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APPLICATION OF STABLE ZINC ISOTOPES TO TRACE ZINC DERIVED FROM ORGANIC FERTILIZERS IN SOIL/FERTILIZER/PLANT SYSTEMS

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ABSTRACT

Zinc (Zn) is an essential micronutrient for all living organisms as this trace element is ubiquitously involved in metabolic reactions of all biological systems. Sufficient Zn supply and its bioavailability are prerequisites on all trophic levels of the human food chain. Zn deficiency impacts the quality and the yield of crops. In intensive livestock production, Zn is supplemented in order to sustain the growth rate and the health of animals. In human, Zn deficiency causes impairments of the immune-, endocrine- and reproductive system. The World Health Organization (WHO) listed Zn deficiency as a major risk factor for human health, especially in developing countries where the daily caloric intake is based on plant-based products, which contain a limited amount of bioavailable Zn. This concern has initiated several research programs with the aim to increase the Zn content and bioavailability in the edible part of crops. The Zn in soils represents the main source for the whole food-chain. Therefore, it is crucial to better understand the Zn-cycle of the soil-plant system. The amount of Zn circulating in a soil-plant system is small, when compared to the total Zn present in soils. Thus, the quantification of Zn fluxes can be challenging. For that purpose, isotope dilution techniques are valuable tools to measure nutrient fluxes and nutrient plant availability in a soil-plant system. This PhD-thesis aimed to measure the transfer of Zn from organic fertilizers and from the soils to the plant by using an isotope dilution approach with stable Zn isotopes.

In the first chapter, we evaluated the suitability of Quadrupole (Q-) Inductively Coupled Plasma Mass Spectrometry (ICPMS) to measure Zn isotope ratios in plant sample extracts in order to calculate Zn contribution from organic fertilizers (source) to plants (sink) using an indirect ^{67}Zn labeling method. One prerequisite of this method is that the mass of tracer added to the soil should not disturb the solid/liquid phases equilibrium. Thus, only a small amount of tracer should be introduced into the system. However, the tracer needs to remain measurable in the source and in the sink. A Q-ICPMS measures isotope ratios with a hundred-fold lower precision (1 % RSD[a]) than a multicollector (MC-) ICPMS (0.01 % RSD[a]). We found that the Q-ICPMS, when compared to the reference instrument (MC-ICPMS) produced an uncertainty of ±0.49 % of the percentage of Zn derived from the fertilizer. By compiling data from previous Zn fertilizer source tracing studies, we concluded that this uncertainty is acceptable to resolve agronomical relevant fertilizer contributions to plant uptake.

In the second chapter, we investigated the impact of organic fertilizers on the Zn plant availability and uptake with the indirect ^{67}Zn labeling method. To this end, a pot experiment was conducted in which Italian ryegrass (*Lolium mulitflorum*) grown on an acidic and an alkaline soil. Cattle manure, poultry manure, dried sewage sludge and water-soluble Zn fertilizer ($ZnSO_4$) were applied at a rate of 1.5 mg of Zn kg^{-1} soil. Soil pH had a significant effect

[a] Relative standard deviation

on the Zn fraction derived from the fertilizer, ranging from 12.2 % in the alkaline soil to 20.4 % in the acidic soil. The amount of Zn derived from the organic fertilizers and from the water-soluble $ZnSO_4$ fertilizer did not differ significantly. However, the isotope dilution technique allowed to highlight an increase of the amount of Zn derived from the alkaline soil in the treatments fertilized with animal manures. This effect was attributed to the organic carbon added with these fertilizers, which might have prevented irreversible Zn sorption on soil particles. The isotope technique also provided a qualitative evaluation of the Zn derived from the ryegrass seeds, which contributed significantly to the Zn uptake in the first ryegrass cut.

In the third chapter, we evaluated the ability of the "diffusive gel gradients in thin films" (DGT) method to predict plant Zn availability and uptake in a fertilized soil-plant system by using the ^{67}Zn indirect labeling method. A soil incubation experiment and a growth trial with Italian ryegrass were conducted using the same soil types and fertilizers as in the second chapter. The results showed that the ^{67}Zn:^{66}Zn ratio in DGT extracts did not significantly differ from the isotope ratio in the ryegrass shoots, which indicated that that DGT sampled only the isotopically exchangeable pool of the soil. The isotope composition of DGT-available Zn was also used to calculate the fraction of Zn derived from the fertilizer in the plant available pool of the soil. The results showed that the percentage of Zn derived from the fertilizer in the plant available pool can be determined with an uncertainty of ± 2.29 %. Even though this resolution of Zn derived from the fertilizer values was low, the DGT technique allowed to compare the effects of the investigated fertilizers on the plant available Zn pool of the soil. This approach might be promising to test the efficiency of fertilizers without the presence of a plant.

This PhD thesis demonstrated that isotope dilution techniques with stable Zn isotopes allow to quantify the transfer of Zn from organic and mineral fertilizer to the plant. Stable Zn isotope methods have a great potential for further development and could contribute to better understand the Zn cycle of the plant-soil system, which are relevant for the food security.

RÉSUMÉ

Le Zinc (Zn) est un micronutriment indispensable pour tous les organismes vivants, car cet oligo-élément est impliqué dans quasi toutes les réactions métaboliques des systèmes biologiques. Une offre suffisante de Zn et sa biodisponibilité sont essentielles à tous les niveaux trophiques de la chaîne alimentaire humaine. La déficience en Zn dans les systèmes agricoles affecte la qualité et le rendement des récoltes. Dans la production animale intensive, le Zn doit être complémenté afin de maintenir le taux de croissance et la santé des animaux. Chez l'homme, la déficience en Zn provoque des altérations au niveau du système immunitaire, endocrinien et reproductif. L'Organisation Mondiale pour la santé (OMS) a classé la carence en Zn comme un facteur à risque majeur pour la santé humaine. Les pays en voie de développement, où l'apport calorifique est basé sur des produits à base de plantes, sont particulièrement concernés, car ces aliments contiennent une quantité limitée de Zn absorbable. Cette préoccupation a initié des programmes de recherche qui ont pour but d'augmenter le contenu en Zn et la biodisponibilité dans les parties comestible des cultures. Le sol représente la principale source de Zn pour l'ensemble de la chaîne alimentaire. Par conséquent, il est important de mieux comprendre le cycle du Zn dans le système sol-plante. La quantité de Zn circulant dans ce système est faible, par rapport au Zn total présent dans ce dernier. De ce fait, la quantification des flux de Zn peut être difficile. Les techniques de dilution isotopiques sont des outils précieux pour mesurer les flux et la disponibilité des nutriments dans un système sol-plante. Cette thèse de doctorat a pour but de mesurer le transfert de Zn des engrais organiques et du sol dans la plante en utilisant une approche de dilution isotopique avec des isotopes stables du Zn.

Dans le premier chapitre, nous avons évalué l'aptitude de la spectrométrie de masse à plasma à couplage inductif avec un analyseur quadripolaire (Q-ICPMS) à mesurer des rapports isotopiques de Zn dans des extraits de plantes, pour calculer le Zn dérivé d'engrais organiques (source) dans la plante (puits), en utilisant une méthode de marquage de sol indirecte avec ^{67}Zn. Le prérequis de l'approche indirecte est que la masse de traceur introduit dans le sol lors du marquage isotopique ne doit pas perturber l'équilibre liquide-solide des phases du sol. Ainsi, la quantité de traceur ajoutée dans le système doit être minimisée. Cependant, le traceur doit rester mesurable/détectable dans la source et dans le puits après sa redistribution dans le système. Le Q-ICPMS a un coefficient de variation (RSD) de 1 % et mesure les rapports isotopiques avec une précision cent fois inférieure à un spectromètre de masse à plasma à couplage inductif à collecteur multiple (MC-ICPMS) qui a un RSD de 0.01 %. Par rapport à notre instrument de référence (MC-ICPMS), le Q-ICPMS nous a permis de déterminer le pourcentage de Zn dérivé de l'engrais avec une incertitude ± 0.49%. En regroupant les données d'études antérieures ayant également utilisé des méthodes isotopiques pour tracer

le Zn dérivé de l'engrais dans la plante, nous avons conclu que cette incertitude était acceptable pour déterminer l'efficacité d'engrais dans le contexte de recherche agronomique. Dans le deuxième chapitre, nous avons utilisé le marquage indirect avec du ^{67}Zn pour étudier l'impact des engrais organiques sur la disponibilité Zn dans le sol et sur la quantité Zn prélevée par la plante. Dans ce but, nous avons réalisé une expérience en pot avec du ray-grass italien (*Lolium mulitflorum*) cultivé sur un sol acide et un sol alcalin. Du fumier bovin, du fumier de volaille, des boues d'épuration séchées et un engrais de Zn solubles ($ZnSO_4$) ont été appliqués à raison de 1,5 mg de Zn kg^{-1} de sol. Le pH du sol a eu un effet significatif sur la fraction de Zn dérivée de l'engrais qui était de 12,2% dans le sol alcalin et de 20,4% dans le sol acide. La quantité de Zn dérivée des engrais organiques et de l'engrais soluble ($ZnSO_4$) ne se différenciait pas de manière significative. Cependant, la technique de dilution des isotopes a permis de mettre en évidence une augmentation de la quantité de Zn dérivée du sol alcalin dans les traitements fertilisés avec des engrais de ferme. Cet effet a été attribué au carbone organique ajouté avec ces engrais, ce qui a pu empêcher une sorption irréversible de Zn sur les particules de sol. La technique isotopique a également fourni une estimation du Zn dérivé des graines, dont la contribution était significative dans la première coupe du ray-grass.

Dans le troisième chapitre, nous avons évalué la capacité des échantillonneurs de sol DGT en gradients de diffusion en couches minces (DGT) à prédire la disponibilité du Zn dans le sol pour la plante dans un système sol-plante fertilisé en utilisant la méthode de marquage indirecte avec du ^{67}Zn. Pour cela, nous avons réalisé une expérience d'incubation de sol pour l'extraction au DGT et une expérience en pot avec du ray-grass avec les deux types de sols et les engrais mentionnés ci-dessus pour le deuxième chapitre. Les résultats ont montré que le rapport ^{67}Zn:^{66}Zn des extraits DGT ne différait pas significativement de celui des pousses de ray-grass. Ces résultats ont prouvé que l'échantillonneur DGT n'a extrait que la fraction de Zn isotopiquement échangeable du sol. La composition isotopique de l'extrait de la DGT a également servi à calculer la fraction de Zn dérivée de l'engrais au pool de Zn disponible dans le sol pour la plante. Les résultats ont montré qu'avec l'extraction DGT le pourcentage de Zn dérivé de l'engrais dans le pool disponible du sol peut être déterminé avec une incertitude de ± 2,29%. Même si cette résolution était faible, la technique DGT a permis de comparer les effets des engrais utilisés sur le pool de Zn disponible du sol. Cette approche pourrait être prometteuse pour tester l'efficacité des engrais sans la présence d'une plante.

Cette thèse de doctorat a démontré que les techniques de dilution isotopique avec des isotopes stables du Zn permettent de quantifier le transfert de Zn d'engrais organiques et minéraux vers la plante. Ces méthodes ont un grand potentiel de développement et pourraient contribuer à mieux comprendre le cycle du Zn dans le système sol-plante qui est essentiel pour la sécurité alimentaire.

GENERAL INTRODUCTION

Zn in the food chain

Zinc is indispensable for all forms of life. Zn has unique chemical properties, which explain its biochemical versatility: under physiological conditions Zn is not redox-active and has a variable coordination sphere which can assume multiple complexation geometries with ligands (Vallee and Falchuk, 1993). Zn is represented in all enzymatic classes and thus present in most metabolic pathways (Sousa et al., 2009). Zn plays an essential role in cell division, development and differentiation (Vallee and Falchuk, 1993) and also in cell signaling (Hershfinkel et al., 2007). For humans, Zn has a low toxicity as it is efficiently regulated to maintain the cell homeostasis over a broad range of exposure (Plum et al., 2010). For plants, the phytotoxic level is high, typically at a concentration of 500 mg kg^{-1} dry matter. However, organisms permanently exposed to elevated Zn supply can suffer from copper deficiency (Mousavi et al., 2012; Prasad et al., 1978) caused by competitive absorption or uptake mechanisms. On the other hand, Zn deficiency occurs frequently in plant, animal and human nutrition. Given the high diversity of biochemical functions in which Zn is involved, the deficiency symptoms are various. “Stunting” is a symptom common to all organisms. Zn is categorized in the nutrients of “Type II”, for which the organism reduces growth in order to maintain the concentration in the tissues (Golden, 1996). In plants, stunted growth is generally coupled with chlorosis and/or necrosis (Broadley et al., 2012). In livestock, Zn deficiency causes reduced growth accompanied by parakeratosis (dysfunction of the epidermis) for swine and ruminants, and by frizzled feathers, shortening and thickening of the long bones for chicken (Nielsen, 2012). In humans, Zn deficiency can affect the immune-, endocrine- and reproductive system (Vallee and Falchuk, 1993). Clinical manifestations of Zn deficiency are diverse: cognitive impairments, immune dysfunction and hypogonadism (Prasad, 2009). Therefore, diagnosis and risk assessment of Zn deficiency in populations is a complex task (Hotz and Brown, 2004). Stein et al. (2014) reported the number of disability-adjusted life-years (DALYs) lost in 2010 due to Zn-deficiency at approximately 10 million. For India alone, Stein et al. (2007) estimated the related economic costs of the impairments of human Zn deficiency at 8.05 billion dollar per year. On a global scale, a large fraction of the world population is exposed to medium or high risks of Zn deficiency (Hotz and Brown, 2004). In human nutrition, Zn deficiency is generally attributed to inadequate intake rates or malabsorption (Solomons and Rosenberg, 1984; Wessells and Brown, 2012). Populations living in regions where the daily caloric intake is mostly based on cereals are particularly exposed to Zn deficiency because plant-based diets have generally low Zn contents (Cakmak

et al., 1999). Recommended daily intakes from dietary sources vary from 2 to 15 mg Zn according to the life stage (age, body weight, pregnancy, lactation) (Hotz and Brown, 2004). Rice, wheat and corn have a median Zn content of respectively 16, 30 and 25 mg kg^{-1} (dry weight) (Welch, 1993). However, the largest Zn fraction of cereal grains is stored in the embryo and the aleurone layer (Cakmak, 2007), which is removed during the polishing or milling processes. Furthermore, antinutrients such as phytic acid, can inhibit the Zn bioavailability in human diets (Graham et al., 2001).

Biofortification aims at combatting Zn malnutrition in human by increasing the Zn content or bioavailability of the edible part of crops. (Bouis and Welch, 2010; Schulin et al., 2009). Modern, high yielding crop varieties developed after the green revolution are particularly Zn-inefficient: crop breeding mainly targeted the enlargement of the storage organs of the plant (carbohydrates), which implied a dilution of the Zn concentration of these parts (Alloway, 2009; Fan et al., 2008). Globally, agricultural intensification has also induced the appearance of Zn deficiency in crop production, especially in regions with low soil Zn availability (Alloway, 2008). Management practices, such as fertilizer or soil amendments can contribute to increase Zn plant availability. This thesis aimed to develop and to use stable isotope techniques to measure the transfer of Zn derived from the soil and from organic fertilizers to the plant.

Zn in plant nutrition

Soil Zn availability for plants

The mean Zn content in soil parent material ranges between 15 and 100 mg kg^{-1} and is manly present in form of minerals such as sulfides, sulfates, oxides carbonates, phosphatases and silicates (Barak and Helmke, 1993). Plant roots mainly take up free Zn^{2+} ions from the liquid phase of the soil. The Zn concentration in the soil solution is at least two or three orders of magnitude lower than the total content of the soil (Harter and Naidu, 2001). In non-contaminated soil, the solid-liquid phase transfer is mainly driven by adsorption/desorption processes and diffusion. The main soil properties affecting the Zn solubility are redox conditions, cation exchange capacity (CEC), organic matter content, soil type and structure, water content and soil pH, the latter being the dominant factor interacting with all the others (Rengel, 2015). Soil pH affects the charge density of inorganic and organic soil particles and thus the adsorption-desorption equilibrium of metals (Harter and Naidu, 2001). In moderate acidic soils a large Zn fraction is bound to organic compounds (Blume et al., 2015). From pH 6 onwards, Zn exchangeability between the solid and the liquid phase is exponentially decreasing, as the affinity of Zn towards Fe- and Mn-oxides as well as clay particles is strongly increasing (Blume et al., 2015). Soils which present a limited Zn availability for plant uptake

can be defined as “Zn-deficient” and mainly occur in regions with alkaline soil conditions and/or low total Zn soil content. Saline or sodic soils are often associated with Zn deficiency. However, high salinity reduces Zn plant uptake rather than Zn availability in soils (Daneshbakhsh et al., 2013). Without Zn fertilization, the Zn balance of agricultural systems is negative because of leaching, runoff or harvest export (Blume et al., 2015). Still, in areas with intensive livestock production, slurry or manure applications can cause an accumulation of Zn (as well as other trace elements: e.g. Cu and Cd) in the soil (Gubler et al., 2015; Nicholson et al., 1999).

