the long-term, contribute to fertilization by bringing essential nutrients to crops, while offering economic and environmental benefits in terms of waste management. Despite these advantages, OF contain contaminants that lead to diffuse contamination of agricultural soils that have been repeatedly amended with OF over a long period (Wassenaar et al., 2014). Trace elements quantitatively appear to be the main type of contaminants affecting agricultural soils following OF amendment (Senesi et al., 1999). The fate of copper (Cu) and zinc (Zn) seems particularly worrisome as Cu and Zn are quantitatively the main trace elements involved in this soil contamination. Indeed, livestock feed is supplemented with Cu and Zn for their growth factor and antibiotic properties, so animal manure may contain high amounts of Cu and Zn (Jondreville et al., 2003). Furthermore, the estimated time necessary to increase the soil concentration from the natural background level to regulatory limits is shorter for Cu and Zn than for other trace elements (Belon et al., 2012; Luo et al., 2009; Nicholson et al., 2003).

The environmental risk related to trace element input in agricultural soils via long-term OF application was recently assessed by a life-cycle assessment (LCA) approach and the findings showed that Cu and Zn were the trace elements that most contributed to aquatic and terrestrial ecotoxicity (Leclerc and Laurent, 2017; Sydow et al., 2018). The country-specific ecotoxicological impacts calculated by LCA were closely correlated with the total mass of trace elements introduced in soils by OF application in the concerned country but not with the trace element availability. This suggests that the environmental risk would mainly be related to the Cu and Zn contamination level rather than to changes in Cu and Zn availability in amended soils. However, this conclusion contradicts the analytical research findings which highlighted substantial changes in Cu and Zn availability in soils following long-term OF amendment.

Depending on the analytical approach adopted, OF application was shown to alternatively decrease or increase Cu and Zn availability in amended soils compared to the corresponding non-amended soils contaminated to the same extent with soluble Cu and Zn salts. Smolders et al. (2012) found that Cu was on average 6-fold less available in OF-amended soils than in the corresponding non-amended soils freshly spiked with Cu^{2+}. By contrast, Plaza et al. (2006, 2005) found that the Cu and Zn binding properties of humic substances were lower in OF-amended soils than in the corresponding non-amended soils, suggesting that Cu and Zn availability would be higher in OF-amended soils than in non-amended soils independently of the soil contamination level. Note that in all of these studies: (i) pH was set at the same level, and (ii) the difference in the organic matter metal binding properties did not account for the higher organic matter concentration usually found in OF-amended soils compared to non-amended soils. Hence, temporal variations in pH and organic matter content and properties usually observed in soil following long-term OF amendment were not accounted for.

Long-term OF application usually alters pH and organic matter levels in soils (Hargreaves et al., 2008; Smolders et al., 2012), i.e. two soil properties that are widely known to substantially influence Cu and Zn availability. The increase in dissolved organic matter (DOM) concentration and aromaticity usually observed in OF-amended soils likely boosts the total Cu concentration in soil solution (Amery et al. 2008; Araujo et al. 2019), while decreasing the Cu^{2+} activity, with the concomitant effect of increasing the pH, as usually observed in OF-amended soils (Bravin et al., 2012; Minnich and McBride, 1987). Zinc availability has been investigated to a much lesser extent than Cu in OF-amended agricultural soils. However, contrary to Cu, Zn is known to have much lower affinity for DOM (Bonten et al., 2008) such that the total Zn concentration and Zn^{2+} activity in soil solution are mainly driven by soil pH rather than by the DOM concentration (Bonten et al., 2008). Accordingly, the pH increase usually observed in OF-amended soils likely decreases the total Zn concentration and Zn^{2+} activity in soil solution.

Note therefore that long-term OF application on agricultural soils not only increases the Cu and Zn contamination level but also changes the pH and DOM concentration and their Cu and Zn binding

properties. So far this issue has, however, only been studied by correlating Cu and Zn availability solely with the increased soil contamination level (e.g. Guan et al., 2018) or by assessing changes in Cu and Zn availability at a given soil contamination level (e.g. Smolders et al., 2012). Consequently, absolute changes in Cu and Zn availability due to long-term OF application on agricultural soils are still very difficult to infer in a comprehensive and mechanistic way.

Our study therefore aimed at comprehensively assessing Cu and Zn availability in soils that had been amended with OF over a decade, by unravelling the respective importance in soil of the increasing level of contamination versus the changes in pH and the concentration and binding properties of organic matter. Hence, 74 soil samples were collected from three field trials with different types of soil upon which no fertilizer, mineral fertilizers or OF had been applied over a decade. The soil samples exhibited a Cu and Zn contamination, pH and organic matter concentration gradient under these conditions. The soil Cu and Zn contamination level was assessed by measuring the total and DTPA-extractable Cu and Zn concentrations. The soil solution chemistry was characterized by measuring the pH and DOM concentration and aromaticity. Cu and Zn availability was comprehensively assessed through three proxies by deploying the diffusive gradient in thin film technique (DGT) on incubated soil samples and measuring the total Cu and Zn concentration and Cu^{2+} activity in soil solution. WHAM was used to estimate Zn^{2+} activity and DOM binding properties in soil solution.

2. Material and Methods

2.1. Soil and field trial characteristics

The three field trials studied were located on the island of Réunion in the Indian Ocean (55°30'E, 21°05'S). They were conducted on soils belonging to three reference soil groups (hereafter called soil type): andosol, andic cambisol, and arenosol (WRB, 2014). The characteristics of each field trial and soil type were previously outlined by Nobile et al. (2018). Considering the mineralogy of the three soil

types (Nobile et al. 2018), their ability to retain trace elements was expected to increase from the arenosol to the andosol. The main characteristics of the three soil types as a function of fertilization types are given in Table 1.

Table 1 Soil physical-chemical properties of the three soil types up to 8 years of either no fertilization, mineral fertilization, or organic fertilization. Mean values are given with their standard deviation in parentheses.

	Fertilization		pH-H_2O[a]	Organic C[b]	CEC	Total Cu[c]	Total Zn[c]
		Units		g/kg	$cmol_+/kg$	mg/kg	mg/kg
		n[d]					
Andosol	No fertilization	5	5.6 (± 0.3)	108 (± 6)	12 (± 2)	84 (± 3)	164 (± 10)
	Mineral fertilization	10	5.1 (± 0.4)	109 (± 9)	9.8 (± 5.1)	79 (± 6)	175 (± 16)
	Organic fertilization	19	6.2 (± 0.4)	121 (± 8)	23.1 (± 5.7)	84 (± 5)	187 (± 16)
Cambisol	Mineral fertilization	6	5.4 (± 0.2)	34 (± 2)	10.1 (± 0.1)[e]	81 (± 2)	242 (± 4)
	Organic fertilization	11	6.2 (± 0.5)	37 (± 3)	19.6 (± 2.7)[e]	85 (± 6)	261 (± 13)
Arenosol	No fertilization	4	6.7 (± 0.1)	10.3 (± 0.5)	5.2 (± 0.3)	73 (± 1)	136 (± 6)
	Mineral fertilization	4	5.9 (± 0.1)	10.4 (± 0.5)	3.7 (± 0.8)	69 (± 2)	134 (± 2)
	Organic fertilization	15	7.3 (± 0.2)	13.6 (± 2)	7.9 (± 1.3)	71 (± 2)	141 (± 5)

[a]Soil:liquid ratio 1:5

[b]NF ISO 10694

[c]NF ISO 14869-1

[d]n = number of soil samples

[e]Data from Djae et al. (2017), n = 2 for mineral fertilization and n = 4 for organic fertilization

The andosol and arenosol were under permanent grassland cover from 2004 to 2012. Plots were arranged in three randomized blocks and were either not fertilized or amended with mineral fertilizer (10 g N m^{-2} y^{-1}), dairy slurry (20 to 55 dm^3 m^{-2} y^{-1}), or dairy manure compost (2.5 to 5 kg m^{-2} y^{-1}) (Table S1; see Appendix A, Supplementary material). The cambisol was cultivated with two market garden crop cycles per year from 2004 to 2011. Plots were arranged in three randomized blocks and amended with mineral fertilizer (40 g N m^{-2} y^{-1}), pig slurry compost (5 kg m^{-2} y^{-1}), or poultry litter compost (7 kg m^{-2} y^{-1}) (Table S1). Soils were sampled once per year from the top soil layer, roughly corresponding to the fertilizer incorporation depth, i.e. 0-15 cm for the andosol and arenosol because there was no tillage and 0-30 cm for the ploughed cambisol. The studied organic fertilizers in the three

field trials were livestock effluents but the results obtained could also be extrapolated to other organic fertilizers (e.g. green waste compost, municipal organic waste, sewage sludge).

Amongst the yearly sampled soils, seventy-four soil samples (andosol, n = 34; cambisol, n = 17; arenosol, n = 23) were selected over time under all fertilization modalities in each field trial to ensure that the collected soil samples would exhibit a Cu and Zn contamination, pH and organic matter concentration gradient that could influence the Cu and Zn availability (Table 1 and Fig. S1). For each field trial, we selected one or more samples for each fertilization modality in each sampling year considered, to ensure that the gradients observed could not be attributed to the comparison of samples from different sampling year. All analytical determinations and modelling calculations were performed without replication on composite samples prepared with an equal amount of soil from the three blocks.

The time-cumulated rate of Cu and Zn inputs by mineral and OF were calculated ($F_{M\text{-}rate}$, mg Cu or Zn kg^{-1} soil) as follows:

$$F_{M-rate} = \frac{n.r.M}{dp.\rho} \quad (1)$$

where *n* is the number of years since the beginning of fertilization, *r* is the yearly rate of OF or mineral fertilizer applied (kg dry matter m^{-2} y^{-1}), *M* is the Cu or Zn concentration in the OF or mineral fertilizer (mg Cu or Zn kg^{-1} dry matter), *dp* is the soil depth (m), and ρ is the bulk soil density (kg m^{-3}).

2.2. Comprehensive framework of Cu and Zn contamination and availability in soils

The Cu and Zn input in soil due to anthropogenic activities and notably fertilizer applications, i.e. soil Cu and Zn contamination, was assessed by measuring the concentration of total and DTPA-extractable Cu and Zn in soil. As Réunion soils are known to exhibit a high natural pedo-geochemical background for Cu and Zn related to the composition of the soil parent material (Doelsch et al., 2006) and the availability of this soilborne Cu and Zn is considered to be low (Doelsch et al., 2008), we assumed that

the soil DTPA-extractable Cu and Zn concentration measurement would be more sensitive to cumulative inputs of Cu and Zn derived from organic fertilization than to the total soil Cu and Zn concentration.

The Cu and Zn availability, as defined by ISO (ISO 17402, 2008), was assessed with three proxies. DGT (DGT Research Ltd) was used to assess the re-supply of trace elements in soil solution from the solid phase (Zhang et al., 2004). The total Cu and Zn concentration in soil solution was measured to assess the size of the pool in which it is considered that soil organisms readily take up Cu and Zn. Cu^{2+} and Zn^{2+} activity in soil solution was measured to assess the size of the pool of the main (if not the sole) species of Cu and Zn that was considered to be taken up by soil organisms. Moreover, the total Cu and Zn concentration and Cu^{2+} and Zn^{2+} activity in soil solutions was used to explain DGT measurement variations.

2.3. DTPA extractions from soils

Soil extraction with diethylenetriaminepentaacetic acid (DTPA) was performed according to Lindsay and Norvell, (1978). Briefly, soil extractions were conducted with a solution composed of 5 mM DTPA, 10 mM $CaCl_2$ and 100 mM triethanolamine buffered at pH 7.3, with a soil:solution ratio of 1:2. The soil-solution mixture was stirred for 2 h and then centrifuged at 1000 *g*, and the supernatant was filtered with ashless medium speed filter paper with an 8 µm pore size. Solutions were analyzed with a flame atomic absorption spectrophotometer. The DTPA extraction procedure was validated by using an uncertified internal reference soil from Réunion when the extracted Cu and Zn recovery was within the 90-110 % range and the coefficient variation on the three replicated internal reference was within 10%.

2.4. DGT soil analysis

Each soil sample was analyzed with the DGT technique using devices equipped with a Chelex binding layer. Soils were incubated at 100% of their water holding capacity at 25°C for 24 h and the devices

were then deployed on the soil surface for 24 h. DGT devices were finally removed from the soil samples and the Chelex layer was eluted in 1.2 ml of 1 M HNO_3 for 24 h. Cu and Zn concentrations in eluates were determined by inductively coupled plasma-mass spectrometry (ICP-MS, ICAP Q, Thermo Scientific). DGT-available Cu and Zn was expressed as the Cu and Zn concentration at the interface between the soil and the DGT surface (C_{DGT}, mol l^{-1}) as follows:

$$C_{DGT} = \frac{m.\Delta g}{M.D.A.t} \quad (2)$$

where *m* is the mass of Cu or Zn accumulated at the resin surface (g), *Δg* is the thickness of the diffusive layer plus filter paper (9.2 10^{-3} dm), *M* is the molar mass of Cu or Zn (g mol^{-1}), *D* is the diffusion coefficient of Cu or Zn (dm^2 s^{-1}), *A* is the active surface area of the DGT device (3.14 10^{-2} dm^2) and *t* is the soil-DGT contact time (s). Eight DGT controls were eluted without any soil exposure to assess the mean Cu and Zn background concentration in the DGT resins.

2.5. Soil solution extraction and analysis

The solution of each soil sample was extracted with 2.5 mM $Ca(NO_3)_2$ and 3.5 mM KNO_3 and analyzed as described by Djae et al. (2017). Each soil solution was subdivided and subsamples were stabilized with either NaN_3 to avoid a shift in metal speciation while preventing DOM degradation for measurement of Cu^{2+} activity and the specific ultraviolet absorbance (SUVA) at 254 nm (A_{254}) or HNO_3 for measurement of the dissolved organic carbon (DOC) and total major cation concentration. Total concentrations of major and trace cations (Ca, Mg, K, Na, Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, and Zn) were determined by ICP-MS. A solid-solution partitioning coefficient (K_D in l kg^{-1}) for Cu and Zn (K_{DCu} and K_{DZn}) was then calculated according to Sauvé et al. (2000):

$$K_D = \frac{M_{DTPA}}{M_{solution}} \quad (3)$$

where M_{DTPA} is the DTPA-extractable Cu or Zn concentration from soil (mg kg^{-1}) and $M_{solution}$ is the total Cu or Zn concentration in the soil solution (mg l^{-1}).

The DOC concentration was determined using a TOC analyzer (Schimadzu, TOC-L). The SUVA at 254 nm was measured by UV-visible spectrometry (Spectronic Unicam, Helios Beta). The SUVA of DOM (l g^{-1} cm^{-1}) was calculated as follows to estimate the DOM aromaticity (Weishaar et al., 2003):

$$SUVA = \frac{A_{254}}{b.DOC} \quad (4)$$

where A_{254} is dimensionless, b is the path length (1 cm) and DOC is the DOC concentration (g l^{-1}).
The pH was measured in parallel to pCu^{2+} (i.e. $-\log_{10}\{Cu^{2+}\}$) using a combined electrode (Radiometer analytical PHC2051-8). Free Cu activity was measured using a Cu^{2+} specific electrode (Thermo Scientific Orion, 9629BNWP).

2.6. Modeling Cu^{2+} and Zn^{2+} activity

Free Cu and Zn activity in each soil solution was predicted using the humic ion-binding model VII included in WHAM (Tipping et al., 2011), as already described by Djae et al. (2017), to estimate the Zn^{2+} activity and compare the experimental Cu^{2+} with theoretical calculations to assess the impact of the variability in DOM binding properties on the determination of Cu and Zn speciation in soil solution. Free Cu and Zn activities were predicted by three approaches, and for each of these the deviation between predicted and measured Cu^{2+} values was determined by calculating the regression slope and coefficient as well as the root mean square residual (RMSR). Free Cu and Zn activity were first predicted as commonly described in the literature with the WHAM default settings (Tipping et al., 2011). Accordingly, the DOM site density (hereafter referred to as *%r-DOM*) was fixed by considering that 65% of DOM was able to bind metal cations with the same binding constants as the colloidal fulvic acid defined in WHAM (e.g. $\log K_{Cu}$ and $\log K_{Zn}$, of 2.16 and 1.68, respectively), with the remaining 35% of DOM being inert. Free Cu and Zn activities were also predicted by optimizing the *%r-DOM* by relating it to the SUVA values of DOM, as previously suggested by Amery et al. (2008). Free Cu activity was

finally predicted by adjusting *%r-DOM* and $\log K_{Cu}$ to within physically meaningful value ranges, i.e. respectively 35-215% and 1.84-2.46, as previously suggested by Djae et al. (2017). Free Zn activity was then calculated on the basis of Cu^{2+} prediction using the same adjusted *%r-DOM*, while $\log K_{Zn}$ remained at the default value. The uncertainty in predicted pCu^{2+} and pZn^{2+} (i.e. ± 2 standard deviation) resulting from analytical uncertainties regarding the pH (± 0.2), total Cu concentration (± 0.4%), total Zn concentration (± 1.1%) and DOM concentration (± 12 and 40% for the first and second modelling approaches, respectively) was calculated using the Monte Carlo procedure (1999 simulations/sample) included in WHAM.

2.7. Data processing and analysis

The whole dataset is accessible via Dataverse (Laurent et al., 2019). All statistical analyses were performed with R software (R Core Team 2016). Differences were considered statistically significant if $p < 0.05$. The effects of the fertilization types (none, mineral, or organic) on the soil variables (i.e. pH, organic carbon, total Cu and Zn concentrations, and DTPA-extractable Cu and Zn) were assessed for each soil by non-parametric tests, i.e. the Kruskal-Wallis rank test and the Dunn post-hoc multiple test comparison, as the residual normality and homoscedasticity conditions were not satisfied. The effects of soil (andosol, cambisol or arenosol) or fertilization types on soil DGT and solution values were assessed by performing either a one-way ANOVA and Tukey HSD post-hoc multiple comparison test when residual normality and homoscedasticity conditions were satisfied, or non-parametric rank tests (the same as above) when the conditions were not satisfied.

Principal components analysis (PCA) was used to visually illustrate the respective effects of soil and fertilization types on DTPA-extractable Cu and Zn concentration, the solution chemistry and Cu and Zn availability in soils without accounting for pH and organic carbon content which were strongly correlated to soil solution pH and DOC concentration. PCA was performed with FactoMineR, factoextra and missMDA packages. Multi-linear regressions were then used to mathematically characterize and hierarchize the respective contributions of pH, DOC, SUVA, DTPA-extractable Cu and Zn concentration

in soils, and K_D on the three proxies of Cu and Zn availability (i.e. DGT measurement in soil as well as the total concentration and free ionic activity in soil solution). Multi-linear regressions were performed using two R packages (olsrr and MASS), thereby combining two approaches based on stepwise regressions and the Akaike information criteria.

3. Results and Discussion

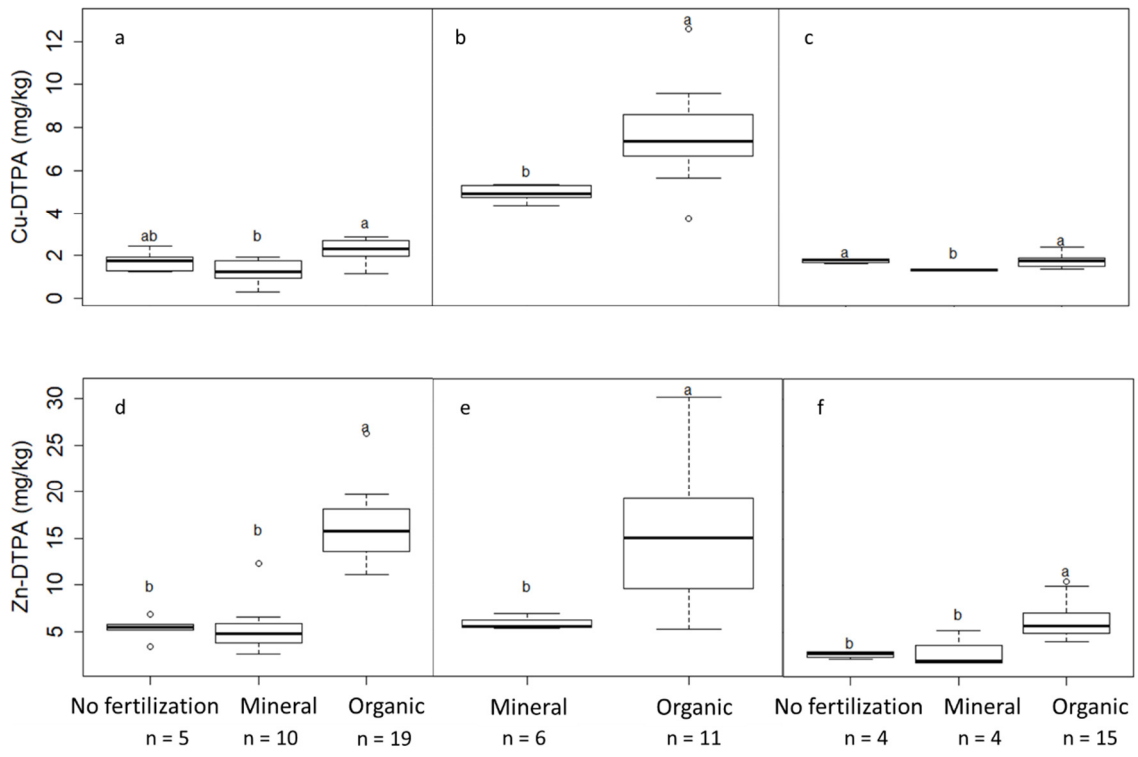
3.1 Organic fertilization over a decade induces copper and zinc contamination in soil

While soil Cu and Zn inputs due to organic fertilization were much higher than those due to mineral fertilization or without any fertilization (Fig. S2), total Cu and Zn concentrations in soils receiving organic fertilization did not clearly increase compared to non-fertilized soils and soil receiving mineral fertilization (Table 1). This could first be explained by the fact that the initial total Cu and Zn contents in the three soils were high (Table 1) and even about 5-fold higher than the cumulative Cu and Zn input due to organic fertilization over a decade in the arenosol for Cu and in the cambisol for Cu and Zn. Réunion soils are indeed known to exhibit a high natural pedo-geochemical background for Cu and Zn (Dœlsch et al., 2006). This could also be explained by the high Cu and Zn loss from the upper soil layer due to erosion and leaching, particularly in the andosol and arenosol in which OF were not mechanically incorporated. The magnitude of these processes was not determined, but the yearly rainfall is known to be high under the tropical climatic conditions that prevail in Réunion, ranging from 1 000 to 2 000 mm on average (Météo France, 2017), which could partly flush away any OF not incorporated in the soil. The cumulative Cu and Zn input due to organic fertilization that actually remained in the soil was thus not sufficient to induce a clear increase in the total soil Cu and Zn concentration.

As soilborne Cu and Zn availability is considered to be low in Réunion soils (Doelsch et al., 2008), we assumed that the DTPA-extractable Cu and Zn concentration, which represented less than 10 % of the total Cu and Zn, would be more sensitive to cumulative inputs of OF-derived Cu and Zn in soil than the

total Cu and Zn concentration. In agreement, OF significantly increased the DTPA-extractable Cu and Zn concentration in soils compared to the non-fertilized treatment or to mineral fertilization (Fig. 1). This result is consistent with the expected moderate but chronic Cu and Zn contamination of soils under OF and this pattern could be better highlighted through DTPA extraction than by measuring the total concentration.

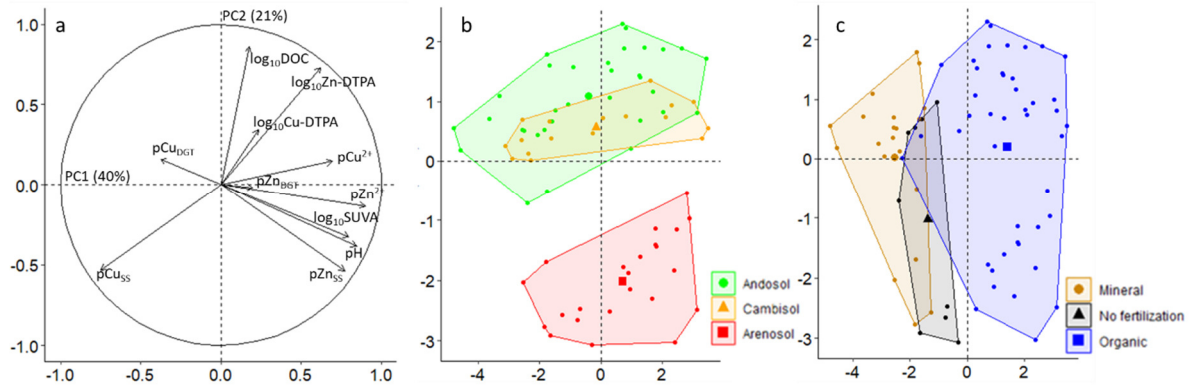
Figure 1 DTPA-extractable concentration of copper (Cu) and zinc (Zn) in the andosol (a and d), the cambisol (b and e) and the arenosol (c and f) as a function of the fertilization type (No fertilization, Mineral or Organic). Different letters indicate a significant difference at the 0.05 level. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5-times the interquartile range above the third quartile or below the first quartile.



3.2 Soil and fertilization types substantially but differently influence the solution chemistry and copper and zinc availability in soils

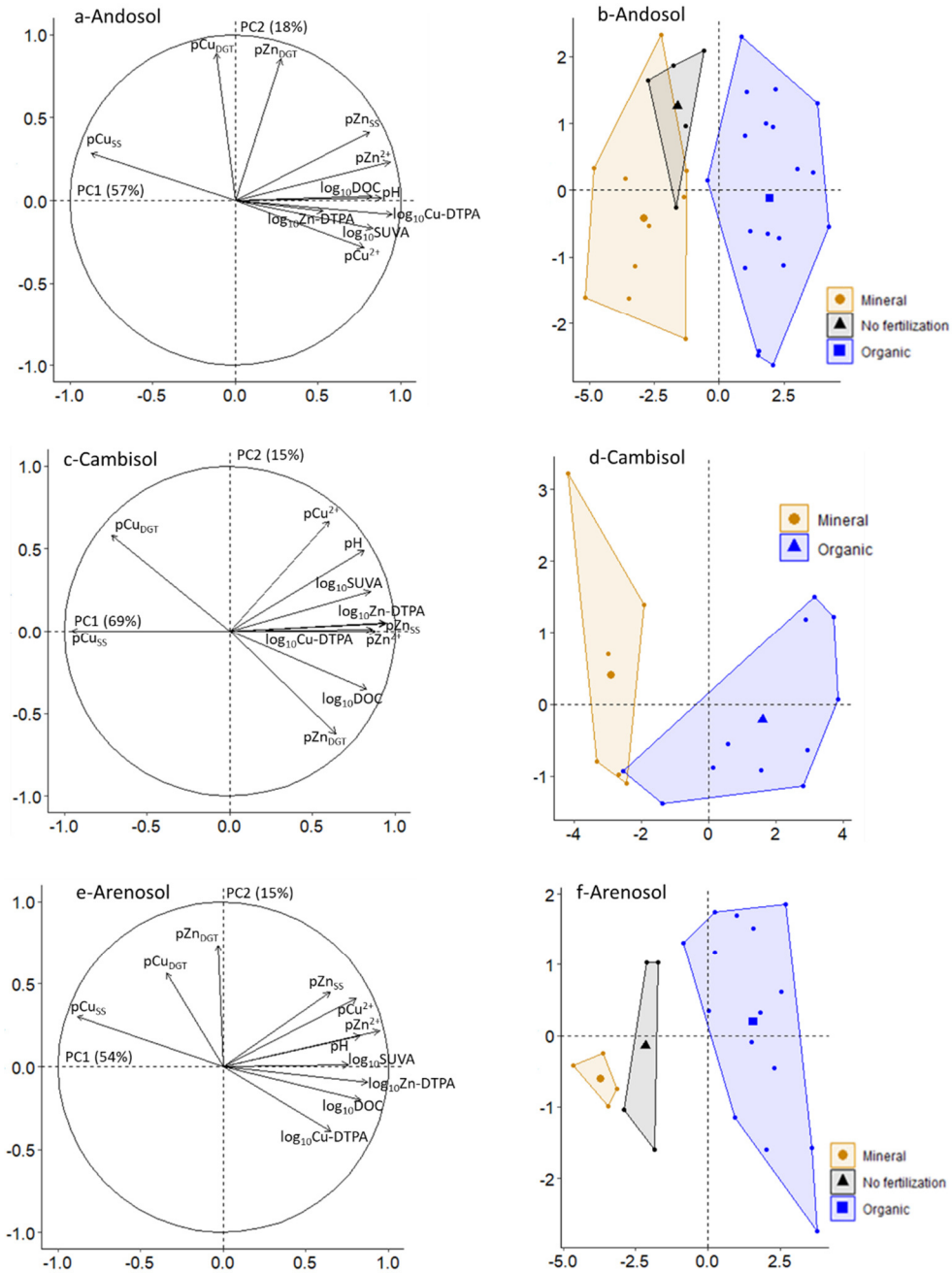
Soil and fertilization types independently structured the distribution of the dataset for the soil solution chemistry and Cu and Zn availability proxies in the PCA (Fig. 2). The first component (PC1, 40% of the explained variance) was mainly determined via the pH, pZn_{ss}, pZn^{2+} and SUVA findings, the second component (PC2, 21% of the explained variance) was mainly determined by DOC and Zn-DTPA, while pCu_{ss}, pCu^{2+} and pZn_{SS} contributed to both PC1 and PC2 (Fig. 2a). However, pCu_{DGT}, Cu-DTPA and even more so pZn_{DGT} contributed very little to PC1 and PC2, in accordance with their small variation ranges. The soil types were clearly dissociated in two clusters along the PC2 (Fig. 2b). This clustering according to soil type was related to the DOC concentration ranges, which significantly increased from 9-30 mg l^{-1} in the arenosol to 23-107 mg l^{-1} in the andosol (Fig. S3b) and similar Zn-DTPA variation ranges in the andosol and cambisol compared to the arenosol (Fig. 1). Conversely, the mineral and organic fertilization types were dissociated in two independent clusters along the PC1 with the no fertilization modality in between (Fig. 2c). Accordingly, these results were consistent with the fact that the soil and fertilization types both had a substantial but different influence on the solution chemistry and Cu and Zn availability proxies in the soils.

Figure 2 Principal component analysis (PCA) of the dataset on the soil solution chemistry and copper (Cu) and zinc (Zn) contamination and availability proxies in soils. The correlation plot (a) shows the contribution of each variable to the two first principal components (PC1 and PC2). Soil samples (n = 74) were clustered (b) as a function of soil types (andosol, cambisol, and arenosol) or (c) as function of fertilization types (No fertilization, Mineral, or Organic). Data points with a larger size in (b) and (c) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, the dissolved organic carbon concentration ($\log_{10}$DOC), the specific UV-absorbance of the soil solutions at 254 nm ($\log_{10}$SUVA), the DTPA-extractable Cu and Zn concentration ($\log_{10}$Cu-DTPA and $\log_{10}$Zn-DTPA), the DGT-available Cu and Zn concentration (pCu_{DGT} and pZn_{DGT}), the total Cu and Zn concentration in soil solution (pCu_{SS} and pZn_{SS}), and the free ionic Cu and Zn activity in soil solution (pCu^{2+} and pZn^{2+}).



By performing PCA separately on each soil type, the effects of the fertilization types on the solution chemistry and on the Cu and Zn availability proxies in soils were even more clearcut (Fig. 3). Both PC1 and PC2 explained 75%, 84% and 69% of the variance for the andosol, cambisol and arenosol, respectively (Fig. 3a, c, and e). Regardless of the soil type, PC1 was mainly determined via the pH, DOC, SUVA, Cu-DTPA, Zn-DTPA, pCu_{SS}, pZn_{SS}, and pZn^{2+} findings, while pCu_{DGT}, pZn_{DGT} and pCu^{2+} contributed to PC1 or PC2, or to both depending on the soil type. For each soil, the fertilization types were clearly dissociated in two (Fig. 3d) or three (Fig. 3b and f) clusters along the PC1 axis. These results highlight the substantial and similar relative influence of the fertilization type on the solution chemistry and Cu and Zn availability proxies in all of the soils, with a clear distinction between mineral and organic fertilization.

Figure 3 Principal component analysis (PCA) on the soil solution chemistry and copper (Cu) and zinc (Zn) contamination and availability proxies in (a) the andosol (a and b; n = 34), (b) the cambisol (c and d; n = 17) and (c) the arenosol (e and f; n = 23). The correlation plots (a, c, and e) show the contribution of each variable to the two principal components (PC1 and PC2). Soil samples were clustered (b, d, and f) as a function of fertilization types (No fertilization, Mineral, or Organic). Data points with a larger size in (b), (d), and (f) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($\log_{10}$DOC), the specific UV-absorbance of the soil solutions at 254 nm ($\log_{10}$SUVA), the DTPA-extractable Cu and Zn concentration ($\log_{10}$Cu-DTPA and $\log_{10}$Zn-DTPA), the DGT-available Cu and Zn concentration (pCu_{DGT} and pZn_{DGT}), the total Cu and Zn concentration in soil solution (pCu_{SS} and pZn_{SS}), and the free ionic Cu and Zn activity in soil solution (pCu^{2+} and pZn^{2+}).



3.3 The increase in pH and DOM induced by organic fertilization mitigates the copper and zinc availability in soil

Organic fertilization significantly increased the soil solution pH for the three soil types compared to mineral fertilization (Table 1; Figs. S4a, S5a and S6a). The soil solution pH increased by up to 0.7 u (unit) with OF compared to no fertilization in the andosol and arenosol, and also compared to mineral fertilization in the cambisol. By contrast, the soil solution pH decreased by up to 0.4 u with mineral fertilization compared to no fertilization in the andosol and arenosol. This increase in pH after long-term OF application has already been reported (Haynes and Mokolobate, 2001; Vanden Nest et al., 2016) and was attributed to a range of processes, notably including the decarboxylation of organic anions during organic C mineralization that releases OH^- and the protonation of organic molecules that consume H^+ (Mkhabela and Warman, 2005; Shi et al., 2019; Yan et al., 1996). A similar decrease in pH with long-term NH_4NO_3 application has also been previously reported (Stroia et al., 2011) and attributed to the production of protons associated with NH_4^+ nitrification that was not completely balanced by OH^- production associated with the incomplete NO_3^- consumption by plants. Accordingly, a decade of organic fertilization strongly alkalinized the soil, while mineral N fertilization tended to acidify the soil.

Organic fertilization increased the soil organic C concentration (Table 1) as well as (by almost two-fold) the DOC concentration and SUVA in the soil solution for the three soil types compared to mineral or no fertilization (Figs. S4b&c, S5b&c, and S6b&c). The increase in DOC concentration following OF application was consistent with previous findings (Cambier et al., 2014; Seo et al., 2018; Wright et al., 2008) and was attributed to the input of organic matter whose solubilization was further favored by the increase in soil pH (Bolan et al., 2011). Araújo et al. (2019) similarly found an increase in SUVA in the solution of a soil that had received dairy manure amendments for 3 years, which suggests an increase in DOM aromaticity. We thus conclude that a decade of organic fertilization increased the soil DOM concentration and aromaticity.

The total Cu concentration in soil solution significantly increased in the three soil types with organic fertilization compared to no fertilization and/or mineral fertilization, but the total Zn concentration in soil solution significantly decreased with organic fertilization (Figs. S4d&g, S5d&g and S6d&g). These two distinct effects could be explained by the fact that the main chemical parameters driving the total Cu and Zn concentration in soil solution differed for Cu and Zn (Table 2). The increase in total Cu concentration in soil solution was mainly driven by the increased DOC concentration and SUVA. Conversely, the decrease in total Zn concentration in soil solution was mainly driven by the increase in the soil solution pH. DTPA-extractable Cu and Zn in soil was only a secondary variable that could explain the variation in the total Cu and Zn concentration in soil solution, except for Zn in the cambisol. In this latter soil, DTPA-extractable Zn was the sole explanatory variable of the total Zn in solution, which had surprisingly decreased as the DTPA-extractable Zn increased. This negative correlation between the DTPA-extractable Zn and the total Zn in solution could, however, be explained by the indirect effect of pH. The concomitant increase in the Zn contamination and pH levels in soil under organic fertilization increased Zn sorption to the soil solid phase and consequently increased the DTPA-extractable Zn, while decreasing the total Zn concentration in soil solution.

Table 2 Multiple linear regression explaining the total copper (Cu) and zinc (Zn) concentration in soil solution (pCu_{SS} and pZn_{SS}) as a function of the DTPA-extractable Cu or Zn concentration in soil ($\log_{10}M_{DTPA}$), pH, $\log_{10}$ of the dissolved organic carbon concentration ($\log_{10}DOC$), and $\log_{10}$ of the specific UV absorbance of soil solutions at 254 nm ($\log_{10}SUVA$) in the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		$\log_{10}M_{DTPA}$	pH	$\log_{10}DOC$	$\log_{10}SUVA$	i	adj R^2[a]	n[b]
pCu_{SS}	3 soils	-0.3^3	ns	-0.41^1	-0.61^2	8.4	0.8***	73
	Andosol	ns	-0.19^2	-0.95^1	ns	8.7	0.78***	34
	Cambisol	ns	ns	-0.55^2	-0.95^1	8.6	0.9***	16
	Arenosol	ns	ns	-1.01^1	-0.28^2	8.4	0.87***	23
pZn_{SS}	3 soils	-0.15^3	0.68^1	ns	0.41^2	2.1	0.68***	73
	Andosol	ns	0.63^1	ns	ns	2.6	0.58***	33
	Cambisol	0.86^1	ns	ns	ns	5.6	0.52***	17
	Arenosol	ns	0.66^1	ns	ns	2.6	0.57***	23

[a]adj R^2 = adjusted R^2 ; [b]n = number of samples

The effects of organic fertilization observed on the total Cu and Zn concentration in soil solution were generally consistent with previously reported results. While some authors have reported that the Cu and Zn concentration in soil solution increased with organic fertilization, they found that the total Cu concentration in soil solution was mainly driven by the increase in DOC concentration, while the total Zn concentration was mainly driven by the soil solution pH (Cambier et al., 2014; De Conti et al., 2016; Pérez-Esteban et al., 2012). Araújo et al. (2019) also found a positive correlation between the total Cu concentration in soil solution and SUVA in a soil after dairy manure application for 3 years. The well-known high affinity of Cu for DOM could explain why the Cu release in the soil solution with organic fertilization was mainly driven by complexation with DOM, which depends on the DOM concentration and aromaticity, despite the concomitant increase in soil solution pH that increased the binding capacity of the soil solid phase for Cu. Conversely, the lower affinity of Zn for DOM as compared to Cu could explain why Zn removal from the soil solution with organic fertilization continued to be driven by deprotonation of the soil solid phase as the soil solution pH increased. This explanation is in line with our results (Fig. S7) and those found in the literature (Bonten et al., 2008), showing that Cu was almost completely complexed with DOM in soil solution, while Zn occurred mainly as Zn^{2+}.

Cu^{2+} and Zn^{2+} activity similarly decreased in the solutions of the three soil types with organic fertilization compared to no fertilization and mineral fertilization (Figs. S4e&h, S5e&h and S6e&h). The decrease in Cu^{2+} and Zn^{2+} activity in soil solution was mainly driven by the decrease in soil solution pH, either directly for Cu^{2+} or indirectly for Zn^{2+}. Indeed, Zn^{2+} activity was mainly driven by total Zn in soil solution, which itself was mainly driven by the soil solution pH (Tables 2 and 3). A similar decrease in Cu^{2+} and Zn^{2+} activity with increasing pH has already been reported (Bravin et al., 2012; Ma et al., 2006; Vulkan et al., 2000), including in the organic fertilization context (De Conti et al., 2016). The deprotonation of DOM and of the soil solid phase with increasing soil solution pH under organic fertilization could likely explain the decrease in Cu^{2+} and Zn^{2+} activity, respectively.

Table 3 Multiple linear regression explaining the activity of free ionic Cu and Zn in soil solution (pCu^{2+} and pZn^{2+}) as a function of the pH, $log_{10}DOC$, $log_{10}SUVA$, and pCu_{SS} or pZn_{SS} in soil solution (pM_{SS}) in the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		pH	$log_{10}DOC$	$log_{10}SUVA$	pM_{SS}	i	adj R^2[a]	n[b]
pCu^{2+}	3 soils	1.98^1	1.51^2	ns	ns	-5.3	0.64***	74
	Andosol	1.6^1	1.64^2	ns	ns	ns	0.77***	34
	Cambisol	2.85^1	ns	ns	ns	-8.8	0.75***	17
	Arenosol	3.03^1	ns	ns	ns	-10.2	0.5***	23
Predicted pZn^{2+}	3 soils	0.33^3	0.68^2	ns	1.14^1	-3.5	0.97***	71
	Andosol	0.41^3	0.68^2	ns	1.20^1	-4.3	0.98***	31
	Cambisol	0.21^2	ns	0.27^3	1.18^1	-2.4	0.99***	17
	Arenosol	0.3^3	0.6^2	ns	0.92^1	-1.6	0.99***	23

[a]adj R^2 = adjusted R^2; [b]n = number of samples

DGT-available Cu and Zn did not significantly change when comparing the different fertilization types in the andosol and arenosol (Figs. S4f&i an d S6f&i). In the cambisol, however, DGT-available Cu increased with organic fertilization compared to mineral fertilization, while DGT-available Zn decreased (Fig. S5f&i). No physical-chemical parameter could significantly explain the DGT-available Cu and Zn, except in the cambisol, where the increase in DGT-available Cu concentration was driven by the increase in total Cu concentration in soil solution and the decrease in SUVA (Table S2).

The measurement of DGT-available metals in soil includes: (i) equilibrium processes that govern the solid-solution partitioning of available metals (K_D), and (ii) kinetic processes that govern metal lability (Degryse et al., 2009). DGT-available Cu and Zn was reported to increase in soils receiving long-term OF applications (Grüter et al., 2017; Guan et al., 2018). In both studies, the substantial increase in the total Cu and Zn concentration in soils likely increased the corresponding K_D and consequently drove the increase in DGT-available Cu and Zn. In our study, K_D was not significantly correlated with DGT-available Cu and Zn (Table S2). Accordingly, it is likely that DGT-available Cu and Zn were driven by kinetic processes rather than equilibrium processes.