Plant uptake

The ion Zn^{2+} is the most available form for plant uptake. Gramlich et al. (2013) found evidence that organic ligand bound Zn can also be taken up as entire complex before being dissociated within the root cells. However, it is still unclear to what extent organic Zn complexes contribute to plant uptake. The Zn concentration of the soil solution, which usually is in the micro-molar range, is relatively low when compared to the plant demand. Therefore roots are mainly supplied with Zn by diffusional processes in the rhizosphere rather than by mass flow (Marschner, 1993). The soil volume explored by the plant roots and root hairs is determining the accessibility to available Zn. Root growth and nutrient uptake induce an acidification of the rhizosphere and thus increase the desorption of exchangeable Zn (Hinsinger, 2001). Plants can also actively respond to Zn deficiency, by releasing organic acids which alter the soil pH or with the exudation of phytosiderophores (Cakmak et al., 1996; Marschner et al., 1986; Zhang et al., 1991). Additionally, plants have the ability to upregulate the gene expression for Zn specific membrane transporters (so called ZIP transporter) in order to increase Zn uptake (Grotz et al., 1998). Furthermore, mutualistic interactions with soil microorganisms can increase Zn supply to plants. Mycorrhizae hyphae allows the host plant to take up Zn from areas inaccessible for roots (Aghili et al., 2014b; Kothari et al., 1991). Besides fungi, Zn solubilizing bacteria might also contribute to increase the plant availability at the soil-root interface (Costerousse, unpublished; Ramesh et al., 2014). In exchange for the nutrient supply, the plant provides the microorganisms with carbohydrates. However, the effectiveness of all uptake mechanisms, root growth, mutualistic interactions, but also the Zn-transport and -translocation within plants rely on sufficient availability of other macro- and micronutrients for the plant. For instance, it was shown that enhanced nitrogen nutrition increased Zn uptake in wheat, root-shoot translocation and remobilization of Zn during grain filling (Erenoglu et al., 2011; Kutman et al., 2010).

Zn fertilization and soil amendments

Zinc fertilization is a common practice in regions with Zn deficient soils. Mineral fertilizers such as Zn sulfate or chelated Zn forms (EDTA-Zn, DTPA-Zn, Zn-fulvate) can either be applied directly to the soil or sprayed on the leaves or both. Several studies reported positive effects of foliar and soil Zn fertilization on yield as well as grain Zn concentration (Kutman et al., 2010; Prasad and Sinha, 1981; Yilmaz et al., 1997; Zou et al., 2012). On a short-term, Zn fertilization strategies provides a direct and efficient effect. Zou et al. (2012), who tested the effect of soil and foliar Zn fertilization of wheat in Zn deficient soil conditions in seven different countries, found that the grain Zn concentration was increased in average by 83.5 % with foliar Zn application and by 12.3 % with soil application. In the same study, both, foliar and soil application had only a minor effect on the yield (+ 3 % for the foliar and + 5.1 % for the soil application). Nevertheless, Zn fertilizer generally present a low recovery in the plant. Prasad et Sinha (1981) used a radioactive tracer (^{65}Zn) and observed a mean Zn recovery of 7 % for Zn-fulvate and only 2 % for $ZnSO_4$. The rest remained in the soil. Depending on soil conditions, chelated Zn fertilizers can stay plant available over several years. However, their elevated price limit their application in crop production (Cakmak, 2008). On the other hand, in alkaline soils, the cheaper water soluble forms (e.g. $ZnSO_4$) can rapidly and strongly be adsorbed on soil particles or precipitate with carbonates and become unavailable for the following crop (Yasrebi et al., 1994).

Organic fertilizers such as animal manure, compost, sewage sludge are potential Zn sources for plant uptake. Bolan et al. (2004) compiled data of the Zn contents of cattle, poultry, swine manure and sewage sludge: the average Zn content was 853 mg kg^{-1} (median 404.5 mg kg^{-1}). The composition as well as Zn-speciation of animal by-products strongly varies according the origin, the food/feed and the pre-application treatment. However, application rates, which are generally determined according to macronutrient demand, can cause a significant accumulation of trace elements in agricultural soils (Gubler et al., 2015; Nicholson et al., 1999). Nevertheless, in Zn-deficient soils, animal by-products can potentially increase plant availability and uptake not only by delivering additional Zn to the system, but also by altering the physicochemical properties of the soil and favoring plant growth. Those side-effects of organic soil inputs will further be developed thereafter and illustrated with the effect of green manures on Zn plant availability.

Fageria (2007) defined green manure as "*plant residues incorporated into the soil while green or at maturity*". Green manures which are generally produced by cover crops can be used for very different purposes: prevention of soil erosion, nitrogen fixation, boost of the biological activity, improve soil aggregation and aeration (Fageria et al., 2005). With green manures, no additional Zn is introduced into the soil-plant system, because they are grown and incorporated on the same surface. However, significant amounts of organic matter and sometimes nitrogen

(with legumes) are added to the soil. Aghili et al. (2014a) used a Zn-deficient soil from Iran to test the effect of sunflower and red clover shoot incorporation on the Zn uptake and Zn content of wheat grains. The results showed a significant effect of the green manure on grain Zn accumulation. As an isotope tracer (^{65}Zn) was used in this study, they could also observe an increase of the Zn derived from the soil in the sunflower treatment. Here, the higher Zn mobilization and uptake from the soil was mainly explained by the additional nitrogen added with the green manure which might have induced elevated root growth and microbial activity in the soil. In Iran, Soltani et al. (2014) tested the effect of several green manures (sunflower, Sudan grass, clover and safflower) in a field experiment in Zn-deficient soil conditions (DTPA-extractable Zn < 0.22 mg Zn kg^{-1} soil) and found a significant increase of the Zn concentration in wheat grains. In this case, the plant uptake was correlated with the amount of dissolved organic carbon (DOC) and amino acids measured in the soil solution. The same experiment was repeated in pots by Habiby et al. (Habiby et al., 2014) who obtained similar outcomes. However, the lack of isotope tracer did not allow to determine the exact contribution of the soil and the green manures to the plant Zn uptake. Grüter et al. (2017) investigated the effect of green manures (clover and mustard) on soil Zn availability (DTPA and DGT extractable Zn) and grain Zn concentration of wheat in a long-term fertilization experiment (ZOFE, Zürich, Switzerland). They also highlighted the importance of long-term carbon addition and nitrogen supply to increase plant available Zn and uptake with cover crops was also highlighted (Grüter et al., 2017).

How to predict plant availability?

When Zn deficiency symptoms appear on a crop, it is generally too late to compensate yield or quality losses with Zn fertilization. Moreover, if Zn deficiency is latent or “hidden”, impairments might even stay unnoticed (Alloway, 2009). For this reason, it is essential to have access to methods allowing to predict Zn plant availability and its optimal range where the plant response is maximized. Total Zn soil content is a poor indicator for plant availability because the major Zn fraction is trapped in the solid phase of the soil and is not representative of the amount of Zn in the liquid phase (Menzies et al., 2007). Various methods have been proposed to predict Zn plant tissue concentrations, Zn uptake or even to estimate the effect of fertilization strategies on Zn availability. Most of these methods are based on chemical soil extractions with diluted acids (e.g. oxalic-, acetic-, hydrochloric acid) or chelating agents (e.g. EDTA, DTPA) (Beckett, 1989; Davis et al., 1995; Fujii and Corey, 1986; Lebourg et al., 1996; Lindsay and Norvell, 1978; Menzies et al., 2007). However, the interactions between the extraction method and the soil properties (pH, organic matter content, soil structure, etc.) influence the amount of extracted Zn (Marzouk et al., 2013; Young et al., 2000). Therefore, the validity of those methods is limited to specific soil conditions for which they were calibrated

for (Degryse et al., 2009), which makes the comparability between different soil types and crops difficult or impossible.

Diffusive gradients in thin films (DGT) sampling has been proposed as an alternative technique to assess trace elements plant availability (Menzies et al., 2007; Zhang et al., 2001). DGT sampler are passive sampler composed of an exposure window with a filter membrane, a diffusion gel and a chelex resin layer successively mounted on a plastic piston (Zhang et al., 1998). The device is directly applied onto wet soil and measures the diffusive Zn supply from the soil solution, which is sorbed onto the resin layer acting as an infinite sink (Degryse et al., 2009). This method does not only take into account the Zn concentration in the soil solution but also the replenishment from the solid phase of the soil (Tandy et al., 2011; Zhang et al., 2001). DGT soil samplers were shown to be promising tools to predict plant Zn concentration (Nolan et al., 2005; Tandy et al., 2011; Zhang et al., 2014; Zhang et al., 2001). Compared to the chemical extraction, the DGT passive sampling, which simulates root uptake does almost not affect the soil conditions and can be generalized to a wide range of soil types. However, the two-dimensional exposure window from DGT samplers only explores a limited and unknown soil volume during its deployment time (24-72 hours). The DGT results reflect the Zn concentration in the soil over the deployment time and cannot be extrapolated to the soil volume.

The use of Zn isotope tracer techniques to measure Zn fluxes from fertilizers to plants

The Zn cycle in a soil-plant system is composed of fluxes which exchange and redistribute the Zn among the organic and inorganic pools (Gao et al., 2012). In non-contaminated soils the fluxes are mainly regulated by adsorption/desorption processes (Brümmer et al., 1983). Compared to the total amount of Zn present in the system, the quantity of Zn circulating between the pools and from the soil to the plant is relatively small and therefore difficult to measure. Isotope dilution techniques have already been used to investigate the phosphorus and Zn cycles in soils (Fardeau, 1993; Frossard et al., 2011; Frossard et al., 1993; Morel and Fardeau, 1991). As for Zn, the solid-liquid phase transfer of phosphorus is controlled by adsorption/desorption processes (Fardeau, 1995). The methodological approaches used for phosphorus are therefore transferable to Zn (Tiller et al., 1972). The principle of isotope dilution techniques in soil-plant systems is based on the introduction of a small amount of an isotope (tracer) into the soil, which is then redistributed among the soil pools (Hamon et al., 2008) and thus also to the plant. For this method, the tracer has to be added in the form which is actually tracked (here Zn^{2+}) and the mass of tracer introduced to the system should not disturb the soil/solution equilibrium, also called steady-state. Also, the tracer must be analytically distinct from the investigated element (the tracee). Regarding plant nutrient availability, several applications can be derived from theses isotope techniques:

- *Isotope exchange kinetics (IEK),* which allow to quantify the isotopically exchangeable pool (E-value) of the soil over time (Frossard et al., 2011). For phosphorus and zinc, the isotopically exchangeable pool represents the plant available pool of the soil (Frossard et al., 1994; Sinaj et al., 2004). Here the tracer is applied to a soil suspension. The liquid phase is sampled at different time points and analyzed to determine the concentration of the tracer, whereas the tracee concentration remains constant in the solution. As the adsorption/desorption processes continuously exchange the tracer and tracee between the solid and the liquid phase of the soil, the concentration of the added tracer in the liquid phase decreases over time. The isotopic composition of the solution allows to calculate the amount of isotopically exchangeable element.
- *L-value*: This method relies on the same principal as the E-value, but in the presence of a plant. Here, the concentrations of the tracer and tracee are not determined in the soil solution, but in the plant (Fardeau and Triboi, 1974). The L-value does not only take into account the plant availability, but also the plant uptake. The comparison of L- and E-values can highlight the access of the plant to non-isotopically exchangeable nutrient sources due to mutualistic interactions or root exudates (Pypers et al., 2006).
- Isotope soil labeling can be used to check the ability of a chemical extraction method to predict plant availability (Demaria et al., 2005; Nanzer, 2012). For instance, Six et al. (2012) compared the specific activity of several phosphorus soil extractions with the specific activity of the plant grown on the same isotope labeled soil and showed that the DGT sampler only extracted phosphorus from the plant available pool.
- *Source tracing* methods with isotopes allow to quantify the transfer of nutrients from a given source to the plant. In the present study, we are focusing on the transfer of Zn derived from fertilizers and from the soil to the plant. As for phosphorus, two source tracing approaches can be used: the direct method and the indirect method. In both cases, the source tracing system is composed of two sources (the soil and the fertilizer) and one sink (the plant). With radioisotopes, the source contribution to the plant is calculated by comparing the specific activity (SA) in the plant with the SA of the labeled source (Morel and Fardeau, 1989). With stable isotopes, the fractions derived from the sources are obtained by mass balance calculations from the isotope ratios (McBeath et al., 2013). In this section describing isotope dilution techniques, the direct method makes an exception, because it is not the soil that is labeled with isotopes but the fertilizer itself, before being incorporated into the soil (Morel and Fardeau, 1989). However, complex fertilizers such as animal or green manure or sewage sludge are difficult to homogenously/intrinsically label with a tracer, because the isotope should previously be metabolized by the organism. Hence, the indirect method represents a convenient alternative. For the indirect method, the isotopically

exchangeable soil pool has to be homogenously labeled with a tracer. This approach was successfully applied to investigate transfer of phosphorus from rock phosphate (Zapata and Axmann, 1995), sewage sludge (Frossard et al., 1996), compost (Sinaj et al., 2002) and animal manure (Oberson et al., 2010) to the plant. For the first time, Nanzer (2012) tested the indirect labeling method with ^{65}Zn to evaluate the transfer of Zn derived from sewage sludge ashes and Zn sulfate to ryegrass and proved its ability to quantify Zn contributions from complex fertilizers to the plant. One year later, McBeath and McLaughlin (McBeath and McLaughlin, 2013) applied the same indirect labeling method with ^{65}Zn to assess the efficiency of different Zn-oxides (ZnO) fertilizers on durum wheat Zn uptake and showed that Zn-oxides can be as effective as water-soluble Zn fertilizers. To our knowledge, the indirect method with stable Zn isotopes has never been used to trace Zn derived from fertilizers in a soil-plant system. Moreover, the transfer of Zn from animal manures, compost and dried sewage sludge has never (as far as we know) been investigated.

Why stable isotopes for source tracing?

Terrestrial Zn is mainly composed of five stable isotopes: ^{64}Zn (0.4917), ^{66}Zn (0.2773), ^{67}Zn (0.0404), ^{68}Zn (0.1845), ^{70}Zn (0.0061), the brackets indicating the fractional abundance (mole mole^{-1}) (Berglund and Wieser, 2011). The radioisotope ^{65}Zn is the only radionuclide commercially available with a sufficient half-life (244 days) to be used in soil or plant studies. ^{65}Zn is a beta (0.33 MeV) and gamma (1.11 MeV) emitter. Therefore, the specific activity of soil and plant samples can be determined either by liquid scintillation counting (Kossert, 2006) or by gamma spectrometry. ^{65}Zn does not naturally occur in the environment, which makes it easier to follow and measure in a soil-plant system. All isotope dilution techniques are based on the assumption that the mass of the introduced tracer does not disturb the steady-state of the studied system (Cobelli et al., 2007; Hamon et al., 2008). Radioisotope labeling is “carrier-free”, which means that the absolute mass introduced to the system is negligible compared to the mass of element to which the isotope is added (the soil solution), as the radiation is strong enough to be measured at extremely low tracer concentrations. Technically ^{65}Zn is a perfect candidate for isotope dilution techniques. Practically, the use of radionuclides is subject to legal regulations and safety issues: first, not everybody feels comfortable to be exposed to radiations, second, only authorized laboratories are allowed to handle radioactive material and finally, legal regulations can prevent the use of radioisotopes in field studies.