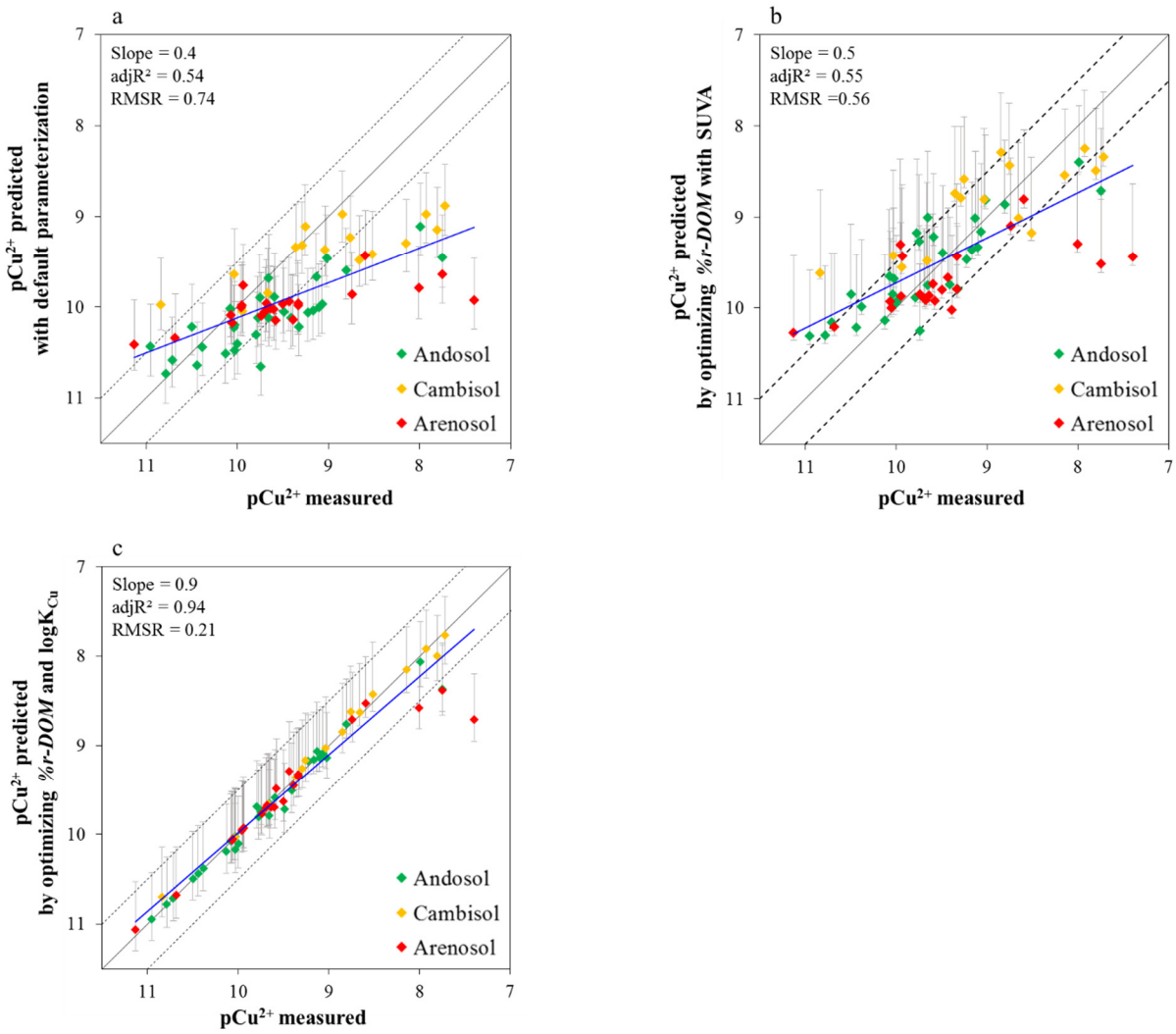
Although we did not perform any kinetic measurements, changes in the total Cu and Zn concentration and activity in soil solution were indicators of Cu and Zn lability in the soil solid phase and in the soil solution, respectively. The total Cu concentration increased while Cu^{2+} activity decreased in the solution of the three soil types with organic fertilization, thus indicating that Cu lability was more or less the same whatever the fertilization type. In addition, the negative correlation between the SUVA and DGT-available Cu in the cambisol, while DGT-available Cu was positively correlated with the total Cu concentration in soil solution, suggests that Cu bound to aromatic DOM, such as phenolic compounds, was weakly labile, in agreement with previous findings obtained under laboratory conditions (Amery et al., 2010; Kalis et al., 2006; Welikala et al., 2018). The total Zn concentration and Zn^{2+} activity decreased in the solution of the three soil types with organic fertilization, implying that Zn lability decreased with organic fertilization. However, DTPA-extractable Zn substantially increased in the three soil types with organic fertilization (Figs. 1d, e&f), which may have counteracted the decrease in Zn lability, resulting in a similar DGT-available Zn concentration regardless of the fertilization type.

3.4 The increase in DOM binding properties induced by organic fertilization drives copper but not zinc speciation in soil solution

Whatever the soil type, measured Cu^{2+} activity in soil solution was partially predicted by multi-linear regression ($adjR^2$ = 0.5-0.64), while Zn^{2+} activity estimated with WHAM was accurately predicted by multi-linear regression ($adjR^2$ = 0.97-0.99; Table 3). In addition, measured Cu^{2+} activity was also partially predicted with WHAM using default parameters, irrespective of the soil type ($adjR^2$ = 0.54 and RMSR = 0.74; Fig. 4a). Indeed, WHAM prediction tended to respectively over- and under-estimate measurements above and below 10 pCu^{2+} units. Djae et al. (2017) and Li et al. (2017) similarly showed, in a broad range of soils, that using the WHAM default settings did not enable them to adequately predict Cu^{2+} activity in soil solution. Moreover, Djae et al. (2017) showed, in a sensitivity analysis, that DOM binding properties (i.e *%r-DOM* and $logK_{Cu}$) were the most sensitive parameters in soil solution to optimize WHAM to achieve adequate Cu^{2+} activity predictions. Accordingly, accounting for the

variability in DOM binding properties in addition to other soil solution parameters could have improved the prediction of Cu^{2+} activity in the soil solutions we studied. However, Zn^{2+} activity is likely much less sensitive to DOM binding properties due to the much lower affinity of Zn^{2+} for DOM compared to Cu^{2+} (see section 3.3), thus explaining why it seems unnecessary to account for the variability in DOM binding properties to adequately predict Zn^{2+} activity in soil solutions.

Figure 4 Free copper activity (pCu^{2+}) predicted with WHAM as a function of pCu^{2+} measured in the soil solution samples (n =70). pCu^{2+} was predicited by (a) using WHAM default parameters with the percentage of dissolved organic matter reactive towards metal cations (*%r-DOM*) equal to 65% and the copper binding constant ($logK_{Cu}$) equal to 2.16 for all soil solutions, (b) optimizing the *%r-DOM* with the SUVA measured in soil solutions, or (c) optimizing the *%r-DOM* and the $logK_{Cu}$ values for each soil solution within a range of physically meaningful values (for b and c, see the *Material and Methods* section for rationale). Vertical error bars stand for the ± 2 standard deviations in predicted pCu^{2+} when considering the analitycal uncertainty on the pH, total Cu concentration in soil solution, and DOM. The blue line, the thick solid line, and the dashed lines represent the regression line, the 1:1 line, and the 1:1 line ± 0.5 pCu^{2+} unit, respectively. The slope and the adjusted coefficient ($adjR^2$) of the regression and the root mean square residual (RMSR) between measured and predicted pCu^{2+} are calculated.



Whatever the soil type, WHAM parameterization by optimizing the *%r-DOM* of each soil solution with their respective SUVA only slightly improved the prediction of measured Cu^{2+} activity (adjR^2 = 0.55 and RMSR = 0.56; Fig. 4b) compared to the prediction obtained with WHAM using default parameters. Contrary to our results, the WHAM parameterization carried out by Amery et al. (2008), whereby *%r-DOM* was optimized with SUVA, improved the prediction of Cu binding to DOM and consequently of Cu speciation in soil solution. Note that Amery et al. (2008) studied soil solutions that were barely impacted by anthropogenic inputs as the solutions were collected at 45-cm depth in soil that was either not fertilized or fertilized with a single application of pig manure at a moderate rate (i.e. 20 m^3 ha^{-1}). Contrary to Amery et al. (2008), our solutions were extracted from soils that received high anthropogenic inputs through OF applications at relatively high rates (see section 2.1) over a decade. Baken et al. (2011) showed that the Cu binding properties of DOM were no longer driven by its aromaticity in freshwaters impacted by high anthropogenic inputs related to the discharge of human effluents and livestock waste. In freshwater systems, such anthropogenic inputs led to the presence of organic molecules such as aminopolycarboxylates exhibiting a high affinity for Cu but low aromaticity compared to humic substances that commonly occur in natural DOM. Based on the findings of Baken et al. (2011), we considered that the application of OF over a decade may have altered the DOM binding properties towards Cu by introducing some organic molecules with a high affinity for Cu but with low aromaticity, which could explain why the DOM binding properties towards Cu were not driven by DOM aromaticity. However, the nature of these added organic molecules and their binding properties are unknown and would require further investigation. Consequently, our results suggest that, although organic fertilization increased DOM aromaticity (see section 3.3), DOM aromaticity was not a good proxy of DOM binding properties towards metals and consequently of Cu speciation in the solution of soils that had received OF over a decade.

Whatever the soil type, WHAM parameterization by manually optimizing the *%r-DOM* and then the $logK_{Cu}$ in each soil solution within physically meaningful ranges substantially improved the prediction of measured Cu^{2+} activity compared to predictions conducted with default parameterization or SUVA

optimization of WHAM (adjR^2 = 0.94 and RMSR = 0.21; Fig. 4c). The optimized *%r-DOM* and $logK_{Cu}$ values came within the variation range we set for these two parameters, as previously observed for the WHAM prediction of Cu^{2+} activity in 72 soil samples exhibiting a very broad range of physical-chemical properties (Djae et al. 2017; Li et al. 2017). These results suggest that organic fertilization induced substantial changes in DOM binding properties, which must be accounted for to adequately predict Cu speciation in soil solution using geochemical models.

Whatever the soil type, the proportion *%r-DOM* higher than 35% and of $logK_{Cu}$ equal to 2.16 (while the other $logK_{Cu}$ values were less than 2.16) optimized in WHAM was higher with organic fertilization than without fertilization or with mineral fertilization, except for the proportion of $logK_{Cu}$ equal to 2.16 in the cambisol, which was equivalent to the proportion with organic and mineral fertilization (Fig. S8). This suggests that organic fertilization over a decade tended to increase the binding properties of DOM compared to no fertilization or mineral fertilization by increasing both the DOM binding site density and the DOM binding affinity towards Cu.

To the best of our knowledge, previous studies on the impact of organic fertilization on DOM binding properties towards soilborne metals were focused on the determination of the Cu binding properties of fulvic and humic acids extracted from OF, OF-amended soils and non-amended soils (Bolan et al., 2011). The comparison of our results with these data appeared particularly relevant as geochemical models such as WHAM have been developed from characterization of the ion binding properties of humic substances (Tipping 1998). It is noteworthy that this approach has led to conflicting results in the literature. In partial agreement with our results, Sebastia et al. (2007) found that fulvic acids in a soil amended with straw or conifer compost over 15 years bound Cu more strongly than fulvic acids in the non-amended soil, while the binding site density of fulvic acids and their Cu binding affinity respectively decreased and increased in the amended soil. Plaza et al. (2005 and 2006) found, however, that fulvic and humic acids in soils amended with either pig slurry or sewage sludge bound Cu less strongly than fulvic and humic acids in the corresponding non-amended soils, with a consistent

decrease in both the binding site density and Cu binding affinity of fulvic and humic acids in the amended soils.

Whatever the reason for the discrepancy between the findings of Sebastia et al. (2007) and Plaza et al. (2005 and 2006), the fact that these authors opted to study the binding properties of soil DOM by focusing on humic substances was recently the focus of intensive debate in the literature (Lehmann and Kleber, 2015; Olk et al., 2019), and this strategy is questionable in the light of recent literature findings. Firstly, Groenenberg et al. (2010) and Ren et al. (2015) showed that fulvic and humic acids represented less than 50 and 5% of soil DOC, respectively. This strongly suggests that humic substances only partially contribute to DOM binding properties, thus indicating that the nature and binding properties of non-humic substances in DOM would require further investigation. Secondly, humic substances are usually extracted from soils with a procedure involving alkaline, acid, and then purification steps (see e.g. Sebastia et al., 2007), which is much harsher than the soft procedure that we used to extract DOM from the soil solution. It is highly suspected that the procedure used to extract soil humic substances may alter the chemical nature of functional groups able to bind metals and even more so the molecular structure of humic substances, although the extent of such alterations is still under debate (Lehmann and Kleber, 2015; Olk et al., 2019), finally resulting in metal binding properties different from those of less disturbed humic substances in extracted soil solution. In agreement with that view, Botero et al. (2017) recently showed that the last step of the soil humic acid extraction procedure (i.e. HF/HCl treatment followed by dialysis) was able to alter the Cd^{2+}, Zn^{2+} and Pb^{2+} concentration by one order of magnitude. This alteration was substantial enough to necessitate a specific optimization of the binding properties of humic acids in the model used to predict metal speciation. While the extent of the bias induced by the extraction procedure used for humic substances requires further investigation, it would be more relevant to study DOM binding properties towards metals directly in extracted soil solutions as we did to maximally reduce potential analytical artefacts.

Note finally that we were unable to adequately predict (i.e. within $\pm$ 0.5 pCu^{2+} unit) measured Cu^{2+} activity in three solutions extracted from the arenosol and one solution extracted from the andosol for which Cu^{2+} was underestimated by up to 1 pCu^{2+} unit (Fig. 4c). To adequately predict Cu^{2+} activity with WHAM in these four soil solutions, it was necessary to set the *%r-DOM* between 5 and 30% while maintaining the $logK_{Cu}$ at 1.84 (Fig. S9). Such low *%r-DOM* values are below the range determined analytically for 401 DOM, including standard and reference fulvic acids, humic acids, and natural organic matter (Ritchie and Perdue, 2003). This could possibly be explained by the fact that the DOM composition in these four soil solutions exhibited a high proportion of molecules with very low Cu binding properties, such as sugars. This hypothesis is supported by the particularly high percentage (i.e. 65-80%) of non-humic substances (including sugars) usually recovered in soil solutions exhibiting the lowest DOM concentration (Groenenberg et al., 2010; Ren et al., 2015), and secondly by the fact that the four soil solutions were collected from the two trials in fields with permanent fodder crop cover. Fodder crops are known to exude a large amount of organic molecules in their rhizosphere, with the bulk of them being composed of free sugars (Jones et al., 2009). This hypothesis underlines the need to further investigate the contribution of non-humic substances to metal binding in soil solution.

4. Conclusion

Organic fertilization over a decade induced, as expected, ongoing soil Cu and Zn contamination compared to no fertilization or mineral fertilization. But we also showed that organic fertilization substantially increased some major soil physical-chemical properties, namely pH, organic C concentration and the concentration and aromaticity of DOM along with its Cu binding properties. The similar alterations in the physical-chemical properties observed for the three studied soil types suggest that the effects of organic fertilization might generally apply to a broad range of initially acidic soils.

Considering the low affinity of Zn for DOM, Zn availability in OF-amended soils was closely related to the increase in soil pH. The increase in soil pH induced by organic fertilization decreased the total Zn concentration and Zn^{2+} activity in soil solution. Accordingly, and despite a marked increase in soil Zn contamination, DGT-available Zn was equal or lower in soils amended with OF compared to soils receiving either no fertilizer or mineral fertilizers.

Considering the high affinity of Cu for DOM, Cu availability in OF-amended soils was closely related to the concomitant increase, in soil solution, of the pH and DOM concentration, aromaticity and binding properties towards Cu. The increase in DOM concentration and aromaticity induced by organic fertilization increased the total Cu concentration in soil solution. By contrast, the increase in pH and DOM binding properties induced by organic fertilization decreased Cu^{2+} activity in soil solution. Accordingly, and despite a marked increase in soil Cu contamination, DGT-available Cu was equal or only slightly higher in OF-amended soils compared to soils receiving either no fertilizer or mineral fertilizers.

Our results consistently suggested that alterations in the soil physical-chemical properties regulate Cu and Zn availability in soils receiving long-term organic fertilization by exerting a protective effect that offsets the concomitant increase in the soil Cu and Zn contamination level. This protective effect of organic fertilization was, however, less clear for Cu than for Zn due to the opposite effect of DOM on the total Cu concentration and Cu^{2+} activity in soil solution. From an ecotoxicological perspective, it remains questionable whether such a protective effect would also be able to regulate Cu and Zn bioavailability and toxicity to organisms living in soils receiving long-term OF applications.

Acknowledgements

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135927.

5. Annexes

Table S1 Characteristics of organic fertilizers applied on the three field trials.

		Andosol and arenosol		Cambisol	
	Unit, dry mass	Dairy slurry	Dairy manure	Pig slurry compost	Poultry litter compost
Dry matter	%	9.1	46	52	47
pH-H_2O		7.5	7.5	7.6	7.1
Organic C	g kg^{-1}	890	232	298	333
Total Cu	mg kg^{-1}	50	96	180	109
Total Zn	mg kg^{-1}	259	383	464	319

Figure S1 Variation range of (a) pH, (b) dissolved organic carbon concentration (DOC), (c) specific UV-absorbance at 254 nm (SUVA), (d) DGT-available copper (Cu) and zinc (Zn) concentrations (pCu_{DGT} and pZn_{DGT}) in soil, total Cu and Zn concentration in soil solution (pCu_{ss} and pZn_{ss}), and free ionic Cu and Zn activity in soil solution (pCu^{2+} and pZn^{2+}) in all of the soil samples (n = 74). The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5-times the interquartile range above the third quartile or below the first quartile.

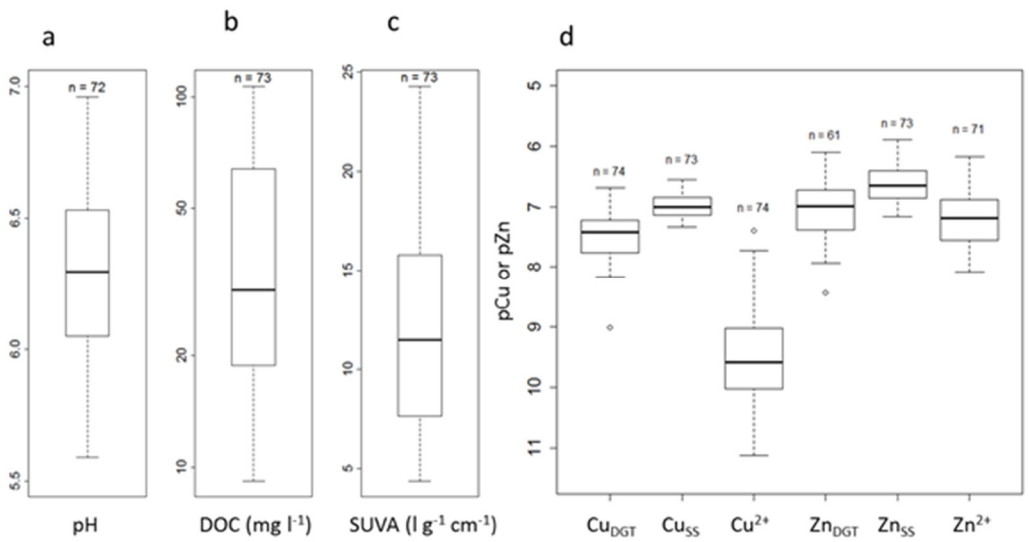


Figure S2 Time-cumulated rate of (a) copper ($F_{Cu\text{-}rate}$) and (b) zinc ($F_{Zn\text{-}rate}$) inputs to the three soil types via mineral or organic fertilization. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5-times the interquartile range above the third quartile or below the first quartile.

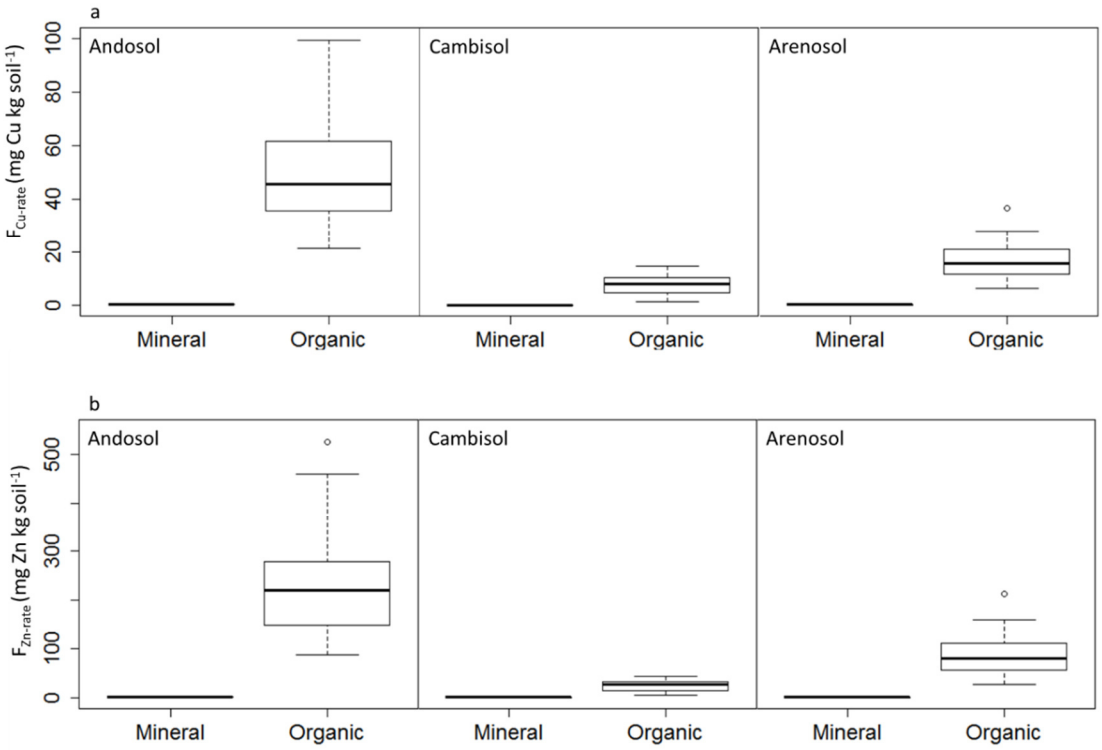


Figure S3 Variations in the (a) pH, (b) DOC, (c) SUVA, (d) DGT-available Cu (pCu_{DGT}), (e) total Cu concentration in soil solution (pCu_{SS}), (f) free ionic Cu activity (pCu^{2+}), (g) DGT-available Zn (pZn_{DGT}), (h) total Zn concentration in soil solution (pZn_{SS}), and (i) predicted free ionic Zn activity (pZn^{2+}) as a function of soil types (Andosol, n = 34; Cambisol, n = 17; Arenosol, n = 23). Different letters indicate a significant difference at the 0.05 level.

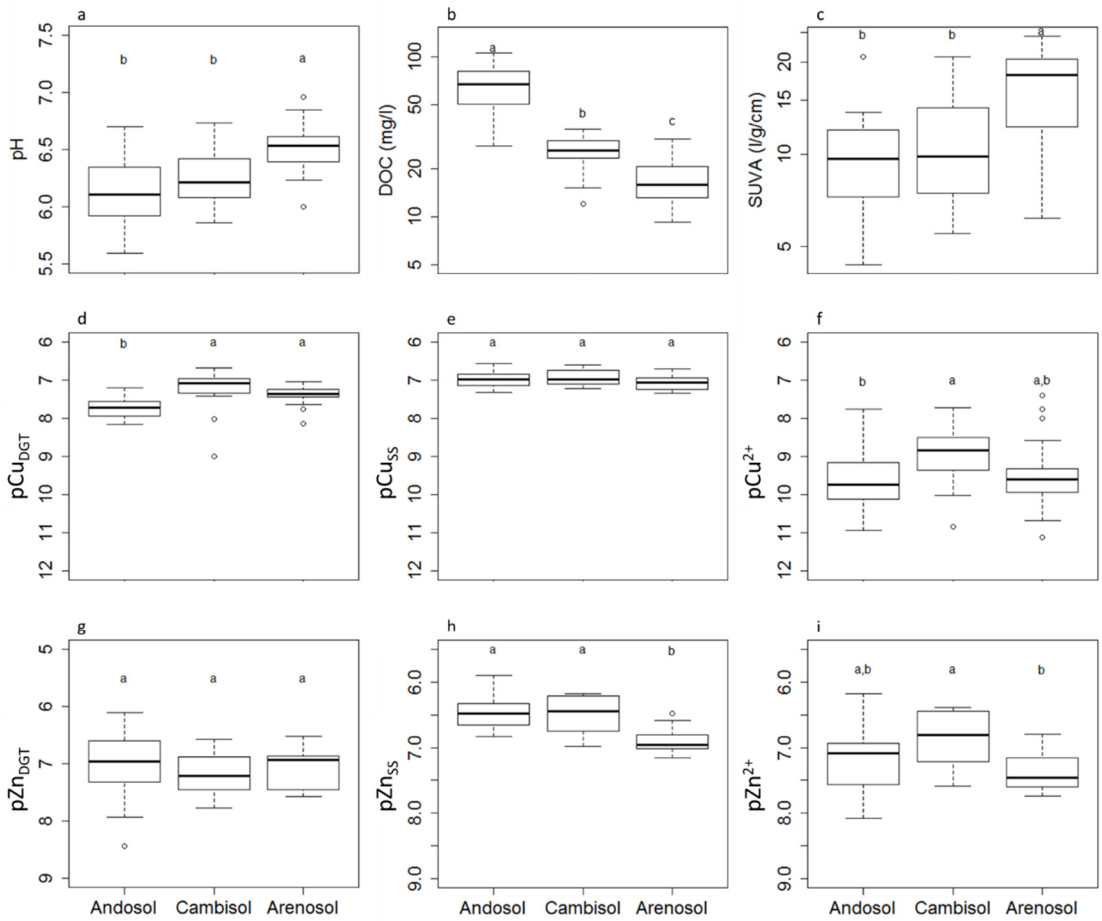


Figure S4 Variations in the (a) pH, (b) DOC , (c) SUVA, (d) total Cu concentration in soil solution (pCu_{SS}), (e) the free ionic Cu activity (pCu^{2+}), (f) DGT-available Cu (pCu_{DGT}), (g) the total Zn concentration in soil solution (pZn_{SS}), (h) the free ionic predicted Zn activity (pZn^{2+}), and (i) DGT-available Zn (pZn_{DGT}) in soil solution of the andosol as a function of the type of fertilization (No fertilization, n = 5; Mineral, n = 10; Organic, n = 19). Different letters indicate a significant difference at the 0.05 level.

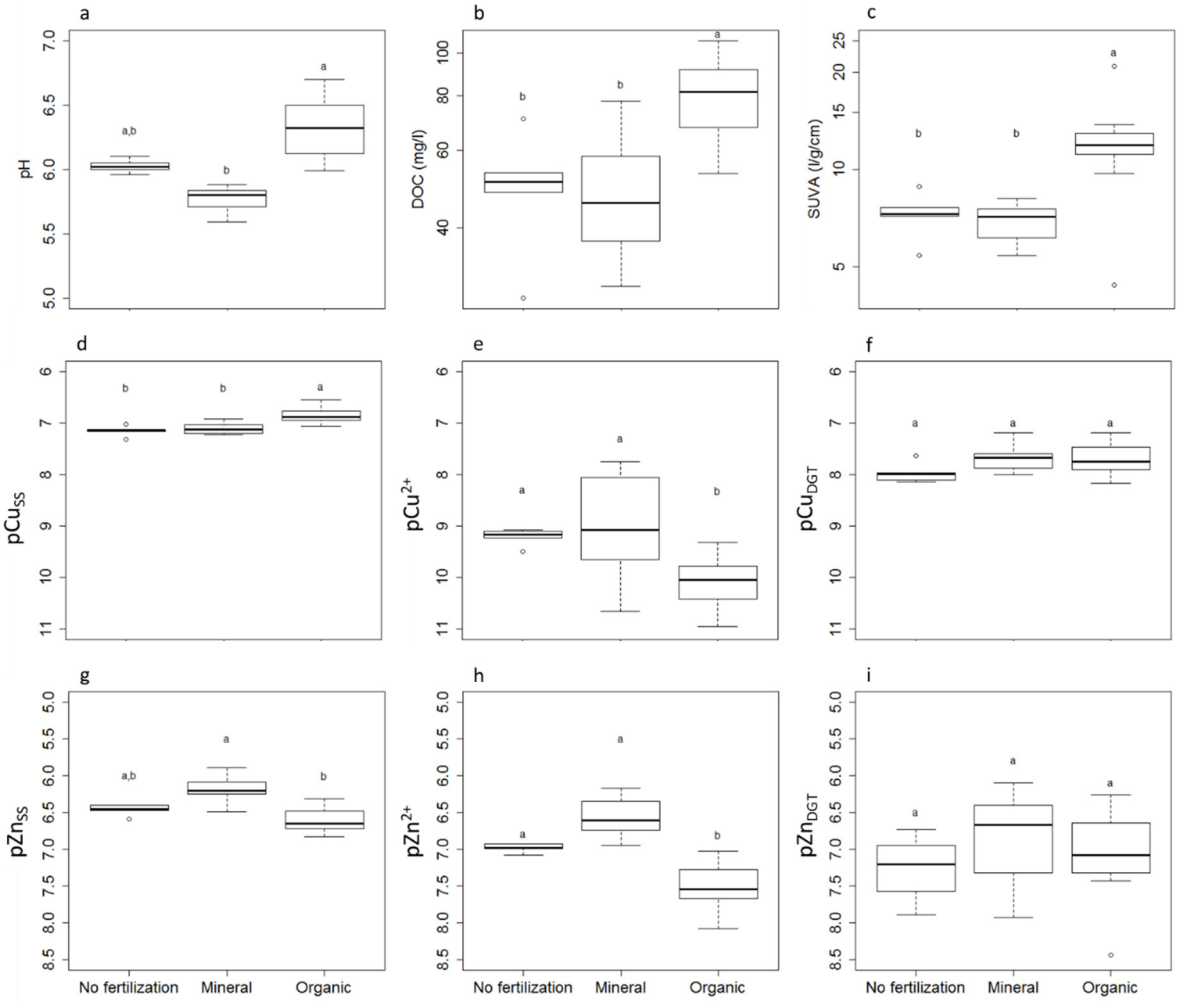


Figure S5 Variations in the (a) pH, (b) DOC, (c) SUVA, (d) total Cu concentration in soil solution (pCu_{SS}), (e) free ionic Cu activity (pCu^{2+}), (f) the DGT-available Cu (pCu_{DGT}), (g) total Zn concentration in soil solution (pZn_{SS}), (h) the free ionic predicted Zn activity (pZn^{2+}), and (i) the DGT-available Zn (pZn_{DGT}) in soil solution of the cambisol as a function of the type of fertilization (Mineral, n = 6; Organic, n = 11). Different letters indicate a significant difference at the 0.05 level.

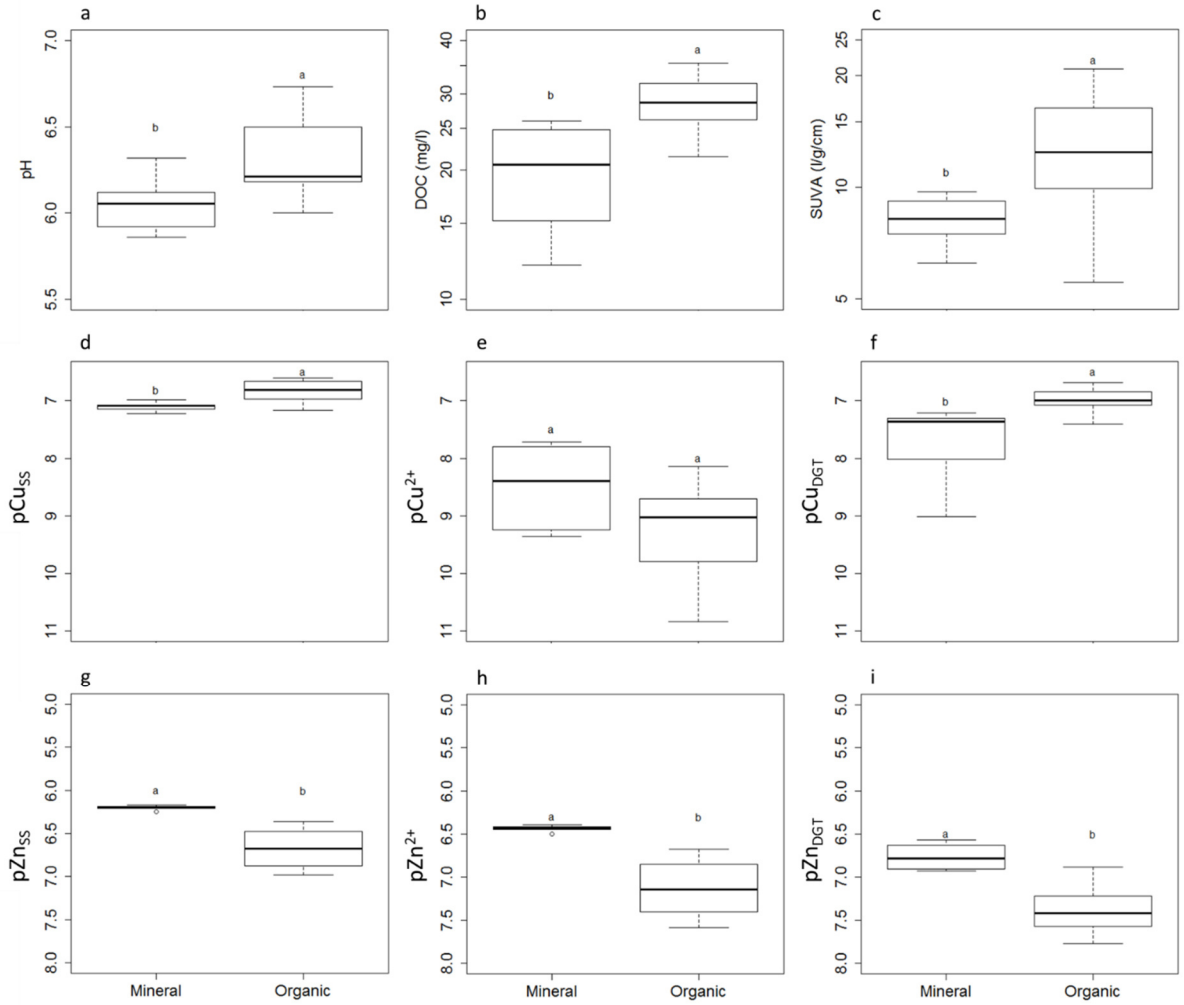


Figure S6 Variations in the (a) pH, (b) DOC, (c) SUVA, (d) total Cu concentration in soil solution (pCu_{SS}), (e) free ionic Cu activity (pCu^{2+}), (f) DGT-available Cu (pCu_{DGT}), (g) total Zn concentration in soil solution (pZn_{SS}), (h) free ionic predicted Zn activity (pZn^{2+}), and (i) DGT-available Zn (pZn_{DGT}) in soil solution of the arenosol as a function of the type of fertilization (No fertilization, n = 4; Mineral, n = 4; Organic, n = 15). Different letters indicate a significant difference at the 0.05 level.

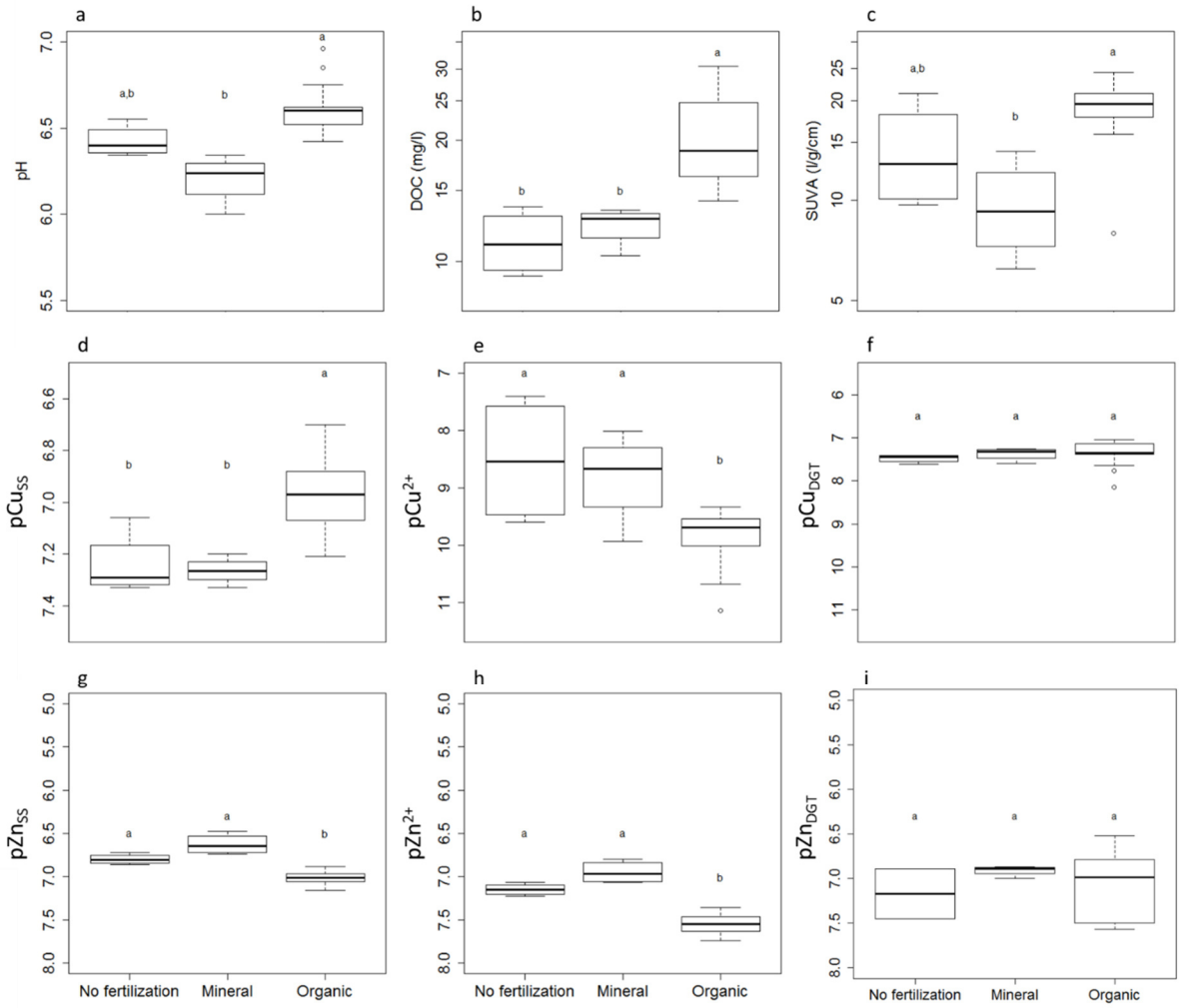


Figure S7 Predicted proportion of (a) free ionic Cu (Cu^{2+}) and Cu bound to DOM (Cu-DOM) and (b) free ionic (Zn^{2+}) and Zn bound to DOM (Zn-DOM) in the soil solution of the three soil types considered together (n = 70). The scale of y-axis is expressed as log.

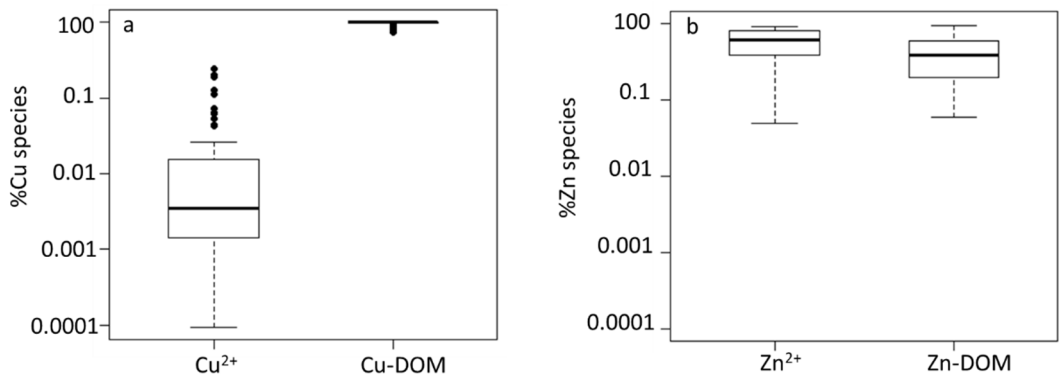


Table S2 Multiple linear regression explaining the concentrations of DGT-available Cu and zinc (pCu_{DGT} and pZn_{DGT}) as a function of $log_{10}Kd_M$, pH, $log_{10}DOC$, log_{10} SUVA, pCu_{SS} or pZn_{SS} (pM_{SS}) and pCu^{2+} or pZn^{2+} (pM^{2+}) in the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		$log_{10}Kd_M$	pH	$log_{10}DOC$	$log_{10}SUVA$	pM_{SS}	pM^{2+}	i	adj R^2[a]	n[b]
pCu_{DGT}	3 soils	ns	ns	0.83^2	ns	1.41^1	ns	-3.62	0.39***	74
	Andosol	ns	ns	ns	ns	ns	ns	ns	< 0.1	34
	Cambisol	ns	ns	ns	5.44^2	5.63^1	ns	-37	0.88***	17
	Arenosol	ns	ns	ns	ns	ns	ns	ns	< 0.2	23
pZn_{DGT}	3 soils	ns	ns	ns	ns	ns	ns	ns	< 0.2	61
	Andosol	ns	ns	ns	ns	ns	ns	ns	< 0.1	31
	Cambisol	ns	ns	ns	ns	ns	ns	ns	< 0.1	12
	Arenosol	ns	ns	ns	ns	ns	ns	ns	< 0.1	18

[a]adj R^2 = adjusted R^2 ; [b]n = number of samples

Figure S8 Proportion of the percentage of dissolved organic matter reactive towards metal cations (*%r-DOM*) and of the copper binding constants ($logK_{Cu}$) optimized in WHAM within ranges restricted to physically meaningful values that were respectively greater than 35% and equal to 2.16 (while the other values were lower than 2.16) for (a) the andosol (n = 31), (b) the cambisol (n = 16), and (c) the arenosol (n = 23) as a function of the fertilization type (No fertilization, Mineral or Organic).