Unlike radioisotopes, stable isotopes do not decay and have the advantage of being applicable without legal or sanitary restrictions. However, before 1990 the measurement of isotope variations of heavy elements (> 40 amu) was limited by the availability of analytical techniques (Weiss et al., 2008). Since then, the progress in mass spectrometry techniques has

democratized the use of stable isotopes to study metal cycles in the environment. In the field of biogeochemistry, Zn isotope variations were principally assessed to study isotope fractionation processes (Hoefs, 1997). Tracer techniques with isotope enriched Zn sources evolved probably the most in biomedical research and human nutrition to investigate metabolism, bioavailability and requirements (Cobelli et al., 2007; Stürup, 2004). Stable Zn isotope labeling for source tracing imposes two main constraints, which are interconnected. On the one hand, the mass added should not disturb the steady-state of the system (Cobelli et al., 2007). Unlike the radioisotopes, all stable Zn isotopes are already present in the system (see the isotope abundances at beginning of this section). Therefore, the amounts of isotopes introduced to the system have to be kept by the minimum. On the other hand, the analytical precision of the mass spectrometer is determining the required enrichment of the source and of the sink, meaning that the amounts of isotopes added to the system has to be high enough to analytically distinguish the source and the sink. Walczyk (2001) called this dilemma: "The mass spectrometry challenge".

Stable Zn isotope measurements can be realized with diverse inductively couple plasma mass spectrometry (ICPMS) techniques, as e.g. multicollector ICP-MS (MC-ICPMS) or quadrupole ICP-MS (Q-ICPMS). The precision of the instrument is given by its ability to eliminate spectral interferences (Mason et al., 2004a) and by the possibility to correct for mass discrimination effects (Mason et al., 2004b). Spectral interferences can be summarized as the measurements of undesirable species (monoisotopic species, agrides, oxides, double-charged species, elemental species) having the same mass than the measured isotope (Mason et al., 2004a). These interferences can be reduced with the parameterization of the instrument and by the separation of the isotope from other elements present in the sample matrix. Matrix separation can be achieved by resin ion exchange chromatography (Durrant et al., 1994). Also, for isotope labeling, it might be advantageous to choose the isotope, that is the least exposed to spectral interferences (Ramanujam et al., 1999). In the present thesis, we selected ^{67}Zn as tracer. Mass discrimination, which occurs through mass-dependent fractionation within the instrument, can be corrected mathematically by using a sample-standard bracketing method (Mason et al., 2004b; Walczyk, 2001). For isotope ratio measurements a MC-ICPMS can achieve a precision of 0.01 % RSD (relative standard deviation), whereas a Q-ICPMS resolves in the range of 1 % RSD (Stürup, 2004). The magnetic fields of the MC-ICPMS separate the masses more efficiently than in a Q-ICPMS. Furthermore, the multicollector-ICPMS, as its name says, can measure several masses at the same time, whereas the Q-ICPMS has a single collector, which measures one isotope after the other. Therefore Q-ICPMS measurements are more sensitive to variations in plasma intensity. In terms of analytical precision, the MC-ICPMS is obviously superior to the Q-ICPMS. However, this precision has a price: the analytical costs for Q-ICPMS are significantly lower than for MC-ICPMS and thus fewer laboratories are equipped with a MC-ICPMS (Stürup, 2004).

Structure and objectives of the thesis

The overall goal of this thesis was to provide a methodology to quantify the transfer of Zn from organic and mineral fertilizers to the plant using stable isotopes techniques. In the first part, we investigated analytical methods for isotope source tracing of Zn in a fertilized soil plant system, in the second part, we quantified the transfer of Zn from complex fertilizers to ryegrass with the indirect ^{67}Zn labeling method, and in the last part we measured this transfer with DGT passive sampler.

The objective of the **first chapter** was to evaluate the suitability of Q-ICPMS technique to measure stable Zn isotope composition in plant sample extracts from a source tracing experiment using the indirect labeling approach. As quality check, the samples were analyzed on a MC-ICPMS. The results of both instruments were used to calculate the Zn derived from the soil and from the fertilizer (wheat straw compost) in ryegrass. The aim was to evaluate if the Q-ICPMS measurements were precise enough to quantify the transfer of Zn from the fertilizer to the plant.

In the **second chapter**, the indirect ^{67}Zn labeling method was applied to measure the transfer of Zn from organic fertilizers to the plant. The first goal was to determine at what magnitude organic fertilizers, such as cattle manure, poultry manure and sewage sludge can contribute to plant Zn uptake, when compared to water-soluble Zn fertilizer ($ZnSO_4$). The second goal, was to measure the impact of organic fertilizers soil inputs on the Zn derived from the soil in the plant. We hypothesized that organic fertilizers are not only significant Zn sources for plant uptake but also that their application leads to changes of the physicochemical and biological properties of the soil, and thus increase the amount of plant available Zn derived from the soil.

In the **third chapter,** we used the indirect ^{67}Zn-labeling method to measure, whether DGT passive samplers and ryegrass extracted the same Zn pool of the soil. The DGT extracts were realized in ^{67}Zn-labeled incubation soils, which were fertilized with water-soluble ($ZnSO_4$) and organic Zn fertilizers. In parallel, a pot experiment with ryegrass was performed with the same treatments. The isotope composition (^{67}Zn:^{66}Zn) of the DGT extract was compared with the isotope composition of ryegrass. The first goal was to check if the DGT technique is suitable to predict plant availability. The second objective was to determine if DGT sampling in combination with indirect soil labeling could provide reliable information on the fertilizer Zn contribution to the plant available soil pool.

I. USING QUADRUPOLE ICPMS TO DETERMINE STABLE ZN ISOTOPE RATIOS FOR ZN SOURCE TRACING IN SOIL-PLANT SYSTEMS

Abstract

The development of inductively coupled plasma mass spectrometry (ICPMS) technique has spawned the use of stable isotopes to trace metals in the environment. Here we investigate the suitability of quadrupole (Q-) ICPMS to determine stable Zn isotope ratios for direct and indirect source tracing of fertilizer-derived Zn uptake by plants. In the direct method, the fertilizer is intrinsically labeled with Zn isotopes, whereas the indirect method is based on soil available Zn labeling. The latter is particularly useful when homogenous labeling of a fertilizer source is difficult to achieve. However, the indirect method with ^{67}Zn has the disadvantage that the mass of isotopes added as spike to the soil affects the plant available Zn status of the latter. Therefore, only a relatively low enrichment of the plant available Zn pool in the soil can be achieved. On the other hand, the enrichment has to be high enough to analytically distinguish the isotope composition of the soil source, the fertilizer source as well as of the sink, where both sources are mixed. In other words, the minimum isotope enrichment required is determined by the analytical precision of the instrument used to measure the isotope ratios. A source tracing pot experiment with Italian Ryegrass grown an acidic (Heiteried) and an alkaline (Strickhof) soils was conducted under controlled conditions. Intrinsically ^{67}Zn labeled (for the direct method) and non-labeled (for the indirect method) wheat straw compost was applied to the soil as Zn fertilizer. Ion exchange chromatography was used to separate the analyte Zn from the sample matrix, in order to reduce spectral interferences during the ICPMS measurements. In order to assess the effect of the analytical precision of the Q-ICPMS (approximately 1 % RSD[b]) on the calculated Zn derived from the fertilizer and from the soil in the plants, the same extracts were also measured on a mulicollector (MC) ICPMS (0.01 % RSD[b]). By pooling the results from the direct and indirect source tracing treatments, we found that the Q-ICPMS could resolve the percentage of Zn derived from the fertilizer ($Zndf_{fertilizer\%}$) with a precision of ±0.49 ($Zndf_{fertilizer\%}$), when compared to the MC-ICPMS. With regard to

[b] Relative standard deviation

previous studies, which evaluated the contribution of Zn fertilizers to plants, this uncertainty was considered as acceptable for the field of agronomic research.

Introduction

Zinc is an essential micronutrient for microorganisms, plants, and animals. Plants take up Zn from the soil solution primarily as free Zn^{2+} ions, but probably also in form of Zn complexes with organic ligands (Broadley et al., 2007; Gramlich et al., 2013). The total Zn soil content, pH and organic matter are the main factors determining the Zn availability of soils for plant uptake. The plant available Zn pool continuously exchanges with the solid phase of the soil through adsorption/desorption processes (Brümmer et al., 1986).

Isotope dilution is a valuable method to quantify nutrient pools and fluxes in soil-plant systems (Frossard et al., 2011). Isotope source tracing allows to determine the contributions of fertilizers ($Zndf_{fertilizer}$) and soil ($Zndf_{soil}$) in plant Zn uptake. Two methods can be used. In the direct method, the fertilizer itself is labeled with a tracer before being added to the unlabeled soil. In the indirect method, the soil is labeled and mixed with unlabeled fertilizer. For all isotope dilution techniques three conditions need to be fulfilled: i) the introduced spike should not perturb the system, ii) the isotopes used for labeling do not differ in their behavior in the system, iii) the isotopic label is homogenously distributed in the source to be traced (Cobelli et al., 2007; Hamon et al., 2008). The indirect method is particularly useful when the third condition is difficult to achieve, for example when complex organic fertilizers need to be labeled.

Zn has multiple isotopes. The radionuclide ^{65}Zn has successfully been used in soil-plant systems with both methods (Aghili et al., 2014a; Diesing et al., 2008; McBeath and McLaughlin, 2013; Sinaj et al., 1999). However, ^{65}Zn is a gamma-emitter with a half-life of 244.4 days: safety issues, legal regulation limits, as well as waste management can be problematic. Thanks to technical improvements of the mass spectrometry during the last decades, stable isotope-ratio measurements have gain attractiveness to trace metals in the environment (Weiss et al., 2008; Wiederhold, 2015). A multicollector ICPMS (MC-ICPMS) achieves a precision of 0.01 RSD (relative standard deviation) which is adequate to study natural biogeochemical fractionation processes (Cloquet et al., 2008; Sturup et al., 2008). The trade-offs are high analytical costs and limited access to MC-ICPMS instruments. Source tracing experiments with enriched systems, however, do not necessarily require the high precision of MC-ICPMS. Quadrupole ICP-MS (Q-ICPMS) with a collision cell is capable of measuring Zn isotope-ratios with a precision of about 1% RSD (Cloquet et al., 2008; Sturup et al., 2008). However, the precision of mass spectrometry isotope measurements can be impeded by spectral interferences (Mason et al., 2004a) and mass discrimination effects

(Mason et al., 2004b). Spectral interferences are due to elemental isobars (species having the same mass as the measured isotope), originating from instrumental and sample matrix components (Mason et al., 2004a). These interferences can be minimized by a thorough tuning of the instrument and by separating the targeted element from the sample matrix with ion exchange chromatography techniques (Durrant et al., 1994; Mason et al., 2004a). Mass discrimination effects are due to mass-dependent fractionation within the instrument and can be corrected mathematically by using a sample-standard bracketing method (Mason et al., 2004b; Walczyk, 2001).

McBeath et al. (2013) successfully used Q-ICPMS in combination with the direct ^{67}Zn-labeling method to assess the efficiency of mineral Zn fertilizer. To our knowledge, there is no study yet showing that Q-ICPMS can also be used in combination with the indirect method and stable Zn isotope labeling. For indirect soil labeling with ^{67}Zn, it is difficult to fulfill the first condition of isotope dilution techniques mentioned above, because ^{67}Zn is naturally occurring in the system (^{67}Zn natural abundance = 4.04 %, mole fraction). The added mass of tracer added to the system has to be minimized so that the disturbance of the system can be considered negligible, while still getting enough uptake of the tracer into the plants to produce a measurable distinct effect. Hence, for the indirect method, the precision of the Q-ICPMS can potentially become an issue.

The objective of this study was to evaluate the suitability of Q-ICPMS for measuring stable Zn isotope ratios in indirect, as compared to direct, ^{67}Zn-enriched source tracing in a fertilized soil-plant system. A growth trial with Italian Ryegrass (*Lolium multiflorum*) was set up with two arable soils. Composted wheat straw was used as model organic fertilizer for the direct and for the indirect method. Shoot samples extracts from the second ryegrass regrowth were processes by ion exchange chromatography and measured on a Q-IPCMS as well as on a MC-ICPMS. The quality of the Q-ICPMS measurements were tested in two steps, by using the Bland-Altman approach, which provides the mean difference (bias) and its uncertainty between two analytical methods (Altman and Bland, 1983). First, we compared the ^{67}Zn:^{66}Zn ratios measurements of both instruments. Also, the sample-matrix effect on the spectral interferences of the Q-ICPMS was tested by measuring sample extracts which were not purified by ion exchange chromatography. In the second phase, the measured isotope ratios were used to calculate, separately for each instrument, the fertilizer contribution to the plant, in order to evaluate the impact of the analytical error on the resulting fraction of Zn in the plant derived from the fertilizer. We quantified the uncertainty of the Zn derived from the fertilizer produced by the lower precision of the Q-ICPMS, in comparison to MC-ICPMS. Finally, we evaluated the impact of this uncertainty to resolve agronomical relevant fertilizer contributions to plant uptake by connecting our results to previous Zn source tracing studies in soil-plant systems.

Material and Methods

Soils

Two arable Swiss soils with different pH and total Zn content were chosen: a slightly acidic, mollic Fluvisol (FAO classification[c]) from Heitenried, and an alkaline, calcaric Cambisol (FAO classification[c]) from Lindau (Table 1). On both sites, the soil was collected from a depth of 0-20 cm and sieved to 7 mm aggregate size. The soil batches used for the indirect method were labeled with ^{67}Zn using the following procedure: four big boxes (100 cm x 120 cm) were filled with a layer of 20 cm of soil above a 30 cm layer of foam glass drainage gravel. The two layers were separated from each other by a fleece mat. Two boxes were filled with the acidic and two with the alkaline soil. One box with each soil was then percolated for 20 weeks with ^{67}Zn-enriched nutrient solution. For the direct method, the other two boxes were treated in the same way with non-enriched solution. The nutrient solutions were obtained from another project, in which wheat was grown in ^{67}Zn-enriched and non-enriched hydroponic systems (Coralie Signorell et al., unpublished). The soil layer was regularly mixed by hand. After the labeling procedure, the four soil batches were air-dried, homogenized using a concrete mixer, sieved to 2 mm, and stored for one year, until they were used for the study presented here. The Zn isotope composition of the total soil Zn (15.5 M HNO_3 microwave digestion) was determined after the labeling.

[c]IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome

Table 1: Selected soil characteristics after the labeling procedure

Soil characteristics	Soil	
Designation	Heitenried	Strickhof
Origin	Heitenried, Switzerland	Lindau, Switzerland
FAO classification[a]	Fluvisol	Cambisol
Clay (g kg^{-1})[b]	146	202
Silt (g kg^{-1})[b]	235	344
Sand (g kg^{-1})[b]	619	454
pH_{H2O}[c]	4.9	7.7
Zn DTPA (mg Zn kg^{-1})[d]	4.1	5.2
Zn total (mg Zn kg^{-1})[e]	54.1	101.1
^{67}Zn-total (%, mol/mol)[f]	4.98	4.47
WHC_{max} (g H_2O kg^{-1})[h]	386.9	446.8

[a]IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.
[b]Gravimetric measurement
[c]pH in H_2O with 1:2.5 solid:liquid ratio
[d]DTPA extractable Zn (Lindsay and Norvell, 1978)
[e]Energy dispersive X-ray fluorescence spectrometry (EDXRF)
[f]HNO_3 microwave digested Zn fraction of the soil
[h]Soil saturation with H_2O without external pressure

Organic fertilizer

The ^{67}Zn-labeled and non-labeled wheat straws used as organic fertilizers in the present study were obtained from the hydroponic experiment from which the nutrient solutions were obtained. Four kilogram of each straw type was hashed and composted in 20 L incubators at 40 °C during four month. In order to accelerate the microbial decomposition of the straw, ammonium nitrate and water (EasyPure^{TM}) was regularly added. After incubation, the compost was dried at 65 °C and finely ground. The resulting powder had a C:N ratio of approximately 5.2.

Growth trial

Ryegrass was grown in pots containing 400 g dry soil moistened to 40 % of the water holding capacity (WHC). For direct source tracing, non-labeled soil was mixed with ^{67}Zn-labeled wheat straw compost, whereas for indirect source tracing ^{67}Zn-labeled soil was mixed with non-labeled wheat straw compost. The compost was added at two doses (Table 2). Reference treatments with labeled and unlabeled soils but without fertilization were established. The function of the latter, was to provide the isotope composition of the plant available Zn pool of the soil. Furthermore, we included a treatment with a ^{66}Zn soil labeling in order to widen the range of ^{67}Zn:^{66}Zn ratios to be measured with the ICPMS. All treatments were prepared in four replicates. The plants were grown in a climate chamber with a daily photoperiod of 14 hours at 25 klx. Temperature and relative humidity (RH) were set to 24 °C and 60 % during daytime and to 18 °C and 65 % RH during night time, respectively.