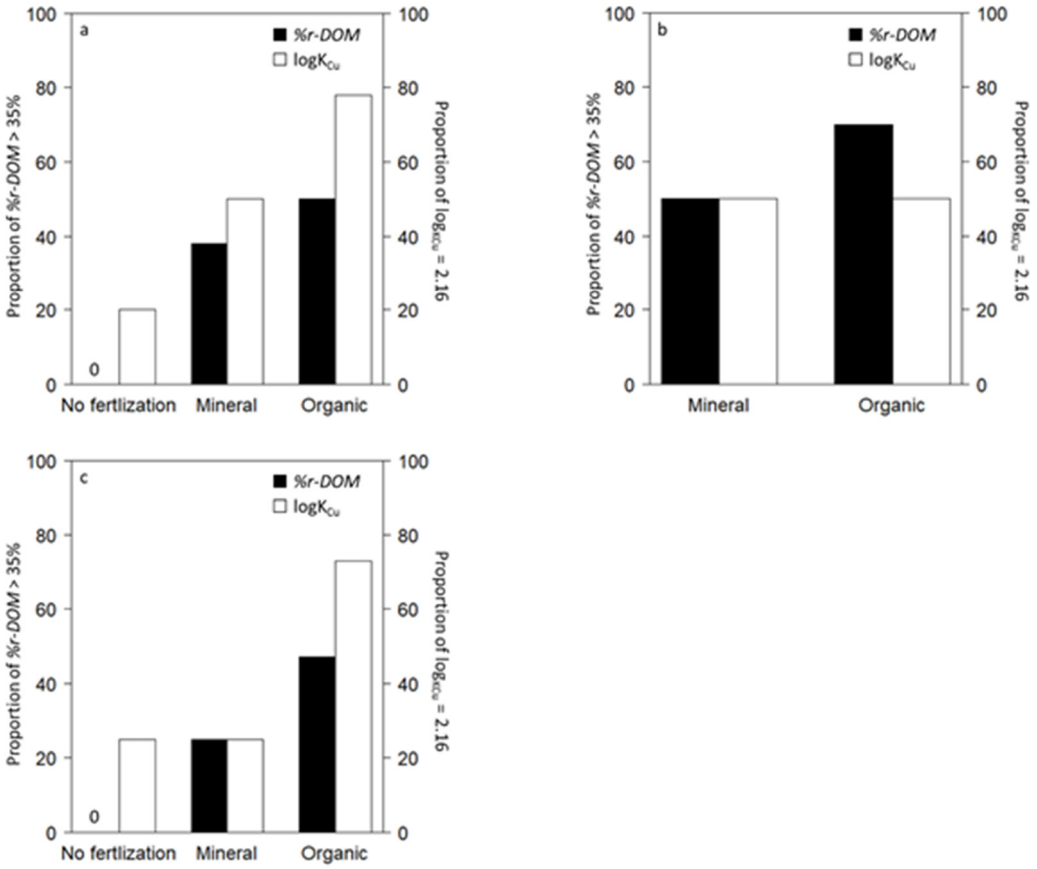
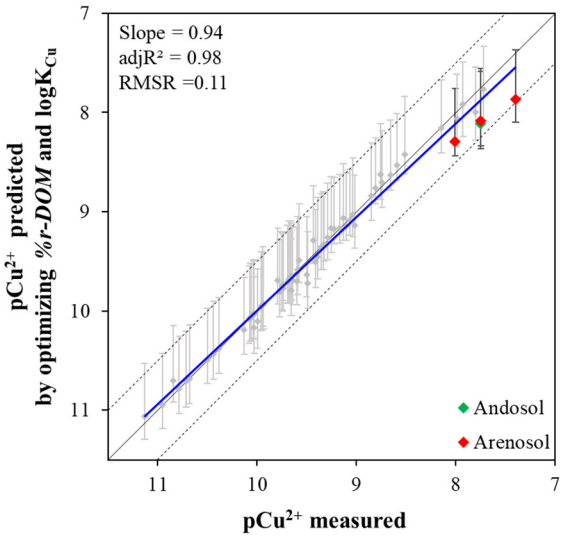


Figure S9 Free copper activity (pCu^{2+}) predicted with WHAM as a function of pCu^{2+} measured in soil solution. Grey diamonds stand for pCu^{2+} predicted by optimizing the *%r-DOM* and the $logK_{Cu}$ values for each soil solution within a range of physically meaningful values (see the *Material and Methods* for rationale). Red and green diamonds stand for pCu^{2+} predicted by optimizing *%r-DOM* below 35% and with $logK_{Cu}$ equal to 1.84. Vertical error bars stand for the ± 2 standard deviations in predicted Cu^{2+} when considering the analytical uncertainty for the pH, Cu concentration in soil solution, and DOM. The blue line, the thick solid line, and the the dashed lines represent the regression line, the 1:1 line, and the 1:1 line ± 0.5 pCu^{2+} unit, respectively. The slope and the adjusted coefficient (adjR^2) of the regression and the root mean square residual (RMSR) between measured and predicted pCu^{2+} are calculated.



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Chapitre III : Biodisponibilité du cuivre et du zinc pour les vers de terre et les plantes

Dans le chapitre précédent, j'ai étudié les effets de l'apport de FO pendant une décennie sur la disponibilité de Cu et Zn au travers d'indicateurs associés aux solutions aqueuses extraites de trois types de sol, et en reliant ces effets à l'évolution temporelle de la contamination, du pH et de la MO des sols. Nos résultats ont montré que l'apport de FO sur une décennie avait augmenté la contamination en Cu et Zn dans les trois types de sols mais aussi le pH, la concentration, l'aromaticité et les propriétés de complexation de la MOD dans les solutions de sols. Les déterminants de la disponibilité de Cu dans la solution du sol ont été identifiés comme le pH, la concentration en MOD et ses propriétés de complexation, tandis que pour Zn le déterminant de sa disponibilité en solution concernait uniquement le pH, du fait d'une plus faible affinité de Zn pour la MO. Les modifications de la chimie de la solution du sol induites par les apports de FO allant dans le sens d'une diminution de disponibilité de Cu et Zn suggèrent que ces apports auraient donc sur le long terme un effet protecteur vis-à-vis de l'augmentation de la contamination en Cu et Zn des sols.

Nous avons vu que les organismes du sol, comme les plantes et les vers de terre, peuvent aussi modifier le pH et la MOD, dans la zone de sol qu'ils bio-influencent (Chapitre 1). Dans le cadre de l'étude de la biodisponibilité de Cu et Zn pour les vers de terre et les plantes, notre objectif était de hiérarchiser l'influence de chacun de ces organismes sur la disponibilité de Cu et Zn dans leur zone de bio-influence comparativement aux effets de l'apport de FO à long-terme, et de préciser le lien entre disponibilité de Cu et Zn dans le sol et biodisponibilité pour les plantes ou les vers de terre, en prenant en compte les concentrations internes des organismes.

En étudiant plus spécifiquement les zones de bio-influence de chaque organisme, le chapitre 3 a pour objectif de distinguer les effets combinés des apports à long-terme de FO et des activités des vers de terre, afin d'évaluer in fine la biodisponibilité de Cu et Zn pour le ver de terre dans la première partie et pour la plante dans la seconde.

I. Do organic fertilizer application to soils for a decade promotes copper and zinc bioavailability to earthworms?

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Abstract

Long-term applications of organic fertilizers (OF) induce moderate but on-going increase in Cu and Zn contamination, in soil pH and in dissolved organic matter (DOM) concentration in agricultural soils. It is also known that earthworms are able to bio-influence soil physical-chemical properties. But the combined effects of earthworm bio-influence and long-term OF applications on the soil physical-chemical properties and their consequence on Cu and Zn bioavailability is poorly known. In chapter II concerning decadal soil OF applications and comparing with non-amended soils or soils amended with mineral fertilizers, we showed that such increases together with increases in aromaticity and binding properties of DOM resulted in similar Cu availability between soils amended with OF or not, but in similar or lower Zn availability. Herein, we aimed to assess if the bio-influence of earthworm changed the effect of OF applications on Cu and Zn availability in soil and therefore on their bioavailability to earthworms. For this purpose an epi-endogeic species of earthworm (*Dichogaster saliens*) was exposed in microcosms to three soil types that had received either organic or mineral fertilizers for a decade, or were never fertilized. The pH, concentration and aromaticity of DOM and the availability of Cu and Zn (total concentration and free ionic activity in solution) were determined at the end of exposure on the soil solution extracted from the zone bio-influenced by the earthworms. Cu^{2+} activity in soil solutions was measured using an ion selective electrode, while Zn^{2+} activity and DOM binding properties were estimated using the Windermere humic aqueous model. Cu and Zn bioavailability for earthworms in the presence or absence of OF application were determined as the internal Cu and Zn contents in earthworms. Whatever the soil samples, we showed that the presence of earthworm buffered the soil solution pH around a mean value of 6.4 ± 0.2 while in the absence of earthworms,

the bulk soil pH ranged from 5.7 to 6.9. The bio-influence of earthworms did not significantly changed Cu and Zn availabilities in soils compared to the absence of earthworms. The Cu and Zn contents in earthworms were not significantly different between the various fertilization types. Our results showed that even if the bio-influence of earthworms induced slight changes in soil solution chemistry compared to the bulk soil, organic fertilization mitigated Cu and Zn availability as observed in the bulk soil, which resulted an absence of effect of OF application for a decade on Cu and Zn availability to earthworms.

Keywords: bioaccessibility, *Dichogaster saliens*, metals, bio-influenced zone, ecotoxicology, pore water

I.1 Introduction

The application of organic fertilizers (OF) in crop fields induces soil contamination with trace elements (Senesi et al., 1999). Cu and Zn are quantitatively the main trace elements concerned by such a soil contamination. Moreover, the estimated time necessary to increase the soil concentration from natural background to regulatory thresholds is the shortest for these two metals, justifying the specific interest for their fate and impact after their addition to soils (Belon et al., 2012; Luo et al., 2009; Nicholson et al., 2003).

The application of OF on the long-term not only increases Cu and Zn soil contamination, but also the pH and organic matter content (Cambier et al., 2014). Vanden Nest et al, (2016) reported a significant increase in pH from 5.6 to 6.5 and an increase in soil organic carbon from 10.5 to 15 g C kg^{-1}, between the control treatment and a soil that received dairy farmyard manure. These two parameters are important factors influencing Cu and Zn availability, i.e. the pool of metals able to interact with soil organisms. In a previous study (Chapter II) we reported that compared to soil that received either mineral fertilizers or were not fertilized, the application of OF for a decade induced modifications not only of pH and concentration of dissolved organic matter (DOM), but also induced modifications of DOM aromaticity and binding properties thus mitigating Cu and Zn availability despite the on-going Cu and Zn soil contamination.

By their activities, earthworms can also modify physical-chemical properties of soil such as pH and DOC concentration in the soil volume they bio-influence. Indeed these organisms, which represent 80 % of the soil living biomass(Yasmin and D'Souza, 2010), are highly involved in different soil processes (Bertrand et al., 2015; Blouin et al., 2013). It was reported that earthworms mainly increased pH and DOM concentration thus enhancing metal availability in soil solution in highly contaminated or spiked soils (Dandan et al., 2007; Udovic et al., 2007; Wen et al., 2004). In their review, Sizmur et Hodson (2009) showed that according to their ecological category earthworms can influence differently metal availability. Epigeic and anecic earthworms were mainly found to increase metal availability (Kizilkaya, 2004 ; Zorn et al., 2005a). Endogeic species were reported to either increase (Coeurdassier et al., 2007; Fritsch et al., 2008) or decrease metal availability (Lukkari et al., 2006 ; Zorn et al., 2005b). Sizmur et al., (2011) assessed the effect of anecic earthworms (*Lumbricus terrestris*) on Cu and Zn availability in two mine soils (one contaminated in Cu and the other in Zn) amended at increasing doses of OF under laboratory conditions. The authors reported an increase in Zn concentration in solution but a decrease in Cu concentration in solution that they related to the impact of earthworms on pH and soluble organic C in the soils compared to soils without earthworms. But the authors did not take into account the possible effects of long-term OF applications on solution chemistry and the consequent impact on

Cu and Zn availability. The role of the earthworm bio-influence on Cu and Zn availability in soils and thus on Cu and Zn bioavailability to earthworms may be difficult to unravel from the role of long-term OF application.

Indeed, applications of OF may also affect Cu and Zn bioavailability for soil organisms, particularly for earthworms. If the internal accumulation of trace elements by earthworms had been extensively studied (Nahmani et al., 2007), it was far less tested in the context of OF applications where organic matter can modify metal bioavailability. Studies assessing effects of OF applications on Cu and Zn bioavailability reported either an increase in Cu or Zn concentration in earthworms (Kızılkaya, 2004; Suthar et al., 2008) or no changes (Centofanti et al., 2016) after exposure to soils that received OF. However, in these studies, the effect of OF application was not distinguish from the effect of earthworms on pH and DOM which both influence metal (bio)availability, probably leading to these contrasted results.

In this context, the aim of our study was twofold: i) to unravel the effects of the presence of earthworms from those of decadal OF application on Cu and Zn availability in soils by focusing on soil solution chemistry in the absence or presence of earthworms and ii) to unravel the effects of the application of OF from those of the earthworm activities on Cu and Zn bioavailability for earthworms by focusing on earthworm Cu and Zn internal contents in the presence or absence of decadal OF inputs. For this purpose, we selected soil samples from three different field trials exhibiting contrasted properties and receiving either no fertilization, mineral fertilization or organic fertilization for a decade. Soil samples were incubated in the presence of an epi-endogeic earthworm species of ecological relevance in the context of OF application of the chosen agricultural soils. We hypothesized that earthworms activities can modify the long-term effects of decadal OF applications on Cu and Zn availability and that OF applications influence Cu and Zn bioavailability for earthworms due to modifications of physical-chemical soil properties.

I.2 Material and Methods

I.2.1 Soil characteristics and incubation

The three decadal field trials were located in Réunion (Indian Ocean, 55°30'E, 21°05'S) and belonging to three reference soil groups (hereafter called soil types): andosol, andic cambisol, and arenosol (WRB, 2014). The characteristics of the field trials were previously reported by Nobile et al. (2018). Briefly, the andosol is the main cultivated soil type in Réunion and is the first step of pedogenic evolution of soils formed on volcanic material. This type of soil contains mainly natural nanoparticles which exhibit a large surface area able to strongly bind trace elements (Levard et al., 2009). The andic cambisol (hereafter called cambisol) represents the second step of the pedogenic evolution of soils in Reunion. It contains minerals with a lower surface area and thus probably with a lower binding affinity for trace elements. The arenosol is the least evolved soil type and contains primary minerals that are expected to bind trace elements in a lesser extent than the cambisol and the andosol.

The andosol and arenosol were under a grassland cover from 2004 to 2012 and plots were arranged in three randomized blocks which were fertilized or amended either with mineral fertilizers (10 g N m^{-2} y^{-1}), or dairy slurries (20 to 55 dm^3 m^{-2} y^{-1}), or dairy manure composts (2.5 to 5 kg m^{-2} y^{-1}) or not fertilized. The cambisol was cultivated with two cycles of market garden crops per year from 2004 to 2011. Plots were arranged in three randomized block and were amended either with mineral fertilizers, pig slurry composts (5 kg m^{-2} y^{-1}) or poultry litter composts (7 kg m^{-2} y^{-1}).

In a previous study (Chapter II), we studied 74 soil samples selected from a soil library of these three field trials taking into account the time between fertilizations and the fertilization modalities . We thus obtained gradients of soil Cu and Zn contaminations, of soil pH and of soil organic matter concentrations related to different Cu and Zn availabilities. For the purpose of the present study, we selected 20 soil samples among these 74 already studies whose main characteristics are given in Table 1. For the andosol and the arenosol, we selected seven soil samples either not fertilized (n = 1), or that received mineral fertilizers (n = 2), dairy slurry (n = 2) or dairy manure compost (n = 2). For the cambisol, we selected six soil samples that received either mineral fertilizers (n = 2), pig slurry composts (n = 2) or poultry litter composts (n = 2). Five replicates of each of these soil samples were pre-incubated in the absence of earthworms for 14 days at 70% of their water holding capacity with a nutrient solution of the following composition (in mM): $Ca(NO_3)_2$ 2, KNO_3 2, $MgSO_4$ 1, KH_2PO_4 0.05.

Table 1 Soil physical-chemical properties as a function of the fertilization types, before the exposition to earthworms. Mean values are given with their standard deviation in parentheses.

		Fertilization	pH-H_2O	Organic C	Total Cu	Total Zn	Cu-DTPA	Zn-DTPA
	n [a]	Units		g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Andosol	1	No fertilization	5.7	115	81	163	1.3	7
	2	Mineral fertilization	5.2 (± 0.4)	111 (± 3)	80 (± 9)	180 (± 11)	1.5 (± 0.3)	8 (± 5)
	4	Organic fertilizaiton	6.2 (± 0.4)	123 (± 6)	83 (± 4)	183 (± 10)	2.3 (0.8)	15 (± 3)
Cambisol	2	Mineral fertilization	5.4 (± 0.2)	34.1 (± 0.1)	80 (± 1)	242 (± 5)	4.5 (± 0.3)	6.01 (± 0.38)
	4	Organic fertilization	6.1 (± 0.6)	36 (± 3)	83 (± 5)	257 (± 14)	7 (± 2)	13 (± 7)
Arenosol	1	No fertilization	6.6	11	74	143	1.5	2
	2	Mineral	5.8 (± 0.1)	10.2 (± 0.7)	70 (± 2)	136 (± 2)	1.2	1.9 (± 0.2)
	4	Organic fertilization	7.2 (± 0.2)	13.4 (± 2.6)	71 (± 2)	143 (± 6)	1.7 (± 0.4)	7 (± 3)

[a] number of soil samples

I.2.2 Earthworms

Sexually mature individuals of *D. saliens* were used for the experiment. *Dichogaster saliens* is an epi-endogeic peregrine species found in different tropical regions, and naturally present in the field trials of the studied cambisol and arenosol but not found in the studied andosol. Individuals of *D. saliens* were collected by hand-sorting in a field margin (cambisol) that did not receive OF nor mineral fertilizers. They were kept in three different batches containing one of the three soil types during 8 days in a climatic room in the dark at 27°C. At the end of this acclimatization period, six earthworms per soil type were collected to determine the initial Cu and Zn contents in earthworms before exposure to the selected 20 soil samples.

I.2.3 Experimental set-up

After pre-incubation, the soil samples were placed in microcosms (5.5 cm diameter, 4 cm height) and the water holding capacity was adjusted to 85%. The quantity of soil in each microcosm was calculated so that all the soil volume was supposed to be explored by earthworms during the incubation period. We thus considered that an earthworm can daily ingest a mass of soil equivalent to its fresh biomass (i.e. around 20 mg for mature individuals) and then adjusted to have a soil layer of 1 cm thick. Three earthworms were placed in each microcosm and five replicates per treatment were performed leading to a total of 100 microcosms (35 for the andosol and arenosol, and 30 for the cambisol). The earthworms were exposed to the soil samples during 21 days and microcosms were stored during this period in a climatic room in the darkness at a constant temperature of 27°C.

I.2.4 Measurements on earthworms

Mortality of individuals during the experiment was followed at 7, 14 and 21 days of exposure period by counting earthworms in the microcosms. At the end of the experiment, the health and activity status (i.e. active or in quiescence) of each individual was visually assessed. Quiescence is a status of reduced activity in response to adverse environmental conditions (Koštál, 2006) during which earthworms remained coiled up in the soil. To evaluate changes in earthworm biomass due to the experiment, individuals were weighed before being introduced into the microcosms, and at the end of the 21 days of experiment. Before being weighed, earthworms were individually placed in petri dishes on damp filter paper for 24 h in the darkness at 27°C to void gut content. At the end of the incubation and after being weighed, earthworms were frozen at -30°C for 24 h, and then freeze-dried for 24h before analysis.

Freeze-dried earthworms were digested in 0.5 ml ultra-pure HNO_3 (Pico pure plus, ChemLAB) in a microwave system (MARSXpress, CEM). The digests were then diluted in 25 ml of ultra-pure water.

Metal quantification in total earthworms was performed by ICP-MS and the reliability of analysis was assessed with blanks and certified reference material (ERM-BB-422, fish muscle). As the earthworm biomass was low, we performed test samples with either 100 mg or 10 mg of the certified reference material to encompass the range of mass of Cu effectively accumulated in earthworms before and after their exposure to soils. The recovery percentage was 96% for Cu and Zn for the 100 mg test sample and 77% and 101% for Cu and Zn respectively for the 10 mg test sample.

The quantity (Q_M, ng) of Cu and Zn in earthworms at the end of the 21 days of experiment were calculated as follows:

$$Q_M = (C_f * m_f) - (C_a * m_a) * 1000 \quad (1)$$

where C_f is the concentration of metal measured in earthworms at the end of the 21 days of experiment (µg g^{-1}), m_f is the dry biomass of earthworms at the end of the 21 days of experiment (g), C_a is the concentration of metal in earthworms at the end of the acclimatization period (µg g^{-1}) and m_a is the dry biomass of earthworms at the end of the acclimatization period (g).

I.2.5 Extraction and analysis of soil solution

Data in the absence of earthworms are presented elsewhere (Chapter II). They will be hereafter called originating from the soils without the influence of earthworms or bulk soils.

At the end of the exposure period in the presence of earthworms, soils from each microcosm were collected. The extraction procedure and analysis were as detailed by Djae et al. (2017). Briefly, the soil solutions were extracted with the nutrient solution used for soil incubation and described in the section I.2.1. The mixture between soil and extracting solution (1:10, soil to nutrient solution ratio) was stirred for 2h, then centrifuged at 4600 rpm for 10 min, and the supernatant was filtered (0.22 µm, polyethersulfone filters). Each sample of soil solution was subdivided and subsamples were stabilized with either NaN_3 for the measurement of Cu^{2+} activity and specific ultraviolet-absorbance at 254 nm or HNO_3 for the measurement of dissolved organic carbon concentration (DOC) and total concentration of Cu and Zn in solution. Total concentration of major and trace cations (Ca, K, Mg, Na, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) were determined by ICP-MS. DOC concentration was determined using a TOC analyzer (Schimadzu, TOC-L). The specific UV-absorbance at 254 nm (SUVA) was measured by UV-visible spectrometry (Spectronic Unicam, Helios Beta) to estimate the aromaticity of DOM (Weishaar et al., 2003). The SUVA (SUVA; l g^{-1} cm^{-1}) was calculated as follows:

$$SUVA = \frac{A_{254}}{\mathrm{b.DOC}} \quad (2)$$

where A_{254} is dimensionless, *b* is the path length (1 cm) and *DOC* is the concentration of DOC (g l^{-1}).

The pH was measured in parallel to pCu^{2+} (i.e. $-log_{10}\{Cu^{2+}\}$) using a combined electrode (Radiometer analytical PHC2051-8). Free Cu activity was measured using a Cu specific electrode (Thermo Scientific Orion, 9629BNWP). The electrode was daily calibrated within the 13.4 and 4.7 range of pCu^{2+}in standard solutions containing imminodiacetic acid as Cu-complexing compound. The calibration curve (r^2 = 0.98, n = 157) showed a slope efficiency of 93% compared to the theoretical Nernstian slope. The deviation in pH and pCu^{2+} between two days of measurement ranged in average between 0.08 ± 0.04 and 0.2 ± 0.2, respectively.

I.2.6 Modeling of copper and zinc speciation in soil solution

Free Cu and Zn activities in each soil solution were predicted using WHAM VII to have an estimation of Zn^{2+} activity and to assess the impact of DOM binding properties on the Cu and Zn speciation in soil solution. Input parameters were those analytically determined in the soil solutions: the pH and the concentration of major and trace cations (Ca, Mg, Na, and K, Cd, Co, Cu, Mn, Ni, Pb, and Zn). The activities of Al^{3+} and Fe^{3+} were calculated by assuming their equilibrium with $Al(OH)_3$ and $Fe(OH)_3$ with solubility products (logKs) at 25°C of 8.5 and 2.5, respectively (Tipping et al., 2003). The concentration of NO_3^- was fixed at the concentration of the extracting solution (see in 1.2.5 for rationale). The charge of the solution was balanced with either NO_3^- or K^+ when the solution exhibited an excess in cation or anion, respectively. The concentration of DOM was calculated from DOC concentration assuming that DOM contained 50% of carbon.

Considering these input parameters, Cu^{2+} and Zn^{2+} activities were predicted by three approaches and for each of these, the deviation between predicted and measured Cu^{2+} activities was determined by calculating the regression slope and coefficient and the root mean square residual (RMSR). Free Cu and Zn activities were first predicted as commonly done in the literature with default settings considering that 65% of DOM able to bind metal cations reacts as the colloidal fulvic acid (*%r-DOM*) as defined in WHAM (with $logK_{Cu}$ and $logK_{Zn}$ of 2.16 and 1.68, respectively) and that the remaining 35% is inert (Tipping et al., 2011). Free Cu activity was secondly predicted by optimizing *%r-DOM* using the SUVA values as previously suggested by Amery et al. (2008) with the value of 37 l g^{-1} cm^{-1} they measured for the Suwannee River fulvic acid provided by the International Humic Substances Society (IHSS). Finally, Cu^{2+} activity was predicted by optimizing in the same way each batch of the five replicates the *%r-DOM* and $logK_{Cu}$ within physically meaningful ranges, i.e. respectively from 35% to 215% and 1.84 to 2.46, as previously suggested by Djae et al. (2017). Free Zn activity was then predicted on the basis of Cu^{2+} prediction using the same adjusted *%r-DOM* while $logK_{Zn}$ remained at the default value.

I.2.7 Data treatment and analysis

All statistical analysis were performed with R (R Core Team, 2016). Differences were considered statistically significant if $p < 0.05$. Data were $\log_{10}$ transformed because pH, DOC concentration, SUVA, total Cu and Zn concentrations in solution and Cu^{2+} and Zn^{2+} activities in solution exhibited different variation ranges.

Principal component analysis (PCA) were performed using the FactoMineR and factoextra packages. PCA was performed to illustrate the distribution of the mean replicates for the variable measured on the soil solution from the bio-influenced soils by earthworms as a function of soil or fertilization types while accounting for the contribution of mean replicates of solution variables (i.e. pH, DOC concentration, SUVA, Cu and Zn total concentration, and Cu^{2+} and Zn^{2+} activity). PCA were also performed to illustrate the effect of fertilization types for the three soil types considered individually.

The effect of the type of soil or of the type of fertilization on the solution variables of the earthworms bio-influenced soils was assessed by performing parametric tests i.e. the one-way Anova and the Tukey HSD post-hoc multiple test comparison, when conditions of residual normality and homoscedasticity were satisfied, or non-parametric rank tests, i.e. the Kruskal-Wallis test and the Dunn post-hoc multiple test comparison when conditions were not satisfied.

Multi-linear regressions were performed on the mean replicates of the solution variables and their variations between earthworm bio-influenced soil and bulk soil to hierarchize the respective contribution of pH, DOC, and SUVA on each Cu and Zn availability proxy (i.e. total concentration of Cu and Zn in soil solution and free ionic activity). To reduce the bias due to unavoidable co-linearity between some explanatory variables (Fig. S1, S2, S3, and S4), multi-linear regressions were built up by using two R packages (olsrr and MASS) to combine two approaches based on stepwise regressions and the Akaike information criteria. The best multi-linear regressions for each proxy of Cu and Zn availability for the whole dataset and for each soil were selected by considering the regression (i) with the highest adjusted coefficient of determination (adj R^2), (ii) which was statistically significant, and (iii) whose regression coefficient of each explanatory variable was statistically significant. In addition, we checked that the sign of each regression coefficient was similar in the corresponding simple and multi-linear regressions (Foucart, 2001). The order of contribution of each explanatory variable was determined according to the results of the stepwise regressions.

Multi-linear regressions were also performed to highlight the respective contribution of pH, DOC concentration, SUVA, total Cu and Zn concentration, experimental Cu^{2+} activity and predicted Zn^{2+} activity on Cu and Zn quantity in earthworms, i.e. to find the determinants of the relationship between soil metal availability and metal earthworm bioavailability. We calculated relative Cu and Zn quantities

in earthworms taking into account the lower quantity of Cu and Zn determined in earthworms on the whole dataset and adding these values to the whole dataset, in order to avoid negative quantities of Cu and Zn that could be sometimes calculated. The relative Cu and Zn contents in earthworms were then log_{10} transformed to perform the multi-linear regressions.

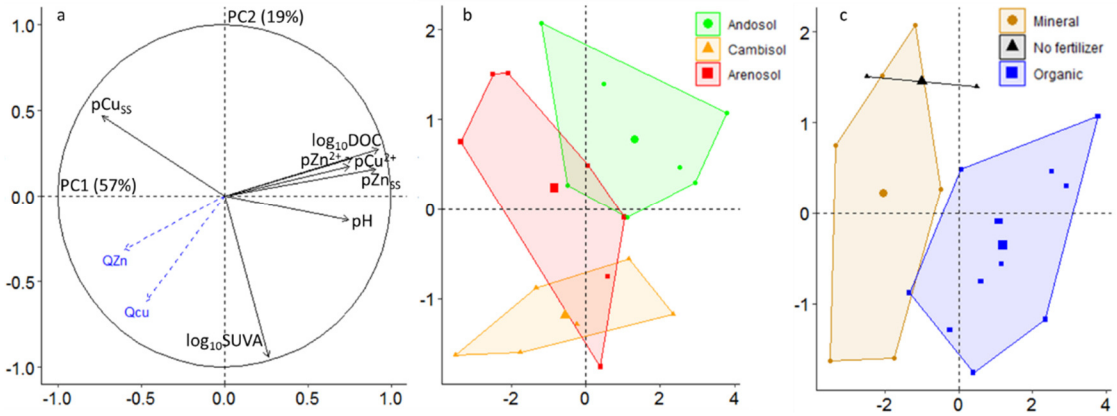
The effect of fertilization types on Cu and Zn quantity in earthworms in each soil type was assessed by performing parametric tests (the same as above) when conditions were satisfied or non-parametric rank tests (the same as above) when conditions were not satisfied.

I.3 Results

I.3.1 Effect of soil and fertilization types on solution chemistry and Cu and Zn availability in the soils bio-influenced by earthworms

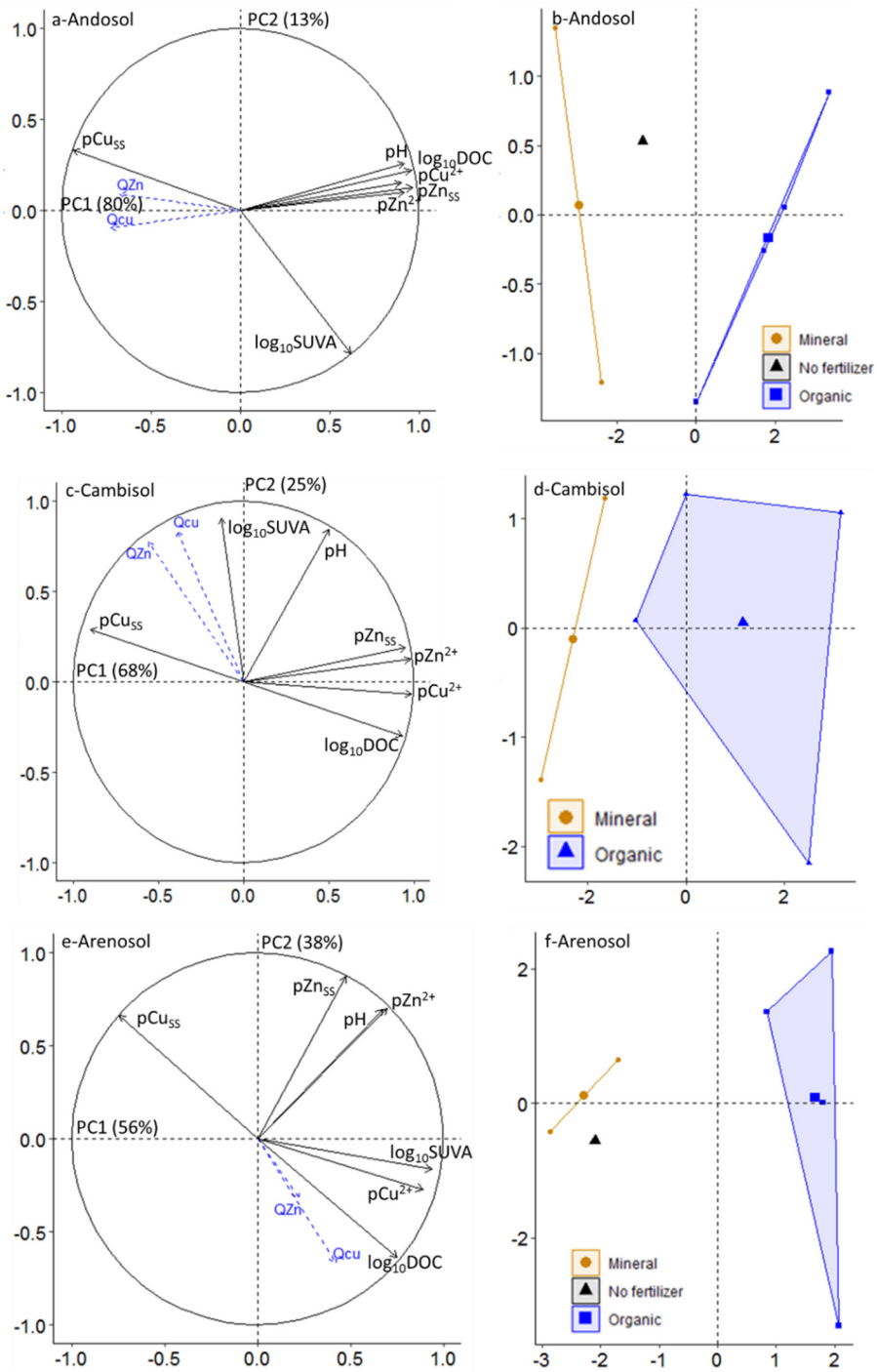
Soil and fertilization types influenced the distribution of the dataset on solution chemistry and Cu and Zn availability in the soil bio-influenced by earthworms (Fig. 1). The first component (PC1 57% of explained variance) was mainly determined by Cu and Zn availability proxies (pCu_{SS}, pZn_{SS},pCu^{2+} and pZn^{2+}) and the DOC concentration, the second component (PC2 19% of explained variance) was mainly determined by SUVA. The pH, pCu_{SS} and pZn_{SS} both contributed to PC1 and PC2 (Fig. 1a). Soil types are clearly dissociated in three clusters along the PC2 (Fig. 1b). This clustering was related to the SUVA which was higher in the cambisol than in the andosol and the arenosol (Fig. S5c). Unlike to soil types, the mineral and organic fertilization types are dissociated in two independent clusters along the PC1 (Fig. 1c). This clustering was related to Cu^{2+} and Zn^{2+} activity which were significantly higher in the cambisol and the arenosol and ranged from 11.6 to 9.3 in pCu^{2+} and from 7.8 to 6.3 in pZn^{2+} (Fig. S5f &g). Accordingly, soil and fertilization types had an influence on the soil solution chemistry and Cu and Zn availability proxies.

Figure 1 Principal component analysis (PCA) of the dataset. The correlation plot (a) shows the contribution of each variable to the two first principal components (PC1, PC2). Earthworm bio-influenced soil solution samples (n = 20) were clustered (b) as a function of soil types (andosol, cambisol, and arenosol) or (c) as a function of the fertilization types (No fertilization, Mineral, and Organic). Data point with a larger size in (b) and (c) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration (log_{10}DOC), the specific UV-absorbance at 254 nm in solution (log_{10} SUVA), the total concentration of Cu and Zn in solution (pCu_{SS} and pZn_{SS}), the activity of free ionic Cu and Zn in solution (pCu^{2+} and pZn^{2+}) of the earthworm bio-influenced soils. Supplementary quantitative variables are the Cu and Zn quantity in earthworms (QCu and QZn) and are represented by blue dashed arrows.



By performing PCA on each soil type individually, a similar effect of fertilization types was observed on solution chemistry and Cu and Zn availability for the three soil types (Fig. 2). The PC1 and PC2 explained 93%, 93% and 94% of the variance for the andosol, the cambisol, and the arenosol, respectively (Fig. 2a, c, and e). For the andosol and the cambisol, the PC1 was mainly determined by pZn^{2+}, DOC concentration, pCu_{SS}, pH, and pZn_{SS} and PC2 was mainly determined by SUVA. For the arenosol the PC1 was mainly determined by SUVA and pCu^{2+}, and PC2 was mainly determined by pZn_{SS}, pZn^{2+}, pH, pCu_{SS}, and DOC concentration. For each soil type, the fertilization types are dissociated in two (Fig. 2d) or three (Fig. 2b and f) distinct clusters along PC1. These findings highlight a clear distinct effect of OF, compared to mineral fertilization and no fertilization, on soil solution chemistry and Cu and Zn availability under earthworm's bio-influence, regardless of the soil type.

Figure 2 Principal component analysis on the earthworm bio-influenced soil solutions of (a, b) the andosol (n = 7), (c, d) the cambisol (n = 6), and (e, f) the arenosol (n =7). The correlation plots (a, c, and e) show the contribution of each variable to the two first components (PC1, PC2).Earthworm bio-influenced soil solution samples were clustered (b, d, and f) as a function of fertilization types (No fertilization, Mineral, and Organic). Data point with a larger size in (b), (d) and (f) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($log_{10}DOC$), the specific UV-absorbance of the solutions at 254 nm (log_{10} SUVA), the total concentration of Cu and Zn in solution (pCu_{SS} and pZn_{SS}), and the activity of free ionic Cu and Zn in solution (pCu^{2+} and pZn^{2+}) of the earthworm bio-influenced soils. Supplementary quantitative variables are the Cu and Zn quantity in earthworms (QCu and QZn) and are represented by blue dashed arrows.



I.3.2 Variation of pH, DOC concentration and SUVA in soils bio-influenced by earthworms

For the three soil types the pH, DOC concentration and SUVA varied in a rather narrow range in the earthworm bio-influenced soils (Fig. S5). The pH ranged from 6.2 to 6.6 and a low but significant increase in pH from not fertilized soil samples to the organic fertilized soil samples was observed in the three soil types (Fig. 3a, 4a, 5a). The DOC concentrations ranged from 3 to 36.7 mg l^{-1} (Fig. S5b) and slightly increased significantly with organic fertilization type in the three soil types (Fig. 3b, 4b, 5b). The SUVA ranged from 13.6 to 36.9 l g^{-1} cm^{-1} (Fig. S5c) and increased in the andosol and arenosol (Fig. 3c, 5c) while in the cambisol, SUVA was not different under organic fertilization compared to mineral fertilization (Fig. 4b).

Compared to values obtained from the bulk soil (Chapter II), soil incubation and the bio-influence of earthworms altered the pH, DOC concentration and SUVA in earthworm bio-influenced soil solutions (Fig S6). The pHs in the earthworm bio-influenced soil tended to be around a mean pH value of 6.3 ± 0.1, so that they were found to increase when initial bulk soil solutions pH were under or equal to 6.3 and to decrease when bulk soil solutions pH was over 6.3 (Fig. S6a). The DOC concentration in the earthworm bio-influenced soil solutions decreased by a 2- to 5-fold compared to the initial bulk soil solutions in the three soil types (Fig. S6b). Compared to the bulk soil solutions, the SUVA in the bio-influenced soil solutions increased by 2-fold (Fig. S6c).