Table 2: Experimental parameters

Method	**Treatment name**	**Zn fertilizer type**	**Zn fertilizer content**	**Application rate**	**Zn input**
			[mg kg^{-1}]	[g kg^{-1} soil]	[mg kg^{-1} soil]
Direct	Reference	-	-	-	-
	High direct	Compost	32.4	33.0	1.069
	Low direct	Compost	32.4	12.5	0.405
	^{66}Zn-spiked	$^{66}ZnSO_4$	$4.4*10^5$	$4.49*10^{-7}$	0.102
Indirect	Reference		-	-	-
	Low indirect	Compost	64	12.5	0.800
	High indirect	Compost	64	33.0	2.112

Each pot was sown with 0.5 g of Italian Ryegrass (*Lolium multiflorum*, var. Gemini). Nutrient solution was applied 14 DAS (days after sowing) at a rate of 460 mg N kg^{-1} (ammonium nitrate), 57 mg P kg^{-1}, 144 mg K kg^{-1}, 32 mg S kg^{-1}, 50 mg Mg kg^{-1}, 6 mg Fe kg^{-1} soil, 232 μg B kg^{-1}, 127 μg Mn kg^{-1}, 31 μg Cu kg^{-1} and 54 μg Mo kg^{-1} soil. For the compost amended treatments N addition was reduced to 321 mg N kg^{-1} soil for the treatments “low direct” and “low indirect” and to 92 mg N kg^{-1} soil for “high direct” and high indirect” in order to supply the same total amount of N. The soils were watered to maintain a WHC between 40 % and 80 %. The shoots were harvested at 21 DAS and 30 DAS, but only the samples of the second cut were used for analysis.

Sample processing and ion exchange chromatography

Composted wheat straw samples collected just before preparing the soil-compost mixtures and the ryegrass shoot samples collected at harvest were dried for 48 hours at 65 °C in bags made of pure cellulose (pergamin) and milled in tungsten bowls. Subsamples of 200 mg dry material were suspended in 2 ml H_2O (EasyPure^{TM}) and 2 ml HNO_3 (Rotipuran® Supra 15.5 M, Zn < 0.5 µg*L^{-1}) in single-use glass tubes and digested in a high-pressure single-reaction microwave chamber (turboWave, MWS microwave system). An aliquot of the extract was used for total Zn analysis by means of inductively coupled plasma atomic emission spectrometry (ICP-OES). The rest of each extract was transferred into PTFE containers, evaporated to dryness and dissolved in 6M HCl (Rotipuran®Supra 11.6 M, Zn < 1 µg*L^{-1}). The Zn was separated from the matrix by ion exchange chromatography following a slightly modified protocol from Pinna et al. (2001). Spectra/Chrom® Minicolumns PP, 7.5ml with 45 µm filter were filled with 2.3 cm of anion exchange resin (AG® 1-X8, 100-200 mesh, chloride form, Bio-Rad laboratories). The resin was cleaned by adding successively 3 x 5 ml HNO_3 2 M, 2ml H_2O and 5 ml of HCl 0.5 M. Then the column was conditioned with 2 x 5 ml HCl 6 M and the samples were loaded onto the resin. Next, the matrix cations (except Cu, Fe and Zn) were washed out with 2 x 5 ml HCl 6M. Cu and Fe were eluted with 5 ml HCl 2.5 M and 3 x 5 ml HCl 0.5 M respectively. In the last step, the Zn was eluted into PTFE containers with 2 x 5 ml HCl 0.005 M. The resulting elution was evaporated to dryness before being dissolved in HNO_3 15.5 M and evaporated twice. Finally, the dry residues were dissolved with 2 ml HNO_3 0.05 M and stored in 2 ml Eppendorf tubes. In order to quantify the additional benefit of the ion exchange chromatography on the spectral interferences the ^{67}Zn:^{66}Zn ratio of the plant extracts were measured on the Q-ICPMS before and after purification.

Sample analysis

All purified extracts were analyzed for Zn isotope composition by means of both Q-ICPMS and MC-ICPMS. For Q-ICPMS we used an Agilent 7500ce with a helium supplied octopole reaction system. Each sample was measured with 12 replicates. For each mass (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn) a three-point peak pattern was selected with an integration time of 0.1 seconds per point. Mass bias correction was performed by standard-sample-standard bracketing with a conventional zinc sulfate heptahydrate solution ($ZnSO_4$•$7H_2O$, Sigma Aldrich) and applying a power law mass fractionation correction (Peel et al., 2008). The sample were diluted to obtain a concentration of approximately 200 µg*L^{-1} in accordance with the $ZnSO_4$-standard. The average of the relative standard deviation (RSD) was <1.3 % for all isotope ratios (^{67}Zn:^{xx}Zn) except ^{67}Zn:^{70}Zn which reached an average RSD of 2.7 %. The MC-ICPMS was a double focusing high resolution Thermo-Finnigan Neptune (Bremen, Germany), equipped with an

Apex desolvation unit (Elemental Scientific, Omaha, USA). Zinc isotopic ratios were analyzed in 3 blocks of 20 measurements for each sample. To correct for instrumental mass bias of the MC-ICPMS, the standard-sample-standard bracketing technique was applied, using a commercial Zn standard solution (Titrisol, Merck Chemicals), where one block of 20 measurements of standard Zn was measured before and after each block of sample. Zn concentrations in sample and standard were matched (approximately 1 $mg*L^{-1}$). Isobaric interference of ^{64}Ni on ^{64}Zn was corrected by simultaneous monitoring of ^{62}Ni. No other ratio normalizations were realized on this data. Regarding the analytical error, the RSD was < 0.05% for all isotopic ratios. The correction factor of the standard-sample-standard bracketing was calculated with the natural Zn isotope abundances (mole fraction in %) reported by the International Union of Pure and Applied Chemistry (IUPAC): 49.17 % for ^{64}Zn, 27.73 % for ^{66}Zn, 4.04 % for ^{67}Zn, 18.45% for ^{68}Zn and 0.61 % for ^{70}Zn (Berglund and Wieser, 2011).

Source tracing calculation

In the present study, the source tracing system was based on two Zn sources, the soil respectively the fertilizer, and a sink which represents the Zn mixture of both sources present in the ryegrass shoots of the Zn-fertilized treatments (Figure 1). The terms of the mass balance formula are composed of the ^{67}Zn:^{66}Zn molar ratio of the sink and the ^{67}Zn and ^{66}Zn abundances (mole fraction in %) of the two sources (Eq. 6). Consequently, all stable Zn isotope ratios (^{67}Zn:^{64}Zn, ^{67}Zn:^{66}Zn, ^{67}Zn:^{68}Zn, ^{67}Zn:^{70}Zn) of the sources needed to be measured in order to calculate the isotope abundances (Figure 1, Eq 6.1 and 6.2). The ryegrass shoots of reference treatments provided the mean isotope composition of the plant available pool of the soil (Figure 1, soil source). The mean isotope composition of the fertilizer (Figure 1, fertilizer source) was obtained from the measurement of four independently processed fertilizer subsamples.

Mass balance calculations

The fraction of plant Zn derived from the fertilizer ($Zndf_{fertilizer\%}$) was determined by mass balance calculations as described by Mc Beath and McLaughlin (2013). For the purpose of this study, the terms (Table 3) have been adapted as follows:

Table 3: Terminology of the mass balance derivation described in Eq. 1-6.

Terms	Definition	Units
$Zn_{sink} = 1$	Total Zn plant shoot uptake of the Zn fertilized treatments derived from the fertilizer and the soil (equals 1)	(mole mole^{-1})
$Zndf_{fert}$	Fraction of Zn derived from the fertilizer	(mole mole^{-1})
$Zndf_{soil}$	Fraction of Zn derived from the soil	(mole mole^{-1})
$^{66}Zn_{fert}$	^{66}Zn abundance of the fertilizer source	(mole mole^{-1})
$^{67}Zn_{fert}$	^{67}Zn abundance of the fertilizer source	(mole mole^{-1})
$^{66}Zn_{soil}$	^{66}Zn abundance of the soil source measured in the plant shoot of the reference treatments	(mole mole^{-1})
$^{67}Zn_{soil}$	^{67}Zn abundance of the soil source measured in the plant shoot of the reference treatments	(mole mole^{-1})
$\left(\frac{^{67}Zn}{^{66}Zn}\right)_{sink}$	^{67}Zn:^{66}Zn isotope ratio of the plant shoot grown of the fertilized treatments	(mole mole^{-1})
$Zndf_{fertilizer\%}$	Percentage of Zn in the plant shoot derived from the fertilizer	(%)

The Zn in the plant shoots from the Zn fertilized treatments (sink) is composed of Zn derived from the fertilizer and from the soil:

$$Zn_{sink} = Zndf_{fert} + Zndf_{soil} = 1 \qquad Eq.\,(1)$$

The mass balance can be decomposed with regard to ^{66}Zn and ^{67}Zn:

$$^{66}Zn_{sink} = Zndf_{fert} * {}^{66}Zn_{fert} + Zndf_{soil}\,{}^{66}Zn_{soil} \qquad Eq.\,(2)$$

$$^{67}Zn_{sink} = Zndf_{fert} * {}^{67}Zn_{fert} + Zndf_{soil}\,{}^{67}Zn_{soil} \qquad Eq.\,(3)$$

The isotope ratio in the sink is obtained by dividing Eq. 3 by Eq. 2:

$$\left(\frac{^{67}Zn}{^{66}Zn}\right)_{sink} = \frac{Zndf_{fert} * {}^{67}Zn_{fert} + Zndf_{soil}\,{}^{67}Zn_{soil}}{Zndf_{fert} * {}^{66}Zn_{fert} + Zndf_{soil}\,{}^{66}Zn_{soil}} \qquad Eq.\,(4)$$

Using Eq. 1, $Zndf_{soil}$ is substituted by ($Zn_{sink} - Zndf_{fert}$):

$$\left(\frac{^{67}Zn}{^{66}Zn}\right)_{sink} = \frac{Zndf_{fert} * {}^{67}Zn_{fert} + (Zn_{sink} - Zndf_{fert})\,{}^{67}Zn_{soil}}{Zndf_{fert} * {}^{66}Zn_{fert} + (Zn_{sink} - Zndf_{fert})\,{}^{66}Zn_{soil}} \qquad Eq.\,(5)$$

As Zn_{sink} represent the total Zn in the plant shoots (Table 3) it can be replaced by 1. By solving the equation for $Zndf_{fert}$ we obtain the Zn fraction derived from the fertilizer:

$$Zndf_{fertilizer\%} = \left(\frac{{}^{66}Zn_{soil} * \left(\frac{^{67}Zn}{^{66}Zn}\right)_{sink} - {}^{67}Zn_{soil}}{\left({}^{67}Zn_{fert} - {}^{67}Zn_{soil}\right) - \left(\frac{^{67}Zn}{^{66}Zn}\right)_{sink} * \left({}^{66}Zn_{fert} - {}^{66}Zn_{soil}\right)}\right) * 100 \qquad Eq.\,(6)$$

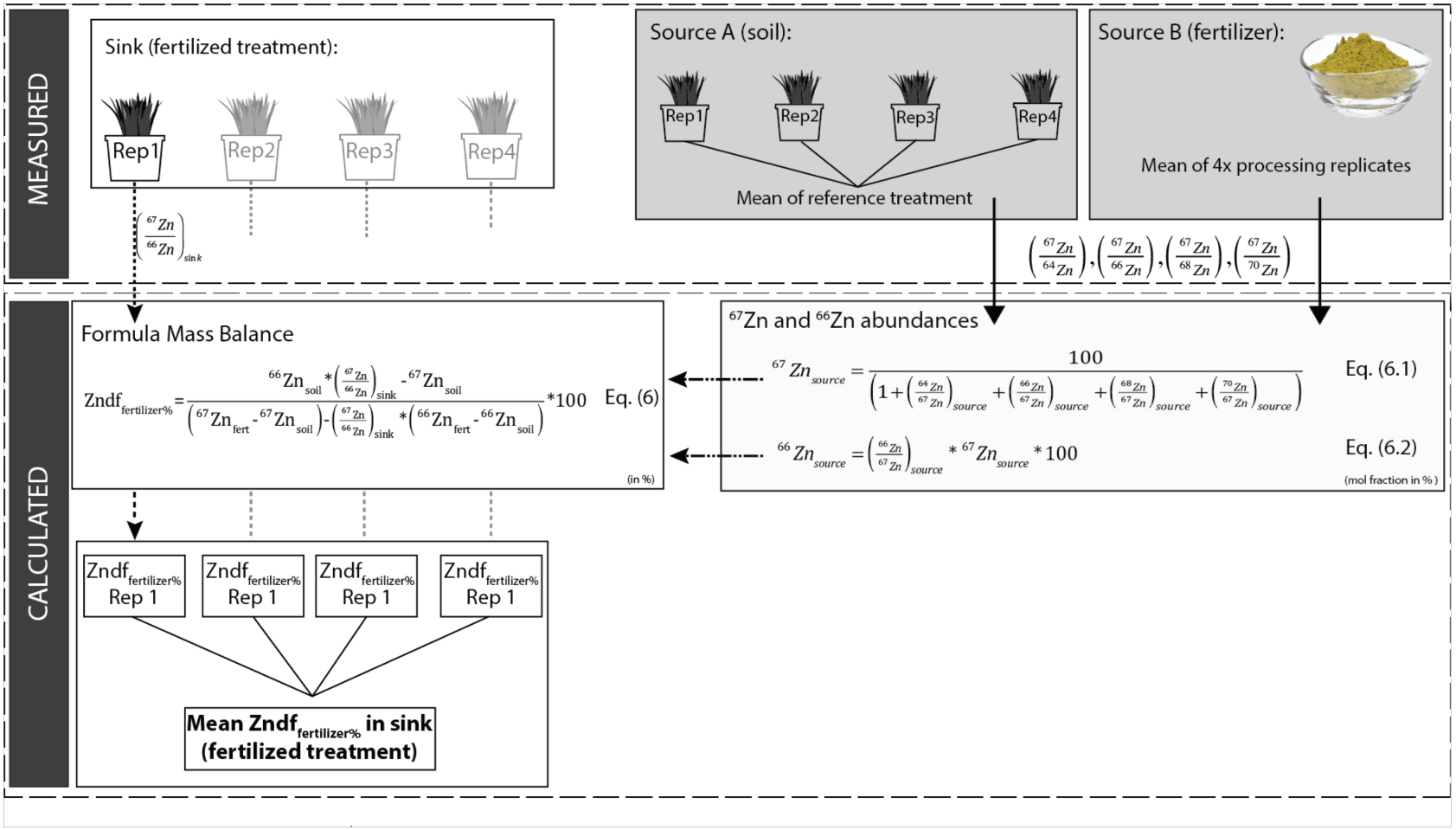

Figure 1: Source tracing calculation system showing how the measured isotope ratios of the sink (fertilized treatment) and of the two sources (soil and fertilizer) were incorporated into the mass balance formula (Eq. 6). For the two sources, all stable Zn isotope ratios were measured to calculate their respective ^{67}Zn and ^{66}Zn abundances (Eq. 6.1 and 6.2) required in the mass balance formula. The isotope composition of the soil source (plant available Zn pool of the soil), was given by the ryegrass shoots from the reference treatment. The isotope composition of the fertilizer source was given by the mean of four processing replicates. The averaged ^{67}Zn and ^{66}Zn abundances of the soil, respectively fertilizer sources were averaged and used to calculate the $Zndf_{fertilizer\%}$ of each individual treatment replicate (Rep 1-4) of the sink (fertilized treatment).

Statistics

To compare the performance of the two analytical methods, the Bland-Altman plots with a 95% confidence interval (±2sd) were computed using the software package R (Version 3.3.2) and the package "BlandAltmanLeh". This method allows to quantify the mean difference (bias) and its uncertainty (95% confidence interval) between two analytical methods (Altman and Bland, 1983; Giavarina, 2015). Data distribution was checked for normality using Bland-Altman plots in combination with the Shapiro-Wilk test. The ^{67}Zn abundance of the soil and fertilizer sources obtained with the Q-ICPMS and the MC-ICPMS of the soil sources were compared with a pairwise t-test and a Bonferroni p-value adjustment. For the analysis, we considered the variability of two different types of replicates: i) processing replicates, which originated from the same sample, being repeatedly processed and measured (e.g the fertilizer) and ii) treatment replicates, originating from four independent pots of the growth trial.

Results

^{67}Zn abundances of the plant available soil- and fertilizer sources

The ^{67}Zn abundance of the soil and fertilizer sources are shown in Table 4. These results did not reveal any significant differences between the analyzes performed on the Q-ICPMS and the MC-ICPMS. However, the confidence interval (±2 sd) of the unlabeled sources was smaller when measured with the MC-ICPMS than with the Q-ICPMS. For both instruments, the confidence interval (±2sd) was higher in the ^{67}Zn-labeled samples. The ^{67}Zn abundances of the unlabeled soil sources were close to the ^{67}Zn natural abundance of 4.04 % (mole fraction) reported by IUPAC (Berglund and Wieser, 2011), whereas the abundance of the fertilizer source was slightly higher (4.12 % ^{67}Zn).

Table 4: Mean and 95% confidence interval (±2sd) of the ^{67}Zn abundances of the soil and fertilizer sources used for the growth trial. The soil values resulted from the isotope composition of the ryegrass shoot of the reference treatments (without fertilization). The standard deviation of the soil sources originates from four treatment replicates, the one of the fertilizer sources from four processing replicates. Letters indicate the significant difference between the two Q-ICPMS and MC-ICPMS measurement technique (pairwise t-test, p-value adjustment method: bonferroni).

Zn-Source	Origin	Labeling	Q-ICPMS			MC-ICPMS		
			^{67}Zn (mole fraction, %)	±	2sd	^{67}Zn (mole fraction, %)	±	2sd
Soil	Heitenried	unlabeled	4.068 [a]	±	0.028	4.082 [a]	±	0.002
		^{67}Zn-labeled	8.108 [a]	±	0.086	8.138 [a]	±	0.064
	Strickhof	unlabeled	4.048 [a]	±	0.032	4.072 [a]	±	0.004
		^{67}Zn-labeled	5.909 [a]	±	0.071	5.937 [a]	±	0.054
Fertilizer	Compost	^{67}Zn-labeled	31.076 [a]	±	0.203	31.442 [a]	±	0.169
		unlabeled	4.121 [a]	±	0.022	4.113 [a]	±	0.007

^{67}Zn:^{66}Zn ratios of the sinks

Table 5 shows the mean ^{67}Zn:^{66}Zn ratios of the fertilized treatments (sink) and the corresponding relative standard deviation (RSD) representing variability of the treatment replicates. The results revealed that the variability within the treatment replicates was identical when measured with the Q- than with the MC-ICPMS. The RSD ranged from 0.31 % to 1.26 % with the Q-ICPMS and from 0.28 % to 1.24 % with the MC-ICPMS.

Table 5: Mean ^{67}Zn:^{66}Zn ratios of the sinks measured on the Q- and on the MC-ICPMS. The relative standard deviation (RSD) indicates the variability of the treatment replicates.

Soil	Treatment	Q-ICPMS		MC-ICPMS	
		^{67}Zn:^{66}Zn ratio mole mole^{-1}	RSD (%)	^{67}Zn:^{66}Zn ratio mole mole^{-1}	RSD (%)
Heitenried	High direct	0.3171	0.36	0.3175	0.39
	Low direct	0.2184	1.08	0.2189	1.05
	Low indirect	0.2873	0.62	0.2877	0.59
	High indirect	0.2626	0.85	0.2626	0.79
Strickhof	High direct	0.2219	0.48	0.2220	0.54
	Low direct	0.1764	1.26	0.1762	1.24
	Low indirect	0.2115	0.33	0.2119	0.34
	High indirect	0.2057	0.31	0.2061	0.28

Source tracing system

The ^{67}Zn:^{66}Zn ratios of the sink and of the soil, respectively the fertilizer sources are graphically represented in Figure 2 for each treatment. The ^{67}Zn:^{66}Zn ratio of the sink is always situated in between the ratios of the two source. Figure 2 provides a visual indication of the relative amount of Zn in the sink derived from the soil and from the fertilizer: the smaller the distance between the sink and a source, the higher the contribution of the latter. In all treatments, the soil source always provided the higher Zn fraction to the sink. For all indirect treatments, the ^{67}Zn:^{66}Zn ratio of the sink could always be statistically distinguished from ratio of the soil source. No significant differences were observed between the ^{67}Zn:^{66}Zn ratios measured on the Q-ICPMS and the MC-ICPMS.

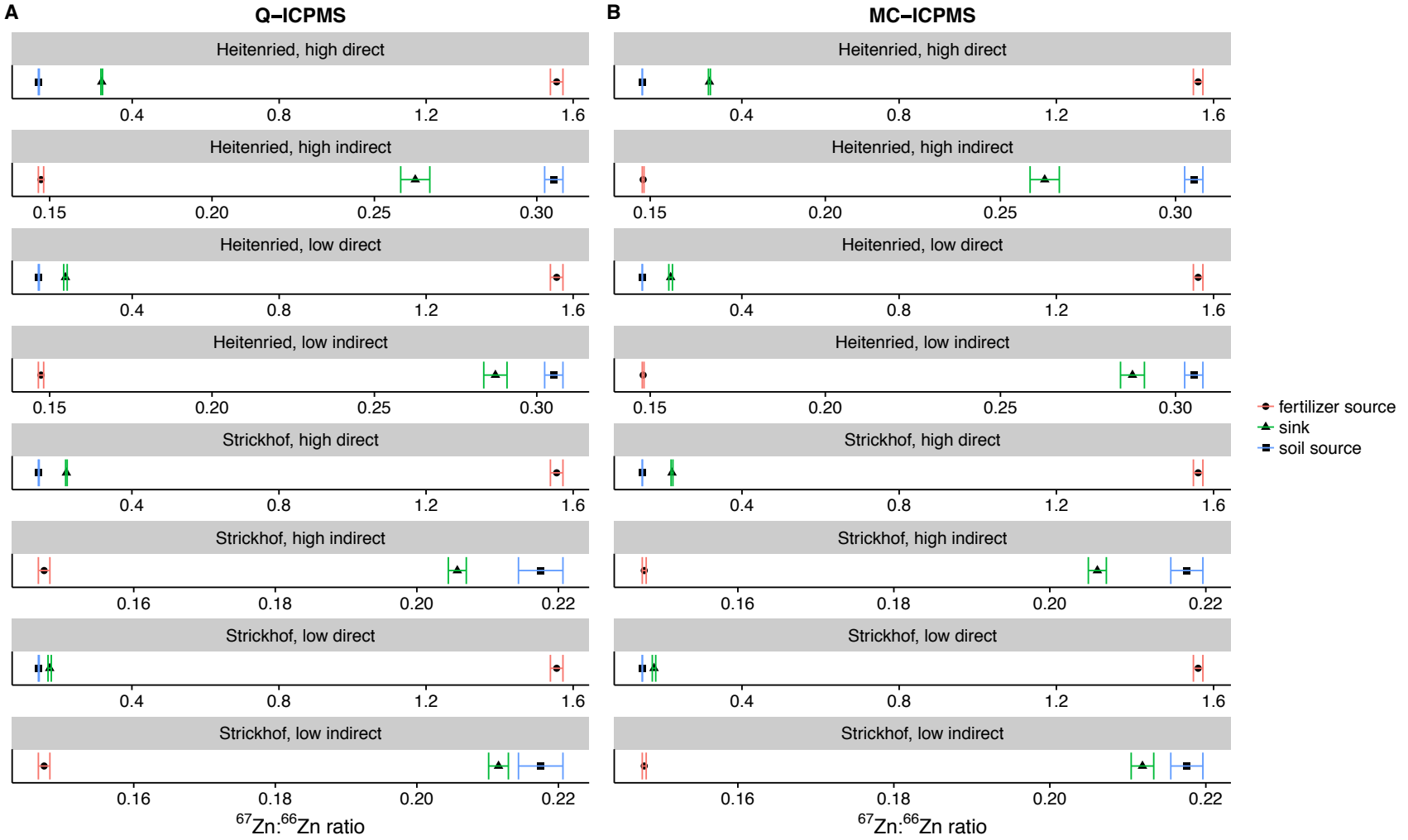

Figure 2: Mean $^{67}Zn:^{66}Zn$-ratios of the plant available soil source (■), the fertilizer source (●) and the sink (▲) measured with the Q-ICPMS (A) and with the MC-ICPMS (B). The $^{67}Zn:^{66}Zn$-ratios from the sink and the soil sources represent the mean (±2sd) from four treatment replicates, whereas for the $^{67}Zn:^{66}Zn$-ratios from the fertilizer sources indicates the mean (±2sd) from four processing replicates.

^{67}Zn:^{66}Zn ratio-comparison between Q- and MC-ICPMS

In total, 57 samples from the Zn fertilized- and reference treatments were analyzed on both instruments. For each individual sample the ^{67}Zn:^{66}Zn ratios obtained with the two analytical methods were averaged and plotted against their differences in the Bland-Altman plot (Figure 3). The plot shows that the ^{67}Zn:^{66}Zn ratios measured with the Q-ICPMS were in average 0.00013 units lower than the values obtained with MC-ICPMS. The uncertainty given by the 95 % confidence interval of the mean difference (bias), was ±0.0013 units.

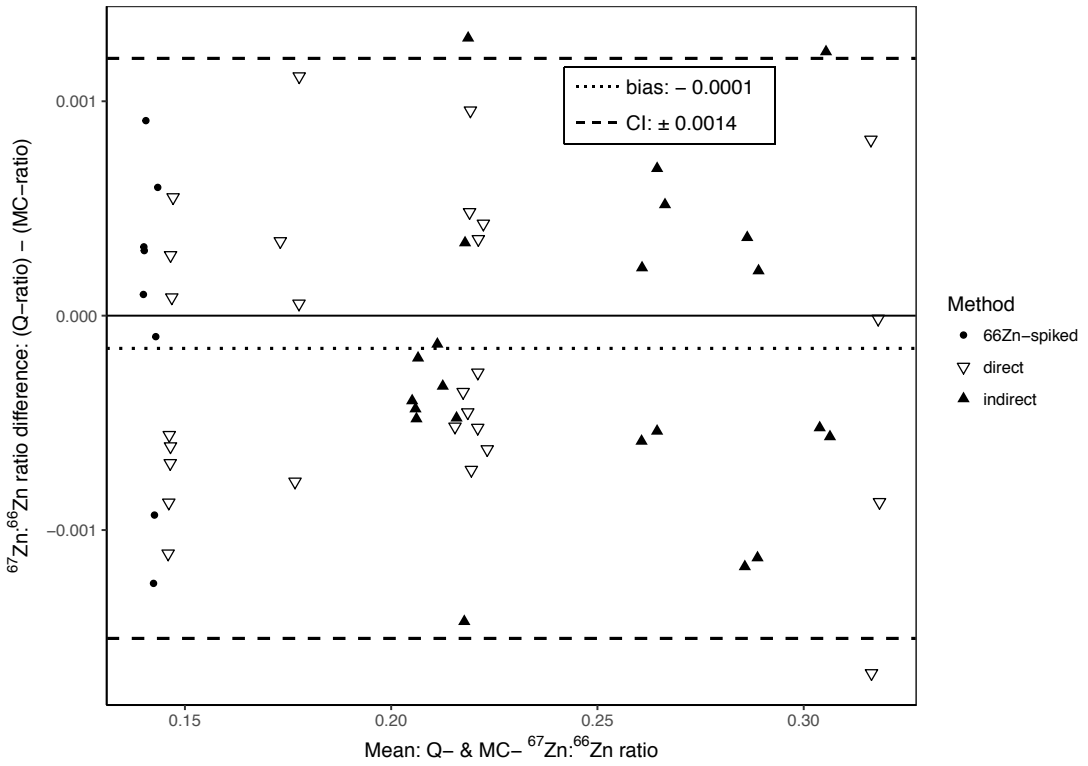

Figure 3: Bland-Altman plot comparing the ^{67}Zn:^{66}Zn ratios measured on the Q-ICPMS and the MC-ICPMS. 57 data points are compared in this plot. The results of each sample measured both instruments were averaged (x-axis) and plotted against their difference (y-axis). Empty triangles (▽) represent the direct treatments, full triangles (▲) the indirect treatments and the dots (•), the ^{66}Zn-enriched treatments. The dotted line represents the bias (-0.00014) and the dashed lines the 95% confidence interval of the bias.

Ion exchange chromatography

The effect of the ion exchange chromatography (IEC) on the ^{67}Zn:^{66}Zn ratios is represented by the Bland-Altman plot in Figure 4. The Zn from the extracts was either separated or not from the sample matrix by resin ion exchange chromatography. The purified and unpurified samples were measured on the Q-ICPMS. In average, the ^{67}Zn:^{66}Zn ratios from the unpurified samples positively deviated from the purified ones. The bias (+ 0.011 units) and its 95% confidence interval (± 0.018 units) was tenfold higher than for the comparison between the Q-ICPMS and the MC-ICPMS (Figure 3). Systematic trends could be identified regarding the response to IEC: all treatments (e.g. the reference and 66Zn spiked treatments) which were not fertilized with compost (-compost) presented a bias above the average bias (> 0.011). Among this group, the samples from the Heitenried (H) soil revealed a stronger bias than those from Strickhof (S).

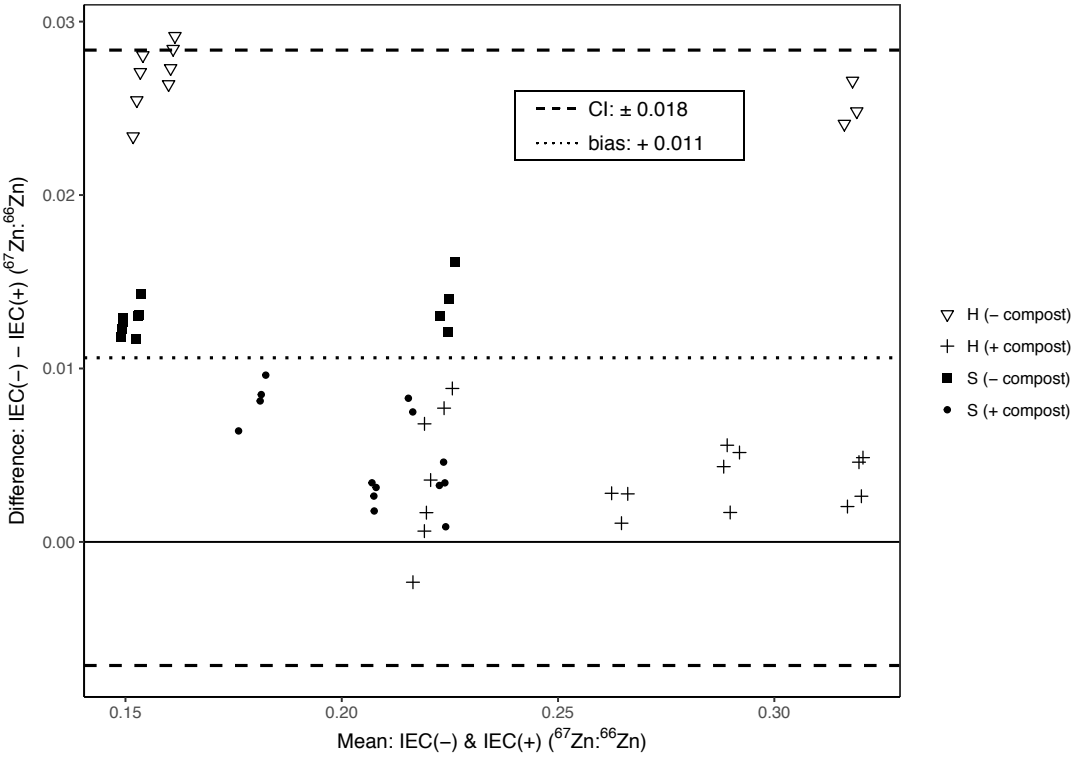

Figure 4: Bland-Altman plot comparing the ^{67}Zn:^{66}Zn ratios from purified and unpurified samples measured on the Q-ICPMS. The purified samples were processed by resin ion exchange chromatography to separate the Zn from the sample matrix. "H" stands for the samples from the Heitenried soil, "S" for the sample from the Strickhof soil. The dotted line represents the bias (+0.011) and the dashed lines the 95 % confidence interval of the bias (±0.018).

Source contribution

The Bland-Altman plot in Figure 5 compares the percentage of Zn in the sink derived from the fertilizer ($Zndf_{fertilizer\%}$, Table 3), calculated separately with the isotope data measured on the Q-ICPMS and on the MC-ICPMS. The mean difference (bias) between the two instruments accounted for +0.16% with an uncertainty of ± 0.49 % (95 % confindence interval). Regarding the distribution of the data points within the confidence interval, it is obvious that the $Zndf_{fertilizer\%}$ results from the indirect method spread wider around the bias, compared to the direct method.