Figure 3 Variation of (a) pH, (b) DOC concentration, (c) the specific UV-absorbance at 254 nm (SUVA), (d) the total Cu concentration in solution (pCu_{SS}), (e) the total Zn concentration in solution (pZn_{SS}), (f) the free ionic Cu activity (pCu^{2+}), and (g) the free ionic Zn activity in solution (pZn^{2+}) of the earthworm bio-influenced soil samples as a function of the fertilization types in the andosol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

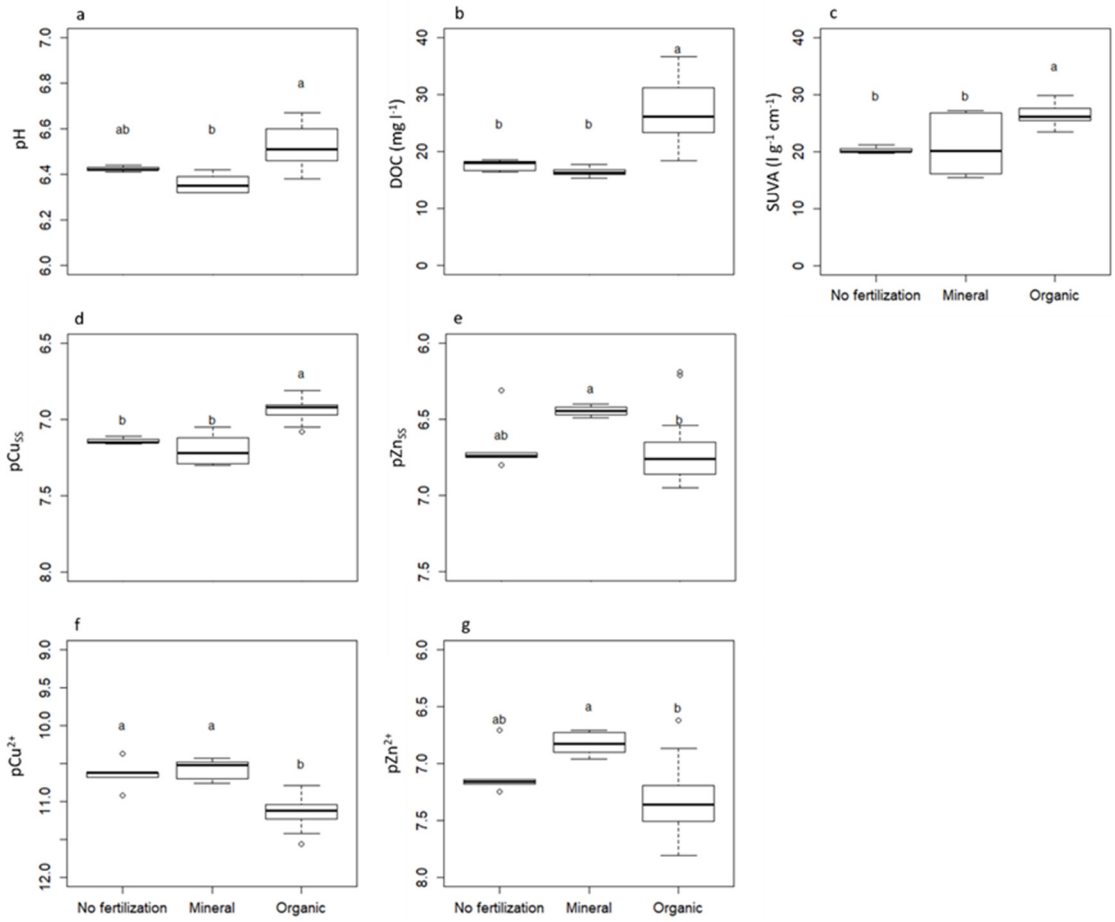


Figure 4 Variation of (a) pH, (b) DOC concentration, (c) the specific UV-absorbance at 254 nm (SUVA), (d) the total Cu concentration in solution (pCu_{SS}), (e) the total Zn concentration in solution (pZn_{SS}), (f) the free ionic Cu activity (pCu^{2+}), and (g) the free ionic Zn activity (pZn^{2+}) in the earthworm bio-influenced soil solutions as a function of the fertilization types in the cambisol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

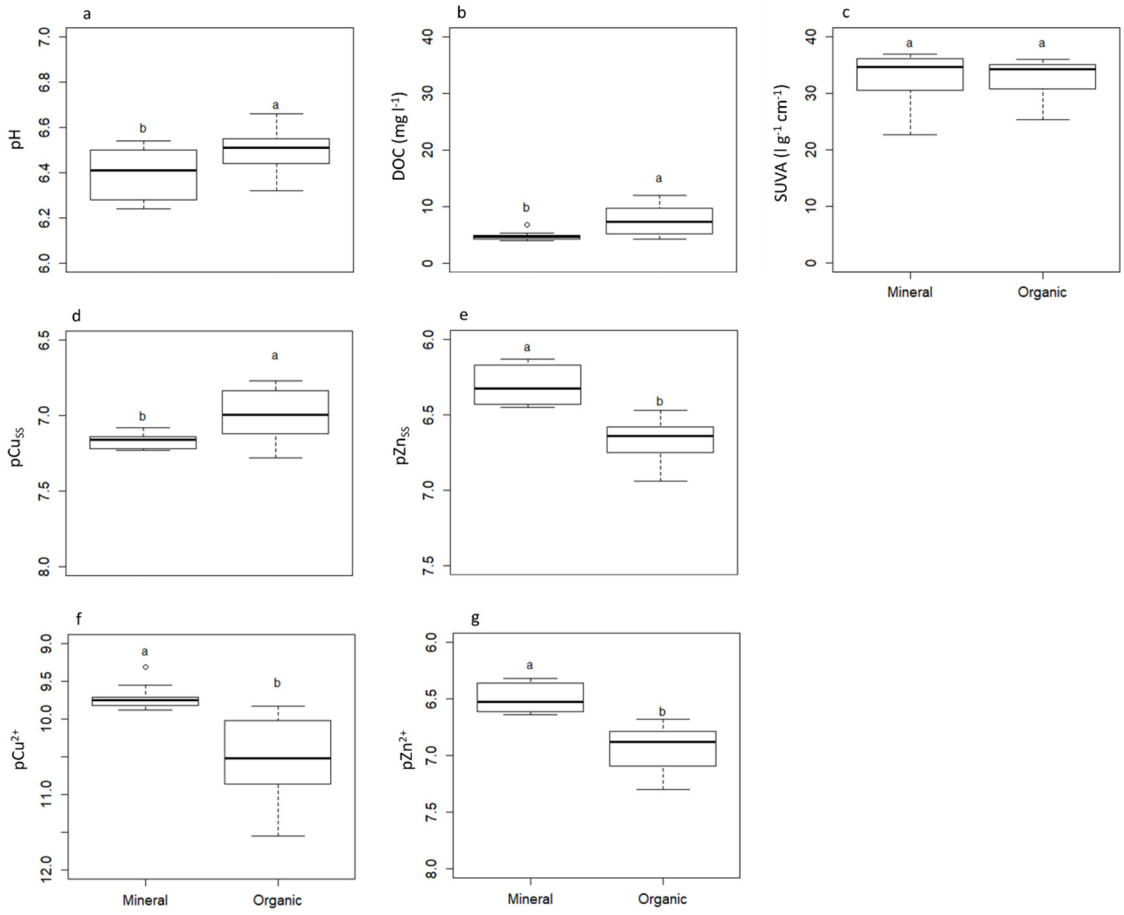
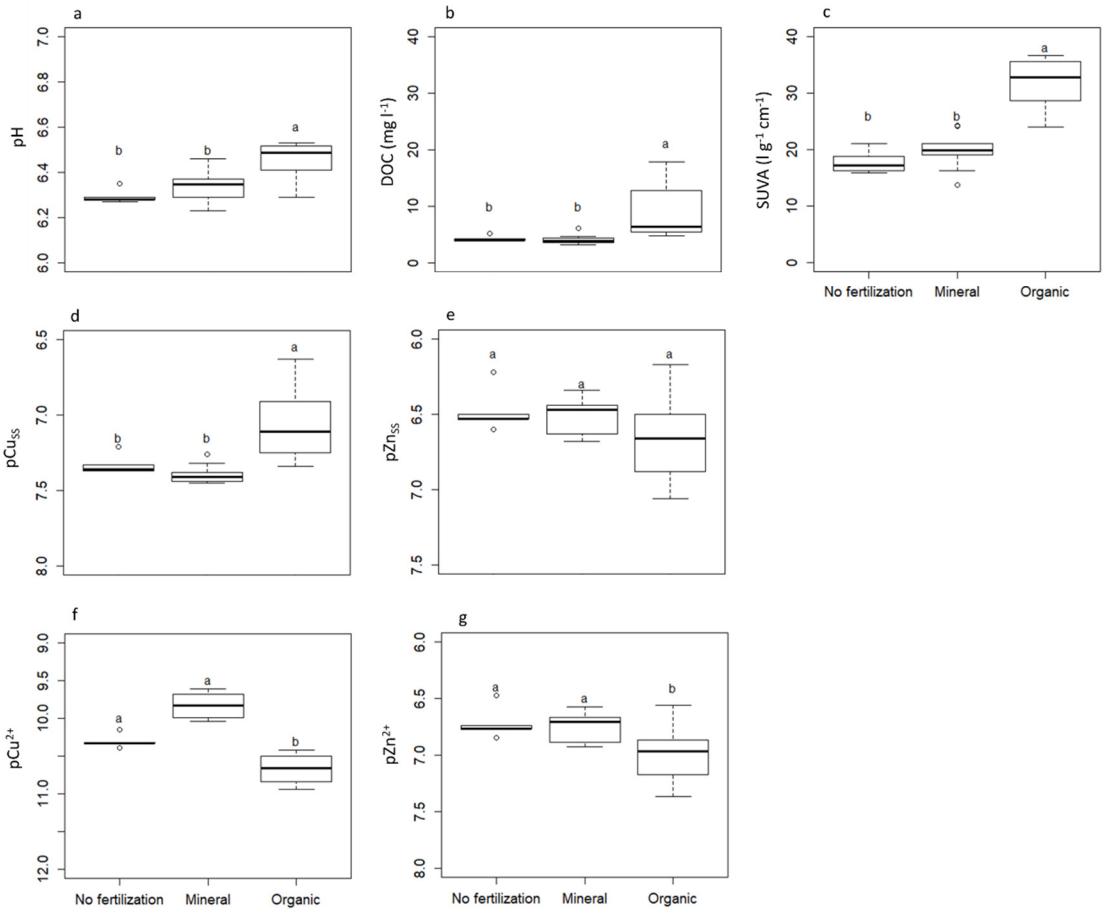


Figure 5 Variation of (a) pH, (b) DOC concentration, (c) the specific UV-absorbance at 254 nm (SUVA), (d) the total Cu concentration in solution (pCu_{SS}), (e) the total Zn concentration in solution (pZn_{SS}), (f) the free ionic Cu activity (pCu^{2+}), and (g) the free ionic Zn activity (pZn^{2+}) in the earthworm bio-influenced soil solutions as a function of the fertilization types in the arenosol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.



I.3.3 Copper and zinc availability in soils bio-influenced by earthworms

The total Cu and Zn concentrations in the solution of the earthworm bio-influenced soils ranged from 7.5 to 6.6 and from 6.1 to 7.1 in pCu_{SS} and pZn_{SS}, respectively (Fig S5 d&e). A low but significant increase in total Cu concentration in the earthworm bio-influenced soils was measured from not fertilized samples to samples which received organic fertilization whatever the soil types (Fig. 3d, 4d, 5d). Unlike the total Cu concentration, the total Zn concentration decreased in the earthworm bio-influenced soils amended with OF compared to soils under mineral fertilization in the andosol and the cambisol (Fig. 3e, 4e) while in the arenosol the total Zn concentration in the soil solution of the earthworm bio-influenced soil amended with OF was equal to soil not fertilized or under mineral fertilization (Fig. 5e).

The free ionic Cu and Zn activities ranged from 11.6 to 9.3 and from 7.8 to 6.3 in pCu^{2+} and pZn^{2+}, respectively (Fig. S5f&g). Regardless of the soil types, Cu^{2+} and Zn^{2+} activities both significantly decreased in the earthworm bio-influenced soil solution of OF amended soils compared to soils samples which were not fertilized or under mineral fertilization (Fig. 3f&g, 4f&g, 5f&g).

Compared to the bulk soil solution, the total Cu concentration in 90% of the earthworm bio-influenced soils decreased to a maximum of 0.2 in pCu_{SS} and slightly increased for two samples to a maximum of 0.1 in pCu_{SS} (Fig. S7a). Compared to the bulk soil solutions, the total Zn concentration decreased in 50% of the earthworm bio-influenced soil solutions to a maximum of 0.3 in pZn_{SS} (Fig. S7b). The decrease in total Zn concentration in solution of the earthworm bio-influenced soils occurred when total Zn concentration in the bulk soil solution was initially higher than 6.6 in pZn_{SS} and increased when Zn concentration in the bulk soil solution was initially higher than 6.6 in pZn_{SS} (Fig. S7b). Compared to the bulk soil, the Cu^{2+} activity has decreased in 85% of the earthworm bio-influenced soil solutions to a maximum of 2.2 in pCu^{2+} and increased in 15% in the arenosol to a maximum of 0.7 (Fig. S8a). Compared to the bulk soil solution, the predicted Zn^{2+} activity in the earthworm bio-influenced soil solutions increased when predicted Zn^{2+} activity in the bulk soil was initially lower than 7 in pZn^{2+} (Fig. S8b). The predicted Zn^{2+} activity slightly decreased in the earthworm bio-influenced soil solutions when predicted Zn^{2+} activity in the bulk soil solutions was initially higher than 7 in pZn^{2+} (Fig. S8b).

The small increase in total Cu concentration values was mainly positively related to the DOC concentration or the SUVA in the three soil types and secondly related to pH only in the andosol under the earthworm influence (Table 2). The total Cu concentration variation between the earthworm bio-influenced soil solutions and the bulk soil solutions was positively related to the variation of DOC concentration and SUVA only in the cambisol (Table S1). The decrease in total Zn concentrations in the earthworm bio-influenced soil solutions compared to the bulk soil was mainly negatively related to the solution pH in the andosol and the arenosol (Table 2). In the cambisol total Zn concentration was mainly driven by DOC concentration (Table 2). The total Zn concentration variation between the earthworm bio-influenced soil solutions and the bulk soil solutions was negatively related to the variation of pH in the andosol and to the variation of SUVA in the cambisol (Table S1). In the cambisol the pH did not explain directly the variation of total Zn concentration due to the significant positive correlation between the variations of pH and SUVA (Fig. S4b). The decrease in Cu^{2+} activity in the earthworm bio-influenced soil solutions was mainly negatively related to the DOC concentration in the andosol and cambisol (Table 3). In the arenosol the Cu^{2+} activity was mainly negatively related to the total Cu concentration in solution (Table 3) which was itself mainly driven by the DOC concentration (Table 2). The variation of Cu^{2+} activity between the earthworm bio-influenced soil solutions and the bulk soil solutions was mainly related to pH only in the andosol and the cambisol (Table S2). The

decrease in predicted Zn^{2+} activity in the earthworm bio-influenced soil solutions was positively related to the total Zn concentration and secondly negatively related to the DOC concentration in the three soil types (Table 3). The variation of predicted Zn^{2+} activity between earthworm bio-influenced soil solutions and bulk soil solutions was mainly related to pH and secondly to DOC concentration in the andosol (Table S2). In the arenosol, the predicted Zn^{2+} activity variation was related to the DOC concentration variation and secondly to the variations of total Zn concentration in soil solution and pH (Table S2).

Table 2 Multi-linear regression explaining the total concentration of copper (Cu) and zinc (Zn) in soil solution (pCu_{SS} and pZn_{SS}) of the earthworm bio-influenced soils as a function of pH, the log_{10} of dissolved organic carbon concentration ($log_{10}DOC$), and the log_{10} of specific UV absorbance of soil solution at 254 nm ($log_{10}SUVA$) for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		pH	$log_{10}DOC$	$log_{10}SUVA$	i	[a]adj R^2	[b]n
pCu_{SS}	3 soils	ns	-0.43^1	-1.1^2	9.12	0.78***	20
	Andosol	-0.83^2	ns	-1.05^1	13.9	0.99***	7
	Cambisol	ns	-1.1^1	ns	7.9	0.95***	6
	Arenosol	ns	-1.04^1	ns	8	0.95***	7
pZn_{SS}	3 soils	1.3^1	ns	ns	ns	0.48***	20
	Andosol	ns	1.02^1	ns	5.3	0.69*	7
	Cambisol	ns	ns	ns	ns	ns	6
	Arenosol	1.9^1	ns	ns	ns	0.76**	7

[a]adj R^2 = adjusted R^2; [b]n = number of samples

Table 3 Multi-linear regression explaining the activity of free ionic Cu and Zn in soil solution (pCu^{2+} and pZn^{2+}) of the earthworm bio-influenced soils as a function of the pH, the $log_{10}DOC$, the $log_{10}SUVA$, and the pCu_{SS} or pZn_{SS} for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

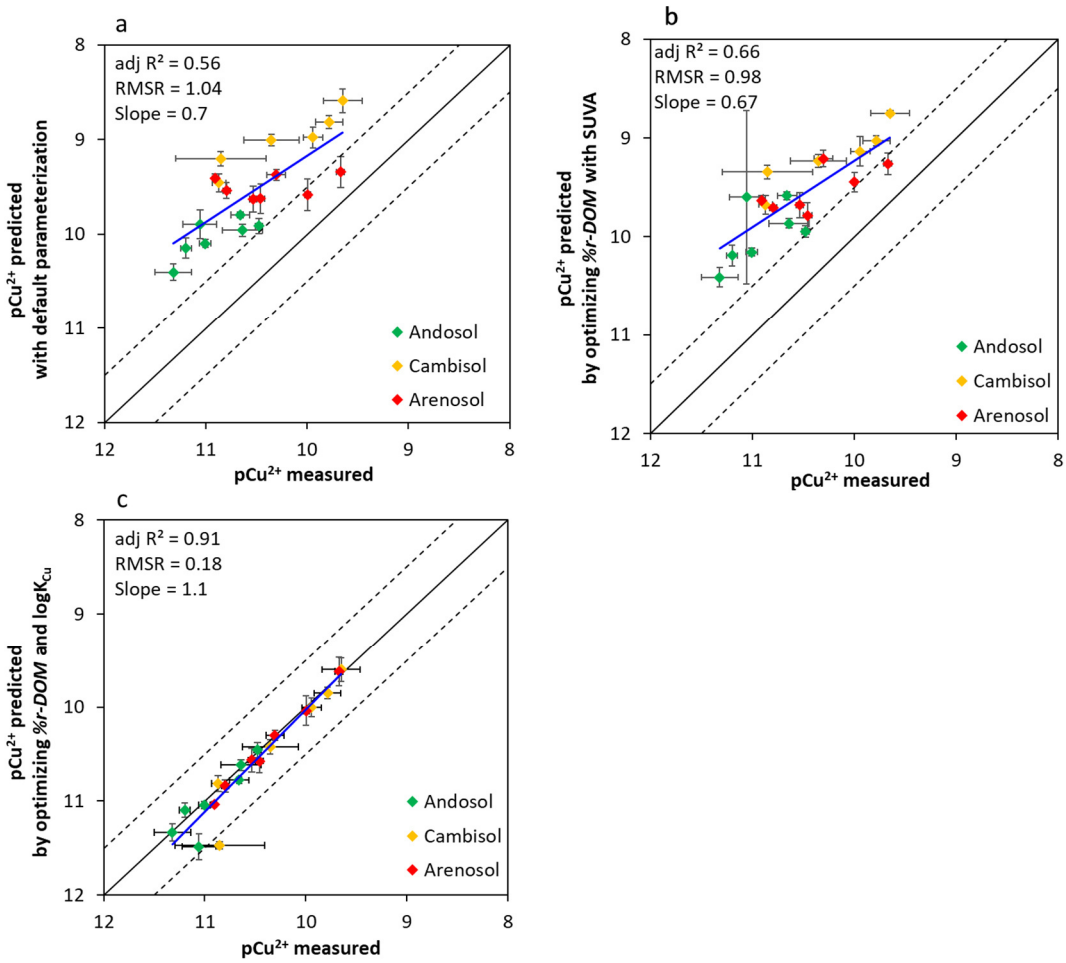
		pH	$log_{10}DOC$	$log_{10}SUVA$	pM_{SS}	i	[a]adj R^2	[b]n
pCu^{2+}	3 soils	1.8^2	ns	-2.8^3	-2.4^1	19.8	0.78***	20
	Andosol	ns	2.3^1	ns	ns	7.8	0.82**	7
	Cambisol	ns	3.03^1	ns	ns	7.8	0.83**	6
	Arenosol	ns	ns	ns	-1.5^1	21.3	0.61*	7
Predicted pZn^{2+}	3 soils	ns	0.44^2	ns	1.2^1	-1.2	0.94***	20
	Andosol	ns	1.1^2	ns	1.1^1	ns	0.95***	7
	Cambisol	ns	0.37^2	ns	1.05^1	ns	0.99***	6
	Arenosol	ns	0.3^2	ns	1.03^1	ns	0.99***	7

[a]adj R^2 = adjusted R^2; [b]n = number of samples

I.3.4 Binding properties of dissolved organic matter in the earthworm bio-influenced soils

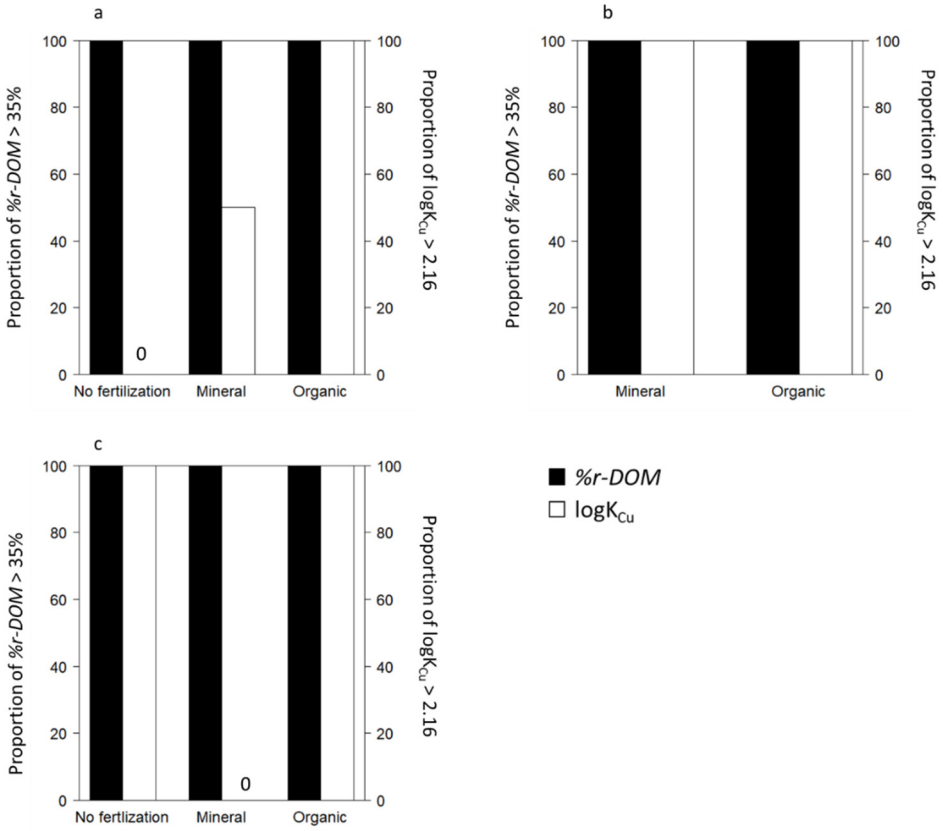
Prediction of Cu^{2+} activity in the earthworm bio-influenced soil solutions with default parameterization of WHAM overestimated the Cu^{2+} activity and poorly fitted the measured Cu^{2+} activities (Fig. 6a). The optimization of site density of DOM (*%r-DOM*) with SUVA still overestimated Cu^{2+} activity and only slightly improved the fitting between predicted and measured Cu^{2+} activities (Fig. 6b). Optimizing *%r-DOM* and the DOM binding affinity towards Cu ($logK_{Cu}$) improved the predicted Cu^{2+} activity (Fig. 6c) except for one sample in the cambisol which was an OF amended soil sample. Under the range of realistic restricted values, we were not able to optimize the *%r-DOM* and $logK_{Cu}$ for this sample whose *%r-DOM* and $logK_{Cu}$ corresponded to the best optimization (i.e. 215 and 2.46) for the 5 replicates of the soil sample (i.e. the lowest RMSR for 5 replicates of one soil sample).

Figure 6 Free copper activity in the earthworm bio-influenced soil solutions (pCu^{2+}) predicted with WHAM as a function of pCu^{2+} measured in earthworm bio-influenced soil solutions (n = 20). pCu^{2+} was predicited by (a) using the default parameters of WHAM for the percentage of dissolved organic matter reactive toward metal cations (*%r-DOM*) equal to 65% and the copper binding constant ($logK_{Cu}$) equal to 2.16 for all bio-influenced soil solutions, (b) optimizing the *%r-DOM* with the SUVA measured in the bion-influenced soil solutions, or (c) optimizing *%r-DOM* and $logK_{Cu}$ values within a range of physically meaningful values for each bacth of five replicates of a sample (see the *Material and methods* section for rationale). The blue line represents the regression line; the thin solid line represents the 1:1 line; the dashed lines represent the 1:1 line ± 0.5 pCu^{2+} unit. Vertical and horizontal error bars stand for the standard deviation on predicted Cu^{2+} activity and measured Cu^{2+} activity, respectively. The abbreviations adjR^2 and RMSR mean adjusted R^2 and root mean square residual, respectively.



In the three soil types, under earthworm influence, the proportion of %r-DOM values higher than 35% that have been optimized in WHAM was equal and reached 100%, whatever the fertilization type (Fig. 7). The proportion of $logK_{Cu}$ values higher than 2.16 (while other values were equal to 2.16) that have been optimized in WHAM increased in the andosol with organic fertilization compared to soil samples without fertilization or with mineral fertilization (Fig. 7a). In the cambisol and the arenosol the proportion of $logK_{Cu}$ values that had to be optimized above the default value of 2.16 was equal between the fertilization types and reached 100% (Fig. 7b&c) except for soil samples under mineral fertilization in the arenosol, whose $logK_{Cu}$ was equal to 2.16 (Fig. 7c).

Figure 7 Proportion of the percentage of dissolved organic matter (DOM) reactive towards metal cations (%r-DOM) and of the copper binding constant ($logK_{Cu}$) optimized in WHAM within ranges restricted to physically meaningful values that were respectively greater than 35% and greater than 2.16 (while the other values were equal to 2.16) in the earthworm bio-influenced soil solutions of (a) the andosol (n = 7), (b) the cambisol (n = 6), and (c) the arenosol (n = 7) as a function of the fertilization type (No fertilization, Mineral or Organic).



Compared to the bulk soil solutions, the value of *%r-DOM* optimized in WHAM all increased, except one, in the earthworm bio-influenced soil solutions of the three soil types (Fig. S9a). In the earthworm bio-influenced soils solutions, 80% *%r-DOM* data were optimized to the maximum value within the physically meaningful range of *%r-DOM* (i.e. 215). The $\log K_{Cu}$ values in the earthworm bio-influenced soil solutions were systematically higher than the $\log K_{Cu}$ optimized in WHAM in the bulk soil solutions (i.e. 2.16) (Fig. S9b). The $\log K_{Cu}$ in the earthworm bio-influenced soil solutions also systematically increased in the three soil types (Fig. S9b).

I.3.5 Bioavailability of copper and zinc for Dichogaster saliens

Between the beginning and the end of the experiment we measured a significant mean biomass loss in the andosol, the cambisol, and the arenosol of 36 %, 22%, and 28%, respectively, whatever the fertilization types (Fig. S10). Earthworm quiescence occurred in the andosol in all fertilization types with a median value of 70% in the not fertilized and OF amended soils (Fig. S11a). Earthworm mortality occurred in the andosol, in the non-fertilized and organic fertilized soil samples with a median value of 38%. Mortality in the soils amended with OF was significantly higher than in the soil samples that received mineral fertilizer (Fig. S12a). In the cambisol quiescence and mortality were low and not significantly different between earthworm in mineral fertilized soil samples and earthworms in soils amended with OF (Fig. S11b, S12b). In the arenosol the quiescence was significantly higher with a median value of 70% in the soils amended with OF, compared to not fertilized soil samples or under mineral fertilization (Fig. S11c). Mortality occurred in the arenosol, in soils amended with OF but the mortality rate of earthworms was not significantly different from soils under mineral fertilization and without fertilization (Fig. S12c).

The Cu and Zn quantity accumulated in earthworms were significantly lower in the andosol compared to the cambisol and the arenosol (Fig. S5h&i). The Cu and Zn quantity accumulated in earthworms slightly decreased as a function of the fertilization types only in the andosol (Fig. 8a and 9a). In the cambisol and arenosol no significant increase in Cu and Zn quantity accumulated in earthworms was measured exposed to soils amended with OF compared to earthworms exposed to soils without fertilization or under mineral fertilization (Fig. 8b&c).

Figure 8 Quantities of Cu in earthworms (*D.saliens*) in (a) the andosol, (b) the cambisol, and (c) the arenosol as a function of the fertilization types. The red line stand for the zero. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

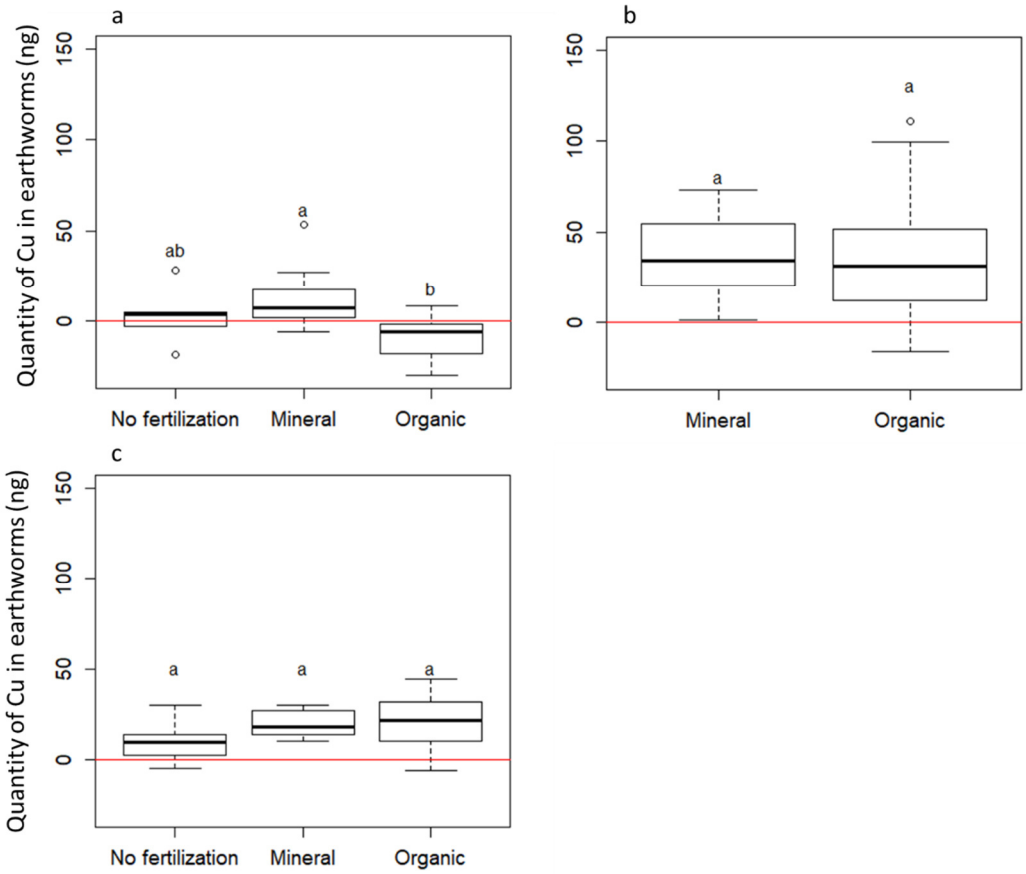
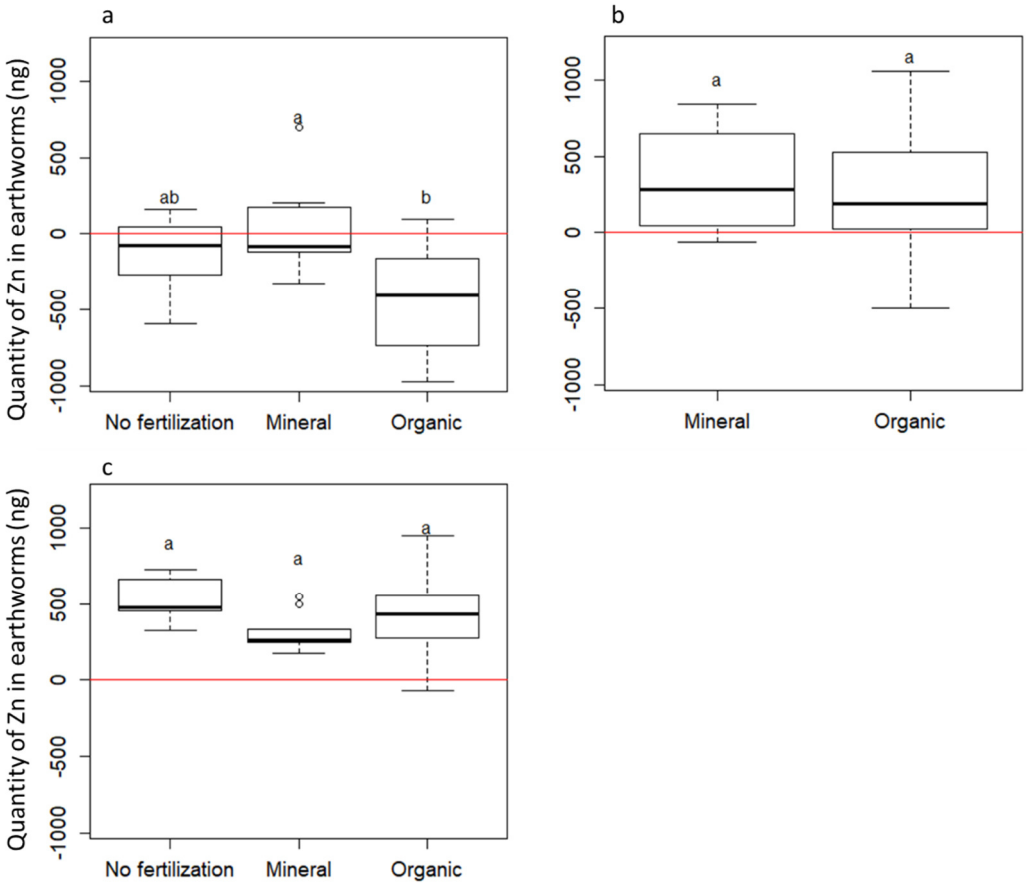


Figure 9 Quantities of Zn in earthworms (*D. saliens*) in (a) the andosol, (b) the cambisol, and (c) the arenosol as a function of the fertilization types. The red line stand for the zero. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.



The accumulation of Cu in earthworms (see 1.2.7 for rationale) was slightly positively related to the Cu^{2+} activity in the earthworm bio-influenced solution for the andosol (Table 4). The accumulation of Zn in earthworms was negatively related to the DOC concentration in the earthworm bio-influenced solution for the cambisol (Table 4). In the arenosol, the relative Zn internal earthworm's quantity was mainly positively related to DOC concentration and secondly to the total Zn concentration in solution and negatively to the Zn^{2+} activity in the solution of the earthworm bio-influenced soils (Table 4).

Table 4 Multi-linear regression explaining the quantity of Cu and Zn in earthworms ($\log_{10}Q_{Cu}$ and $\log_{10}Q_{Zn}$) as a function of pH, the $\log_{10}$DOC, the $\log_{10}$SUVA, the pCu_{SS} or pZn_{SS}, and the pCu^{2+} or pZn^{2+} for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		pH	$\log_{10}$DOC	$\log_{10}$SUVA	pM_{SS}	pM^{2+}	i	[a]adj R^2	[b]n
$\log_{10}Q_{Cu}$	3 soils	ns	ns	0.74^2	ns	-0.31^1	3.8	0.52***	20
	Andosol	ns	ns	ns	ns	-0.57^1	7.6	0.62*	7
	Cambisol	ns	ns	ns	ns	ns	ns	< 0.6	6
	Arenosol	ns	ns	ns	ns	ns	ns	< 0.4	7
$\log_{10}Q_{Zn}$	3 soils	ns	-0.7^1	ns	ns	ns	3.6	0.36**	20
	Andosol	ns	ns	ns	ns	ns	ns	ns	7
	Cambisol	0.6^2	-0.64^1	ns	ns	ns	ns	0.95**	6
	Arenosol	ns	1.7^1	ns	6.2^2	-6.02^3	2.5	0.83*	7

[a]adj R^2 = adjusted R^2; [b]n = number of samples

I.4 Discussion

I.4.1 A small and similar effect of soil incubation and earthworm bio-influence on soil solution chemistry and copper and zinc availability

In our study, the main difference between the soil samples under earthworm influence and the bulk soil samples is the incubation: bulk soil samples were not incubated while soil samples with earthworms were pre-incubated during 14 days prior inoculation then incubated during 21 days. It is noteworthy that the incubation period may have, together with earthworm bio-influence, induced changes in soil solution chemistry compared to the bulk soil.

It was interesting to note that in our case in the presence of earthworms and whatever the initial bulk soil solution pH, the pH of the bio-influenced zone tended to be buffered around a mean value of 6.3 in the three soil types. When incorporating soils with OF Tella et al. (2016) reported in the absence of earthworm an increase in soil solution pH followed by a decrease from 0.1 to 1.4 units during the incubation time which was attributed to the primary production of ammonium followed by a production of nitrate (Usman et al., 2004). But most of studies in the literature reported an increase in soil pH under the influence of earthworms compared to soil with no adding of earthworms (e.g. Leveque et al., 2014; Udovic et al., 2007; Wen et al., 2006). Barois and Lavelle. (1986) noted that the pH in the gut of tropical earthworms *Pontoscolex corethrurus* was higher than the soil pH (6.8 compared to 4.6) while the casts had lower pH than in the gut but still higher than the soil pH (+ 0.2 pH units). In our case, the slight variations we observed on solution pH in the earthworm bio-influenced soils compared to the bulk soil solution may be attributed to the combination of both processes: soil incubation which tends to decrease pH and earthworm's activity which tends to increase pH.

The DOC concentration decreased in the earthworm bio-influenced soil solutions by 2- to 5-fold compared to soils without earthworm. But the DOC concentration may also be altered only by soil incubation (Amery et al., 2007; Merckx et al., 2001). After two cycles of drying-rewetting soil, Merckx et al. (2001) first reported a DOC flush followed by a 2- to 3-fold decrease in DOC concentration with incubation time. The DOC flush was attributed to the rewetting of the soil which promoted C mineralization and induced solubilization of soil organic matter. The authors suggested that the removed flush DOM was mainly composed of non-aromatic compounds (e.g. sugars). Such easily degradable DOM is preferentially utilized by microorganisms (Kalbitz et al., 2003), that could explain the subsequent decrease in DOC concentration. In the presence of earthworms in the soil it has been sometimes reported an increase in the DOC concentration until 1.5-fold compared to the soil without earthworm (Dandan et al., 2007; Wen et al., 2004) which is contradictory to our findings. A hypothesis supporting the decrease in DOC concentration may be that earthworm activity increase the activity of

microorganisms (Binet et al., 1998; Toyota and Kimura, 2000) stimulating the degradation of easily degradable organic compounds resulting DOC concentration decrease. Finally our observed decrease in DOC concentration in the earthworm bio-influenced soil solutions is likely due to first an effect of soil incubation followed by a hypothetic priming effect related to the bio-influence of earthworms.

Our findings on SUVA in the earthworm bio-influenced soil solutions compared to the bulk soil solutions may be related to this DOC concentration decrease, with the hypothesis that low organic molecules first disappear leaving high molecules less biodegradable in soil solution. Amery et al. (2007) reported an increase in SUVA with soil incubation time, and suggested that this increase was related to the degradation by microorganisms of easily degradable compounds with low aromaticity, leading to concentrate aromatic compounds that are per se less degradable. Zhu et al. (2016) reported higher SUVA in vermicomposting residues compared to compost residues suggesting that the biodegradation activity of earthworms (*Eisenia fetida*) increased relatively the aromaticity of DOM. Thus, both soil incubation and earthworm's activity result in an increase in DOM aromaticity and can explain our observed results.

Compared to bulk soil solutions, in the presence of earthworms a slight decrease of total Cu in soil solution was observed while only low variations of total Zn concentration were noted. Usman et al. 2004 reported that the incubation of soil with sewage sludge or compost at low dose (15 t ha^{-1}) did not increased the mobility of Cu and Zn in soil during the soil incubation compared to the same soil without OF application. Several studies in the literature rather reported an increase in Cu and Zn in solution due to earthworm activity (Udovic et al., 2007; Wen et al., 2004). This was attributed either to direct effects like mucus production, burrowing activity, or DOC alteration, or to indirect effects of earthworm activity after stimulation of microorganisms (Sizmur et Hdson, 2009). In our case, the variation between total Cu and Zn concentrations in earthworm bio-influenced solutions and bulk soil solutions was poorly related to the variation of pH, DOC concentration or nature of organic matter expressed as SUVA. This could be attributed to the narrow range of variations observed in our solutions in the presence of earthworms. Compared to bulk soil solutions, in the presence of earthworms a slight decrease in Cu^{2+} activity in soil solution was observed while only low variations of Zn^{2+} activity were noted. The variations between Cu^{2+} and Zn^{2+} activities in earthworm bio-influenced soil solutions and bulk soil solutions were mainly related to the variation of pH in solution between earthworm bio-influenced soil solutions and bulk soil. Sizmur et al. (2011a) predicted Cu^{2+} and Zn^{2+} activity with WHAM and reported that compared to the bulk soil, the predicted Cu^{2+} activity in the soil with earthworm decreased with a decrease in organic carbon solubility due to earthworm activity. An increase in Zn^{2+} activity was predicted and was attributed to the decrease in pH in the presence of earthworm inducing an increase in total Zn in solution. Our findings suggest that the effect of soil incubation and the effect

of earthworms on Cu and Zn availability proxies were weak and of the same order of magnitude hence the difficulty of clearly distinguishing them.

I.4.2 A major effect of organic fertilizers on solution chemistry and copper and zinc availability in earthworm bio-influenced soils

The bio-influence of earthworms did not change the effect of OF applications on pH, DOC concentration, SUVA, and Cu and Zn availability proxies in soil solutions when comparing with the effect of OF applications observed in the absence of earthworms (Chapter II). The only difference is that variation ranges of pH, DOC concentration, SUVA, and Cu and Zn availability proxies were lower with earthworms than without.

The total Cu concentrations in solution were found mainly driven by DOC concentration and aromaticity while the total Zn concentrations were found mainly driven by pH due to the lower affinity of Zn for DOM (Bonten et al., 2008). In a Zn contaminated mine soils amended with OF, Sizmur et al. (2011a) reported an increase in Zn solubility in soils inoculated with earthworms (*Lumbricus terrestris*) compared to bulk soil. The authors explained the increase in Zn solubility by a decrease in pH in the presence of earthworms compared to the soil amended with OF but without earthworms. The authors also showed that the increase in the solubility of Zn in the presence of earthworms was reduced when increasing the OF rate applied to the soil due to a significant increase in pH. In our study the decrease in Cu^{2+} activity was mainly driven by DOC concentration. Sizmur et al. (2011a) reported in a Cu contaminated mine soil amended with OF and inoculated with earthworms, a decrease in predicted Cu^{2+} activity compared to bulk soil. This finding was attributed to the lower solubility of organic C in the presence of earthworms, allowing for less Cu to be soluble in soil solution. In our context, the Cu and Zn availability proxies were mainly mitigated by the OF fertilization application rather than by the bio-influence of earthworm in soils.

I.4.3 Organic fertilization did not increase copper and zinc bioavailability to earthworms

Our findings revealed a decrease in earthworm biomass during the 21 days of experiment, regardless of soil and fertilization types. Leveque et al. (2014) reported a decreasing trend in biomass of *Aporrectodea caliginosa* and *L. terrestris* during their incubation experiment (6 weeks). The authors suggested that in the absence of clear dose-response between metal concentration in soil and earthworm biomass the metal contamination was not the main cause for the decrease in earthworm biomass. Sizmur et al. (2011b) reported a decrease in biomass of *L. terrestris* between the beginning and the end of their experiment (28 days). According to the authors that may be due to the fact that

no food was added to soil during the experiment to make sure that observed changes in soil chemistry were due to earthworms burrowing activity and not due to their effect on mixing food within the soil. In the present study the earthworms were not fed during the experiments thus it may explain the earthworm biomass loss. The results on the health status (i.e. quiescence and mortality) of *D. saliens* suggested that the fertilization types were not the main cause of the quiescence and mortality but rather the soil type. Andosol and arenosol were not enough suitable for the proper development of *D. saliens*. Indeed the andosol was the only soil type in which *D. saliens* did not occurred naturally in the field trial and in the arenosol fewer individuals were found than in the cambisol. However, in the andosol, the rate of mortality was significantly higher in soil samples that received OF compared to soil samples that received mineral fertilizers. This is probably due to the fact that the application of OF increased the organic C in the soil and thus the retention of water, so that for the same water content applied to moist the andosol samples, the soil samples that received OF tend to be drier to earthworms. Plus, the Cu and Zn quantities in earthworms in OF soil samples did not increase thus there was no relation between Cu and Zn quantities in earthworms and mortality. If there is toxicity in the andosol, it is not related to Cu and Zn contamination in soil.