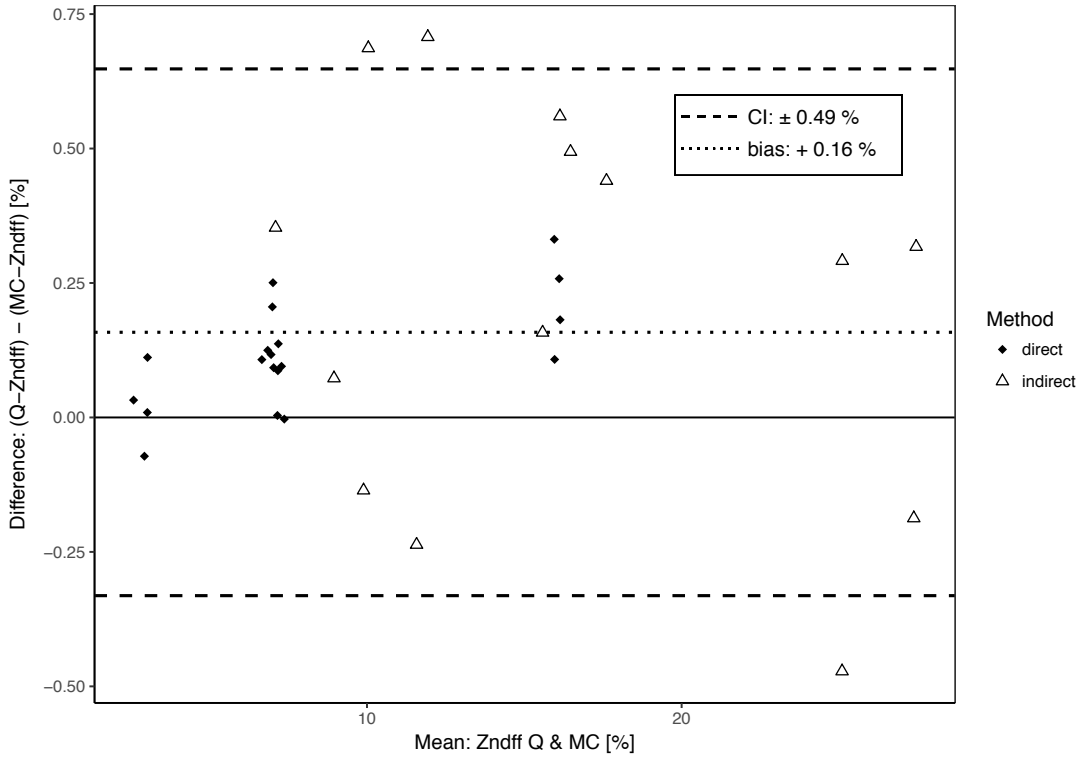

Figure 5: Bland-Altman plot for fraction of Zn derived from the fertilizer ($Zndf_{fertilizer\%}$). 35 data points are compared in this plot: diamonds (◆) stand for the direct method, empty triangles (△) for the indirect. The averaged $Zndf_{fertilizer\%}$ (x-axis) resulting from the ratios measured on the Q-ICPMS and MC-ICPMS is plotted against the differences (y-axis) obtained with these two instruments. The dotted line indicates the bias (+ 0.16) and the dashed lines the 95% confidence interval.

Discussion

Sources of error

The precision of the ^{67}Zn:^{66}Zn ratio measurements was in the range of typical relative standard deviations (RSD) values for both analytical methods: approximately 1% for Q-ICPMS and 0.01% for MC-ICPMS (Sturup et al., 2008). Compared to Q-ICPMS, the advantage of MC-ICPMS in terms of precision was still noticeable when analyzing samples at natural abundance (Table 4). However, with increasing ^{67}Zn enrichment, the variability among treatment replicates increased (Table 4 and 5). The RSD of the treatment replicates (Table 5) were between one and two orders of magnitude higher the analytical precision of the MC-ICPMS, and close or even identical to the precision of the Q-ICPMS. These results indicated that the experimental variability was larger than the analytical precision of the MC-ICPMS. The variability within the ^{67}Zn-enriched treatments might have been caused by contamination with Zn at natural abundance. Potential sources of contamination are mainly dust and Zn-impurities in water and acids used for the sample processing. The dilution effect of natural abundance Zn added to a sample increases with the enrichment. For instance, considering two ^{67}Zn enriched samples containing both 99 µg Zn and having a ^{67}Zn abundance of 8.11 % and 90.59 %. If we add 1 µg Zn at natural abundance (^{67}Zn 4.04 %) to these samples, the mixture results in a ^{67}Zn abundance of 8.07 % and 89.70 % respectively. Here, the same amount of Zn induced a change of -0.04 % in the first and -0.89 % in the second sample. Contaminations in this order of magnitude could have occurred during the growth trial and the sample processing. This hypothesis is furthermore supported by the amount of Zn measured in the processing blanks (data not shown) which contained about 2 µg Zn L^{-1} whereas the plant extracts were measured at the order of magnitude of 200 µg Zn L^{-1}. As the Zn content of the processing blanks was varying and generally below the limit of quantification, their results were not considered for the data correction.

As the matter of fact, the variability among treatment replicates enriched with ^{67}Zn outweighed the analytical precision of the MC-ICPMS. The impact of the error of the ^{67}Zn:^{66}Zn ratios from the two sources and the sink accounted for an uncertainty of the calculated $Zndf_{fertilizer\%}$ of ±0.49 as illustrated by the Bland-Altman approach in Figure 5. This resolution might be sufficient to investigate source contributions, which are relevant for agronomic purposes. By grouping the data from three similar Zn source tracing studies (Aghili et al., 2014a; McBeath et al., 2013; Nanzer, 2012) covering several soil and fertilizer types, over 95 % of the obtained $Zndf_{fertilizer\%}$ values could be resolved with an uncertainty of ± 0.49 % (Figure 6).

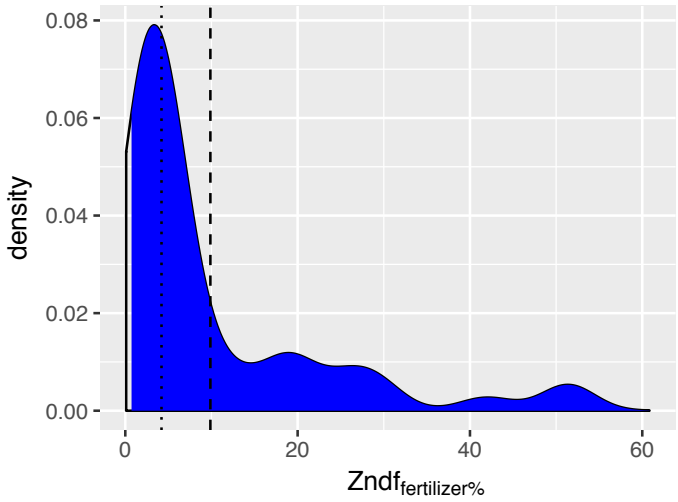

Figure 6: Density curve regrouping the Zn derived from fertilizer found in similar source tracing studies (Aghili et al., 2014a; McBeath et al., 2013; Nanzer, 2012). The curve area starts at the 5% quantile ($Zndf_{fertilizer\%} > 0.72$), the dotted line represents the median (4.2) and the dashed line the mean (9.87).

Direct versus indirect method

As mentioned in the previous section, the higher ^{67}Zn:^{66}Zn ratios of a sample, the higher the impact of Zn contamination. Therefore, this type of error mainly concerns the direct method, as frequently the fertilizer source is strongly enriched. However, the weight of the uncertainty in the mass balance calculation is inversely proportional to the isotope enrichment difference between the ^{67}Zn enriched and the natural abundance sources. In other words, the same errors occurring in a highly-enriched source tracing system have less impact on the calculated $Zndf_{fertilizer\%}$ values than in weakly-enriched systems. Therefore, the indirect method is more affected by the instrumental precision, as the soil can only be spiked with a limited amount of ^{67}Zn. This effect could clearly be observed in Figure 5, where the samples from the indirect method spread wider compared to the direct method. However, the analytical precision of the Q-ICPMS was sufficient to resolve the fertilizer contribution ($Zndf_{fertilizer\%}$) of the indirect method within an acceptable range of +0.49 %.

Ion exchange chromatography

Error minimization is a major task when working with stable isotopes. With inductive coupled plasma mass spectrometry, special attention has to be paid to spectral interferences. Monoisotopic and molecular (argides, oxides, double-charged and elemental) species can significantly bias the isotope measurements (Mason et al., 2004a). Thoroughly tuning of the

instrument, collisions cells and standardization can partially eliminate this type of error. However, our results showed that sample matrix separation by ion exchange chromatography remains an essential step to accurately measure ^{67}Zn:^{66}Zn ratios with a Q-ICPMS (Figure 4). The bias between purified and unpurified samples was by far too high to use the results for the source quantification. Furthermore, the Bland-Altman approach used in Figure 4 pointed out treatment specific clusters, which highlight the relevance of the matrix effect on the mass bias. As a complete elemental analysis was not performed for theses samples, it was not possible to link the different clusters to specific elements, which could have induced the interferences.

Conclusions

The precision of the Q-ICPMS, when compared to MC-ICPMS allowed us to determine the percentage of Zn derived from the fertilizer in the plant with an uncertainty of ±0.49 %. From an agronomic point of view this precision is high enough to resolve most relevant source contributions, when compared to previous studies. The resolution of the Q-ICPMS was also sufficient to perform the source tracing with the indirect method, even though this method is more responsive to analytical errors. The variability among the ^{67}Zn-enriched treatment replicates predominated the analytical error of the Q-ICPMS. The magnitude of this variability outweighed the analytical precision of the MC-ICPMS. An important source of error was probably due to Zn contamination, which occurred during the sample processing. Even though it is technically possible to reduce the risk of contamination, the costs would be unproportioned to the benefit. Spectral interferences of the Q-ICPMS measurements can easily be overcome by ion exchange sample processing.

II. THE PLANT ZINC AVAILABILITY OF ORGANIC FERTILIZERS AS ASSESSED BY INDIRECT ^{67}ZN LABELING.

Abstract

Organic fertilizer application can impact the availability of Zn for plants as i) they can add significant amounts of Zn to soils and ii) they can modify soil physicochemical and biological properties controlling soil Zn availability. In the present study, we determined the fractions of Zn taken up by ryegrass derived from organic fertilizers, soil and seeds using soil that had been labeled with ^{67}Zn. This method, also called indirect labeling is particularly useful to trace elements with complex fertilizers, which are difficult to label homogenously. As soil pH is a major factor affecting Zn plant availability, a pot experiment was conducted with one acidic (Heitenried) and one alkaline (Strickhof) ^{67}Zn-labeled soil. We selected the following fertilizers: cattle manure, poultry manure, dried sewage sludge (DSS) and water soluble Zn ($ZnSO_4$). The fertilization rate was 1.5 mg Zn kg^{-1} soil. The model plant, Italian ryegrass (*Lolium multiflorum*), was harvested four times. Zinc uptake was significantly lower on the alkaline soil, but it did not affect biomass production. The soil effect on Zn uptake was mainly explained by soil pH. The average fraction of Zn in ryegrass shoots derived from the fertilizers ranged, without correction for seed derived Zn, from 12.2 % in the alkaline soil to 20.4 % in the acidic soil. The amount of Zn derived from the organic fertilizers and from $ZnSO_4$ did not differ significantly. In the alkaline soil, $ZnSO_4$ addition decreased the amount of Zn derived from the soil, compared to a reference treatment that did not receive any Zn fertilization. Organic carbon added with the organic fertilizers might have led to more production of Zn-organic complexes in the alkaline soil hindering its sorption on soil surfaces. The recovery of Zn (RecZn) derived from the fertilizers measured with the isotopic approach was compared to the apparent use efficiency (AUE) which is the difference in Zn uptake between a fertilized and a non-fertilized treatment divided by the amount of applied Zn. The AUE was in most cases lower than the RecZn, and in one case higher. This discrepancy between AUE and ZnRec was attributed to the fact that AUE did not consider that fertilizer input leads to simultaneous changes in Zn derived from the soil and the fertilizers. The isotope technique also provided a qualitative evaluation of the Zn derived from the ryegrass seeds, which was significant in the first cut.

Introduction

Livestock manure, sewage sludge, composts and crop residues are frequently used as organic fertilizers and soil amendments in agricultural systems. These inputs can improve soil physicochemical properties and nutrient contents (Westerman and Bicudo, 2005). In developing countries, these inputs sometimes represent the only available resource to preserve soil fertility (Douxchamps et al., 2011). The application of organic fertilizers on agricultural soils does also affect the cycle of micronutrients such as Zn (Bolan et al., 2004). Farmyard manures, composts and sewage sludges can contain up to several grams of Zn per kilogram dry matter and their application can lead to significant Zn accumulation in topsoil layers (Gubler et al., 2015; Nicholson et al., 1999). Organic fertilizers can also impact soil physicochemical and biological properties leading to an increased Zn plant availability (Aghili et al., 2014a; Habiby et al., 2014). The aim of the present work was to study the transfer of Zn added as organic inputs, to the shoots of ryegrass grown in two soils with a stable Zn isotope dilution technique.

Two approaches can be applied to evaluate the fate of Zn derived from fertilizers in the soil-plant system. The first is the apparent use efficiency (AUE) (Muñoz et al., 2004). The AUE indicates the additional plant nutrient uptake in a fertilized treatment divided by the added amount of nutrient when compared to an unfertilized control. This method is based on the assumptions that the fertilizer does neither affect biotic or abiotic soil processes, nor plant physiology, and that the amount of Zn derived from the soil remains constant (Schindler and Knighton, 1999). However, organic fertilizers can alter the amount of nutrients derived from the soil, as these inputs change the carbon dynamics in the soil and affect plant growth (Sinaj et al., 2002). For phosphorus and nitrogen, it has been shown that the AUE often over- or underestimates the fertilizer recovery (Jenkinson et al., 1985; Oberson et al., 2010). Aghili et al (2014) showed that the addition of green manure labeled with radioactive Zn was increasing Zn uptake derived from the soil in wheat, invalidating the use of the AUE approach to assess the Zn use efficiency of fertilizer. These disadvantages can be overcome by using isotope tracers. Isotope dilution techniques are valuable tools to assess nutrient plant availability in soils and to quantify nutrient fluxes in soil-plant systems (Frossard et al., 2011; Hamon et al., 2008). Isotope source tracing allows to quantify and distinguish the nutrient fraction derived from the soil and the fertilizer within plant shoots. Zn source tracing in a fertilized soil-plant system can be conducted using two alternative approaches. The isotope tracer can either be introduced with the fertilizer source, here called *direct labeling method,* or in the soil-source, here called *indirect labeling method*. The radionuclide ^{65}Zn has been used to study the transfer of Zn from fertilizers to plant using the direct labeling method with green manures (Aghili et al., 2014a; McBeath and McLaughlin, 2013; Nanzer, 2012). However, the transfer of Zn from manure, compost or sewage sludge to plant has not yet been studied with isotopic techniques.

The use of radio-isotopes is complicated by the practical and legal limitations related to their radiations. Alternatively, Zn source tracing can also be conducted with enriched stable isotope systems. Terrestrial Zn is composed of five isotopes: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn with a respective mole fraction of 49.17 %, 27.73 %, 4.04 %, 18.45 % and 0.61 % also called natural abundances (Berglund and Wieser, 2011). Isotope dilution techniques with stable isotopes have been developed during the last decades. First, the technical and methodological development has increased the accessibility to sensitive analytical instruments as the inductively coupled plasma mass spectrometry (Sturup et al., 2008; Weiss et al., 2008; Wiederhold, 2015). Second, the use of stable isotopes is not subject to legal restrictions or safety issues relating to radiation risks. McBeath et al. (2013) have applied the direct labeling method with ^{67}Zn to determine the uptake of Zn applied as $ZnSO_4$ by wheat. To our knowledge, the indirect labeling method with stable Zn isotopes has never been tested in a soil plant system. Complex and insoluble fertilizers cannot easily be labeled with isotopes, as the targeted element is present in multiple molecular species. For a direct labeling method, the tracer has to be metabolized by the organism to obtain a homogenously and intrinsically labeled organic fertilizer. Bosshard et al. (2011) fed a sheep with ^{15}N enriched ryegrass to obtain intrinsically labeled urine and feces. Ostermann et al. (2015) fed a pig with $^{65}CuCl_2$ to produce ^{65}Cu enriched manure. Even though intrinsic labeling of organic fertilizers is possible, its production is time- and labor-intensive. For this reason, the indirect method is more suitable to trace Zn derived from organic fertilizers. Here, the isotope is applied to the soil source and not the fertilizer. This method is based on the assumption that the isotopically exchangeable Zn pool of the soil can be homogenously labeled. However, special attention has to be paid to the amount of tracer added to the soil source. The ^{67}Zn spike should only cause minimal steady-state disturbances within the system and not become a third source. Therefore, the added mass of ^{67}Zn has to be kept to a minimum. On the other hand, the labeling needs to be high enough to remain measurable. Moreover, the tracer should be equilibrated during sufficient time in the soil to label the soil available fraction as much as possible (Wiggenhauser, 2017). For the source quantification, the measured isotopic composition of i) the soil source, ii) the fertilizer source and iii) the sink need to be statistically distinguishable from each other. The indirect method can involve another minor, but non-negligible constraint, especially in soils that contain little available nutrient: the nutrient contribution from the seeds (Brookes, 1982; Pypers et al., 2006). With the indirect method, the Zn from the seeds and the fertilizer have the same isotopic composition (natural abundance). The Zn derived from the seeds induces therefore an isotope dilution in the plant shoots (sink), which implies an overestimation of the Zn derived from the fertilizer. If the proportion of the Zn derived from the seeds to the total Zn present in the shoot is significant it has to be accounted for and corrected accordingly. In the present study, we apply the indirect labeling method to quantify the amount of Zn derived from the fertilizer and from the soil in the shoots of a plant grown in a ^{67}Zn-enriched soil

fertilized with $ZnSO_4$, cattle manure, poultry manure and dried sewage sludge (DSS). Furthermore, we compared the fraction of Zn derived from the fertilizer recovered in the plant shoots with the AUE calculated from the Zn uptake of fertilized and unfertilized plants. We also take advantage of the continued regrowth of the model plant (Italian ryegrass*, Lolium multiflorum*) to estimate the Zn derived from the seeds.