The OF application to soils did not induce an increase in Cu and Zn accumulation in earthworms. Kizilkaya (2004) reported an increase in Cu and Zn concentration in earthworms as a function of the rate of sewage sludge (0 to 800 t ha^{-1} soil) added to the soil and attributed this increase to the content of metals and organic matter induced by OF applications. Centofanti et al. (2016) reported that Cu concentration in *L. terrestris* did not increase with OF amendments (112 or 224 t ha^{-1}) while Zn concentrations in *L. terrestris* exposed to soils amended with OF were similar to known Zn concentration in *L. terrestris* exposed to non-contaminated or forest soils. The authors suggested that earthworms, which are selective feeders, preferentially feed on decomposed organic matter when dead roots and plant debris are present, thus less contaminated soil was introduced in their body.

The pH was reported in the literature to be the main factor determining the metal accumulation in earthworms: as pH increased the metal accumulation in earthworms decreased (Nahmani et al., 2007). Our findings showed that Cu and Zn bioavailability was poorly explained by pH, DOC concentration, aromaticity and Cu and Zn availability proxies except for the andosol in the case of Cu and in the cambisol in the case of Zn. The variations of pH between the earthworm bio-influenced soil solution and bulk soil solution were low, indicating that earthworm and soil incubation buffered soil solution pH to a value around 6.3 in the three soil types. Also the DOM concentration in the soil bio-influenced by earthworms varied on a lower range than in the bulk soil and the estimated binding properties of DOM have increased in the presence of earthworms. Hence the total Cu and Zn concentration and Cu^{2+} and Zn^{2+} activity did not much vary in the earthworm bio-influenced soil solution. Thus, first the

changes induced by OF application and secondly the combined of low effect of *D. saliens* and soil incubation on soils did not change Cu and Zn content compared to non-amended or soils receiving mineral fertilizers. In our experimental conditions, this resulted in a protective effect for earthworms on Cu and Zn bioavailability.

The low influence of solution chemistry and Cu and Zn availability in the earthworm bio-influenced soils on Cu and Zn content in earthworms suggests that other mechanisms proper to the earthworm may occurred. According to Spurgeon et Hopkins (1999) earthworms are able to regulate metal body contents, and excretion of metals may be a regulation mechanism in the case of essential elements such as Cu and Zn.

1.5 Conclusion

Using the collection of soil samples of three different type of soils, and with three different types of fertilization (either no fertilization, and mineral or organic fertilization) our experimental results showed that under the influence of soil incubation with earthworms, the variation ranges of pH and DOC concentration in solution were reduced compared to bulk soil samples. Nevertheless, the effects of OF application on Cu and Zn availability in soil solution for the earthworm bio-influenced soils were similar to those in the bulk soil but in a lesser extent. Finally compared to situations without earthworm, the combination of the attenuated effect of OF application on the solution chemistry and the buffered effect of the activity of earthworms in soils together with soil incubation mitigated Cu and Zn availability. Hence, in our experimental conditions, internal Cu and Zn contents in earthworms were not affected by the OF application on soils for a decade whatever the increase in contamination, probably due to a major effect of organic fertilization combined with the low effect of earthworms and soil incubation on Cu and Zn availability.

Earthworms are considered to be ecosystem engineers due to their ability to modify resources and make it available for other organisms. It should be interesting, however, to confirm such results in the presence of other organisms like plants. Furthermore, considering interactions between earthworms and other key organisms in soils such as plants, it could be interesting to study the effect of this biotic interaction on Cu and Zn availability and phytoavailability in soils that received OF during a decade.

Acknowledgements

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1.6 Annexes

Figure S1 Correlation matrix of the pH, the log_{10} of dissolved organic carbon concentration ($log_{10}DOC$), the log_{10} of specific UV absorbance of the solution at 254 nm ($log_{10}SUVA$), the total concentration of copper (Cu) and zinc (Zn) in solution (pCu_{SS} and pZn_{SS}), the free ionic Cu or Zn activity (pCu^{2+} and pZn^{2+}) activity in the earthworms bio-influenced soil solutions, and the log_{10} of the Cu or Zn quantity in earthworms ($log_{10}Q_{Cu}$ and $log_{10}Q_{Zn}$) on the whole dataset. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***

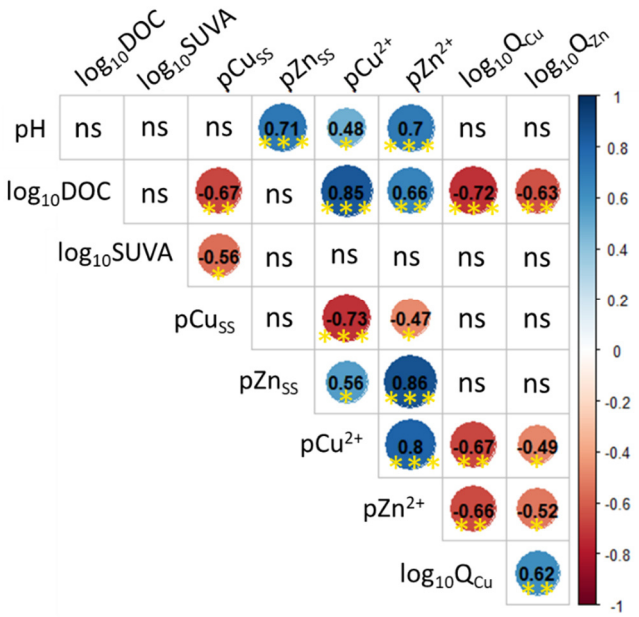


Figure S2 Correlation matrix of the pH, the $\log_{10}$ of dissolved organic carbon concentration ($\log_{10}$DOC), the $\log_{10}$ of specific UV absorbance at 254 nm ($\log_{10}$SUVA), the total Cu and Zn concentration in solution (pCu_{SS} and pZn_{SS}), and the Cu^{2+} and Zn^{2+} activity (pCu^{2+} and pZn^{2+}) in the earthworm bio-influenced soil solutions of (a) the andosol, (b) the cambisol, and (c) the arenosol. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: $0.01 < p < 0.05$***; $0.001 < p < 0.01$**; $p < 0.001$***.

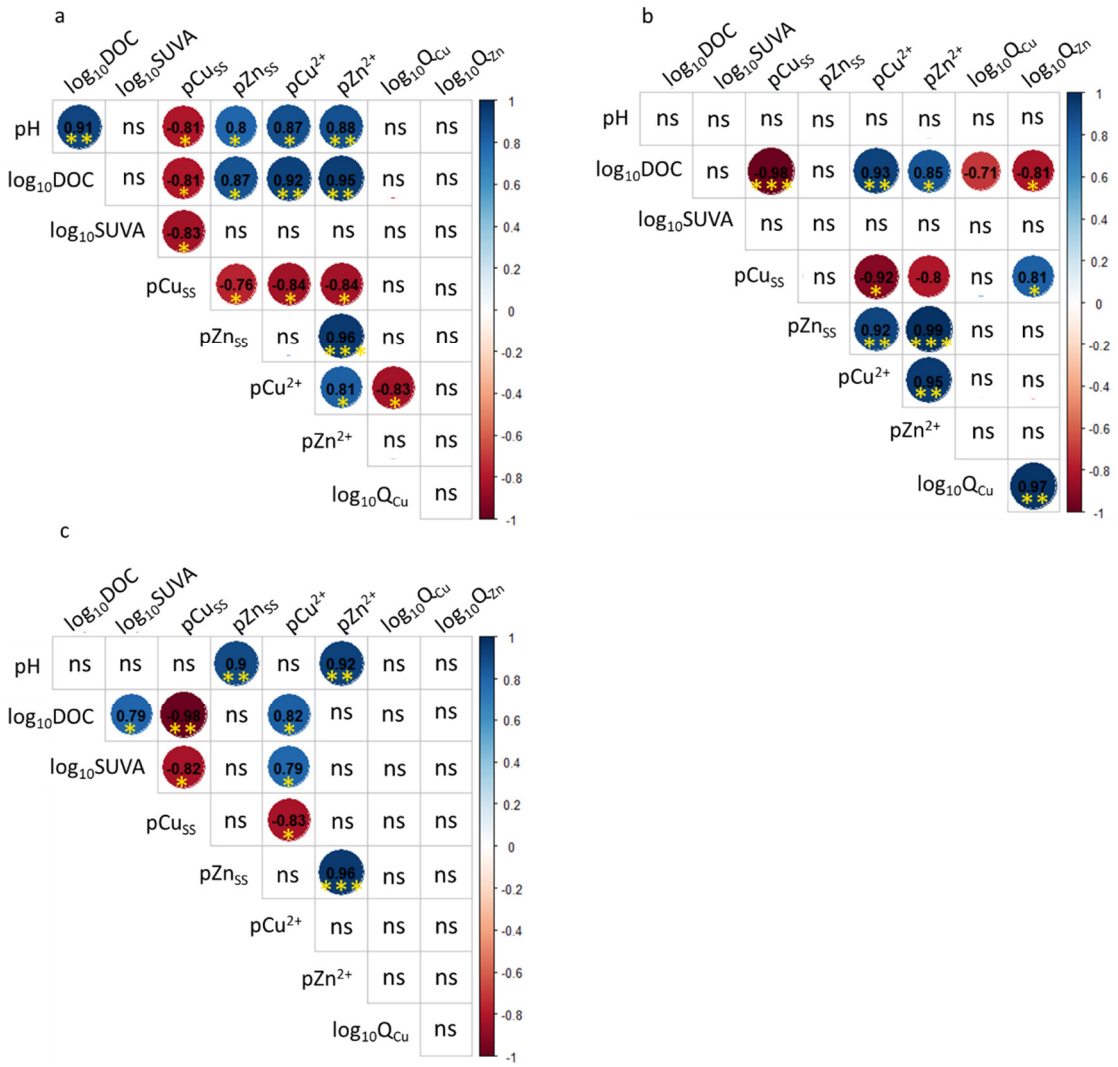


Figure S3 Correlation matrix of the variation of pH (ΔpH), log_{10}DOC (Δlog_{10}DOC), log_{10}SUVA (Δlog_{10}SUVA), pCu_{SS} or pZn_{SS} (ΔpCu_{SS} and pZn_{SS}), pCu^{2+} or pZn^{2+} (ΔpCu^{2+} and pZn^{2+}) between earthworm bio-influenced soil solutions and the bulk soil solutions on the whole dataset. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: $0.01 < p < 0.05$***; $0.001 < p < 0.01$**; $p < 0.001$***.

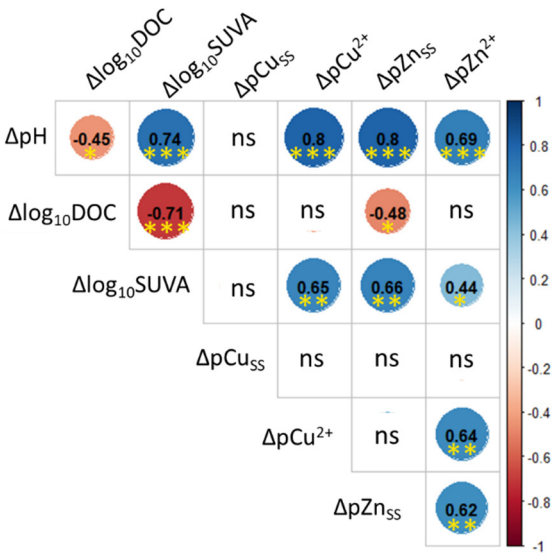


Figure S4 Correlation matrix of the variation of pH (ΔpH), log_{10}DOC (Δlog_{10}DOC), log_{10}SUVA (Δlog_{10}SUVA), pCu_{SS} or pZn_{SS} (ΔpCu_{SS} and pZn_{SS}), pCu^{2+} or pZn^{2+} (ΔpCu^{2+} and pZn^{2+}) between earthworms bio-influenced soil solutions and the bulk soil solutions in (a) the andosol, (b) the cambisol, and (c) the arenosol. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***.

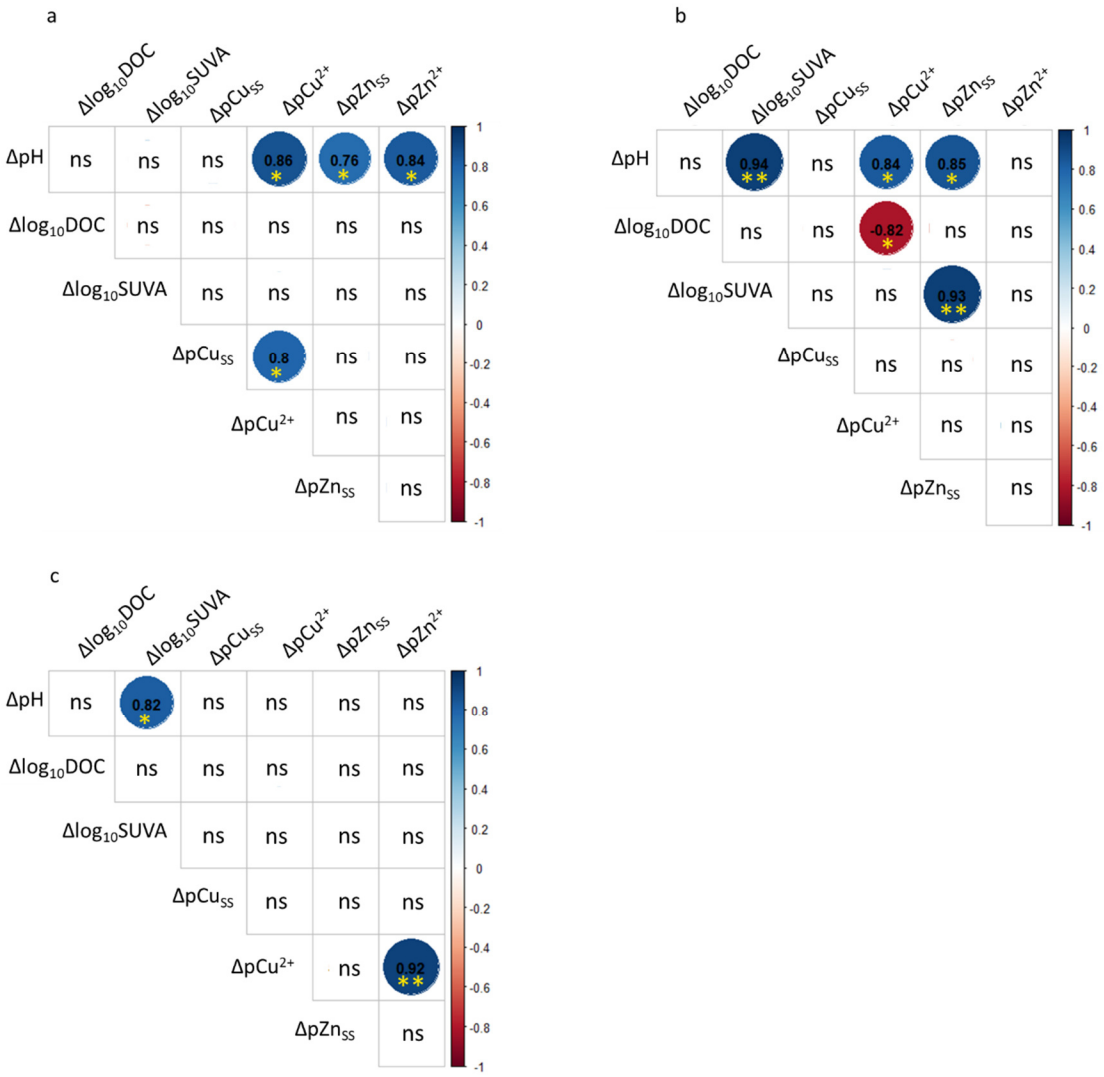


Figure S5 Variation of (a) pH, (b) DOC concentration, (c) the specific UV-absorbance at 254 nm (SUVA), (d) the total Cu concentration in solution (pCu_{SS}), (e) the total Zn concentration in solution (pZn_{SS}), (f) the free ionic Cu activity (pCu^{2+}), and (g) the free ionic Zn activity (pZn^{2+}) in the earthworm bio-influenced soil solutions as a function of soil types. Andosol n = 35, cambisol n = 30, and arenosol n = 35. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

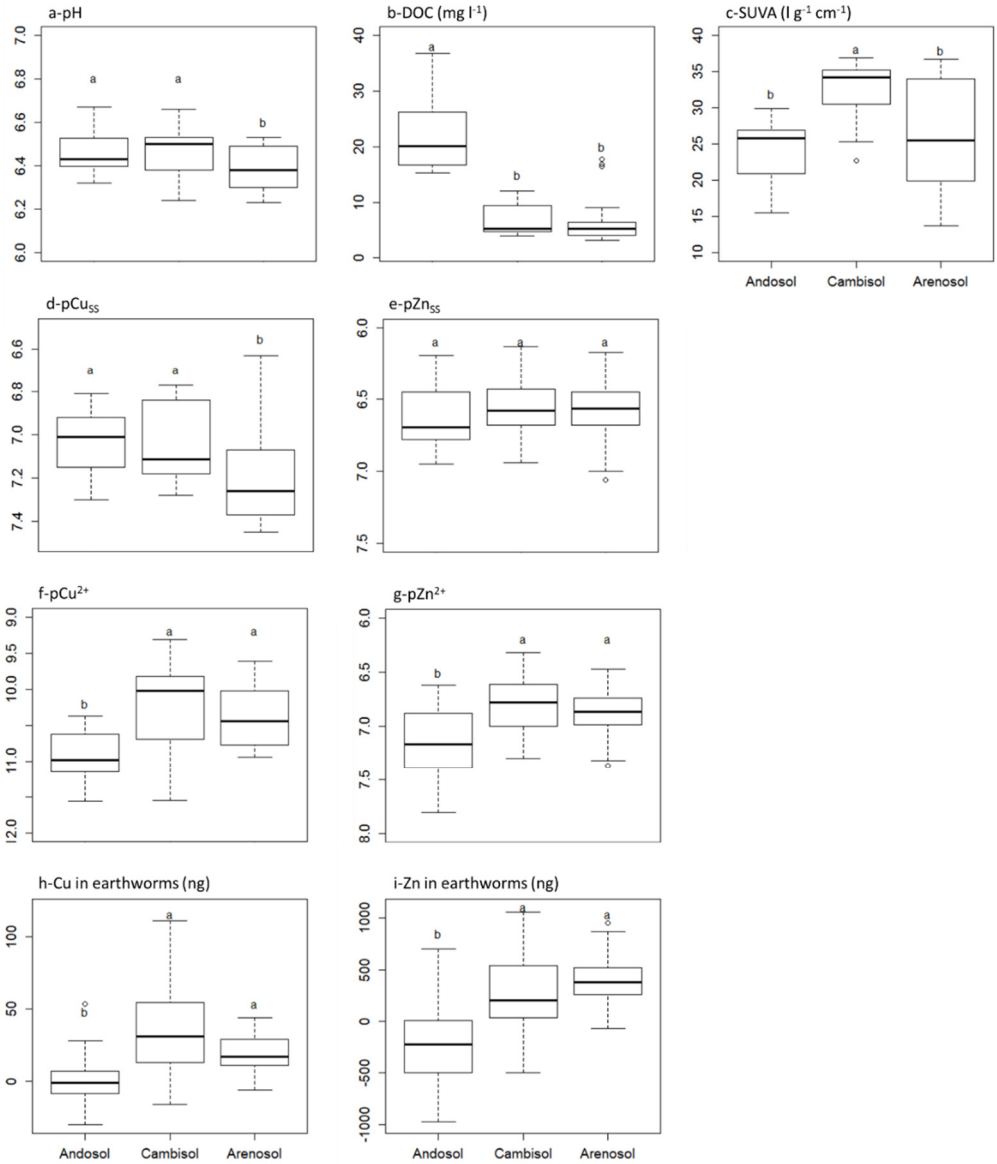


Figure S6 Mean values of (a) pH, (b) DOC concentration, and (c) SUVA in the earthworm bio-influenced soil solution as a function of pH, DOC concentration or SUVA in the bulk soil solution in the three soil types. Vertical error bars stand for standard deviation. The thin line represent the 1:1 line. The scale in (b) is expressed as $\log_{10}$.

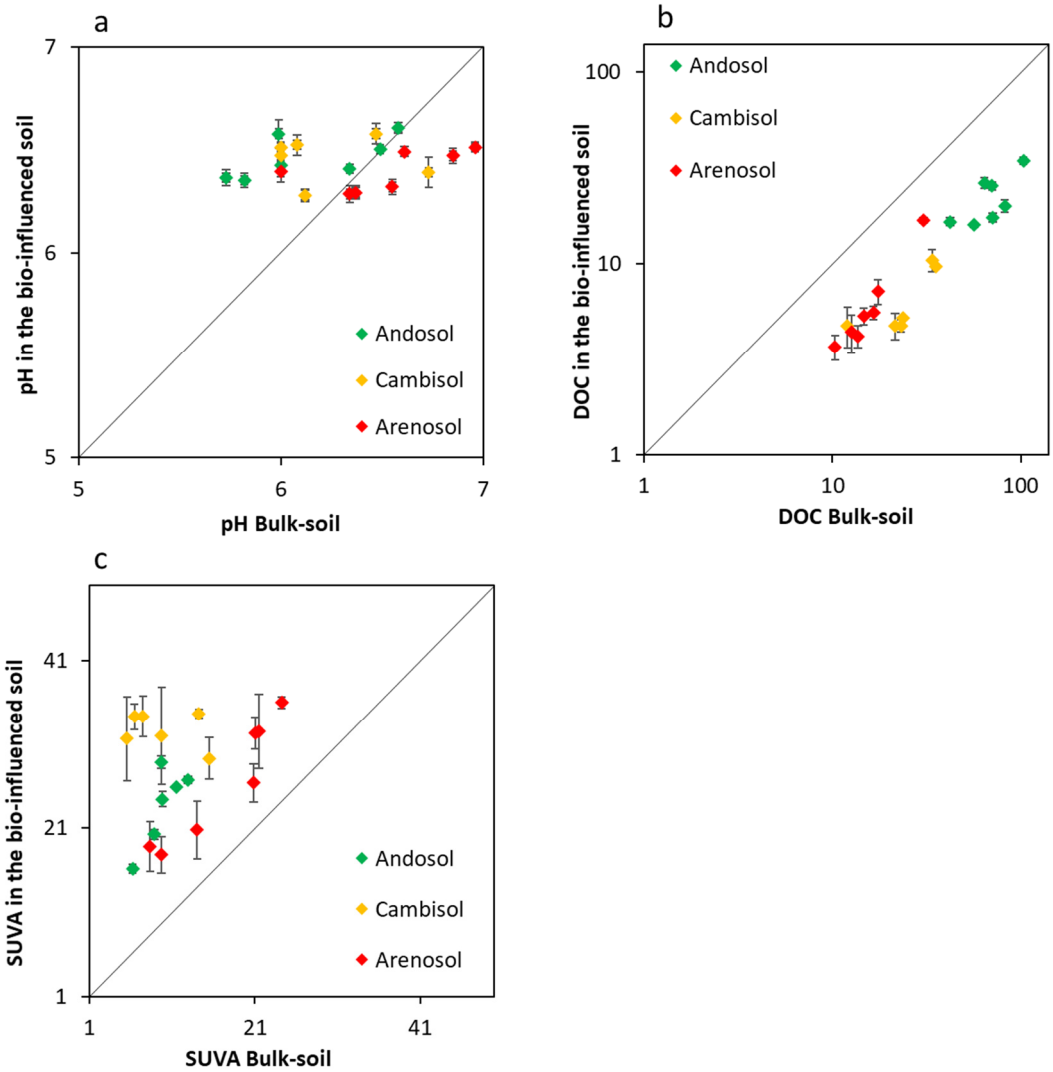


Figure S7 Mean values of (a) total Cu concentration (pCu_{SS}) and (b) total Zn concentration (pZn_{SS}) in the earthworm bio-influenced soil solution as a function of total Cu or Zn concentration in the bulk soil solution in the three soil types. Vertical error bars stand for standard deviation. The thin line represent the 1:1 line.

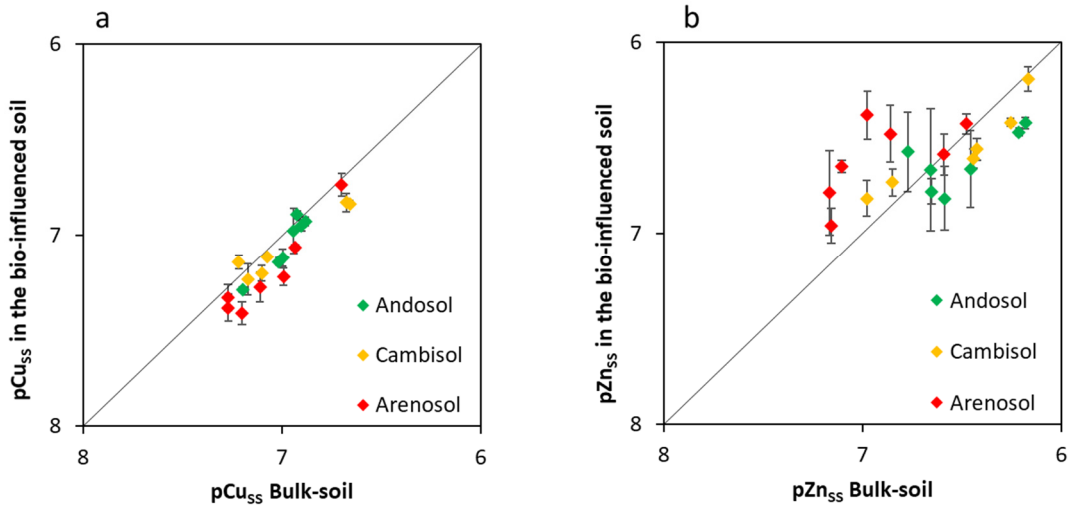


Figure S8 Mean values of (a) the free ionic Cu activity (pCu^{2+}) and (b) the predicted free ionic Zn activity (pZn^{2+}) in the earthworm bio-influenced soil solutions as a function of the free ionic Cu or Zn activity in the bulk soil solution in the three soil types. Vertical error bars stand for standard deviation. The thin line represent the 1:1 line.

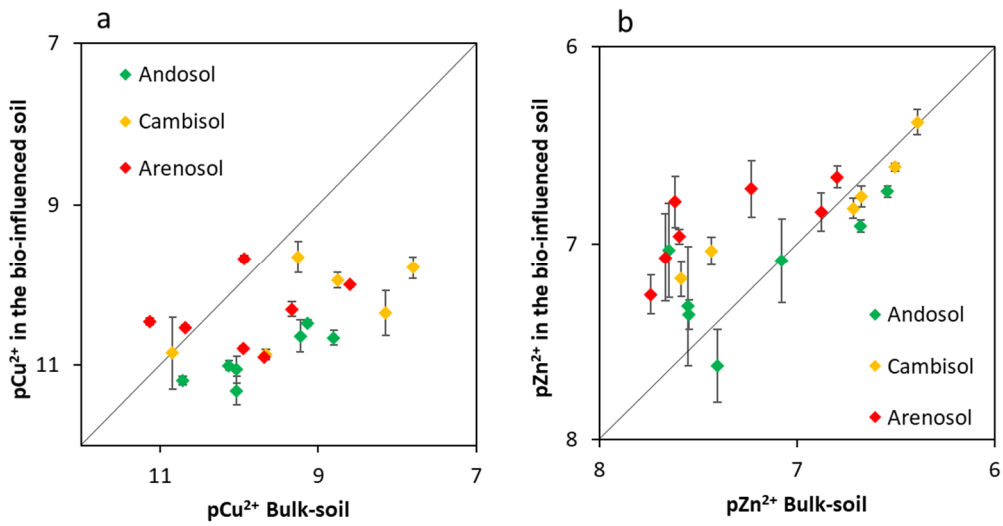


Table S1 Multi-linear regression explaining the variation of the total concentration of copper (Cu) and zinc (Zn) concentration in solution (ΔpCu_{SS} and ΔpZn_{SS}) between the earthworm bio-influenced soil and the bulk soil solutions as a function of the variation of pH (ΔpH), log_{10} of the dissolved organic carbon concentration ($\Delta log_{10}DOC$), and log_{10} of specific UV-absorbance of soil solution at 254 nm ($\Delta log_{10}SUVA$) for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		ΔpH	$\Delta log_{10}DOC$	$\Delta log_{10}SUVA$	i	[a]adj R^2	[b]n
ΔpCu_{SS}	3 soils	ns	-0.54^{2}	-0.38^{1}	ns	0.36**	20
	Andosol	ns	ns	ns	ns	< 0.4	7
	Cambisol	ns	-0.93^{2}	-0.56^{1}	ns	0.91*	6
	Arenosol	ns	ns	ns	ns	< 0.1	7
ΔpZn_{SS}	3 soils	0.59^{1}	ns	ns	-0.13	0.62***	20
	Andosol	0.5^{1}	ns	ns	ns	0.49*	7
	Cambisol	ns	ns	0.7^{1}	-0.35	0.83**	6
	Arenosol	ns	ns	ns	ns	< 0.3	7

[a]adj R^2 = adjusted R^2; [b]n = number of samples

Table S2 Multiple linear regression explaining the variation of free ionic Cu and predicted Zn activity (ΔpCu^{2+} and ΔpZn^{2+}) between the earthworm bio-influenced soil and the bulk soil solutions as a function of the variation of pH (ΔpH), log_{10} of the dissolved organic carbon concentration ($\Delta log_{10}DOC$), log_{10} of specific UV-absorbance at 254 nm ($\Delta log_{10}SUVA$), and total Cu and Zn concentration (ΔpCu_{SS} and ΔpZn_{SS}) for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		ΔpH	$\Delta log_{10}DOC$	$\Delta log_{10}SUVA$	ΔpM_{SS}	i	[a]adj R^2	[b]n
ΔpCu^{2+}	3 soils	1.77^{1}	ns	ns	ns	0.69	0.62***	20
	Andosol	0.94^{1}	ns	ns	ns	0.74	0.7*	7
	Cambisol	2.2^{1}	ns	ns	ns	ns	0.62*	6
	Arenosol	ns	ns	ns	ns	ns	< 0.6	7
ΔPredicted pZn^{2+}	3 soils	0.71^{1}	ns	ns	ns	3.3	0.45***	20
	Andosol	0.8^{1}	1.4^{2}	ns	ns	4.1	0.94**	7
	Cambisol	ns	ns	ns	ns	ns	< 0.1	6
	Arenosol	0.7^{3}	0.6^{1}	ns	-0.8^{2}	3.2	0.98**	7

[a]adj R^2 = adjusted R^2; [b]n = number of samples

Fig S9 Values of (a) the site density of DOM (*%r-DOM*) optimized in WHAM and (b) the copper binding constant ($logK_{Cu}$) in the earthworm bio-influenced soil solutions as a function of *%r-DOM* or $logK_{Cu}$ in the bulk soil solutions in the three soil types. The thin line represent the 1:1 line.

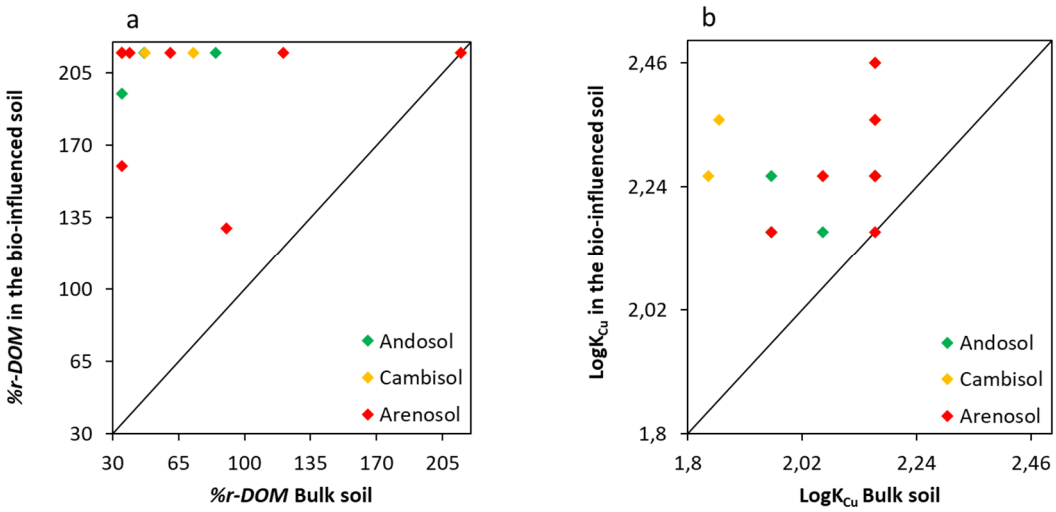


Figure S10 Earthworm fresh biomass at the beginning (white boxes) and at the end (grey boxes) of the 21 days of exposure in (a) the andosol, (b) the cambisol, and (c) the arenosol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

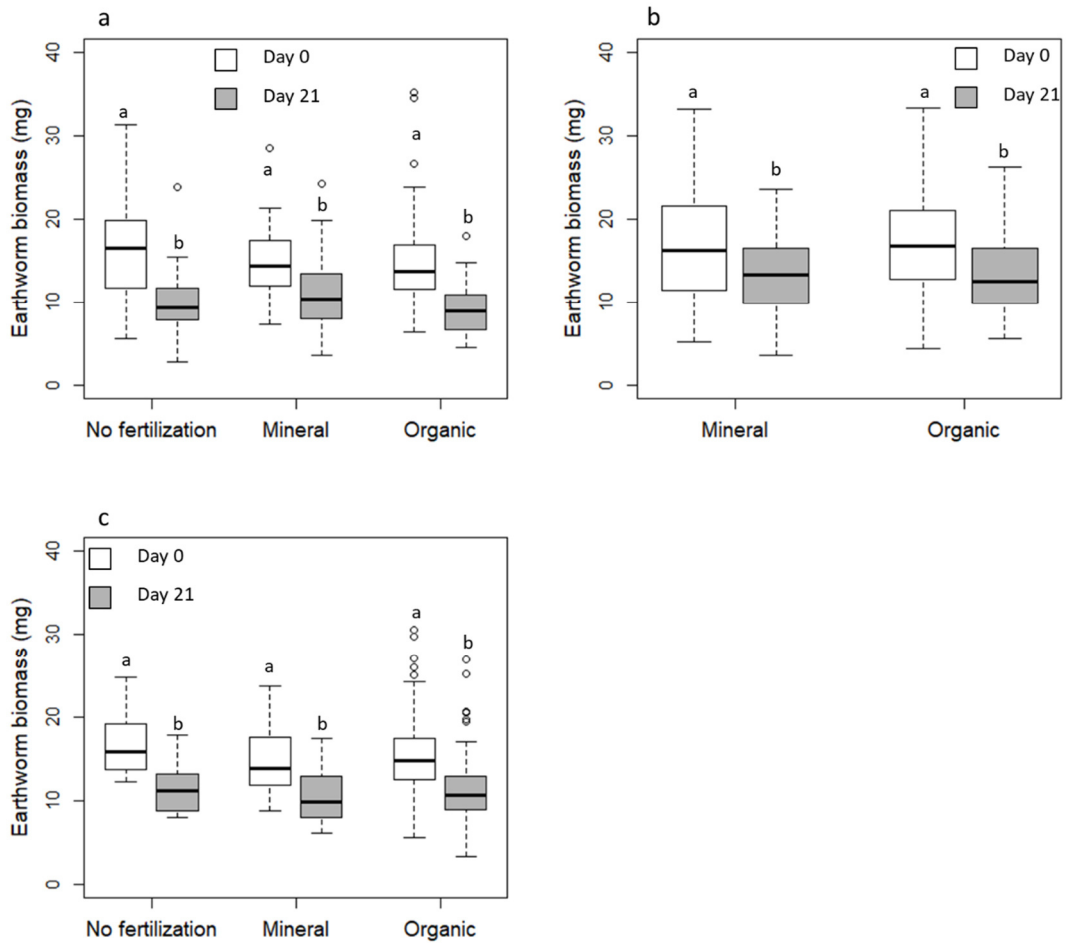


Figure S11 Rate of earthworms in quiescence in (a) the andosol, (b) the cambisol, and (c) the arenosol as a function of the fertilization types. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

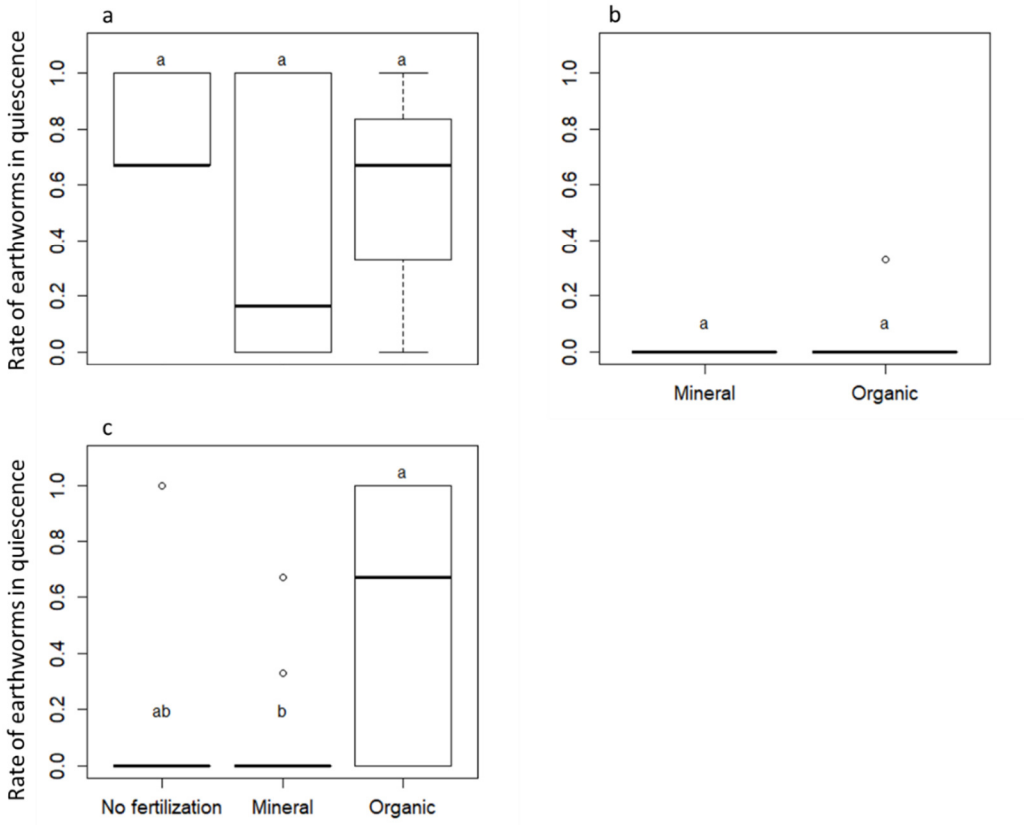
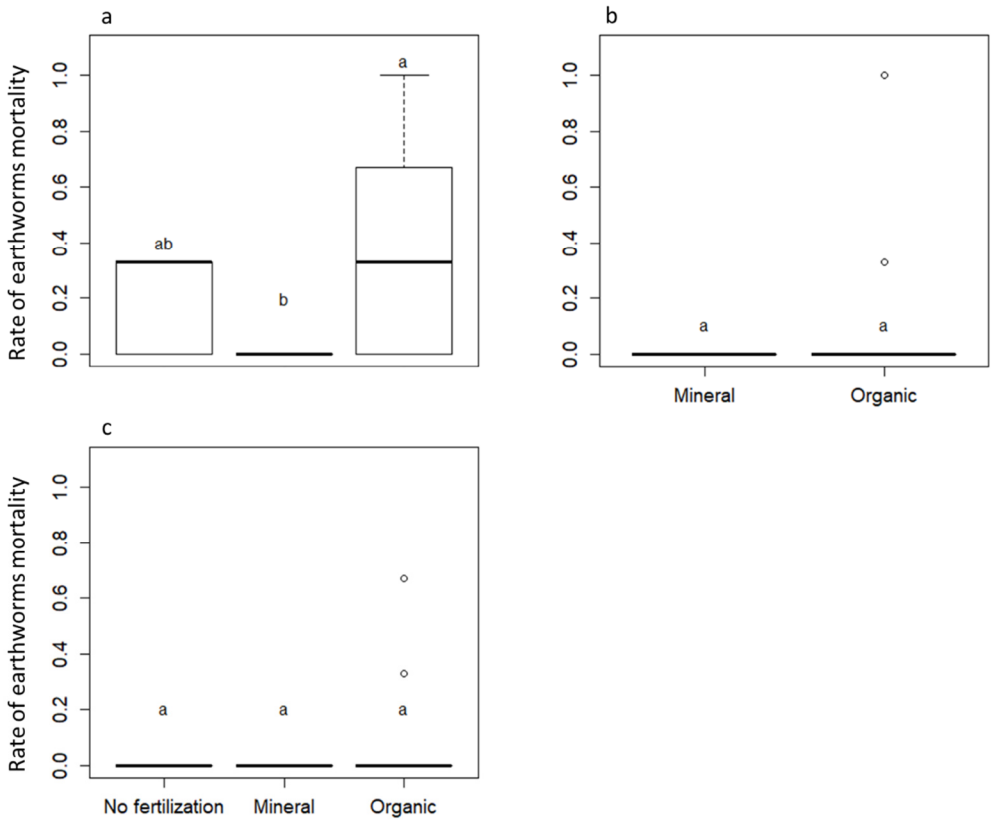


Figure S12 Rate of earthworms mortality in (a) the andosol, (b) the cambisol, and (c) the arenosol as a function of the fertilization types. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.



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Dans la partie II de ce troisième chapitre, nous aborderons la biodisponibilité de Cu et Zn pour les plantes. L'objectif de cette partie est d'étudier les effets combinés d'une plante et de l'apport de FO sur une décennie sur la chimie de la solution du sol avec un focus sur la rhizosphère et sur le flux de Cu et Zn dans la plante. L'étude a été menée sur les trois types de sols et les types de fertilisants précédemment étudiés (Chapitre II et Chapitre III partie I) et une espèce végétale, la fétuque (*Festuca arundinacea*).