Material and Methods

Soils

Two arable soils with different pH and total Zn content were selected for the pot experiment. The main soil characteristics are detailed in Table 6. On both sites, the soil was collected from a depth of 0-20 cm and sieved to 7 mm. The soil was labeled with ^{67}Zn as follows. Soil samples were transferred into big-boxes (100 x 120 cm) above a layer of foam glass drainage gravel separated by a fleece mat. A nutrient solution enriched with $^{67}Zn^{2+}$ derived from the dissolution of ^{67}ZnO in H_2SO_4 was percolated during 20 weeks into the soil layer, which was regularly mixed. The nutrient solution was provided by a partner project, growing ^{67}Zn-enriched wheat in hydroponic systems. After the labeling procedure, the soil was homogenized with a concrete mixer, air-dried and sieved at 2 mm mesh size before being stored during two years. The Zn isotope composition of the total soil Zn (15.5 M HNO_3 microwave digestion) was determined after the labeling.

Table 6: Selected soil characteristics after the labeling procedure

Soil characteristics	Soil	
Designation	Heitenried	Strickhof
Origin	Heitenried, Switzerland	Lindau, Switzerland
FAO classification[a]	Fluvisol	Cambisol
Clay (g kg^{-1})[b]	146	202
Silt (g kg^{-1})[b]	235	344
Sand (g kg^{-1})[b]	619	454
pH_{H2O}[c]	4.9	7.7
Zn DTPA (mg Zn kg^{-1})[d]	4.1	5.2
Zn total (mg Zn kg^{-1})[e]	54.1	101.1
^{67}Zn-total (%, mol/mol)[f]	4.98	4.47
WHC_{max} (g H_2O kg^{-1})[h]	386.9	446.8

[a]IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.
[b]Gravimetric measurement
[c]pH in H_2O with 1:2.5 solid:liquid ratio
[d]DTPA extractable Zn (Lindsay and Norvell, 1978)
[e]Energy dispersive X-ray fluorescence spectrometry (EDXRF)
[f]HNO_3 microwave digested Zn fraction of the soil
[h]Soil saturation with H_2O without external pressure

Growth trial

The source contribution from soil and fertilizer was quantified with a pot experiment. The treatments and their respective Zn application rates are listed in Table 7. Commercial pelleted cattle and poultry manure fertilizers were used. The dried sewage sludge (DSS) was provided by a local water treatment plant (Zurich, Switzerland). The amount of zinc, carbon and nitrogen added with the organic fertilizers are indicated in Table 7. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) solution was applied in the mineral treatment ($ZnSO_4$). A reference treatment without Zn fertilization was also established. The function of this last treatment was to provide the isotope composition of the exchangeable Zn pool of the labeled soils and to check if Zn was a limiting factor for plant growth. All treatments were prepared in four replicates. Before being applied, the organic fertilizers were finely ground in order to assure a homogeneous distribution in the soil. Pots of 400 g of soil (dry weight equivalent) were mixed with the respective fertilizers and moistened to 40 % of the water holding capacity (WHC, Table 6). Per pot, 0.5 g of Italian ryegrass (*Lolium multiflorum, var. Gemini*) seeds were sown. Nutrient solution was applied the first time 10 days after sowing (DAS) at a rate of 506 mg N (ammonium nitrate), 55 mg P, 138 mg K, 31 mg S, 23 mg Mg, 5 mg Fe kg^{-1}, 232 μg B kg^{-1}, 127 μg Mn kg^{-1}, 31 μg Cu kg^{-1} and 54 μg Mo kg^{-1} soil. The plants were watered to maintain the WHC between 40% and 80%. The shoots of ryegrass were cut four times (21, 30, 38 and 48 DAS). The day after each cut, the pots were fertilized at rate of 1/5 of the initial nutrient load. The plants were grown in a climate chamber with a daily photoperiod of 14 hours at 25 klx. Temperature and relative humidity (RH) were set to 24 °C and 60% during daytime and to 18 °C and 65% RH during night time, respectively.

Table 7: Growth trial treatment list and corresponding fertilizer Zn content and total Zn (Zn_{input}), carbon (C_{input}) and nitrogen (N_{input}) inputs. Dry matter (DM).

Treatment/Fertilizer	Zn content	Zn_{input}	C_{input}	N_{input}
	mg Zn kg^{-1} DM	mg Zn kg^{-1} soil	mg Zn kg^{-1} soil	mg Zn kg^{-1} soil
Reference	-	-	-	-
$ZnSO_4$	$4.4*10^5$	1.42	-	-
Cattle manure	353	1.56	1704.3	225.3
Poultry manure	443	1.51	1090.1	87.8
Dried sewage sludge	634	1.52	733.9	90.3

Incubation experiment

In parallel of to the growth trial, an incubation experiment was set up. The aim was to characterize the plant available Zn pool of the soil via extraction methods in the absence of plant. One pot per treatment was prepared as described in the section "*Growth trial*". However, no ryegrass was sown. The pots were kept in the same conditions as the growth trial except that they were weekly watered up to 80 % WHC. At the end of the growth trial, the incubated soils were used to determine the free Zn concentration of the soil solution (C_{DGT}) by diffusive gradients in thin films (DGT) (Hooda et al., 1999). The DTPA-extractable Zn was measured following the protocol described in Lindsay and Norvell (1978) and in Quevauviller et al. (1998).

Sample processing

The samples collected in separate bags made of pure cellulose (pergamin) and dried at 65°C during 48h. The biomass was then milled with tungsten bowls to avoid any Zn contaminations. Subsamples of 200 mg plant material were digested with 2 ml H_2O (EasyPure^{TM}) and 2 ml HNO_3 (Rotipuran® Supra 15.5 M, Zn < 0.5 µg*L^{-1}) in single use glass tubes in a high pressure single reaction chamber microwave system (turboWave, MWS microwave system). An aliquot of the plant extract was collected to determine the Zn concentration by ICP-OES. The remaining was transferred into PTFE containers, evaporated to dryness and dissolved into 6 M HCl (Rotipuran®Supra 11.6 M, Zn < 1 µg*L^{-1}). The Zn was separated from the matrix by ion exchange chromatography following a slightly modified protocol from Pinna et al. (2001). Spectra/Chrom® Minicolumns PP, 7.5 ml with 45 µm filter were filled with 2.3 cm of anion exchange resin (AG® 1-X8, 100-200 mesh, chloride form, Bio-Rad laboratories). The resin was cleaned by adding successively 3 x 5 ml HNO_3 2 M, 2ml H_2O and 5 ml of HCl 0.5 M. Then the column was conditioned with 2 x 5 ml HCl 6 M and the samples were loaded onto the resin. Next, the cations were washed out with 2 x 5 ml HCl 6 M. Cu and Fe were eluted with 5 ml HCl 2.5 M and 3 x 5 ml HCl 0.5 M respectively. In the last step, the Zn was eluted into PTFE container with 2 x 5 ml HCl 0.005 M. The resulting solution was evaporated to dryness before being dissolved in HNO_3 15.5 M and evaporated twice. Finally, the dry residues were dissolved with 2 ml HNO_3 0.05 M and stored in 2ml Eppendorf tubes.

Sample analysis

After the Zn separation by column chromatography, the Zn isotope composition of the extracts was determined by a quadrupole inductively coupled plasma mass spectrometer (Q-ICPMS). The analytical approach was established and verified in the first chapter of the present thesis. The instrument was an Agilent 7500ce with a helium supplied octopole reaction system. Each

sample was measured with 12 replicates. For each mass (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn) a three-point peak pattern was selected with an integration time of 0.1 seconds per point. The mass bias correction was realized by standard-sample-standard bracketing with a conventional zinc sulfate heptahydrate solution ($ZnSO_4 \cdot 7H_2O$, Sigma Aldrich) and applying a power law mass fractionation correction (Peel et al., 2008). The extracts were diluted to obtain a concentration of approximately 200 µg L^{-1} in accordance with the $ZnSO_4$-standard. The average of the relative standard deviation (RSD) was < 1.3 % for all isotope ratios (^{67}Zn:^{xx}Zn) except ^{67}Zn:^{70}Zn which reached an average RSD of 2.7 %. The Zn concentration of all extracts was determined by ICP-OES.

Calculations: Zn derived from fertilizer and soil

Zinc is composed of five stable isotopes (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn). The isotope abundances of each isotope are given in mole fractions. The sum of the abundances of all five isotopes equals 1. The source contribution of the fertilizer ($Zndf_{fertilizer\%}$) within the plant of the Zn fertilized treatments, here called "sink", was calculated by mass balance following Eq. 3. For the derivation of this equation readers are referred to McBeath et al. (McBeath et al., 2013) and the first chapter of this thesis.

In Eq. 3, the ^{66}Zn and ^{67}Zn abundances (mole fractions, %) of the two sources (soil and fertilizer) are required. In order to calculate them, all five stable Zn isotopes had to be measured to be used in Eq. 1 and 2. For the "sink", only the ^{67}Zn:^{66}Zn isotope ratio were required.

$$^{67}Zn_{source} = \frac{100}{1 + \left(\frac{^{64}Zn}{^{67}Zn}\right)_{source} + \left(\frac{^{66}Zn}{^{67}Zn}\right)_{source} + \left(\frac{^{68}Zn}{^{67}Zn}\right)_{source} + \left(\frac{^{70}Zn}{^{67}Zn}\right)_{source}} * 100 \quad (\%) \qquad \text{Eq. (1)}$$

$$^{66}Zn_{source} = \left(\frac{^{66}Zn}{^{67}Zn}\right)_{source} * \ ^{67}Zn_{source} \ * 100 \ \ (\%) \qquad \text{Eq. (2)}$$

In Eq. 1 and 2, $^{66}Zn_{source}$ and $^{67}Zn_{source}$ represent the ^{66}Zn and ^{67}Zn abundance (mole fraction, %) and $\left(\frac{^{67}Zn}{^{xx}Zn}\right)_{source}$ the Zn isotope ratios of the soil or the fertilizer sources. In Eq. 3 the subscript "source" used in Eq. (1) and (2) is replaced by the respective sources: "*soil*" and "*fertilizer*". The shoot from the fourth cut of the reference treatment was selected to provide the isotope composition of the exchangeable Zn pool of the soil as it was thought to be free from interference from Zn derived from the seed. The terms of Eq. 3 to Eq. 6 are defined in

Table 8. First, we calculated the percentage of Zn in the shoots derived from the fertilizer (Eq. 3).

$$Zndf_{fertilizer\%} = \frac{{}^{66}Zn_{soil} * \left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink} - {}^{67}Zn_{soil}}{\left({}^{67}Zn_{fertilizer} - {}^{67}Zn_{soil}\right) - \left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink} * \left({}^{66}Zn_{fertilizer} - {}^{66}Zn_{soil}\right)} * 100\ (\%) \quad \text{Eq. (3)}$$

In a second step, we calculated the amount of Zn derived from the fertilizer (Eq. 4).

$$Zndf_{fertilizer} = Zndf_{fertilizer\%} * Zn_{uptake} \qquad (\mu g\ kg^{-1} soil) \quad \text{Eq. (4)}$$

The counterpart of the results obtained with Eq. 3 gave the percentage of Zn in the shoot derived from the soil (Eq. 5) which was used in Eq. 6 to calculate the amount of Zn derived from the soil.

$$Zndf_{soil\%} = 100 - Zndf_{fertilizer\%} \qquad (\%) \quad \text{Eq. (5)}$$

$$Zndf_{soil} = Zndf_{soil\%} * Zn_{uptake} \qquad (\mu g\ kg^{-1} soil) \quad \text{Eq. (6)}$$

Table 8: Terminology of the mass balance derivation described in Eq. 3-6.

Terms	Definition	Units
$Zndf_{fertilizer\%}$	Zn fraction in the plant shoot derived from the fertilizer	(%)
$Zndf_{fertilizer}$	Quantity of Zn derived from the fertilizer	(µg kg^{-1} soil)
$Zndf_{soil\%}$	Zn fraction in the plant shoot derived from the soil	(%)
$Zndf_{soil}$	Quantity of Zn derived from the soil	(µg kg^{-1} soil)
${}^{66}Zn_{fertilizer}$	^{66}Zn abundance of the fertilizer source (Eq. 2)	(mole fraction in %)
${}^{67}Zn_{fertilizer}$	^{67}Zn abundance of the fertilizer source (Eq. 1)	(mole fraction in %)
${}^{66}Zn_{soil}$	^{66}Zn abundance of the soil source measured in the plant shoots from the fourth cut of the reference treatments (Eq. 2)	(mole fraction in %)
${}^{67}Zn_{soil}$	^{67}Zn abundance of the soil source measured in the plant shoots from the forth cut of the reference treatments (Eq. 1)	(mole fraction in %)
$\left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink}$	^{67}Zn:^{66}Zn isotope ratio of the plant shoot grown of the fertilized treatments	(mole $mole^{-1}$)

The fertilizer Zn recovery (ZnRec, %) in the plant was calculated (Eq. 7) by comparing the $Zndf_{fertilizer}$ (µg kg^{-1} soil) to the application rate (Zn_{input}, µg kg^{-1} soil).

$$ZnRec = \frac{Zndf_{fertilizer}}{Zn_{input}} * 100 \quad (\%) \qquad \text{Eq. (7)}$$

For the comparison, the apparent use efficiency (AUE) was calculated (Eq. 8). The AUE is obtained by dividing the Zn uptake difference between the fertilized plants (U_f in µg kg^{-1} soil) and the reference plants (U_0 in µg kg^{-1} soil) by the Zn application rate (Zn_{input} in µg kg^{-1} soil, Table 7).

$$AUE = \frac{U_f - U_0}{Zn_{input}} * 100 \quad (\%) \qquad \text{Eq. (8)}$$

Statistics

The data set was analyzed separately for the two soil types (Heitenried and Strickhof) using variance analysis (ANOVA) testing the factor treatment (T) and cut (C). All statistical analyses were performed with the software "R" (version 3.3.2). Multiple comparison tests were realized with the Tukey's HSD test. When the data distribution did not allow the use of a variance analysis, a Kruskal-Wallis test was applied. Linear regressions were fitted using the linear model function (lm) in the software "R".

Results

Shoot dry matter: productivity

Figure 7 shows the cumulated shoot dry matter (DM) for four cuts. For the Heitenried soil, the mean DM production ranged from 13.1 g kg^{-1} in the reference treatment to 15.9 g kg^{-1} in the poultry manure treatment. For the Strickhof soil, it was stable for all treatments around a mean of 16.2 g kg^{-1}. There was no significant correlation between the yield and the amount of additional nitrogen applied with the organic fertilizers. Compared to Strickhof, the mean DM productivity of the Heitenried was significantly lower (Kruskal-Wallis test, $p<0.001$).