II. Do organic fertilizer application to soils for a decade promotes copper and zinc phytoavailability?

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Abstract

It is known that in the soil volume they bio-influence, i.e. the rhizosphere, plant-roots can induce changes in the soil solution chemistry concerning parameters like pH, concentration of dissolved organic matter (DOM) and DOM aromaticity. These modifications can result in changes in trace elements availability in the rhizosphere as well as their phytoavailability. Trace elements in soils can originate from long-term organic fertilizations which are known to increase both Cu and Zn soil contamination and soil pH, soil organic matter content and its binding properties. In the case where plants are growing on soils amended by long-term organic fertilizer (OF) application, the combined effects of changes in the rhizosphere and of OF incorporation on Cu and Zn availability and phytoavailability are not straightforward. In this study we used tall fescue (*Festuca arundinacea*) as a model plant cultivated on three different soil types exhibiting three different types of fertilization. The aim was to assess the combined effects of long-term OF application and of plant on solution chemistry and on Cu and Zn availability in the rhizosphere, as well as on Cu and Zn phytoavailability. To this end, we selected 34 soil samples from three decadal field trials which received either mineral fertilizers or OF for a decade, or were not fertilized and used a design experiment with the tall fescue to easily isolate the rhizosphere compartment (RHIZOtest). In each rhizosphere solution we measured the pH, the DOM concentration and its aromaticity, and the Cu and Zn availability expressed either by the total

metal concentration in solution or by their free ionic activity. The Zn^{2+} activity and binding properties of DOM in the rhizosphere solution were estimated with the Windermere humic aqueous model (WHAM). Phytoavailability was estimated by measuring the Cu and Zn contents in the whole plant. Results showed that the tall fescue buffered all pHs in the rhizosphere around a mean value of 7 ± 0.2 while without the plant influence (i.e. in the bulk soil) the pH ranged from 5.6 to 6.9 in the soil solution. The range of variation of DOM concentration decreased by 10-fold in the rhizosphere compared to the soil without plant bio-influence. The total Cu and Zn concentration in the rhizosphere and the Cu^{2+} activity were weakly influenced by pH and DOM concentration. The optimization of DOM binding properties using WHAM modelling suggested important changes in DOM binding properties in the rhizosphere whatever the soil and the type of fertilization compared to the soil without plant bio-influence. No specific effect of organic fertilization application was observed on the soil solution chemistry nor on the Cu and Zn availability in the rhizosphere compared to non-amended soils and soils amended with mineral fertilizers. Finally, the combination of the effects of plant activities in the rhizosphere rather than the effect of organic fertilization resulted in a similar Cu and Zn phytoavailability whatever the fertilization types for each soil. These findings suggested a great impact of plant on rhizosphere solution chemistry overcoming the effect of OF initially observed in the bulk soil.

Keywords: bioavailability; long-term; metal; organic residues; soil-root interface

I.1. Introduction

Long-term organic fertilizer (OF) application is known to increase Cu and Zn contamination in soils. Recent studies used life cycle assessment (LCA) methods to assess the environmental risk related to the long-term application of OF on soils and showed that Cu and Zn were the main contributors to the terrestrial ecotoxcity of this practice (Leclerc et Laurent, 2017; Sydow et al., 2018). The findings of these studies revealed that ecotoxicological impact of Cu and Zn was related to total amount of Cu and Zn bring with OF application on soils. Meanwhile, it is known that OF application also increase the pH and organic C content in soil (Cambier et al., 2014; Vanden Nest et al., 2016; Smolders et al., 2012). In a previous study (chapter II), we showed that the application of OF increased also soil solution pH, and the concentration and the binding properties of dissolved organic matter (DOM). DOM and pH were the main factors that mitigated Cu and Zn availability, i.e. the pool of metals able to interact with organisms, in soil solution, with proxies of availability taken as their total concentration in solution or their free ion activities. Interestingly the total Cu concentration increased proportionally to the increase in DOM concentration and aromaticity, while Cu^{2+} activity decreased with the increase in pH. This led to a similar Cu availability whatever the fertilization type when compared to soils not fertilized. For Zn, due to its lower affinity for DOM, total Zn concentration and Zn^{2+} activity in soil solution were shown to decrease with the increase in pH. This resulted in a similar or lower Zn availability with organic fertilization compared to soils not fertilized. Thus, despite changes in the soil solution chemistry, the impact of long-term OF did not induced changes in Cu and Zn availability. Thus these findings and the results obtained with LCA methods raise the question that Cu and Zn toxicity and thus Cu and Zn bioavailability, i.e. the uptake of metals by an organism during an exposure period to the soil, is directly determined by the mitigation of Cu and Zn availability induced by OF application or if Cu and Zn (bio)availability is altered by biological regulation processes.

It is notably well known that plant-roots can also induce changes in soil chemical properties such as modifications of pH and DOM concentration in the rhizosphere (i.e. the volume of soil bio-influenced by plant-roots) (Hinsinger et al., 2009, 2003; NGuyen, 2003). The modifications induced by the bio-influence of plant-roots on soil can induce changes in Cu and Zn availability and changes in Cu and Zn phytoavailability. Bravin et al. (2009a) reported in an acidic soil a decrease in Cu phytoavailability to durum wheat with on-going rhizosphere alkalization. De Conti et al. (2018) reported in a soil contaminated with increasing doses of Cu (0, 40, 80 mg Cu kg^{-1}) an increase in total Cu concentration and a decrease in Cu^{2+} in the rhizosphere of black oat and ascribed these changes in Cu availability to the increase in pH and DOM concentration they measured in the rhizosphere. Plant-roots can also exude organic compounds such as sugar, vitamins, enzymes, amino acids and organic anions in the rhizosphere inducing an increase in microbial activity and presumably an increase in DOM

concentration (Jones et al., 2009). The exudation of these organic compounds modifies also the global DOM binding properties towards metals. For example, Jones (1998) reported that citrate, malate and oxalate are often present in the rhizosphere and can form stable complexes with Cu^{2+}. Plants can also produce phytosiderophores having high affinity towards metals (Dell'Mour et al., 2010; Schenkeveld et al., 2014). Thus, Degryse et al. (2008) found that spinach and tomato produced root-exudates with high Zn affinity in conditions of a Zn deficient soil. Specific pH and DOM content in the rhizosphere appear thereby as main parameters controlling bioavailability for plants

But in the context of the application of OF, Cu and Zn phytoavailability was reported to either increase or decrease (Singh et al., 2010; Wang et al., 2016; Zheljazkov and Warman, 2004). For long-term organic fertilization, Benedet et al. (2016) reported accumulation of Zn in maize but without phytotoxic effects. In another study, Benedet et al. (2019) did not reported changes in solution chemistry between rhizosphere and bulk soil but showed an increase in Cu and Zn accumulation in roots and shoots of white oat with application of OF for a decade without toxic effects on plants. It is therefore still not clear from the literature whether Cu and Zn bioavailability is determined either primarily by the effect of long-term OF application or rather by the effect of plant bio-influence on Cu and Zn availability in the rhizosphere.

To unravel the respective impacts of long-term OF and plant bio-influence, we aimed in this study to assess the modifications induced by long-term organic fertilizer application compared to, or in association with, plant bio-influence on solution chemistry in the rhizosphere and on Cu and Zn (phyto)availability. For this we used a model plant, the tall fescue (*Festuca arundinacea* cv Callina) cultivated on three soil types exhibiting contrasted physical-chemical properties and using a specific design, the RHIZOtest, that allowed to isolate specifically the rhizosphere compartment. The soil samples used originated from three decadal field trials receiving either no fertilization, mineral fertilization or organic fertilization. To assess the changes in the rhizosphere solution chemistry we measured the pH, the DOC concentration and the specific UV absorbance (SUVA) as a proxy of the nature of the organic matter, related to its aromaticity. Availability was assessed through two proxies: total metal concentrations and their ionic activity which were measured (for Cu^{2+}) in the rhizosphere solution. For the estimation of Zn^{2+} activity in solution and of the binding properties of DOM towards Cu in the rhizosphere solution we used a geochemical model (WHAM). Phytoavailability was ascribed to the Cu and Zn flux in plants.

II.2. Material and methods

II.2.1 Soil and field trial characteristics

The three decadal field trial were located in Reunion Island in the Indian Ocean (55°30'E, 21°05'S). They were conducted on soils belonging to three soil groups (hereafter called soil type): andosol, cambisol, and arenosol (WRB, 2014). The characteristics of the three field trials and soil types were already detailed in (Nobile et al., 2018). Briefly, the andosol and arenosol were under grassland cover from 2004 to 2012. Plots were arranged in randomized block with three replicates and were either not fertilized or fertilized with mineral fertilizer (10 g N m^{-2} y^{-1}), dairy slurry (20 to 55 dm^3 m^{-2} y^{-1}), or dairy manure compost (2.5 to 5 kg m^{-2} y^{-1}). The cambisol was cultivated under market garden crop from 2004 to 2011. Plots were arranged in randomized block with three replicates and were fertilized either with mineral fertilizer (40 g N m^{-2} y^{-1}), pig slurry compost (5 kg m^{-2} y^{-1}), or poultry litter compost (7 kg m^{-2} y^{-1}). Soils were sampled once per year on the top soil layer corresponding to depth of fertilizer incorporation, i.e. between 0-15 cm for the andosol and the arenosol because there was no tillage and between 0-30 cm for the cambisol which was ploughed.

We selected 34 soil samples among the 74 soil samples from a previous study (Chapter II). The 34 soil samples were selected as a function of pH, DOC concentration and Cu and Zn availability gradient measured in soil solution in a previous study (Chapter II). For each soil type, the soil samples were prepared on composite samples with an equal amount of soil from the three blocks. We selected 14 soil samples for the andosol which were not fertilized (n = 2), or received mineral fertilizer (n = 4), dairy slurry (n = 4) or dairy manure compost (n = 4). For the cambisol we selected 8 soil samples which either received mineral fertilizer (n = 2), pig slurry compost (n = 3), or poultry litter compost (n = 3). For the arenosol we selected 12 soil samples which were not fertilized (n = 2), or received mineral fertilizer (n = 2), dairy slurry (n = 4) or dairy manure compost (n = 4). The soil samples characteristics and soil contamination are given in Table 1. The contamination was assessed in our previous study (Chapter II) by two measurements: total Cu and Zn concentration in soil and DTPA-extractable Cu and Zn concentration in soils. Organic fertilization significantly increased the DTPA-extractable Cu and Zn concentration in soils even if the contamination in Cu and Zn induced by organic fertilization was moderate. Indeed the cumulative Cu and Zn input due to organic fertilization did not induced significant increase in total Cu and Zn concentration in soils due to high initial stock of Cu and Zn in soils (Chapter II).

Table 1 Physical-chemical properties of soil samples before plant exposure as a function of the fertilization types. Mean values are given with their standard deviation in parentheses.

		Fertilization	pH-H_20	Organic C	Total Cu	Total Zn	Cu-DTPA	Zn-DTPA
	n [a]	Units		g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Andosol	2	No fertilization	5.9 (± 0.3)	111 (± 5)	81 (± 1)	164 (± 1)	1.3 (± 0.0)	6 (± 1)
	4	Mineral fertilization	5.3 (± 0.3)	114 (± 4)	78 (± 6)	176 (± 10)	1.3 (± 0.4)	7.3 (± 3.5)
	8	Organic fertilizaiton	6.4 (± 0.4)	126 (± 5)	82 (± 4)	185 (± 17)	2.3 (± 0.7)	17 (± 5)
Cambisol	2	Mineral fertilization	5.4 (± 0.2)	33.7 (± 0.6)	82 (± 4)	242 (± 6)	5.0 (± 0.4)	5.6 (± 0.2)
	6	Organic fertilization	6 (± 0.5)	36 (± 3)	84 (± 5)	257 (± 11)	7 (± 2)	12 (± 6)
Arenosol	2	No fertilization	6.6 (± 0.1)	10.6 (± 0.3)	73 (± 2)	137 (± 8)	1.6 (± 0.1)	2.5 (± 0.5)
	2	Mineral	5.8 (± 0.1)	10.2 (± 0.7)	70 (± 2)	136 (± 1)	1.2	1.9 (± 0.2)
	8	Organic fertilization	7.2 (± 0.2)	14 (± 2)	71 (± 2)	141 (± 5)	1.6 (± 0.3)	6 (± 2)

[a] number of soil samples

II.2.2 Plant growth and soil incubation

This study was performed using the RHIZOtest (Bravin et al., 2010; NF EN ISO 16198, 2015), a standardized bioassay to assess the environmental bioavailability of trace elements to plants using the root-mat approach. A monocot plant, i.e. the tall fescue (*Festuca arundinacea*, cv. Callina) came from RAGT-Semences (France) was used for the experiment as it is one of the standardized species recommended to use in RHIZOtest and also it was shown to modify the most (among the species recommended to use in RHIZOtest) chemical properties in the rhizosphere (Djae, 2017).

Briefly, tall fescue was pre-grown for 14 days in hydroponics. To promote the development of a dense root-mat, 80 seeds were put in a cylinder closed at the bottom with a 30 µm polyamide mesh (thereafter referred as to plant pot). Seeds were germinated for 7 days in $CaCl_2$ 600 µM and H_3BO_3 2 µM. Fescue was then grown for 7 days in a nutrient solution of following composition (in µM): $Ca(NO_3)_2$ 2000, KNO_3 2000, $MgSO_4$ 1000, KH_2PO_4 500, NaFe(III)ETDA 100, H_3BO_3 10, $MnCl_2$ 2, $CuCl_2$ 1, $ZnSO_4$ 1, and Na_2MoO_4 0.05. At the end of hydroponics five plant pots were harvested as control to determine Cu and Zn concentration in plants before exposure to soil samples. During the 14 days of plant preculture, the soil samples were incubated at 70% of their water holding capacity with a nutrient solution of the following composition (in µM): $Ca(NO_3)_2$ 2000, KNO_3 2000, $MgSO_4$1000, KH_2PO_4 50.

Plants pots were then transferred on pre-incubated soils (6 mm thick, and equivalent of 9 g dry soil packed at 1.2 g cm^{-3}) for 8 days period (hereafter called exposure period). The soil was connected via a filter paper mesh to a nutrient solution (same composition as the solution used during soil incubation) during the exposure period. For each soil samples three replicates were performed leading to a total of 102 soil samples. The preculture period and the exposure period were conducted in a growth chamber with controlled climatic conditions (day/night) (16/8 h, 200-400 µmol of photons m^{-2} s^{-1} during the day, 25/20°C, and 75/70% of relative humidity).

At the end of the exposure period, the whole plants were harvested (i.e. shoots and roots) and dried at 50°C to determine their dry biomass and for measurements of total Cu and Zn concentration in plants (shoots and roots not dissociated). The plant showed a significant increase in biomass between the hydroponics and the soil exposure period and this increase was homogenous between the three soil types (Fig. S8). The plants were then burned in platinum cup at 500°C for 2 hours. Ashes were then mineralized in 5 ml HNO_3 and 2ml HF. The concentrations of Cu and Zn were determined by inductively coupled plasma-mass spectrometry (ICP-MS, ICAP Q, Thermo Scientific). The flux (F_M, ng m^{-2} s^{-1}) of Cu and Zn uptake by plants during the exposure period was calculated as follow:

$$F_M = \frac{(C_f.m_f - C_p.m_p).1000}{A.t} \quad (1)$$

where C_f is the measured concentration of metal in plants at the end of the exposure period (µg g^{-1}), m_f the dry biomass of plants at the end of the exposure period (g), C_p is the concentration of metal in plants at the end of the hydroponic period (µg g^{-1}), m_p is the dry biomass of plants at the end of the hydroponic period, A is the surface area of contact between root-mat and soil (m^2), and t is the duration of exposure period (s). The quality of measurements of Cu and Zn concentrations in plants was validated using the certified reference materials INCT-TL-1 (tea leaves) for Cu and Zn and LGC71162 (strawberry leaves) only for Zn. The recovery percentage for tea leaves was of 95% and 94% for Cu and Zn respectively, and was of 92% for Zn for strawberry leaves (no certified value for Cu in strawberry leaves).

II.2.3 Extractions and analysis of soil solution

We considered the whole soil layer recovered in the RHIZOtest at the end of the exposure period as rhizosphere. To evaluate the influence of plant roots on rhizosphere chemical properties, the corresponding soil samples not incubated and not exposed to the plants were considered as reference bulk soil (see Chapter II). Similar measurements were done in the rhizosphere and bulk-soil samples.

At the end of the exposure period soil from each device was collected. The extraction procedure and analysis were as detailed by Djae et al. (2017). The soil solutions were extracted with the nutrient solution used for the soil incubation and described in section 2.2.2. The mixture between soil and extracting solution (1:10 nutrient solution ratio) was stirred for 2h, then centrifuged at 4600 rpm for 10 min, and the supernatant was filtered (0.22 µm, polyethersulfone filters). Each sample of soil solution was subdivided and subsamples were stabilized with either NaN_3 for measuring Cu^{2+} activity and specific ultraviolet-absorbance at 254 nm, or HNO_3 for measuring dissolved organic carbon concentration (DOC, using a TOC analyzer Schimadzu, TOC-L) and total concentration of Cu and Zn in

soil solution. Total concentration of major and trace cations (Ca, K, Mg, Na, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) were determined by ICP-MS. The UV-absorbance at 254 nm of DOM (SUVA) was measured by UV-visible spectrometry (Spectronic Unicam, Helios Beta) to estimate the aromaticity of dissolved organic matter (DOM) (Weishaar et al., 2003). The SUVA of DOM (l g^{-1} cm^{-1}) was calculated as follow:

$$SUVA = \frac{A_{254}}{\text{b.DOC}} \quad (2)$$

where A_{254} is dimensionless, *b* is the path length (1 cm) and DOC is the concentration of DOC (g l^{-1}).

The pH was measured in parallel to pCu^{2+} using a combined electrode (Radiometer analytical PHC2051-8). Free Cu activity was measured using a Cu specific electrode (Thermo Scientific Orion, 9629BNWP). The electrode was daily calibrated within the 13.4 and 4.7 range of pCu^{2+} (i.e. $-\log_{10}\{Cu^{2+}\}$) in standard solutions containing imminodiacetic acid as Cu-complexing compound. The calibration curve ($r^2 = 0.98$, $n = 113$) showed a 92% slope efficiency compared with the theoretical Nernstian slope. The deviation in pH and pCu^{2+} between two days of measurement ranged in average between 0.08 ± 0.08 and 0.3 ± 0.2, respectively.

II.2.4 Modeling Cu^{2+} and Zn^{2+} activity

Free Cu and Zn activity in each soil solution was predicted using WHAM VII (Tipping et al., 2011) to have an estimation of Zn^{2+} activity and to assess the impact of DOM binding properties on the determination of Cu and Zn speciation in soil solution. Input parameters that were analytically determined in soil solution were pH and the concentration of major cations (Ca, Mg, Na, and K) and trace metals (Cd, Co, Cu, Mn, Ni, Pb, and Zn). The activities of Al^{3+} and Fe^{3+} were calculated by assuming their equilibrium with $Al(OH)_3$ and $Fe(OH)_3$ with solubility products (logKs) at 25°C of 8.5 and 2.5, respectively (Tipping et al., 2003). The concentration of NO_3^- was fixed at the concentration of the extracting solution (see in 2.2.2 for rationale). The charge of the solution was balanced with either NO_3^- or K^+ when the solution exhibited an excess in cation or anion, respectively. The concentration of DOM was calculated from DOC concentration assuming that DOM contained 50% of carbon.

Considering these input parameters, Cu^{2+} and Zn^{2+} activities were predicted by four approaches and for each of these, the deviation between predicted and measured Cu^{2+} values was determined by calculation the regression slope, its coefficient and the root mean square residual (RMSR). Free Cu and Zn activities were first predicted as commonly done in the literature with default settings considering that 65% of DOM able to bind metal cations reacts as the colloidal fulvic acid (*%r-DOM*) defined in WHAM (with $\log K_{Cu}$ and $\log K_{Zn}$ of 2.16 and 1.68, respectively) and the remaining 35% being inert

(Tipping et al., 2011). Free Cu activity was secondly predicted by optimizing %r-DOM with SUVA as previously suggested by (Amery et al., 2008) with the SUVA value of 37 l g^{-1} cm^{-1} they found for the Suwannee River fulvic acid provided by the International Humic Substances Society (IHSS). Free Cu activity was thirdly predicted by adjusting *%r-DOM* and $logK_{Cu}$ in the same way for each batch of three replicates within physically meaningful ranges, i.e. respectively from 35% to 215% and 1.84 to 2.46, as previously suggested by Djae et al. (2017). Free Zn activity was then predicted on the basis of Cu^{2+} prediction using the same adjusted *%r-DOM* while $logK_{Zn}$ remained at the default value with the %r-DOM fixed at 65% for each soil. Finally, free Cu activity was predicted by optimizing the $logK_{Cu}$ within a range from 2.46 to 3 and the *%r-DOM* was set by default in WHAM (i.e. 65%). Note that %r-DOM and $logK_{Cu}$ or $logK_{Zn}$ stand for the binding site density and the binding affinity of DOM towards Cu or Zn.

II.2.5 Data treatment and analysis

All statistical analyses were performed with R (R Core Team 2016). Differences were considered statistically significant if $p < 0.05$. As solution variables exhibited different range of variation, data were log_{10} transformed.

Principal components analysis (PCA) were performed with FactoMineR and factoextra packages. PCA was performed to illustrate the distribution of rhizosphere solution samples as a function of the soil or fertilization types while accounting for the contribution of mean replicates of solution variables. PCA analysis were performed to illustrate the effect of fertilization types for the three soils considered individually.

The effect of soil (andosol, cambisol, and arenosol) on the rhizosphere solution variables (i.e. pH, DOC concentration, SUVA, Cu and Zn total concentration, and free Cu and Zn activity) was assessed by performing parametric tests i.e. the one-way Anova and the Tukey HSD post-hoc multiple test comparison, when conditions of residual normality and homoscedasticity were satisfied, or non-parametric rank tests, i.e. the Kruskal-Wallis test and the Dunn post-hoc multiple test comparison when conditions were not satisfied. The effect of soil and fertilization types on Cu and Zn content measured in plants in each soil type was assessed by performing parametric tests (the same as above) when conditions were satisfied or non-parametric rank tests (the same as above) when conditions were not satisfied.

Multi-linear regressions were performed on the mean replicates of the solution variables and their variation between rhizosphere and bulk soil solutions to hierarchize the respective contribution of pH, DOC, and SUVA on each Cu and Zn availability proxy (i.e. total concentration of Cu and Zn in soil solution and free ionic activity). To reduce the bias due to unavoidable co-linearity between some explanatory

variables (Fig. S1, S2, S3, and S4), multi-linear regressions were built up by using two R packages (olsrr and MASS) to combine two approaches based on stepwise regressions and the Akaike information criteria. The best multi-linear regressions for each proxy of Cu and Zn availability for the whole dataset and for each soil were selected by considering the regression (i) with the highest adjusted coefficient of determination (adj R^2), (ii) which was statistically significant, and (iii) whose the regression coefficient of each explanatory variable was statistically significant. In addition, we checked that the sign of each regression coefficient was similar in the corresponding simple and multi-linear regressions (Foucart, 2001). The order of contribution of each explanatory variable was determined according to the results of the stepwise regressions. Multi-linear regressions were also performed, based on the same method as above, to unravel the respective contribution of pH, DOC concentration, SUVA, Cu and Zn total concentration, and Cu^{2+} and predicted Zn^{2+} activity to Cu and Zn fluxes in plants.

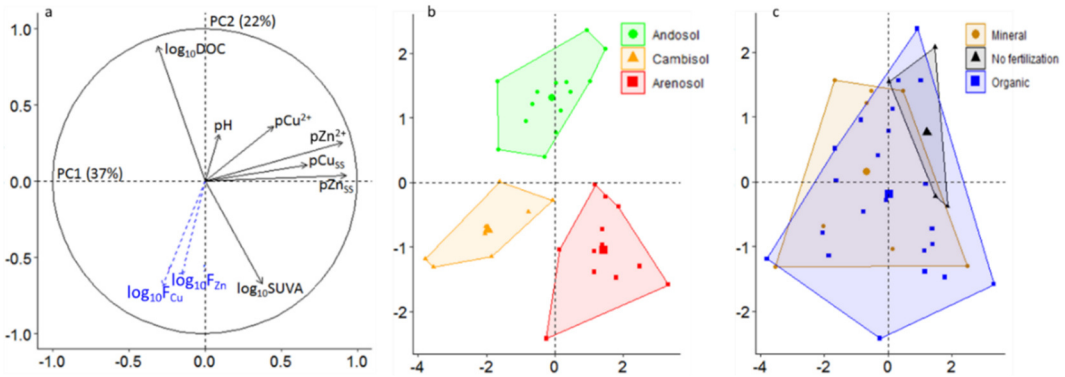
As explained in 2.2.3, the soil samples referred as bulk soil samples in 2.3 and 2.4 are the soil samples studied in Chapter II, corresponding to the same soil and fertilization types of the soil samples of the present study but without plant-roots influence.

II.3 Results

II.3.1 Effect of soil and fertilization types on solution chemistry and Cu and Zn availability in the rhizosphere

Soil types influenced the distribution of the dataset of rhizosphere solution chemistry and of Cu and Zn availability proxies (Fig. 1). The first component (PC1, 35% of explained variance) was mainly determined by pZn_{SS}, pZn^{2+} and secondly by pCu_{SS}, i.e. parameters that refer to metals. The second component (PC2, 21% of explained variance) was mainly determined by $log_{10}DOC$ and secondly by $log_{10}SUVA$ (Fig. 1a) i.e. parameters that refer to organic matter. Soil types were dissociated in three distinct clusters, with the andosol clustered along the PC2 while the cambisol and the arenosol were clustered along the PC1 (Fig 1b). The andosol clustering was due to the DOC concentration which was significantly higher in the andosol compared to the cambisol and the arenosol (Fig. S5b). The clustering along PC1 was related to total Zn concentration and Zn^{2+} activity in the solution which were significantly higher in the arenosol than in the cambisol (Fig S5 e&g). Performing PCA on the three soil type taken together or separately did not allow to show any influence of the type of fertilization on rhizosphere solution chemistry neither on Cu and Zn availability (Fig. 1c and S6). Our findings highlight that soil type mainly influenced the soil solution chemistry and the Cu and Zn availability proxies in the rhizosphere while the type of fertilization did not.

Figure 1 Principal component analysis (PCA) of the dataset. The correlation plot (a) shows the contribution of each variable to the two first principal components. Rhizosphere samples (n = 34) were clustered (b) as a function of soil type (andosol, cambisol, and arenosol) or (c) as function of fertilization type (No fertilization, Mineral or Organic). Data point with a larger size in (b) and (c) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($log_{10}DOC$), the specific UV-absorbance of the soil solutions at 254 nm (log_{10} SUVA), the total concentration of Cu and Zn in soil solution (pCu_{SS} and pZn_{SS}), the activity of free ionic Cu and Zn in soil solution (pCu^{2+} and pZn^{2+}). Supplementary quantitative variables are the Cu and Zn fluxes in plants ($log_{10}F_{Cu}$ and $log_{10}F_{Zn}$) and are represented by blue dashed arrows.



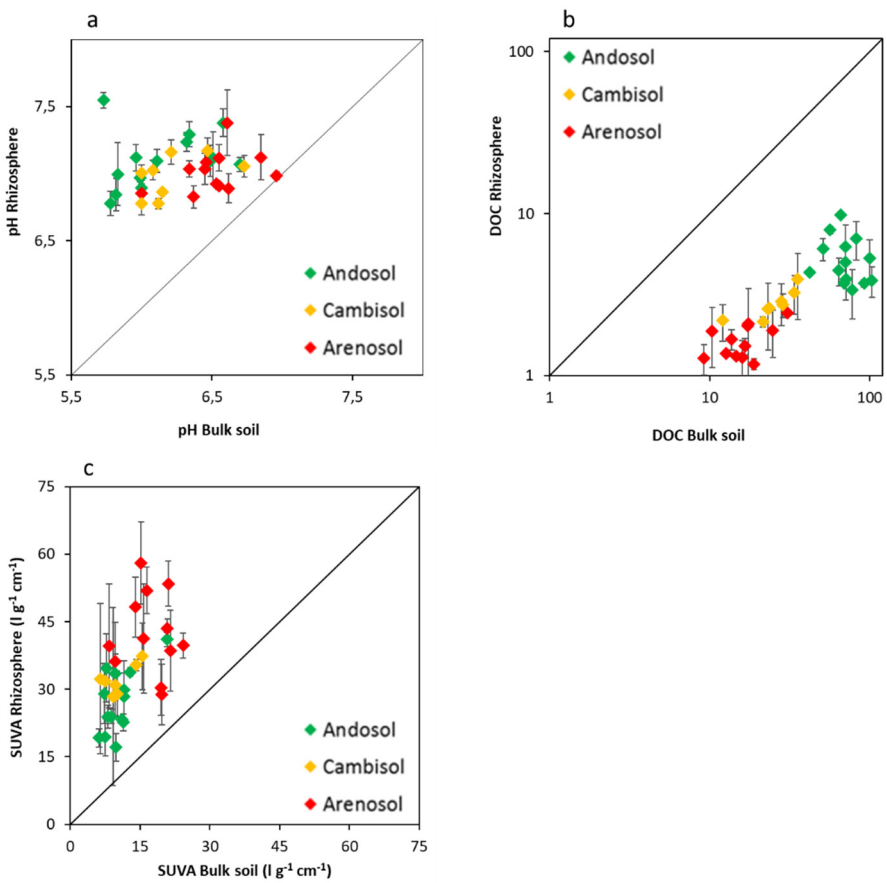
II.3.2 Variation of pH, DOC concentration and SUVA in the rhizosphere compared to the bulk soil

Compared to the bulk soil, the pH increased in all rhizosphere solution samples to a maximum of 1.8 pH units, the median pH value being of 7 ± 0.2 (Fig. 2a). The pHs in the rhizosphere varied in a narrow range from 6.7 to 7.7 (Fig. S5a).

Compared to the bulk soil, the DOC concentration in the rhizosphere decreased by 10-fold in the rhizosphere (Fig. 2b). The DOC concentrations ranged from 0.94 to 9.9 mg l^{-1} in the rhizosphere (Fig. S5b).

Compared to the bulk soil, the SUVA increased by 2-fold in the rhizosphere solution (Fig. 2c). The SUVA ranged from 6.2 to 67.2 l g^{-1} cm^{-1} in the rhizosphere solution (Fig. S5c).

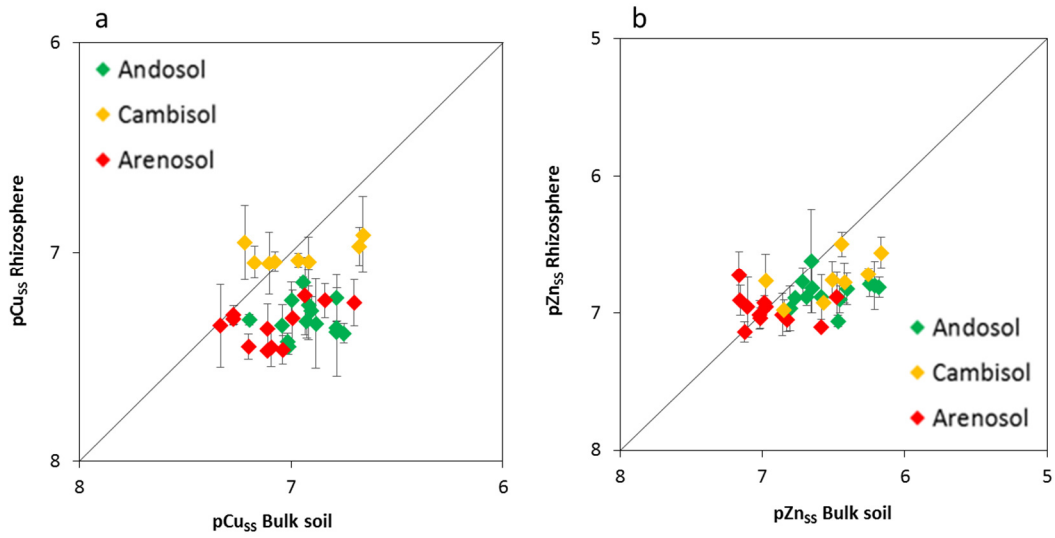
Figure 2 Mean values of (a) pH, (b) DOC concentration, and (c) SUVA in the rhizosphere solutions as function of pH or DOC concentration in the bulk-soil solutions for each soil type. Vertical error bars stand for standard deviation of mean pH in the rhizosphere. The scale in (b) was expressed as log_{10}.



II.3.3 Variation of Cu and Zn availability proxies in the rhizosphere compared to the bulk soil

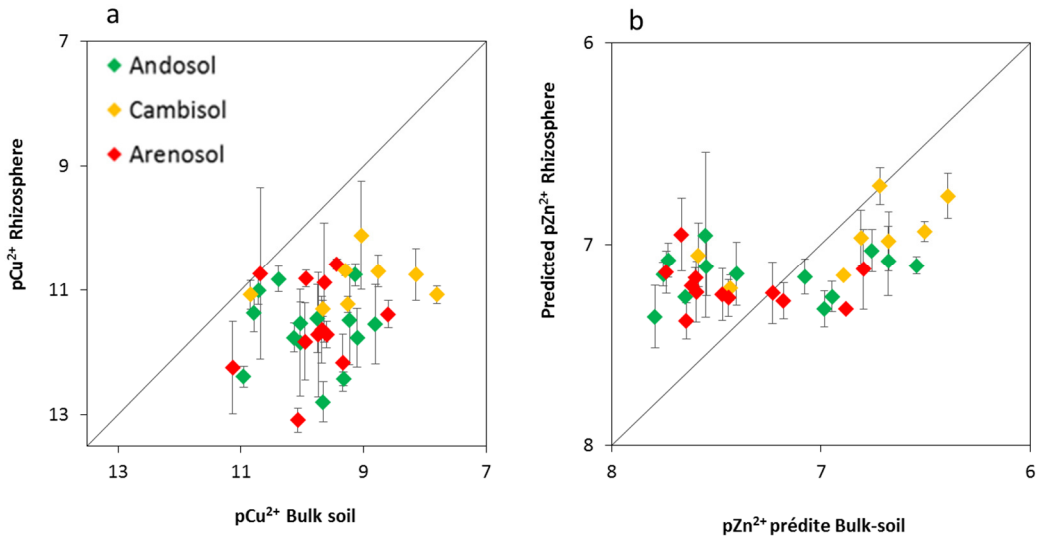
In the rhizosphere the total Cu and Zn concentration in solution ranged from 7.6 to 6.7 in pCu_{SS} and from 7.2 to 6.4 in pZn_{SS} (Fig. S5d&e). The total Cu concentration in rhizosphere solutions decreased in 88% of rhizosphere solutions and up to 0.6 in pCu_{SS} compared to the bulk-soil solution (Fig. 3a). But compared to the bulk-soil solution, the total Cu concentration in the rhizosphere increased to a maximum of 0.26 in pCu_{SS} for four soil samples only in the cambisol (Fig. 3a). Compared to the total Zn concentration in the bulk-soil solutions, we observed in 76% of the rhizosphere solution samples a decrease in the total Zn concentration in solution and up to 0.6 in pZn_{SS} (Fig. 3b). For the other samples, and compared to the bulk-soil solution, the total Zn concentration in rhizosphere solutions increased and up to 0.4 in pZn_{SS} (Fig. 3b). We identified a kind of threshold: the total Zn concentration in the rhizosphere solution decreased when the total Zn concentration in the bulk-soil solution was higher than 7 in pZn_{SS} and increased when the total Zn concentration in the bulk-soil was higher than 7 in pZn_{SS} (Fig. 3b).

Figure 3 Mean values of (a) total Cu concentration (expressed as pCu_{SS}) and (b) total Zn concentration (expressed as pZn_{SS}) in the rhizosphere solutions as a function of total Cu or Zn concentration in the bulk-soil solution for each soil type. Vertical error bars stand for standard deviation of mean Cu and Zn concentration in the rhizosphere.



The Cu^{2+} and Zn^{2+} activity ranged from 13.2 to 9.4 in pCu^{2+} and 7.5 to 6.5 in pZn^{2+} (Fig. S5f&g). Compared to the bulk-soil solutions, the Cu^{2+} activity was lower in the rhizosphere solutions and up to 3.28 in pCu^{2+} (Fig. 4a). Compared to the bulk soil solutions, the Zn^{2+} activity increased in the rhizosphere solutions and up to 0.7 in pZn^{2+} when Zn^{2+} activity in the bulk soil was lower than 7.2 in pZn^{2+} (Fig. 4b). Conversely, the Zn^{2+} activity decreased in the rhizosphere solutionsand up to 0.6 in pZn^{2+} when Zn^{2+} activity was higher than 7.2 in pZn^{2+} (Fig. 4b).

Figure 4 Mean values of (a) Cu^{2+} activity (expressed as pCu^{2+}) and (b) Zn^{2+} activity (expressed as pZn^{2+}) in the rhizosphere solutions as a function of free Cu and Zn activity in the bulk-soil solution for each soil type. Vertical error bars stand for standard deviation of mean Cu^{2+} and Zn^{2+} activity in the rhizosphere.



In the andosol and the arenosol, the total Cu concentration in the rhizosphere solution was positively driven by DOC concentration (Table 2). The changes in total Cu concentration between the rhizosphere and the bulk soil were not driven by the variations of the measured variables in solution (i.e. pH, DOC concentration and SUVA) (Table S1). Regardless of the soil type, the total Zn concentration in the rhizosphere was not driven by any of the measured variables in solution (Table 2). The changes in total Zn concentration between the rhizosphere and the bulk soil were however weakly driven by the variation of pH for the regression considering the three soil types together or for the arenosol when the regression was performed on soil types independently (Table S1).

Table 2 Multi-linear regression explaining the total copper (Cu) and zinc (Zn) concentration in the rhizosphere solution (pCu_{SS} and pZn_{SS}) as a function of pH, log_{10} of the dissolved organic carbon ($log_{10}DOC$), and log_{10} of the specific UV absorbance at 254 nm ($log_{10}SUVA$) for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		pH	$log_{10}DOC$	$log_{10}SUVA$	i	[a]adj R^2	[b]n
pCu_{SS}	3 soils	ns	ns	ns	ns	< 0.1	34
	Andosol	ns	-0.44[1]	ns	7.6	0.44**	14
	Cambisol	ns	ns	ns	ns	< 0.2	8
	Arenosol	ns	-0.89[1]	ns	7.5	0.79***	12
pZn_{SS}	3 soils	ns	ns	ns	ns	< 0.1	34
	Andosol	ns	ns	ns	ns	< 0.1	14
	Cambisol	ns	ns	ns	ns	< 0.1	8
	Arenosol	ns	ns	ns	ns	< 0.1	12

[a]adj R^2 = adjusted R^2; [b]n = number of samples

The Cu^{2+} activity in the rhizosphere was mainly negatively driven by the total Cu concentration in soil solution in the cambisol and the arenosol (Table 3). In the arenosol the Cu^{2+} activity was secondly positively driven by the DOC concentration in soil solution and the soil solution pH (Table 3). Only for the cambisol we found that changes in Cu^{2+} activity in the rhizosphere were significantly driven first by the variations of DOC concentrations and secondly by variations of total Cu concentrations between the rhizosphere and the bulk soil solution, (Table S2). Regardless of the soil type, the Zn^{2+} activity in the rhizosphere was mainly driven by the total Zn concentration in the rhizosphere solution and secondly by the DOC concentration, pH or SUVA in the rhizosphere (Table 3). In the three soil types, the changes in Zn^{2+} activity in the rhizosphere were mainly driven by the variations in total Zn concentration between the rhizosphere and the bulk soil solution (Table S2).

Table 3 Multi-linear regression explaining the free ionic Cu and Zn in the rhizosphere solution (pCu^{2+} and pZn^{2+}) as a function of pH, $log_{10}DOC$, $log_{10}SUVA$ and pCu_{SS} or pZn_{SS} (pM_{SS}) for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

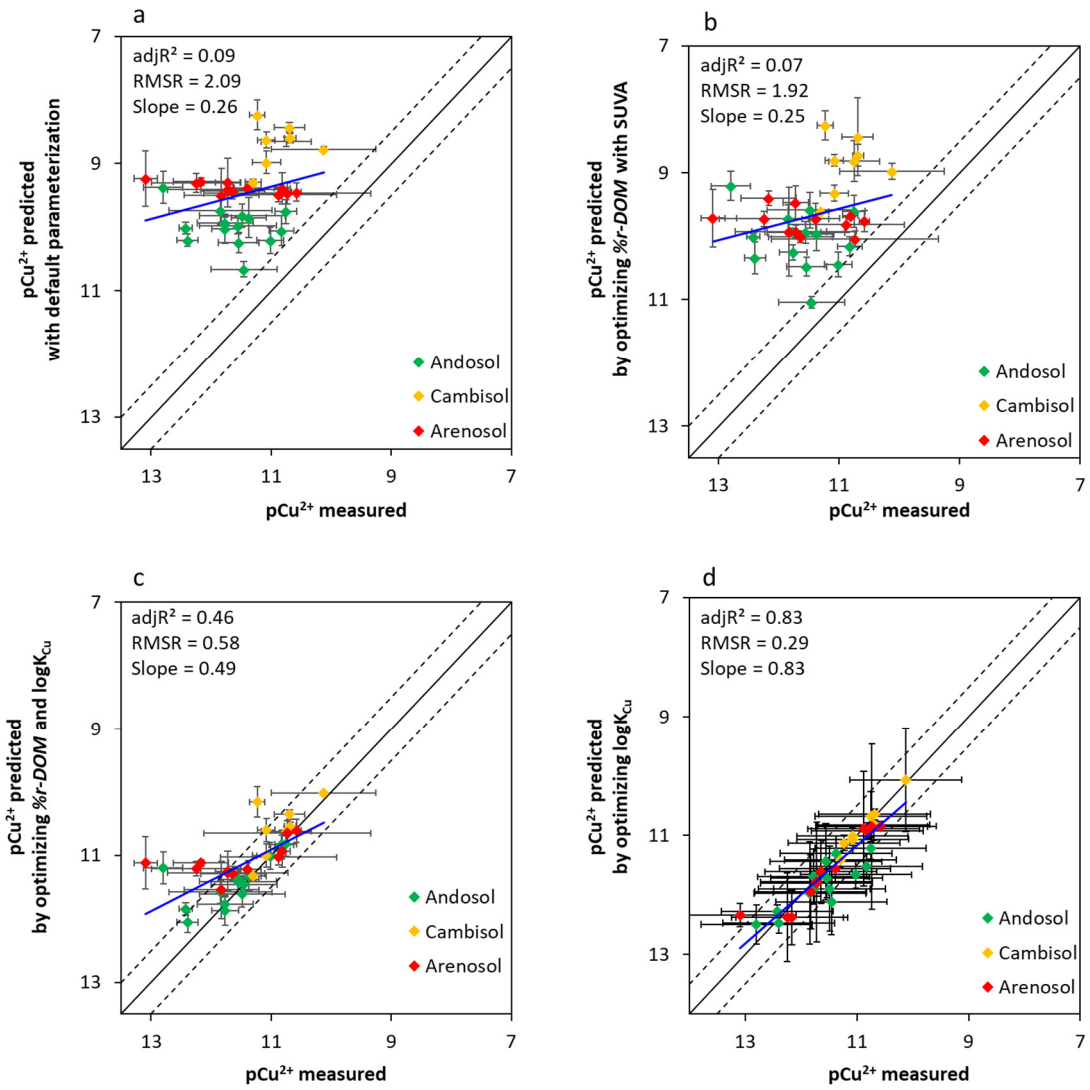
		pH	$log_{10}DOC$	$log_{10}SUVA$	pM_{SS}	i	[a]adj R^2	[b]n
pCu^{2+}	3 soils	ns	ns	ns	ns	ns	< 0.1	34
	Andosol	ns	ns	ns	ns	ns	< 0.1	14
	Cambisol	ns	ns	ns	-5.2^{1}	47.6	0.46*	8
	Arenosol	-2.6^{3}	-9.7^{2}	ns	-14.1^{1}	136	0.72***	12
Predicted pZn^{2+}	3 soils	0.15^{3}	0.11^{2}	ns	1.1^{1}	-1.2	0.93***	34
	Andosol	ns	0.4^{2}	ns	0.8^{1}	ns	0.73***	14
	Cambisol	ns	0.3^{2}	ns	0.99^{1}	ns	0.99***	12
	Arenosol	0.09^{2}	0.07^{3}	0.06^{4}	1.05^{1}	-0.94	0.99***	8

[a]adj R^2 = adjusted R^2; [b]n = number of samples

II.3.4 Binding properties of dissolved organic matter in the rhizosphere

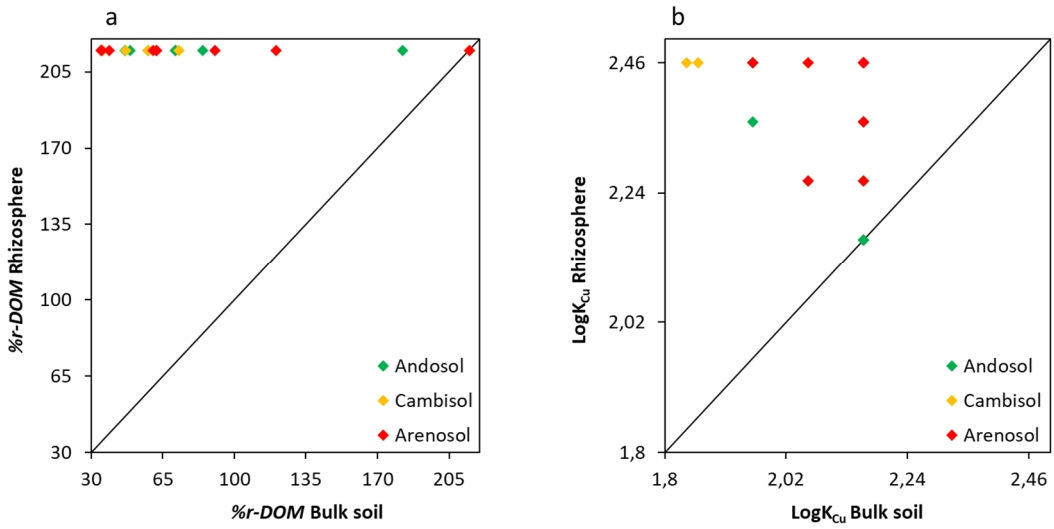
For the whole dataset, the prediction of Cu^{2+} activity in the rhizosphere with default WHAM parameterization was poorly correlated to the measured Cu^{2+} activity which was strongly overestimated (adj R^2 = 0.09 and RMRS = 2.09, Fig. 5a). The optimization of the DOM site density (*%r-DOM*) with SUVA values did not improve the prediction of Cu^{2+} activity (Fig. 5b). The optimization of *%r-DOM* and $logK_{Cu}$ within ranges restricted to physically meaningful values (see section 2.2.4 for rationale) improved the prediction of Cu^{2+} activity but still poorly fitted 20 % of the data points that showed a discrepancy higher than 0.5 pCu^{2+} units compared to measured Cu^{2+} activity (adj R^2 = 0.46, RMSR = 0.58, Fig. 5c). Finally the additional optimization of $logK_{Cu}$ within a range of values higher than 2.46 (the upper limit of values initially considered as physically meaningful) and setting the *%r-DOM* to its default parameterization (i.e *%r-DOM* = 65%) enabled to predict Cu^{2+} activity (adj R^2 = 0.83, RMSR = 0.29, Fig. 5d).

Figure 5 Free copper activity in the rhizosphere solution (pCu^{2+}) predicted for each batch of three replicates for each sample with WHAM as a function of pCu^{2+} measured in rhizsophere solution (n = 34). pCu^{2+} was predicited by (a) using the default parameters of WHAM for the percentage of dissolved organic matter reactive toward metal cations (*%r-DOM*) equal to 65% and the copper binding constant ($logK_{Cu}$) equal to 2.16 for all rhizosphere solutions, (b) optimizing *%rDOM* with the SUVA measured in the rhizosphere solutions, (c) optimizing *%r-DOM* and $logK_{Cu}$ values within a range of physically meaningful values for each bacth of three replicates of a sample, and (d) optimizing $logK_{Cu}$ with values between 2.46 and 3 (for b, c and d see the *Material and methods* section for rationale). The blue line represents the regression line; the thick solid line represents the 1:1 line; the dashed lines represent the 1:1 line ± 0.5 pCu^{2+} unit. Vertical and horizontal error bars stand for the standard deviation on mean predicted Cu^{2+} activity and measured Cu^{2+} activity, respectively. The abbreviations adjR^2 and RMSR mean adjusted R^2 and root mean square residual, respectively.



Compared to the same data set obtained with the bulk soil, the *%r-DOM* and the $logK_{Cu}$ optimized in WHAM increased in the rhizosphere of the three soil types (Fig. 6a). Compared to the bulk soil, the *%r-DOM* were optimized to the maximum value within the physically meaningful range of *%r-DOM* (i.e. 215) and no $logK_{Cu}$ values were under the default logKCu in WHAM (i.e. 2.16).

Figure 6 Values of (a) the site density of DOM (*%r-DOM*) optimized in WHAM and (b) the copper binding constant ($logK_{Cu}$) in the rhizosphere as a function of *%r-DOM* or $logK_{Cu}$ in the bulk soil solution in the three soil types. The thin line represent the 1:1 line.



II.3.5 Bioavailability of copper and zinc to fescue

Whatever the soil type, the organic fertilization for a decade did not affect the Cu and Zn fluxes measured in plants, thus did not affect the Cu and Zn phytoavailability (Fig. 7, 8). The Cu and Zn fluxes in plants ranged from 3.3 to 19.7 ng Cu m^{-2} s^{-1} (Fig.S5h) and from 12.5 to 85.6 ng Zn m^{-2} s^{-1} (Fig. S5i), respectively. The Cu and Zn fluxes in plants were significantly higher in the cambisol and the arenosol compared to the andosol (Fig. S5h&i).

Figure 7 Variation of Cu fluxes in plant as a function of fertilization types (no fertilization, mineral, and organic) in (a) the andosol, (b) the cambisol, and (c) the arenosol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

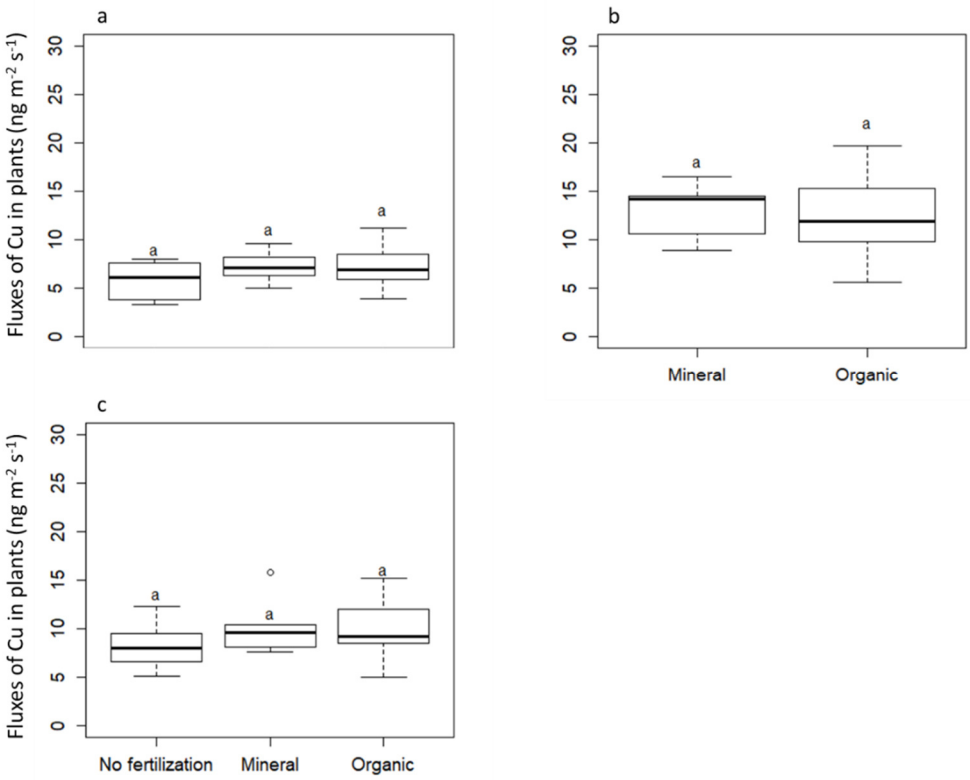
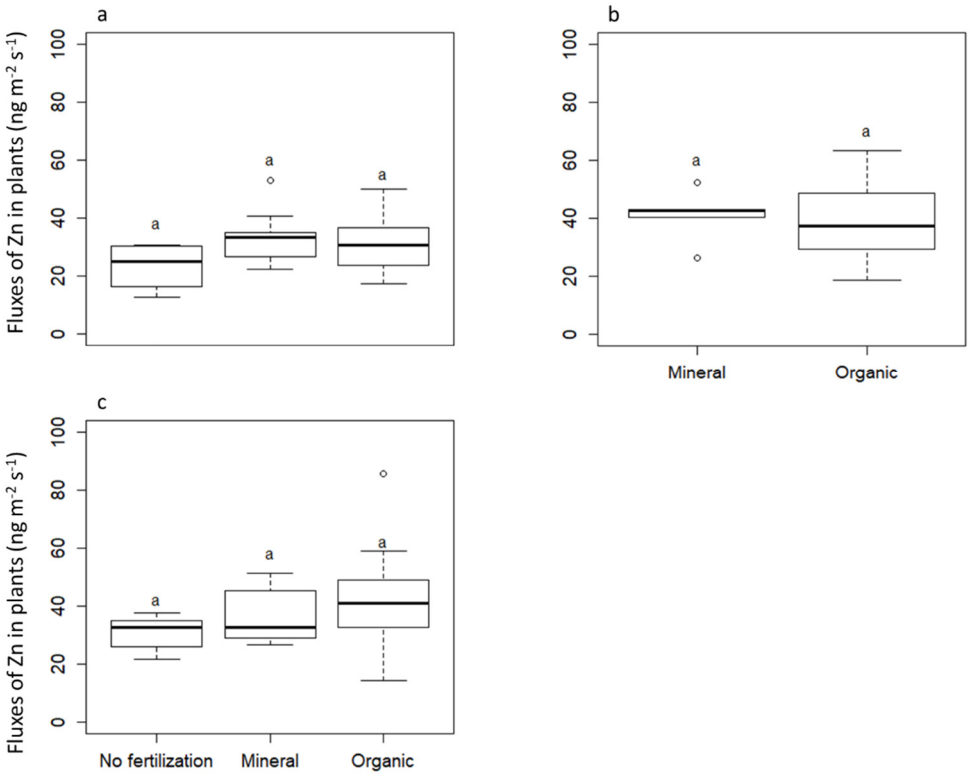


Figure 8 Variation of Zn fluxes in plant as a function of fertilization types (no fertilization, mineral, and organic) in (a) the andosol, (b) the cambisol, and (c) the arenosol. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.



Only for the arenosol the Cu fluxes in plants were positively related first to the SUVA and secondly to the Cu^{2+} activity and the total Cu concentration in the solution of rhizosphere (Table S3). For the andosol and the cambisol, the Cu fluxes in plants were not driven by the solution chemistry of the rhizosphere nor by Cu availability proxies (Table S3). For the three soils the Zn fluxes in plants were positively driven by the Zn^{2+} activity and the SUVA in the rhizosphere solution while in the arenosol the Zn fluxes were only weakly driven by the SUVA (Table S3).

II.4 Discussion

II.4.1 Plant influence rather than organic fertilization drives solution chemistry and Cu and Zn availability in the rhizosphere

The rhizosphere solutions we isolated after fescue growing using the RHIZOtest were very informative to analyse the availability process when comparing with the bulk soil solutions. However, we have to take into account the experimental difference of incubation that differentiate the rhizosphere soil samples and the bulk soil samples, these latter being not incubated. Thus the conditions in the rhizosphere may have been modified by both the effects of soil samples incubation and plants. These two factors (plant activity and soil incubation) are known to induce changes in soil solution chemistry but not necessarily in the same direction (e.g. Amery et al., 2007; Hinsinger et al., 2006; Merckx et al., 2001).

For pH for example, under laboratory conditions, the soil solution pH of soils incubated with organic fertilizers increased followed by a decrease from 0.1 to 1.4 pH units (Tella et al., 2016). This decrease in pH was attributed to the acidic effect of decomposable organic fertilizers products (Usman et al., 2004). But an increase in pH in the presence of plants was reported in the literatture and was mainly attributed to the balance of cation/anion uptake (Bravin et al., 2009a; Hinsinger, 2003). In our study, fescue (monocot) were supplied with a nutrient solution composed of nitrate (NO_3^-) as a source of nitrogen. For monocot species, the NO_3^- utpake by roots is usually counteracted by OH^- efflux in the rhizoshpere (Jarvis and Robson, 1983). Bravin et al. (2009a) reported alkalization of durum wheat (monocot) rhizosphere when supplied with NO_3^-, and even more so in acidic bulk soil pH (an increase up to + 2.8 pH units). On a set of 55 soil samples of contrasted physical-chemical properties, Djae, (2017) reported alkalization of cabbage (dicot), tomato (dicot) and fescue rhizosphere. This latter species markedly increased the rhizosphere pH compared to the other two dicots when bulk soil pH was acid. These results suggest that incubation is expected to induce lower variation in soil pH compared to the expected effect on pH due to the growth of plants in the rhizosphere. Thus in our study, the alkalization in the rhizosphere solutions might be mainly the result of the plant-roots effect.

In our experiments, the DOC concentration decreased by 10-fold in the rhizosphere compared to the bulk soil solution (Fig. 2b). Soil incubation alone can induce a decrease in DOC concentration (Amery et al., 2007; Merckx et al., 2001). After two cycles of soil drying-rewetting, Merckx et al. (2001) first reported a rapid DOC flush followed by a 2- to 3-fold decrease in DOC concentration with incubation time. The DOC flush was attributed to the rewetting of the soil which promoted the C mineralization and resulted in the solubilization of soil organic matter. The authors suggested that the DOM released with the flush was mainly composed of low aromatic organic compounds (e.g. sugars) thus easily

degradable which could explain the decrease in DOC concentration. Contrarily, in the presence of plants, it was reported in the literature an increase in DOC concentration in the rhizosphere (Kim et al., 2010; Bravin et al., 2012). This increase in DOC concentration was attributed to the exsudation of organic compounds followed by the enhancemet of microbial activity in the rhizosphere (Khalid et al., 2007). However, Djae (2017) reported a decrease in DOC concentration to a maximimum of 10-fold in the fescue rhizosphere, on a set of 55 soil samples and three plant species. The authors suggested that the decrease in DOC concentration might be due to indirect effect of rhizodeposition corresponding to mineralization promotion of soil organic matter by rhizospheric microorganisms (i.e. a rhizosphere priming effect) (Kuzyakov, 2002). The high DOC varation range between rhizosphere and bulk soil samples observed by (Djae, 2017) compared the DOC concentration decrease induced by soil incubation in (Merckx et al., 2001) suggests that in our study, the decrease in DOC concentration could be mainly due to the rhizosphere effect.

The SUVA increased in the rhizosphere solution compared to the bulk soil solution (Fig. 2c). Amery et al. (2007) reported an increase in SUVA with soil incubation. The authors suggested that with soil incubation the increase in SUVA was due to the decrease in DOC concentration related to the degradation by soil microorganisms of easily degradable organic compounds with low aromotacity (e.g. sugars). Plant-roots can exude organic compounds of high (e.g. mucilages) and low (e.g. sugars, organic anions and amino-acids) molecular weight (NGuyen, 2003). The exudation of low molecular weight compounds can promote the rhizosphere priming effect corresponding to the promotion of soil organic matter degradation by rhizospheric microrganisms (Kuzyakov, 2002). An hypothesis can be that molecules such as sugar which have low aromaticity will be first degraded by microorganisms in the rhizosphere. Thus it remains organic compounds such as organic anions and mucilages, which have a higher aromaticity, inducing a relative increase in SUVA in the rhizosphere compared to the bulk soil. In that case, both soil incubation and plant growth can be evoke to explain the changes in the nature of DOM towards more aromaticity as seen by the increase in SUVA.

In our study using fescue, the total Cu concentration in the rhizosphere solution mainly decreased compared to the bulk soil solution (Fig. 3a). In the same experimental conditions (i.e. RHIZOtest), Bravin et al. (2012) reported an increase in total Cu concentration in the rhizosphere solution compared to the bulk soil, while Djae (2017) reported mainly a decrease in total Cu concentration in the rhizosphere. The increase in total Cu concentration in the rhizosphere was mainly correlated to the increase in DOC concentration (Bravin et al., 2012; Cattani et al., 2006; Otero et al., 2012; Zhao et al., 2007). For two of the three soil, our results are consistent with the literature.

For zinc, we highlighted two patterns: the total Zn concentration in the rhizosphere mainly decreased when the total Zn concentration in the bulk soil solution was higher than 7 in pZn_{SS} and increased when total Zn concentration in the bulk soil was lower than 7 in pZn_{SS} (Fig. 3b). Otero et al, (2012) reported an increase in total Zn concentration compared to the bulk soil but Kim et al, (2010) reported a decrease in Zn concentration after plant growth in an acidic soil. (Kim et al., 2010) related this decrease in Zn concentration in the rhizosphere to the increase in pH after plant growth. In our study, however, the total Zn concentration in the rhizosphere solution was not related to any of the measured variables, and the variation of total Zn concentration between the rhizosphere and the bulk soil solution was weakly explained by pH, DOC concentration or SUVA. The weak variation range of pH and DOC concentration and SUVA in the rhizosphere may induced a weak variation of total Cu and Zn concentration in the rhizosphere solutions. This may explain the poor explanation of total concentration in solution of Cu and Zn with the solution chemistry (pH, DOC concentration and SUVA).

Concerning the Cu^{2+} activity in the rhizosphere, Bravin et al. (2012, 2009a) and (Djae, 2017) also reported a decrease in Cu^{2+} activity in the rhizosphere solutions. In our experiment, the variation of Cu^{2+} activities between the rhizosphere and the bulk soil solutions was significantly related first to the variation of DOC concentration and secondly to the variation of total Cu concentration in soil solution, but only in the cambisol. Bravin et al. (2012) reported that pCu^{2+} in the rhizosphere was correlated to its alkalization and the increase in DOC concentration while Djae, (2017) reported poor correlations between pCu^{2+} in the rhizosphere and the pH and DOC concentration. In our study, similarly to the total Cu concentration in the rhizosphere, the low variation of pH, DOC concentration, and total Cu concentration in the rhizosphere may induced poor explanation of Cu^{2+} activity in the rhizosphere by solution chemistry and total Cu concentration in the rhizosphere solution.

The predicted Zn^{2+} activity in the rhizosphere solution was mainly explained by the total Zn concentration in solution while the variation of Zn^{2+} activity between the rhizosphere and the bulk soil was mainly explained by the variations of total Zn concentration, the pH, and the DOC concentration which were the input data of the model. As the Zn^{2+} activity was predicted with WHAM, it is not surprising that the predicted Zn^{2+} activity or its variations between rhizosphere and bulk-soil solution were significantly correlated to the data input in WHAM.

Finally, the Cu and Zn availability proxies were poorly explained by the rhizosphere solution variables (except for the predicted Zn^{2+}). This poor relationships suggest that other parameters in the rhizosphere solution may have an effect that we did not took into account with the multi-linear regressions. In chapter II, we already show the importance of the binding properties of dissolved organic matter (DOM) on Cu speciation. The use of WHAM was thus relevant to assess the potential

effect of DOM binding properties on Cu speciation in the rhizosphere. As in the bulk soil, the prediction of Cu^{2+} activity with the default WHAM parameterization or by optimizing the DOM site density (%r-DOM) with SUVA did not improve the fit between predicted Cu^{2+} activity and Cu^{2+} activities measured in the rhizosphere solution (Fig. 5a&b). The optimization of both the site densities and the binding affinities of DOM ($logK_{Cu}$) suggested that compared to the bulk soil, the %r-DOM and $logK_{Cu}$ may increase in the rhizosphere regardless of the soil type (Fig. 6).

Thus optimizing only the $logK_{Cu}$ with higher values than in the range fixed showed a better fit between predicted and measured Cu^{2+} activities in the rhizosphere. This modification of DOM binding properties can be due to the effect of plant roots in the rhizosphere. One hypothesis to explain the increase in DOM binding properties towards Cu may be the production by plant roots of organic compounds with higher affinity for Cu than the DOM binding affinity constant for Cu we optimized within the range of restricted physical meaningful values (i.e. from 2.16 to 2.46). Indeed plants can exudate organic acids (e.g. citrate, malate, and oxalate) which were found in the rhizosphere and can form stable complexes with Cu^{2+} (Jones, 1998). Graminous species such as fescue can also produce phytosiderophores which are known to have a very high affinity towards Cu (Dell'Mour et al., 2010; Schenkeveld et al., 2014). The changes in DOM binding properties were only assessed through optimization of binding constants using modelling, it will thus require further analytical characterization of DOM to validate the hypothesis that root exudates influenced DOM binding properties.

II.4.2 Copper and zinc phytoavailability does not increase with the application of organic fertilizers for a decade

Whatever the soil types, the decadal OF application did not affect the Cu and Zn fluxes towards fescue measured to assess Cu and Zn phytoavailability (Fig.7, 8) despite the Cu and Zn contamination in soils. The Cu and Zn fluxes towards fescue were poorly correlated to the pH, DOC concentration, SUVA, and Cu and Zn availability proxies measured in the rhizosphere solution. Michaud et al. (2007) reported that root Cu content of durum wheat was not related to the bulk soil pH and hypothesized that it was related to the rhizosphere alkalization. Bravin et al. (2009a) reported that whatever the initial bulk soil pH, the Cu fluxes of durum wheat did not vary much and was related to the rhizosphere alkalization which was around 7.3 pH unit. In our experiment although there was an increase in pH in the rhizosphere compared to the bulk-soil solution pH, the variation of pH within the rhizosphere samples was low because the pH was buffered by the plants around a mean value of 7. These little pH changes may induced non-significant changes in Cu and Zn fluxes between the different fertilization types.

Indeed, the variation of total Cu and Zn concentration and Cu^{2+} and Zn^{2+} activities in solution were low in the rhizosphere and thus exhibited a low variation between the fertilization types.

The only significant difference concerned Cu and Zn phytoavailability according to the soil type (Fig. S5h&i). This could be related to the different soil characteristics. Indeed in the andosol, fescue had lower Cu and Zn fluxes compared to the cambisol and the arenosol. The andosol, which represents the first step of the pedological evolution of soil formed on volcanic material, predominantly contains nano particles such as allophane and protoimogolite which present a large surface area able to strongly bind trace elements (Levard et al., 2009). The cambisol represents the second step of pedological evolution, and the arenosol represents the least evolved soil type. The cambisol and the arenosol contain minerals with a lower surface area than in the andosol and thus presumably with a lower trace element binding affinity. Thus our results showing higher Cu and Zn phytoavailability for fescue grown on the cambisol and the arenosol compared to the andosol are consistent with the intrinsic properties of these soils.

II.5 Conclusion

Under the influence of plant, the pH increased and was strongly buffered. Moreover in the rhizosphere, the DOC concentration decreased by 10-fold, the SUVA increased by 2-fold and the DOM binding properties were increased compared to the bulk soil. By contrast with our study performed with earthworms (Chapter III, part I) where the organism had a weak effect on mitigation of Cu and Zn availability, the plant overcame the effect of OF application on Cu and Zn availability in the rhizosphere. Finally, due to a major effect of the bio-influence of plant in the rhizosphere, the phytoavailability was not influenced by the type of fertilizations despite the contamination. Thus the earthworm seem to be more dependent to conditions in its bio-influenced zone rather than the plant. This study highlight the need to assess the environmental risk considering the effect of the organisms in its bio-influence zone and different levels of organisms.

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1.6 Annexes

Figure S1 Correlation matrix of the pH, the log_{10} of dissolved organic carbon concentration ($log_{10}DOC$), the log_{10} of specific UV absorbance of the solution at 254 nm ($log_{10}SUVA$), the total concentration of copper (Cu) and zinc (Zn) in solution (pCu_{SS} and pZn_{SS}), the free ionic Cu or Zn activity (pCu^{2+} and pZn^{2+}) activity in the rhizosphere, and the log_{10} of the Cu or Zn fluxes in plants ($log_{10}F_{Cu}$ and $log_{10}F_{Zn}$) on the whole dataset. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***.

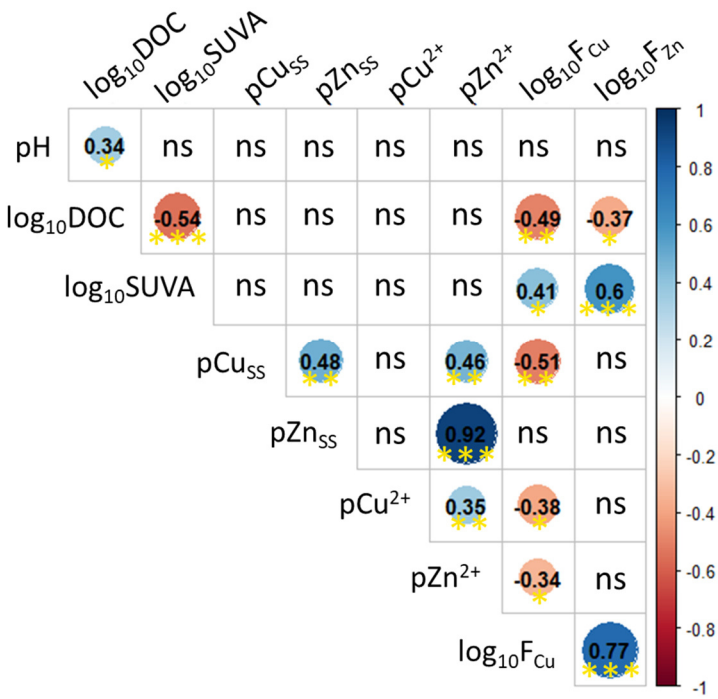

Figure S2 Correlation matrix of the pH, the log_{10} of dissolved organic carbon concentration ($log_{10}DOC$), the log_{10} of specific UV absorbance of the solution at 254 nm ($log_{10}SUVA$), the total concentration of copper (Cu) and zinc (Zn) in solution (pCu_{SS} and pZn_{SS}), the free ionic Cu or Zn activity (pCu^{2+} and pZn^{2+}) activity in the rhizosphere, and the log_{10} of the Cu or Zn fluxes in plants ($log_{10}F_{Cu}$ and $log_{10}F_{Zn}$) on (a) the andosol, (b) the cambisol, and (c) the arenosol. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***.

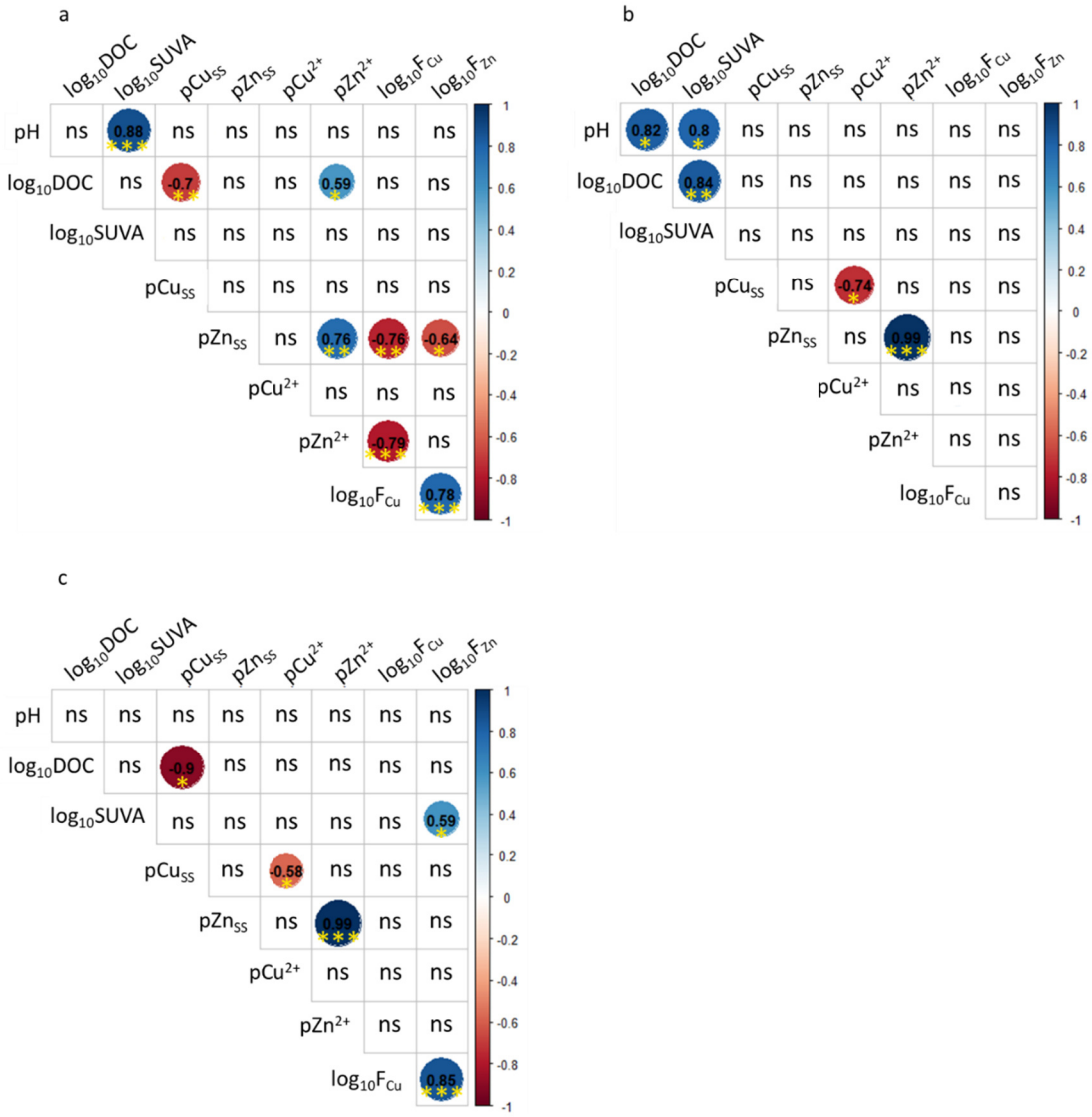

Figure S3 Correlation matrix of the variation of pH (ΔpH), log_{10}DOC (Δlog_{10}DOC), log_{10}SUVA (Δlog_{10}SUVA), pCu_{SS} or pZn_{SS} (ΔpCu_{SS} and pZn_{SS}), pCu^{2+} or pZn^{2+} (ΔpCu^{2+} and pZn^{2+}) between the rhizosphere and the bulk soil solution on the whole dataset. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***.

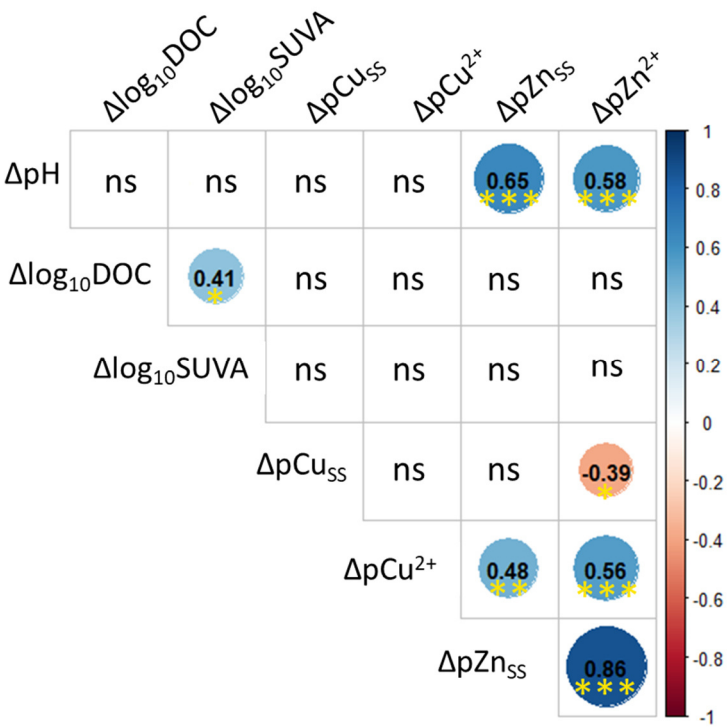

Figure S4 Correlation matrix of the variation of pH (ΔpH), log_{10}DOC (Δlog_{10}DOC), log_{10}SUVA (Δlog_{10}SUVA), pCu_{SS} or pZn_{SS} (ΔpCu_{SS} and pZn_{SS}), pCu^{2+} or pZn^{2+} (ΔpCu^{2+} and pZn^{2+}) between the rhizosphere and the bulk soil solution on (a) the andosol, (b) the cambisol, and (c) the arenosol. Non-significant Pearson correlation coefficients are labeled as ns. The significance level of the correlation is indicate as follows: 0.01 < p <0.05***; 0.001< p < 0.01**; p < 0.001***.

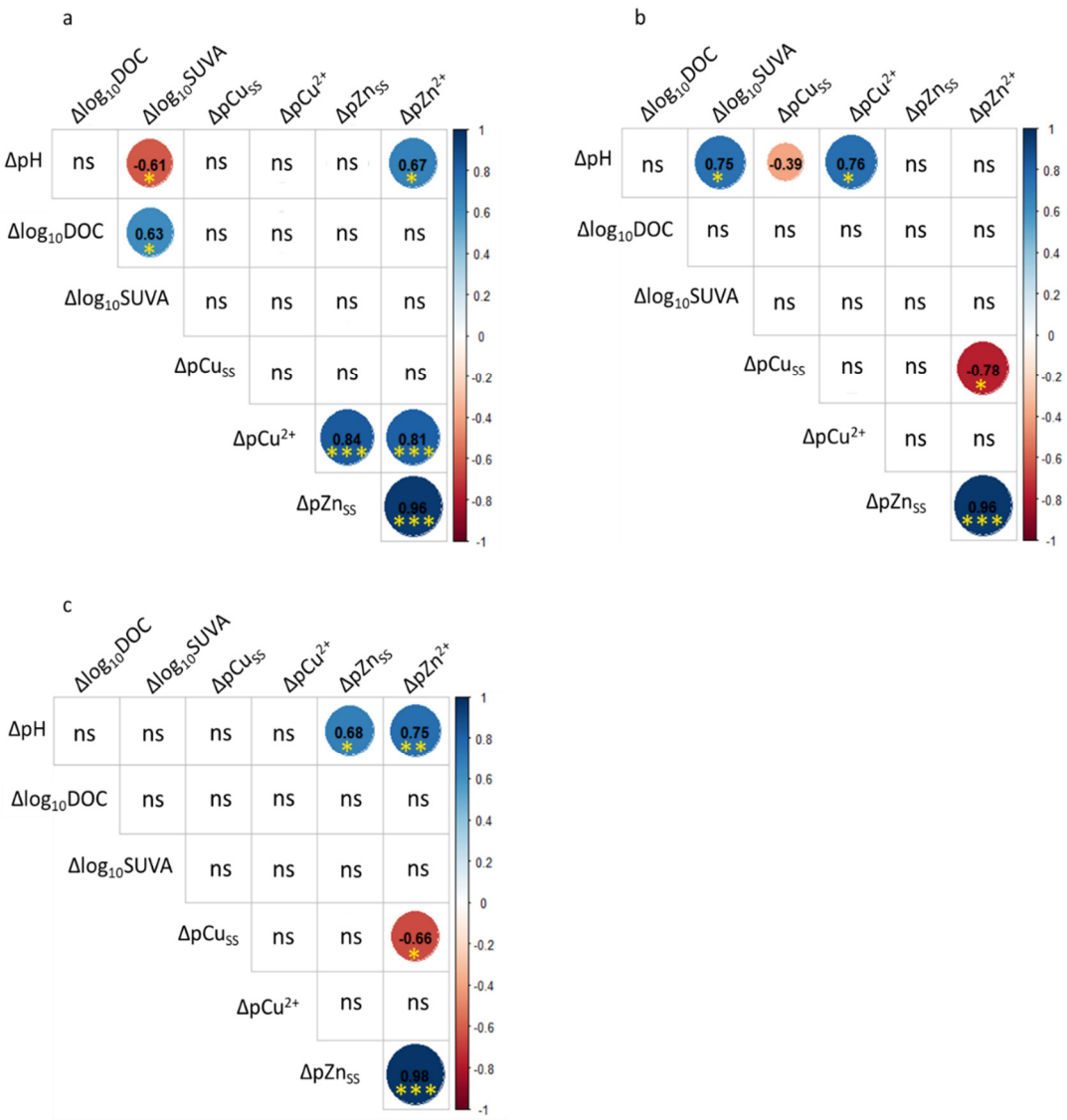

Figure S5 Variation of (a) DOC concentration, (b) total Zn concentration (pZn_{SS}), and (c) Zn^{2+} activity (pZn^{2+}) in the rhizosphere solutions as a function of soil types. Andosol n = 42, cambisol n =24, and arenosol n = 36. Different letters indicate significant difference at the level of 0.05. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile.

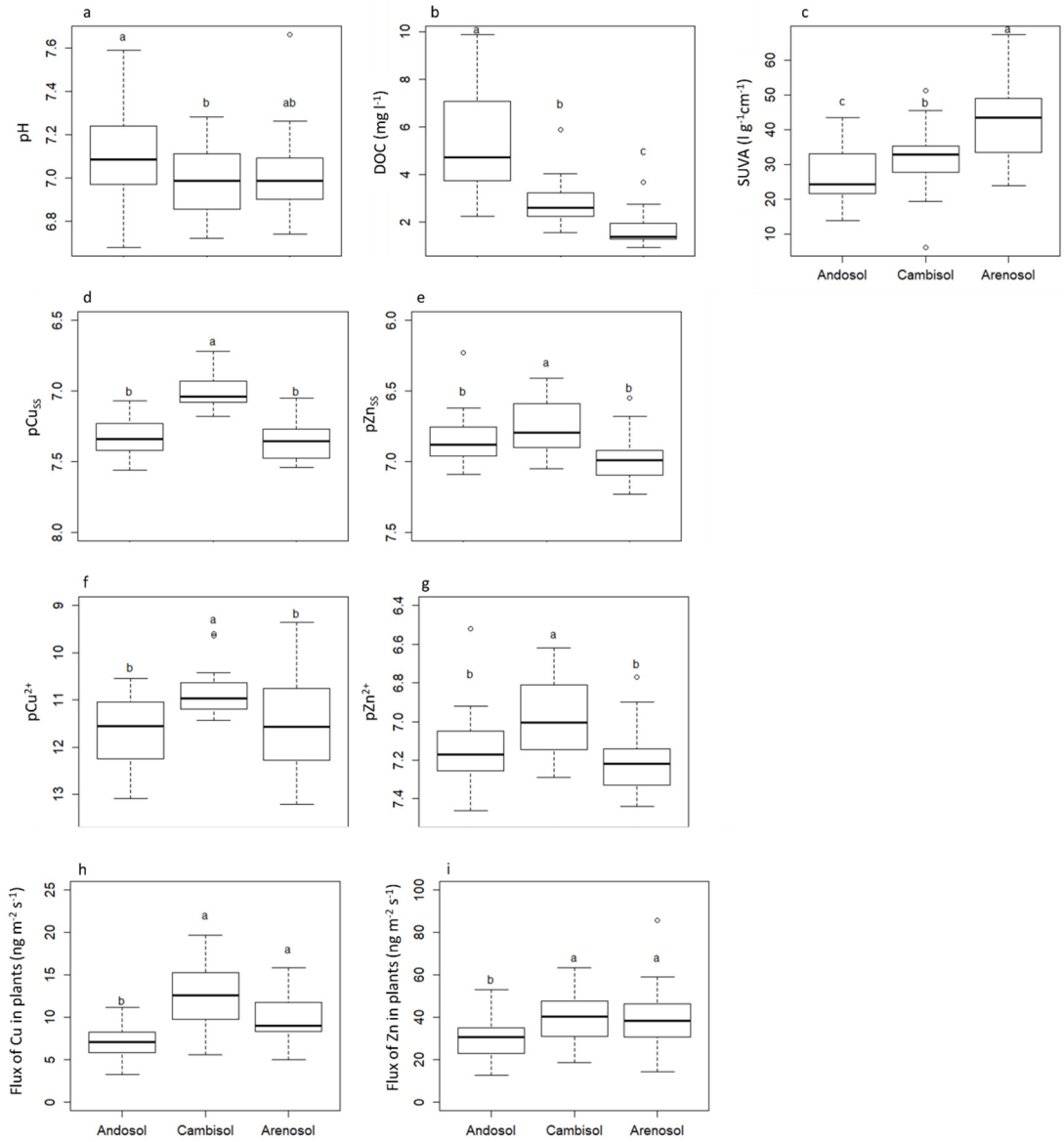

Figure S6 Principal component analysis (PCA) on soil solution of (a and b) the andosol (n = 14), (c and d) the cambisol (n = 8), and (e and f) the arenosol (n = 12). The correlation plot (a, c, and e) show the contribution of each variable to the two first principal components. Rhizosphere samples were clustered as a function of the fertilization types (No fertilization, Mineral and Organic). Data point with a larger size in (b), (d), and (f) correspond to the barycenter of each cluster. The quantitative variables considered in the PCA are pH, dissolved organic carbon concentration ($log_{10}DOC$), the specific UV-absorbance of the soil solutions at 254 nm (log_{10} SUVA), the total concentration of Cu and Zn in soil solution (pCu_{SS} and pZn_{SS}), the activity of free ionic Cu and Zn in soil solution (pCu^{2+} and pZn^{2+}). Supplementary quantitative variables are the Cu and Zn fluxes in plants ($log_{10}F_{Cu}$ and $log_{10}F_{Zn}$) and are represented by blue dashed arrows.

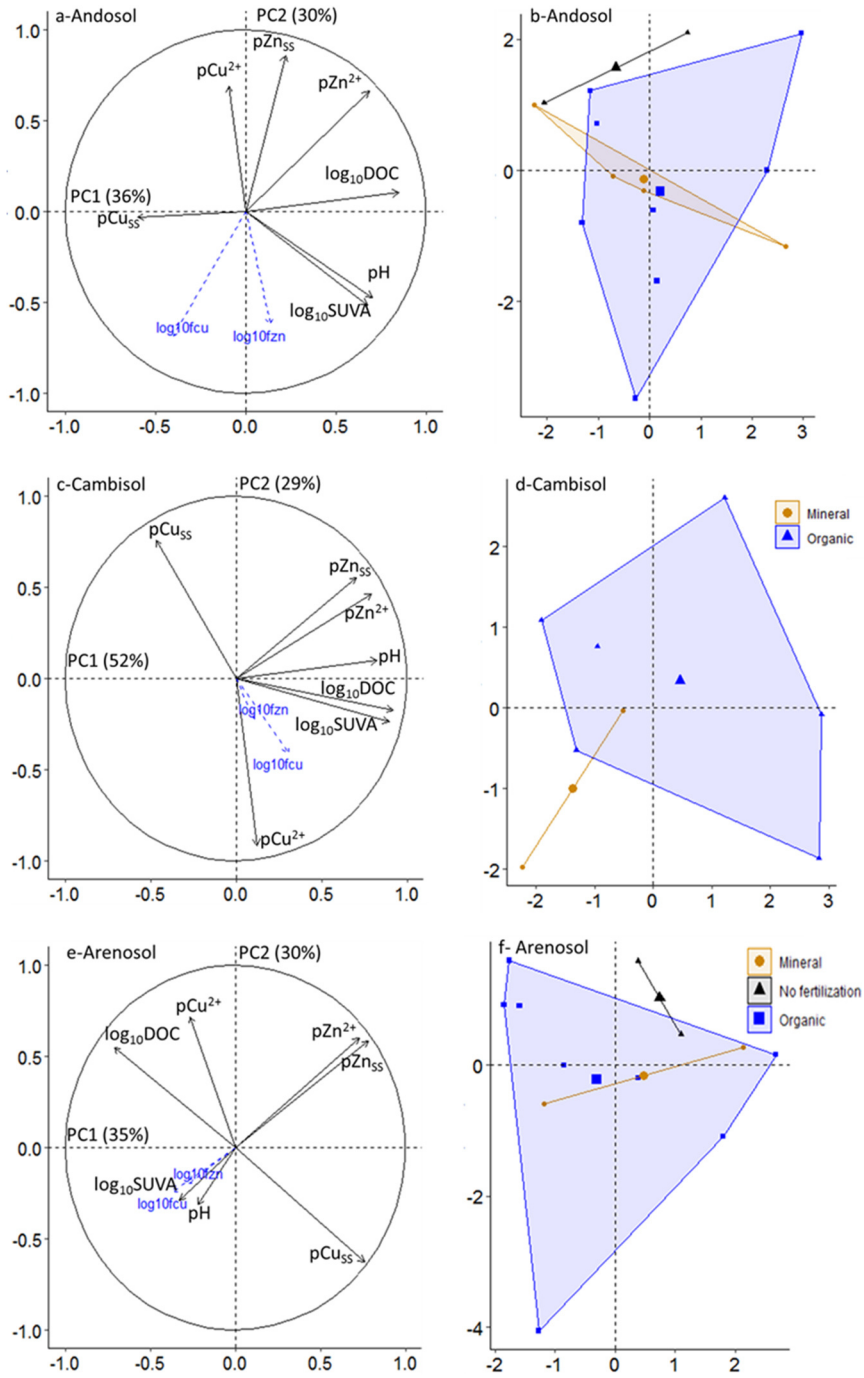

Table S1 Multi-linear regression explaining the variation of the total Cu and Zn concentration (ΔpCu_{SS} and ΔpZn_{SS}) in the rhizosphere as a function of ΔpH, $\Delta log_{10}DOC$, and $\Delta log_{10}SUVA$ for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		ΔpH	$\Delta log_{10}DOC$	$\Delta log_{10}SUVA$	i	[a]adj R^2	[b]n
ΔpCu_{SS}	3 soils	ns	ns	ns	ns	< 0.1	34
	Andosol	ns	ns	ns	ns	< 0.1	14
	Cambisol	ns	ns	ns	ns	< 0.1	8
	Arenosol	ns	ns	ns	ns	< 0.3	12
ΔpZn_{SS}	3 soils	0.5[1]	ns	ns	-0.18	0.4***	33
	Andosol	ns	ns	ns	ns	< 0.3	13
	Cambisol	ns	ns	ns	ns	< 0.1	8
	Arenosol	0.76[1]	ns	ns	-0.33	0.41*	12

[a]adj R^2 = adjusted R^2; [b]n = number of samples

Table S2 Multi-linear regression explaining the variation of the activity of free ionic Cu and Zn (ΔpCu^{2+} ΔpZn^{2+}) between the rhizoshpere as a function of ΔpH, $\Delta log_{10}DOC$, $\Delta log_{10}SUVA$, and ΔpCu_{SS} for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		ΔpH	$\Delta log_{10}DOC$	$\Delta log_{10}SUVA$	ΔpM_{SS}	i	[a]adj R^2	[b]n
ΔpCu^{2+}	3 soils	ns	ns	ns	ns	ns	< 0.1	34
	Andosol	ns	ns	ns	ns	ns	< 0.2	14
	Cambisol	ns	6.55[1]	ns	-2.72[2]	8.1	0.71*	8
	Arenosol	ns	ns	ns	ns	ns	<0.1	12
ΔPredicted pZn^{2+}	3 soils	ns	ns	ns	1.3[1]	- 0.34	0.73***	33
	Andosol	0.33[2]	ns	ns	1.62[1]	-0.94	0.96***	13
	Cambisol	ns	ns	ns	1.42[1]	-0.23	0.92***	12
	Arenosol	ns	ns	ns	1.31[1]	-0.24	0.95***	8

[a]adj R^2 = adjusted R^2; [b]n = number of samples

Figure S8 Dry weight of whole fescue as a function of soil types (andosol, cambisol, and arenosol). The control treatment represents the plants cultivated in hydroponics to compare with the growth of fescue on the different soil types. The values represented from the bottom to the top indicate the minimum, first quartile, median, third quartile, and maximum values. Where there are outliers, they are represented by circles and the related vertical dashed bars represent the whiskers, i.e. 1.5 times the interquartile range above the third quartile or below the first quartile. Different letters indicate significant difference at the level of 0.05.

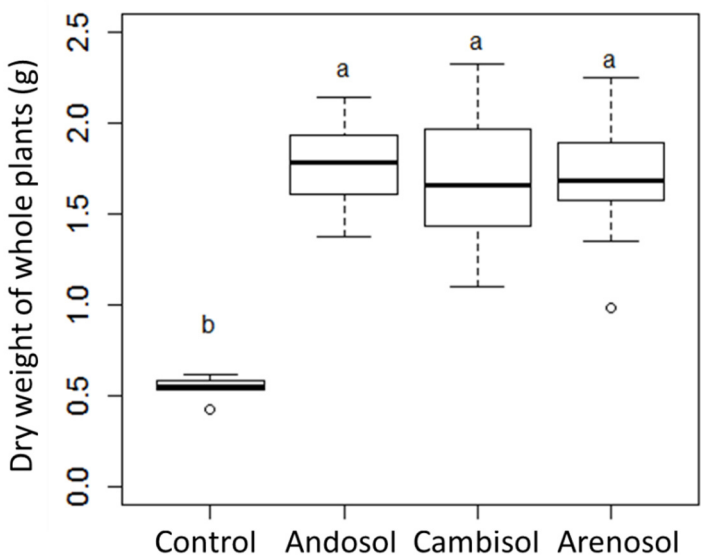

Table S3 Multi-linear regression explaining the $\log_{10}$ of the Cu and Zn fluxes in plants ($\log_{10}F_{Cu}$ and $\log_{10}F_{Zn}$) as a function of pH, $\log_{10}$DOC, $\log_{10}$SUVA, the total Cu or Zn concentration in solution (pM_{SS}), and the free ionic Cu or Zn activity (pM^{2+}) in the rhizosphere solution for the three soil types considered together or independently. Exponents indicate the order of contribution of explanatory variables to the regression. Non-significant regression coefficients and intercepts (i) were labelled as ns ($p \geq 0.05$).

		pH	$\log_{10}$DOC	$\log_{10}$SUVA	pM_{SS}	pM^{2+}	i	[a]adj R^2	[b]n
$\log_{10}F_{Cu}$	3 soils	ns	-0.3^2	ns	-0.5^1	ns	4.7	0.58***	34
	Andosol	ns	ns	ns	ns	ns	ns	< 0.2	14
	Cambisol	ns	ns	ns	ns	ns	ns	ns	8
	Arenosol	ns	ns	0.36^1	-0.48^3	-0.07^2	4.7	0.73**	12
$\log_{10}F_{Zn}$	3 soils	ns	ns	0.54^1	ns	-0.23^2	2.4	0.45***	34
	Andosol	ns	ns	ns	ns	-0.5^1	4.4	0.59***	14
	Cambisol	ns	ns	ns	ns	ns	ns	ns	8
	Arenosol	ns	ns	0.62^1	ns	ns	ns	0.28*	12

[a]adj R^2 = adjusted R^2; [b]n = number of samples

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Conclusion générale et perspectives scientifiques

L'objectif général de ma thèse a été d'évaluer les effets respectifs (i) de la contamination du sol, (ii) de l'évolution du pH et de la MO du sol et (iii) des plantes et des vers de terre (organismes clés du sol) dans leur volume de sol respectif qu'ils bio-influencent sur la disponibilité de Cu et Zn dans le sol et in fine leur biodisponibilité pour ces organismes en contexte d'apports de FO sur le long-terme. Les effets sur la disponibilité de Cu et Zn ont été principalement étudiés dans la solution des sols non bio-influencés par les organismes, des sols bio-influencés par les vers de terre et de la rhizosphère. Les effets sur la biodisponibilité ont été évalués par des mesures biologiques sur les plantes et les vers de terre.

1. Conclusion générale

Dans cette thèse, trois études ont été menées pour répondre à cet objectif. Les résultats de ces différentes études ont montré que l'apport de FO contamine les sols en Cu et Zn mais modifie également les propriétés physico-chimiques des sols telles que pH et MOD qui sont les principaux déterminants de la disponibilité de Cu et Zn. Ces modifications de pH et de la MOD ont ainsi contrebalancé l'augmentation concomitante de la contamination en Cu et Zn dans les sols, en se traduisant par une disponibilité de Cu et Zn globalement identique dans les sols ayant reçu ou non des apports FO. Les effets de l'apport de FO sur le pH et la MOD et les modifications de ces deux paramètres induites par l'activité des plantes (fétuque, *Festuca arundinacea cv* Callina) et des vers de terre (*Dichogaster saliens*) dans le volume de sol qu'ils bio-influencent ont induit des effets contrastés en termes de disponibilité de Cu et Zn dans la zone bio-influencée. La biodisponibilité de Cu et Zn n'a pas augmenté dans les sols amendés avec des FO en comparaison des sols fertilisés avec des engrais minéraux ou non fertilisés.

1.1 L'augmentation du pH et des propriétés de la matière organique dissoute du sol induit par l'apport de fertilisants organiques régule la disponibilité du cuivre et du zinc

L'apport de FO à long-terme a, comme attendu, contaminé en Cu et Zn les trois types de sols étudiés (i.e. andosol, cambisol et arénosol) mais a également augmenté le pH et la concentration, l'aromaticité et les propriétés de complexation de la MOD dans les solutions de sol. Ces résultats suggèrent une généricité des effets des apports de FO pour une grande gamme de sols, même lorsque la disponibilité diffère au départ en fonction du type de sol.

Bien que l'apport de FO ait induit une contamination en Zn du sol, la disponibilité de Zn dans la solution du sol (i.e. concentration totale de Zn et activité de Zn^{2+}) est principalement influencée par le pH (Chapitre II, Tableau 2). Dans les sols amendés avec des FO, l'augmentation de pH a ainsi induit une diminution de la disponibilité de Zn quel que soit le type de sol. Au contraire, la concentration et les propriétés de complexation de la MOD (approchées par modélisation WHAM) n'ont pas montré d'influence sur la disponibilité en Zn du fait de la faible affinité de la MOD vis-à-vis de Zn (Bonten et al., 2008).

En ce qui concerne le Cu, la disponibilité dans la solution du sol (i.e. concentration totale de Cu et activité de Cu^{2+}) est principalement influencée par le pH, la concentration et les propriétés de complexation de la MOD pour les trois types de sol (Chapitre II, Tableaux 2 et 3). Nous avons mis en évidence que la MOD a un effet contrasté sur la disponibilité de Cu selon le type de mesure. Une augmentation de la MOD augmente la concentration totale de Cu et diminue l'activité de Cu^{2+} dans la solution du sol. L'utilisation du modèle géochimique WHAM pour estimer les propriétés de complexation de la MOD (densité de site -*%r-MOD*- et constante d'affinité de Cu -$logK_{Cu}$-) vis-à-vis de Cu a suggéré que l'apport de FO à long-terme a entraîné une augmentation du *%r-DOM* et du $logK_{Cu}$ par rapport aux sols non fertilisés ou fertilisés avec des engrais minéraux (excepté pour le $logK_{Cu}$ dans le cambisol qui n'a pas été modifié).

Malgré des effets contrastés de la MOD sur la disponibilité de Cu, l'ensemble des résultats obtenus dans cette première étude a montré que les modifications de pH et de la MOD induites par l'apport de FO sur une décennie ont régulé la disponibilité de Cu et Zn en induisant un effet protecteur qui a contrebalancé la contamination en Cu et Zn induite par ces apports.

1.2 Les plantes et les vers de terre bio-influencent la disponibilité du cuivre et du zinc dans le sol

Dans les études 2 et 3, l'objectif était d'évaluer les effets de l'apport de FO à long-terme sur la disponibilité de Cu et Zn dans la zone bio-influencée par les vers de terre (étude 2) et les plantes (i.e. la rhizosphère) (étude 3) et en conséquence sur la biodisponibilité de Cu et Zn pour ces organismes.

1.2.1 La bio-influence des vers de terre ne modifie pas l'effet de l'apport de fertilisants organiques

De façon similaire aux résultats obtenus dans l'étude 1, les résultats de l'étude 2 montrent qu'en présence de vers de terre le pH, la concentration en COD et le SUVA augmentent dans les sols fertilisés avec des FO comparés aux échantillons de sols non fertilisés et fertilisés avec des engrais minéraux. La disponibilité de Cu et Zn a été déterminée par les mêmes facteurs de la solution du sol mis en évidence dans les sols non bio-influencés par les vers de terre dans l'étude 1 (i.e. pH et MOD). Cependant en ce

qui concerne l'activité de Cu^{2+} dans le sol bio-influencé par les vers de terre, le facteur principal qui l'a déterminée n'est plus le pH mais la concentration de COD ou la concentration totale de Cu (par ailleurs elle-même corrélée à la concentration de COD). L'estimation des propriétés de complexation de la MOD vis-à-vis de Cu dans le sol bio-influencé par les vers de terre a montré que le *%r-DOM* et le $logK_{Cu}$ augmentaient quel que soit le type de fertilisation et le type de sol. Cette optimisation suggère que Cu est plus fortement complexé à la MOD en présence de vers de terre dans le sol. Une plus forte complexation de Cu à la MOD pourrait induire une plus faible disponibilité de Cu dans les sols.

L'amplitude de variation de pH, de concentration de COD et de SUVA qu'il est attendu d'observer lors d'incubation de sols tend à être dans la même gamme de variation que ce que nous avons observé pour ces paramètres entre le sol bio-influencé par les vers de terre et le sol non bio-influencé. Il est ainsi difficile de différencier l'effet dû à l'incubation du sol de l'effet des vers de terre dans leur zone de bio-influence. Nos résultats suggèrent que la présence du ver de terre *D.saliens* a eu une faible influence sur la chimie du sol. Par conséquent cela a induit une faible variation de la disponibilité de Cu et Zn comparé aux sols de l'étude 1, non bio-influencés par les vers de terre.

1.2.2 La bio-influence des plantes efface l'effet de l'apport de fertilisants organiques

Comme dans l'étude 1, le type de sol a eu une influence sur la chimie des solutions (i.e. pH, concentration de COD, SUVA) de sols bio-influencés par les plantes (solution de sol de la rhizosphère) et sur la disponibilité de Cu et Zn tandis que les effets induits par l'apport de FO ne sont plus visibles. Les effets attendus de la fertilisation organique sur la chimie de la solution du sol et la disponibilité de Cu et Zn obtenus dans l'étude 1 ont été gommés par l'effet de la plante au niveau de sa rhizosphère. Dans la rhizosphère, le pH a été tamponné à une valeur moyenne de 7,04 ± 0,19, la concentration de COD a diminuée d'un facteur 10 et le SUVA a augmenté d'un facteur 2 comparé aux mêmes sols non bio-influencés de l'étude 1. D'après la littérature, les variations de pH, concentrations de COD et SUVA induites par des incubations du sol semblent moins importantes que celles attendues, suite à l'effet des racines des plantes. Nos résultats suggèrent donc que les racines ont eu un effet majeur sur la chimie de la solution dans la rhizosphère. Ces résultats suggèrent donc qu'une plante comme la fétuque est capable de modifier fortement les conditions physico-chimiques dans la rhizosphère contrairement au ver de terre comme *D.saliens* qui semble beaucoup plus dépendant des conditions physico-chimiques du sol.

En parallèle, une diminution des indicateurs de disponibilité de Cu et Zn (concentrations totales dans la solution et activité ionique) a été observée dans la solution de sol de la rhizosphère comparé aux échantillons de sols de l'étude 1, non bio-influencés par les plantes. Néanmoins, les indicateurs de la

disponibilité de Cu et Zn ont été faiblement reliés à la chimie de la solution du sol, du fait de la faible variation de pH, de la concentration de COD et du SUVA dans la rhizosphère. L'estimation des propriétés de complexation de la MOD vis-à-vis de Cu dans la rhizosphère a montré que le *%r-DOM* et le $\log K_{Cu}$ augmentaient dans tous les échantillons de sols, quel que soit le type de sol et le type de fertilisation. Cela suggère que dans la rhizosphère les propriétés de complexation de la MOD sont fortement modifiées et que la MOD dans la rhizosphère est susceptible de complexer plus fortement Cu que sans influence de la plante dans le sol. Les modifications de la chimie de la solution du sol dans la rhizosphère ont ainsi induits de faibles variations de la disponibilité de Cu et Zn dans la rhizosphère et qui n'ont pas entraîné de différence de disponibilité de Cu et Zn en fonction du type de fertilisation apporté.

1.3 La biodisponibilité du cuivre et du zinc pour les vers de terre et les plantes n'augmente pas avec l'apport de fertilisants organiques

La biodisponibilité de Cu et Zn n'a pas été modifiée ou a diminuée dans les sols ayant reçu des FO par rapport aux sols fertilisés avec des engrais minéraux ou non fertilisés, quel que soit le type de sol. Néanmoins, la biodisponibilité de Cu et Zn pour *D.saliens* a été faiblement influencée par les variables de la solution du sol (i.e. pH, concentration de COD, Cu et Zn total dans la solution de sol, activité de Cu^{2+} et Zn^{2+}). Les modifications de la chimie de la solution du sol induisant l'effet protecteur de la fertilisation organique, combinées à l'effet des vers de terre (même si faible) régulent la disponibilité de Cu et Zn dans le sol bio-influencé par les vers de terre. Par conséquent, la biodisponibilité de Cu et Zn pour les vers de terre est constante ou faiblement diminuée en fonction du type de fertilisation, et ceci quel que soit le type de sol. La faible explication de la biodisponibilité de Cu et Zn par les variables de la solution du sol suggèrent que d'autres mécanismes peuvent intervenir, comme par exemple des mécanismes de régulation/excrétion de Cu et Zn chez les vers de terre (Beaumelle et al., 2015; Spurgeon and Hopkins, 1999).

Alors que la phytodisponibilité de Cu et Zn n'a pas été modifiée quel que soit le type de fertilisation, des différences de phytodisponibilité de Cu et Zn en fonction du type de sol ont été observées (Chapitre III, Figures 7, 8 et S5h&i). Néanmoins, la phytodisponibilité de Cu et Zn a été très faiblement corrélée aux variables de la solution du sol dans la rhizosphère, ce qui peut être expliqué par l'alcalinisation du pH dans la rhizosphère (Bravin et al., 2009). L'influence des plantes sur la chimie de la solution du sol suggère que l'effet attendu de la fertilisation organique dans la rhizosphère a été gommé par l'effet de l'activité racinaire. La présence de la plante, ayant induit un pH moyen (autour de 7) dans les rhizosphères, implique de faibles amplitudes de variations dans la chimie du sol et la disponibilité en

Cu et Zn. Par conséquent, la phytodisponibilité n'a pas été modifiée quel que soit le type de fertilisation pour un sol donné. De plus, l'estimation des propriétés de complexation de la MOD dans la rhizosphère, en particulier l'optimisation de la constante d'affinité de Cu ($logK_{Cu}$), au-delà de la limite que l'on s'était imposé dans l'étude 1 et cela quel que soit le type de sol et de fertilisation, suggère que le Cu est fortement complexé dans la rhizosphère et donc peu disponible, expliquant par conséquent pourquoi l'on n'a pas observé de différence de phytodisponibilité entre les différents types de fertilisation.

2. Perspectives scientifiques

Mes travaux de thèse ont permis de mettre en évidence que l'apport de FO sur une décennie n'a pas induit d'augmentation de la disponibilité de Cu et Zn malgré la contamination des sols, ce qui s'est traduit par une absence d'augmentation de la biodisponibilité de Cu et Zn pour les plantes et les vers de terre. Néanmoins certaines limites sont apparues :

- l'effet protecteur de l'apport de FO a été impacté par des facteurs confondants puisque le rôle du pH et de la MOD sur la disponibilité de Cu et Zn n'ont pas pu être complètement dissociés afin de hiérarchiser l'importance de ces deux paramètres sur l'effet protecteur des FO ;
- les effets de l'incubation des sols sur les modifications de pH, de la MOD et des indicateurs de la disponibilité de Cu et Zn n'ont pas pu être clairement dissociés des effets liés à la bio-influence des plantes et des vers de terre puisqu'aucun dispositif sans organisme n'a été incubé en parallèle des dispositifs en présence des organismes ;
- alors que dans les agroécosystèmes les plantes et les vers de terre sont en interaction, les études de la bio-influence sur la (bio)disponibilité de Cu et Zn ont été menées séparément sur les plantes et les vers de terre ;
- les propriétés de complexation de la MOD (*%r-MOD* et $logK_{Cu}$) n'ont pas été mesurées, à l'exception de la mesure du SUVA, mais estimées par modélisation dans les différentes études ;
- la biodisponibilité a été étudiée à travers le flux de Cu et Zn dans la plante ou la quantité de Cu et Zn dans les vers de terre sans prendre en compte les phénomènes de régulation interne via la compartimentation de Cu et Zn dans les organismes ;
- du point de vue des conséquences environnementales de mes travaux, l'arrêt des apports de FO aux sols pourrait remettre en cause leur effet protecteur en lien avec de possibles retour en arrière des modifications de pH et de la MOD à court ou moyen-terme, alors que la contamination en Cu et Zn restera présente sur le très long-terme ; cela interroge sur les conséquences d'un tel retour en arrière sur la biodisponibilité de Cu et Zn pour les organismes.

2.1 Valider les déterminants bio-physico-chimiques de la disponibilité du cuivre et du zinc et évaluer l'effet de l'interaction plante-vers de terre

Dans le cadre de ma thèse, une dernière expérimentation a été réalisée afin de (i) hiérarchiser le rôle respectif des modifications du pH et de la MOD dans l'effet protecteur induit par l'apport de FO, (ii) distinguer la bio-influence de la plante dans la rhizosphère et du ver de terre dans sol de l'effet de l'incubation d'un sol initialement sec, et (iii) tester l'effet de l'interaction plante-vers de terre en contexte d'apport de FO à long-terme. Ce travail n'a pas été présenté dans le manuscrit car le travail de traitement et d'interprétation des données est encore en cours.

Dans cette étude, nous avons étudié un seul type de sol (i.e. l'arénosol) et deux échantillons issus de parcelles ayant reçu des engrais minéraux ou du lisier de porc pendant 15 ans ont été utilisés. La plante étudiée était la fétuque et le ver de terre *D.saliens*. Nous avons utilisé le dispositif RHIZOtest afin d'étudier la biodisponibilité pour les plantes et les vers de terre, en adaptant le RHIZOtest en introduisant des vers de terre dans le dispositif. Nous avons mesuré le pH, la concentration et l'aromaticité de la MOD, les indicateurs de la disponibilité de Cu et Zn (concentrations totales et activités ioniques dans la solution du sol) dans la solution des sols. La biodisponibilité de Cu et Zn a été évaluée par la mesure du flux de Cu et Zn dans la plante entière (parties aériennes et racinaires) et les concentrations en Cu et Zn dans les vers de terre.

2.1.1 Hiérarchiser le rôle respectif des modifications du pH et de la MOD dans l'effet protecteur induit par l'apport de fertilisants organiques

Afin de comprendre le rôle du pH et de la MOD sur la disponibilité de Cu et Zn, en lien avec l'apport de FO et l'activité des organismes, nous avons contrôlé le pH dans les échantillons de sol. Cela nous permettra de mieux expliquer les mécanismes déterminants la (bio)disponibilité de Cu et Zn pour les organismes et d'établir une hiérarchisation des effets de l'apport de FO à long-terme et de la bio-influence des plantes et des vers de terre sur la biodisponibilité de Cu et Zn. Pour cela nous avons étudié les deux échantillons de sol dans les conditions de pH initiales des échantillons puis en alcalinisant (ou en acidifiant) le pH d'un échantillon pour étudier au même pH de sol l'effet de la MO entre les deux échantillons sans exposition des organismes. En présence de plantes ou de vers de terre, nous avons également bloqué le pH du sol dans les deux échantillons avec une solution nutritive prévue à cet effet, afin d'empêcher l'alcalinisation du pH du sol par les organismes. Ces manipulations ont permis de découpler l'évolution du pH et de la MOD. Nos hypothèses sont que (i) empêcher l'organisme d'alcaliniser le pH du sol va induire une augmentation de la biodisponibilité de Cu et Zn pour l'organisme exposé au sol et (ii) la biodisponibilité de Cu et Zn pour les plantes ou les vers de terre

dans le sol fertilisé avec des engrais minéraux va augmenter par rapport au sol ayant reçu des FO pour un même niveau de pH du sol, du fait de l'effet protecteur de l'apport de FO.

2.1.2 Distinguer la bio-influence de la plante dans la rhizosphère et du ver de terre dans sol de l'effet de l'incubation d'un sol initialement sec

Dans les études 2 et 3 nous n'avons pas complètement pu conclure quant à l'effet de la bio-influence des vers de terre et des plantes sur les modifications de pH et de la concentration, de l'aromaticité de la MOD et de la résultante de ces modifications sur la disponibilité de Cu et Zn. Afin de dissocier les effets liés à l'incubation de ceux liés à la bio-influence du ver de terre ou de la plante, des dispositifs RHIZOtest contenant le sol incubé et les plantes ou les vers de terre exposés au sol ainsi que des dispositifs témoins contenant uniquement le sol incubé ont été réalisés. Le pH, la concentration et l'aromaticité de la MOD, les concentrations totales de Cu et Zn ainsi que les activités de $Cu2^{+}$ et Zn^{2+} ont été mesurés dans les témoins et dans les sols bio-influencés puis comparés. Cela permettra d'observer si les organismes bio-influencent le sol par rapport au sol non-bio-influencé mais incubé.

2.1.3 Tester l'effet d'interaction plantes-vers de terre en contexte d'apport de fertilisants organiques à long-terme

Les effets de l'interaction plante-vers de terre sur la disponibilité de Cu et Zn ont été évalués sur la solution du sol et comparé aux résultats testant les effets de la plante et du ver de terre seul. Une étude récente a été réalisée en RHIZOtest pour évaluer l'effet du ver de terre *Lumbricus terrestris* sur la phytodisponibilité des éléments traces. Dans cette étude, les vers de terre ont tout d'abord été exposés au sol afin que le sol soit bioturbé par les vers. La plante a ensuite été exposée, en RHIZOtest, au sol bioturbé par les vers. Dans notre étude, nous avons introduits les vers de terre dans la galette de sol, cela permettant d'évaluer le contact direct entre la plante et le ver de terre. Jana et al. (2010) ont montré que le contact entre les vers de terre et les racines pouvait induire une augmentation de la transcription de gènes signalant un stress qui pourrait conduire à une réduction de la croissance racinaire. L'effet de la plante sur la chimie de la solution dans la rhizosphère pourrait être modifié ainsi que les effets sur la biodisponibilité de Cu et Zn pour la plante. Dans l'étude de Lévêque et al. (2018), la catégorie écologique de ver de terre est différente (anécique) par rapport à celle utilisée dans notre étude (épi-endogée). Cela pourrait également induire des effets différents en termes de biodisponibilité de Cu et Zn pour les plantes et les vers de terre.

2.2 Caractérisation de la matière organique dissoute pour évaluer ses propriétés de complexation

Dans les trois études, nous avons mis en évidence l'importance de la variabilité des propriétés de complexation de la MOD dans des sols ayant reçu des apports de FO à long-terme, et d'autant plus en fonction de la bio-influence des plantes et des vers de terre. Cependant à part le SUVA, les propriétés de complexation de la MOD n'ont été estimées que via la modélisation et après optimisation manuelle de ces propriétés dans le modèle WHAM. L'utilisation du modèle avec une paramétrisation par défaut ou après l'optimisation de la densité de site de la MOD avec la mesure de SUVA n'ont pas permis d'améliorer les prédictions de l'activité de Cu^{2+} par rapport à l'activité de Cu^{2+} mesurée dans nos solutions.

La méthode par potentiométrie spectrophotométrique semble être une méthode réalisable avec des échantillons dont la concentration de MOD est assez faible (Janot et al., 2010), comme dans nos solutions de sol (concentrations de COD variant de 0.9 à 107 mg l^{-1}). D'autres travaux utilisant la potentiométrie spectrophotométrique comme technique analytique ont permis d'estimer les constantes de complexation entre des métaux, dont Cu, et un acide fulvique de référence (Yan et al., 2013a,b). Le protocole à mettre en œuvre semble possible mais peut-être dans un premier temps à appliquer sur un jeu de quelques échantillons afin de caractériser des échantillons ayant des propriétés de complexation de la MOD contrastées comme par exemple parmi notre jeu de données : un échantillon de sol non fertilisé (ou fertilisé avec des engrais minéraux), deux échantillons de sol ayant reçu des FO pendant des temps différents (un de l'étude 1 et un de l'étude présenté en 2.1), deux échantillons de sol ayant été bio-influencés par les plantes (un ayant reçu des FO et l'autre non fertilisé), deux échantillons de sol ayant été bio-influencés par les vers de terre (un ayant reçu des FO et l'autre non fertilisé). Cela nous permettrait une meilleure optimisation de la prédiction des propriétés de complexation de la MOD à partir d'échantillons de solution de sol.

Il pourrait également être envisagé de doser les acides organiques ainsi que les phytosidérophores qui sont des ligands capables de complexer les métaux. Cela pourrait être réalisé dans le sol non bio-influencé, la rhizosphère et le sol bio-influencé par les vers de terre, pour des sols ayant reçus des FO à long-terme, afin de déterminer la concentration de ces éléments qui peut être modifiée sous l'effet de la bio-influence des organismes.

2.3 Mesure de la compartimentalisation du cuivre et du zinc dans les organismes du sol exposés

Dans les études 2 et 3 nous avons seulement évalué l'effet de l'apport de FO à long-terme sur la biodisponibilité environnementale de Cu et Zn par la mesure de leur concentration dans les plantes et les vers de terre. A des niveaux de contaminations modérés, il est possible de ne pas observer d'effet sur la concentration interne des organismes exposés à ce sol (e.g. Beaumelle et al., 2015). Compte tenu

du niveau de contamination en Cu et Zn modéré avec l'apport de FO, les organismes ont pu réguler le Cu et Zn. L'évaluation de la biodisponibilité environnementale pourrait alors ne pas se faire par une approche de recherche de la concentration dans l'organisme mais plutôt d'étudier la répartition de Cu et Zn dans l'organisme exposé à différents type de fertilisants. Concernant les plantes, l'étude du compartiment racinaire, en particulier de l'apoplasme pourrait être d'intérêt. En effet, les métaux, en fonction de leur affinité pour les ligands organiques peuvent être retenus dans ce compartiment et ne pas être transférés vers les parties aériennes (Page et Feller, 2015). Chez les vers de terre, les métaux sont transportés par les métallothionéines. L'évaluation du niveau d'expression de gènes codant les métallothionéines pourrait être un indicateur de biodisponibilité de Cu et Zn pour les vers de terre (Pérès et al., 2011).

2.4 Arrêt de l'apport de fertilisants organiques

Si les modifications de pH et de matière organique dues à l'apport de FO sur les sols agricoles ont un effet protecteur vis-à-vis de la contamination en Cu et Zn, on peut se demander dans quelle mesure cet effet peut être maintenu après l'arrêt de l'apport de FO qui peut intervenir pour de multiples raisons (contraintes réglementaires, disponibilité des FO, coût d'épandage…). Par exemple, à La Réunion, les sols qui ne sont pas souvent amendés avec des FO ou qui ne sont pas régulièrement chaulés avec des amendements minéraux se ré-acidifient en quelques années. L'apport de FO augmentant le contenu en C organique du sol, l'arrêt de l'apport de FO pourrait également le modifier. Le pH étant un déterminant principal de la disponibilité de Cu et Zn dans les sols, l'acidification du sol pourrait entrainer une ré-augmentation de la disponibilité de Cu et Zn dans les sols et de la biodisponibilité de Cu et Zn pour les organismes.

Il faudrait donc étudier l'effet de l'arrêt de l'apport de FO à différents temps (pour des échantillons collectés sur le terrain après l'arrêt et plusieurs années après l'arrêt d'apport de FO) sur les déterminants (pH, concentration et propriétés de complexations de la MOD) et les différents proxies de la disponibilité de Cu et Zn au laboratoire, sur la même base que nos travaux précédents. En parallèle, il faudrait mettre en œuvre des tests biologiques afin d'étudier l'effet des organismes sur la biodisponibilité de Cu et Zn lors de l'arrêt de l'apport de FO. Ces tests pourrait être améliorés au regard de ce qui est proposé dans la perspective 2.2. L'étude de différents types de sols pourrait être envisagée afin de voir si les effets induits par l'arrêt de la FO présentent une généricité.

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Titre : Biodisponibilité du cuivre et du zinc pour les plantes et les vers de terre : interactions entre les effets de fertilisants organiques sur le long-terme et des organismes sur l'évolution des propriétés physico-chimiques du sol

Mots clés : Bioaccessibilité, Extraction, Matières fertilisantes d'origine résiduaire, Métaux, Test biologique

Résumé : La valorisation agronomique de fertilisants organiques (FO) est une source majeure de contamination des sols agricoles en éléments traces. Ce constat est particulièrement prégnant pour le cuivre (Cu) et le zinc (Zn) dont l'écotoxicité vis-à-vis des organismes du sol et plus largement l'impact sur la fertilité du sol posent question sur le long-terme. La littérature scientifique des deux dernières décennies ne permet cependant pas de trancher quant à l'évolution de la biodisponibilité pour les organismes du sol de Cu et Zn ainsi apportés. En effet les travaux antérieurs ne tiennent pas compte de la modification de la disponibilité de Cu et Zn pouvant être induite (i) par l'évolution temporelle du pH et de la matière organique (MO) observée avec l'apport de FO et (ii) par les activités des organismes dans le sol qu'ils sont susceptibles de bio-influencer. L'objectif de mon travail de thèse a donc été d'évaluer les effets respectifs de la contamination du sol, de l'évolution du pH et de la MO du sol et d'organismes clés du sol (i.e. les plantes et les vers de terre) dans le volume de sol bio-influencé, sur la biodisponibilité de Cu et Zn pour ces organismes en contexte d'apports de FO sur le long-terme.
Ce travail a été basé sur l'étude de sols provenant d'essais de terrain conduits à La Réunion, amendés pendant une décennie avec des fertilisants minéraux ou organiques ou jamais fertilisés. L'évolution du pH ainsi que de la concentration, de l'aromaticité de la matière organique dissoute (MOD) et de la disponibilité de Cu et Zn (i.e. concentration totale et activité de l'ion libre) a été mesurée dans la solution du sol non bio-influencé ainsi que du sol bio-influencé, soit par les activités racinaires (i.e. la rhizosphère), soit par l'activité des vers de terre (i.e. la drilosphère). La biodisponibilité de Cu et Zn a été évaluée par la mesure de leur concentration dans une espèce épi-endogée tropicale de vers de terre (*Dichogaster saliens*) et une espèce végétale tempérée (*Festuca arundinacea*, la fétuque), exposées aux sols en mésocosmes au laboratoire.
En l'absence de bio-influence, l'apport de FO a non seulement augmenté la contamination en Cu et Zn dans les sols, mais également le pH ainsi que la concentration et l'aromaticité de la MOD dans la solution des sols. La concentration totale de Cu dans la solution du sol a augmenté dans les sols ayant reçu des FO, proportionnellement à la concentration en MOD alors que l'activité de Cu^{2+} a diminué principalement du fait de l'augmentation du pH, se traduisant par une disponibilité de Cu identique entre les sols ayant reçu des FO ou non. Le Zn ayant une plus faible affinité pour la MOD, la concentration totale en Zn et l'activité de Zn^{2+} ont diminué dans les sols ayant reçu des FO sous l'effet de l'augmentation du pH, se traduisant par une disponibilité de Zn identique ou inférieure, dans ces sols par rapport aux sols non amendés. Ainsi, les modifications de pH et de MOD semblent réguler la disponibilité de Cu et Zn dans les sols non bio-influencés par un effet protecteur vis-à-vis de la contamination en Cu et Zn.
Sous l'influence des vers de terre ou des racines des plantes, la gamme de variation du pH et de la concentration en MOD quel que soit la modalité de fertilisation s'est réduite par rapport aux sols non bio-influencés. Cela s'est traduit dans la drilosphère par un effet de l'apport de FO sur la disponibilité de Cu et Zn similaire à celui observé dans le sol non bio-influencé mais de plus faible ampleur. Du fait d'une forte intensité des activités racinaires, cela s'est en revanche traduit dans la rhizosphère par une absence d'effet de l'apport de FO sur la disponibilité de Cu et Zn. La combinaison des effets protecteurs induits par l'apport de FO et l'activité des organismes a permis d'expliquer pourquoi la biodisponibilité de Cu et Zn pour les vers de terre et les plantes n'a pas augmenté avec les apports décennaux de FO malgré la contamination en Cu et Zn.

Title: Bioavailability of copper and zinc for plants and earthworms: interactions between the effects of organic fertilization on long-term and organisms on the evolution of soil physical-chemical properties

Keywords : Bioaccessibility, Biological assay, Extraction, Metals, Organic wastes

Abstract : The agronomic valorization of organic fertilizers (OF) is one of the main source contamination of agricultural soils in trace elements. This is particularly significant for copper (Cu) and zinc (Zn) for which the ecotoxicity and more generally their impact on soil fertility on the long-term is a matter of concerns. The scientific literature of the two last decades did not enable to state clearly whether Cu and Zn bioavailability for soil organisms will be impacted by OF application. Indeed, previous studies did not take into account the modifications of Cu and Zn availability in soils induced by (i) pH and organic matter (OM) temporal evolution observed with OF application and (ii) organism activities in the soil they can bio-influence. My PhD work thus aimed at assessing the respective effects of soil contamination, soil pH and OM evolution and key soil organisms (i.e. plants and earthworms) in the bio-influenced volume of soil, on Cu and Zn bioavailability for these organisms in the context of OF applications.This work was based on soils sampled from field trials in Réunion and amended for a decade with mineral or organic fertilizers, or never fertilized. The evolution of pH, concentration and aromaticity of dissolved organic matter (DOM), and Cu and Zn availability (i.e. totale concentration and free ionic activity) was measured in soil solutions of the non-bio-influenced soils and the soil bio-influenced by the activities of either plant roots (i.e. rhizosphere) or earthworm bioturbation (i.e. drilosphere). Copper and Zn bioavailability was assessed by the measurement of Cu and Zn concentration in a tropical epi-endogeic earthworm species (*Dichogaster saliens*) and a temperate plant species (*Festuca arundinacea*, fescue) exposed to soils in mesocosms under laboratory conditions.
In the absence of bio-influence, OF application did not only increase the level of Cu and Zn contamination in soils, but also pH and the concentration and aromaticity of DOM in soil solutions. Total Cu concentration in soil solution increased in soils amended with OF proportionally to DOM concentration while Cu^{2+} activity decreased mainly proportionally to pH increase, which resulted in a similar Cu availability between soils amended with OF or not. Due to the lower affinity of Zn for DOM, total Zn concentration and Zn^{2+} activity decreased in soils with OF application proportionally to pH increase, which resulted in Zn availability equal or lower in soils amended with OF than in non-amended soils. Thus, the modification of pH and DOM seems to regulate Cu and Zn availability in non-bio-influenced soils by exerting a protective effect that counteracts Cu and Zn contamination.
Under the influence of earthworms or plants, the variation range of pH and DOM concentration was reduced regardless of fertilization type in bio-influenced soils compared to non-bio-influenced soils. In the drilosphere, this resulted in a similar effect of OF application on Cu and Zn availability but in a lesser extent than the effect of OF application in non-bio-influenced soils. In the rhizosphere, the intense root activities resulted in the absence of OF effect on Cu and Zn availability. The combination of the protective effects induced by OF application and organism activity explained why Cu and Zn bioavailability for earthworms and plants did not increase with OF application for a decade despite Cu and Zn contamination in soil.