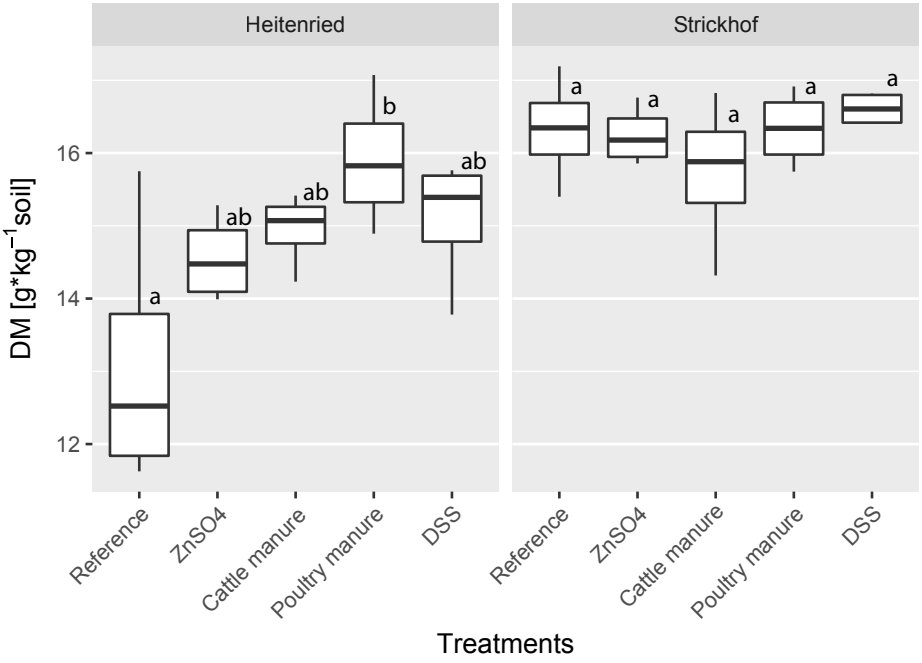

Figure 7: Cumulated shoot dry matter production per treatment obtained on the two experimental soils. Letters indicate significant differences between the treatments within a soil (Tukey's HSD, $\alpha = 0.05$). Dried sewage sludge (DSS).

Shoot Zn concentration

The Zn concentration of the shoots (Figure 8) was higher in ryegrass growing on the Heitenried soil when than on the Strickhof soil (Kruskal Wallis test, $p < 0.001$). For the Heitenried soil, there was no significant difference in shoot Zn concentrations between the treatments. The overall mean for Heitenried was 55.8 Zn mg kg^{-1} DM. For the Strickhof soil, the mean shoot Zn concentration ranged from 38.3 mg kg^{-1} DM for the "reference" treatment to 46.1 mg kg^{-1} DM for the "cattle manure". The overall mean for Strickhof was 41.3 mg kg^{-1} DM. The Zn concentration increased from the first cut to the third, whereas the fourth cut recorded the lowest values. These tissue concentrations were close to typical ryegrass Zn concentrations reported in the literature (Davis and Beckett, 1978; Römheld, 2012), excluding Zn deficiency and Zn toxicity.

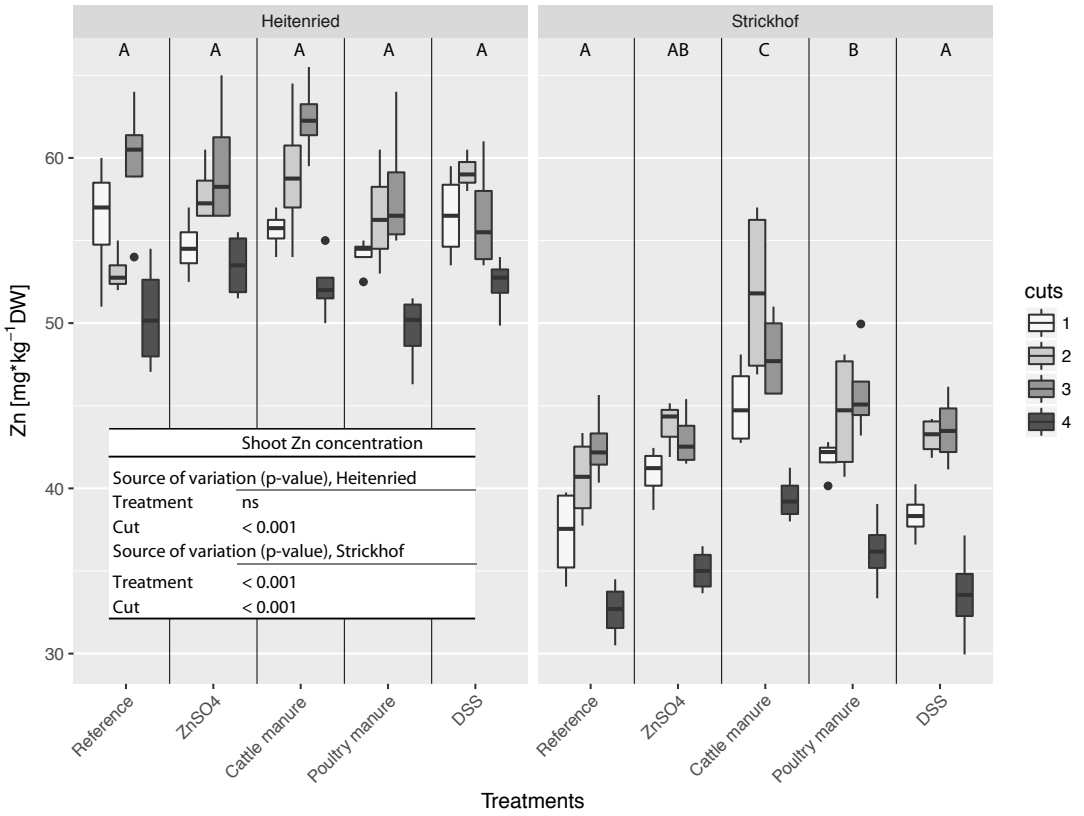

Figure 8: Zn concentration in ryegrass shoots per treatment and per cut in the two soils. Capital letters indicate significant differences between the treatments within a soil (Tukey's HSD, $\alpha = 0.05$). Black dots (•) represent the outliers. Not significant (ns). Dried sewage sludge (DSS).

Zn isotope composition of the shoots

Figure 9 shows the ^{67}Zn:^{66}Zn ratio of the ryegrass shoots per treatment and per cut for both soil types. The mean ^{67}Zn:^{66}Zn ratio of the reference treatments, which represents the isotope composition of the exchangeable Zn pool of the soil, reached 0.298 and 0.211 (mole mole^{-1}) in the Heitenried and Strickhof soils, respectively. The isotope dilution induced by the Zn derived fertilizer could be observed in all Zn fertilized treatments, as all of them showed a significantly lower ^{67}Zn:^{66}Zn ratio compared to the non-fertilized reference treatment. The ^{67}Zn:^{66}Zn ratio of all treatments was significantly lower in the first cut, when compared to the three other cuts.

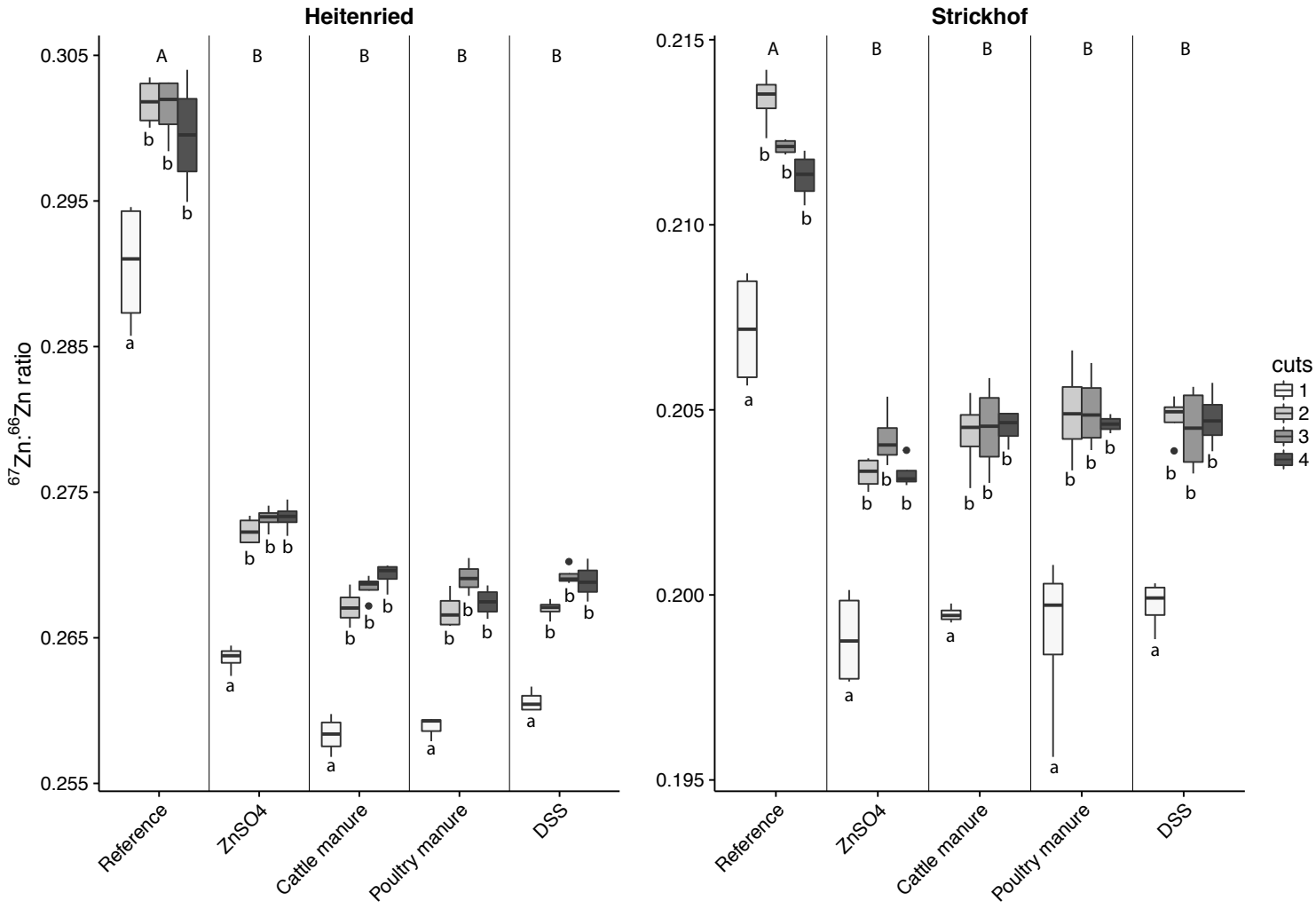

Figure 9: $^{67}Zn:^{66}Zn$ ratios of ryegrass shoots per treatment and per cut for Heitenried (left) and for Strickhof (right). Capital letters indicate significant differences between the treatments of the respective soils (Kruskal-Wallis test, $\alpha = 0.05$). Lower case letters indicate the significant difference between the cuts with the respective soils (Kruskal-Wallis test, $\alpha = 0.05$). Black dots (•) represent the outliers. Dried sewage sludge (DSS).

Percentage of Zn in plant shoots derived from the fertilizers

The Zn fraction derived from the fertilizer ($Zndf_{fertilizer\%}$) in the shoot is presented in Figure 10. The $Zndf_{fertilizer\%}$ was in average significantly higher in ryegrass growing on the Heitenried as on the Strickhof soil (Kruskal Wallis test, $p < 0.001$). The overall mean of $Zndf_{fertilizer\%}$ was 20.4 % for the Heitenried and 12.2 % for the Strickhof soil. The $ZnSO_4$ treatment had the lowest $Zndf_{fertilizer\%}$ in the Heitenried soil (18.2 %) compared to the other fertilizers while it had the highest $Zndf_{fertilizer\%}$ in the Strickhof soil (13.4 %). In all treatments, the $Zndf_{fertilizer\%}$ of the first cut was significantly higher than in the three following cuts (Kruskal-Wallis, $p< 0.001$).

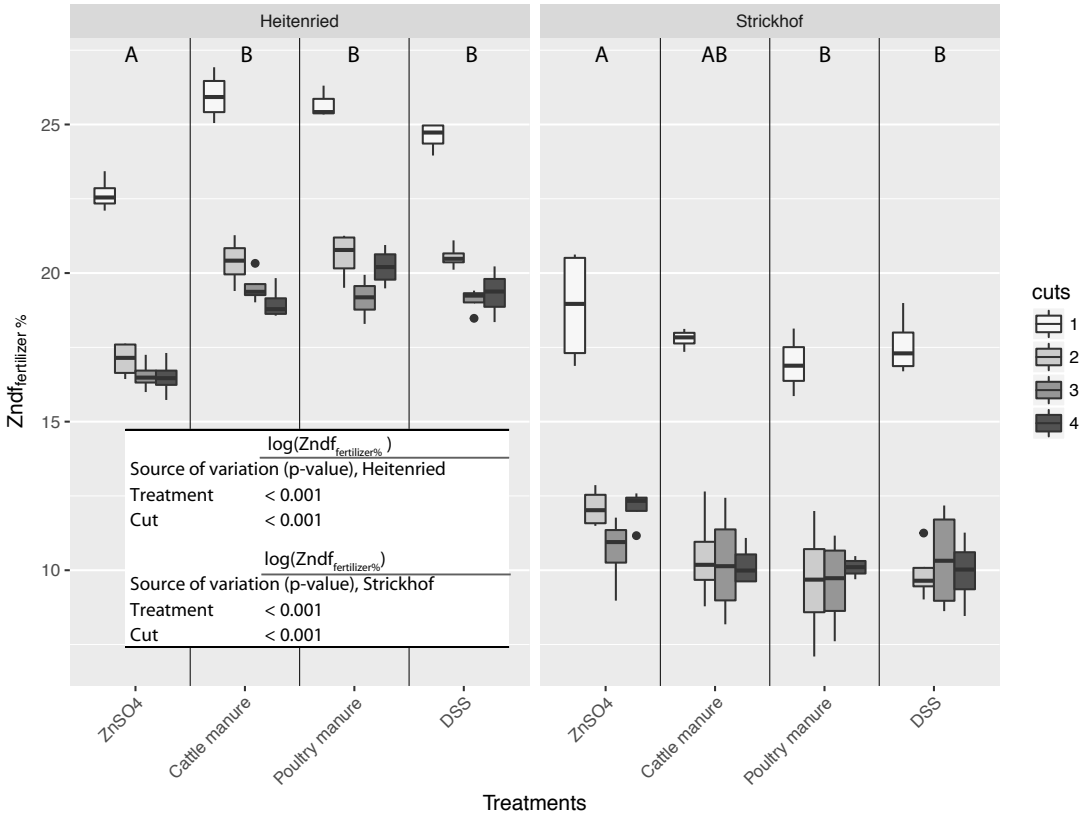

Figure 10: Fraction of Zn in plant shoots derived from the fertilizer ($Zndf_{fertilizer\%}$) per treatment and per cut for each soil. Capital letters indicate the significant difference between the treatments within a soil (Tukey's HSD, $\alpha = 0.05$). Black dots (•) represent the outliers. Dried sewage sludge (DSS).

Amounts of Zn taken up in plant shoots derived from the fertilizers

The amount of Zn derived from the fertilizer ($Zndf_{fertilizer}$, $\mu g*kg^{-1}$ soil) in shoots is shown for each cut and each soil in Figure 11. The amounts of $Zndf_{fertilizer}$ differed significantly between Heitenried and Strickhof (Kruskal Wallis test, $p < 0.001$). The overall average calculated across the 4 cuts was 44.0 $\mu g\ kg^{-1}$ soil for Heitenried and 23.0 $\mu g\ kg^{-1}$ soil for Strickhof. The treatment effects were similar to the ones observed for the $Zndf_{fertilizer\%}$ in Figure 10. The $ZnSO_4$ treatment of Heitenried showed the lowest $Zndf_{fertilizer}$ mean (38.1 $\mu g\ kg^{-1}$ soil). The treatment effect in the Strickhof soil effect was significant but less pronounced than for Heitenried. The average $Zndf_{fertilizer}$ ranged from 22 $\mu g\ kg^{-1}$ soil in the DSS treatment to 24 $\mu g\ kg^{-1}$ soil in the $ZnSO_4$ treatment. The amount of $Zndf_{fertilizer}$ decreased between the first and the third cut within all treatments of both soil types. For all Heitenried treatments as well as the Strickhof $ZnSO_4$ treatment, the $Zndf_{fertilizer}$ was stable after the third cut whereas in the Strickhof soil the $Zndf_{fertilizer}$ of the cattle and poultry manures and of the dried sewage sludge (DSS) continued to decrease in the fourth cut.

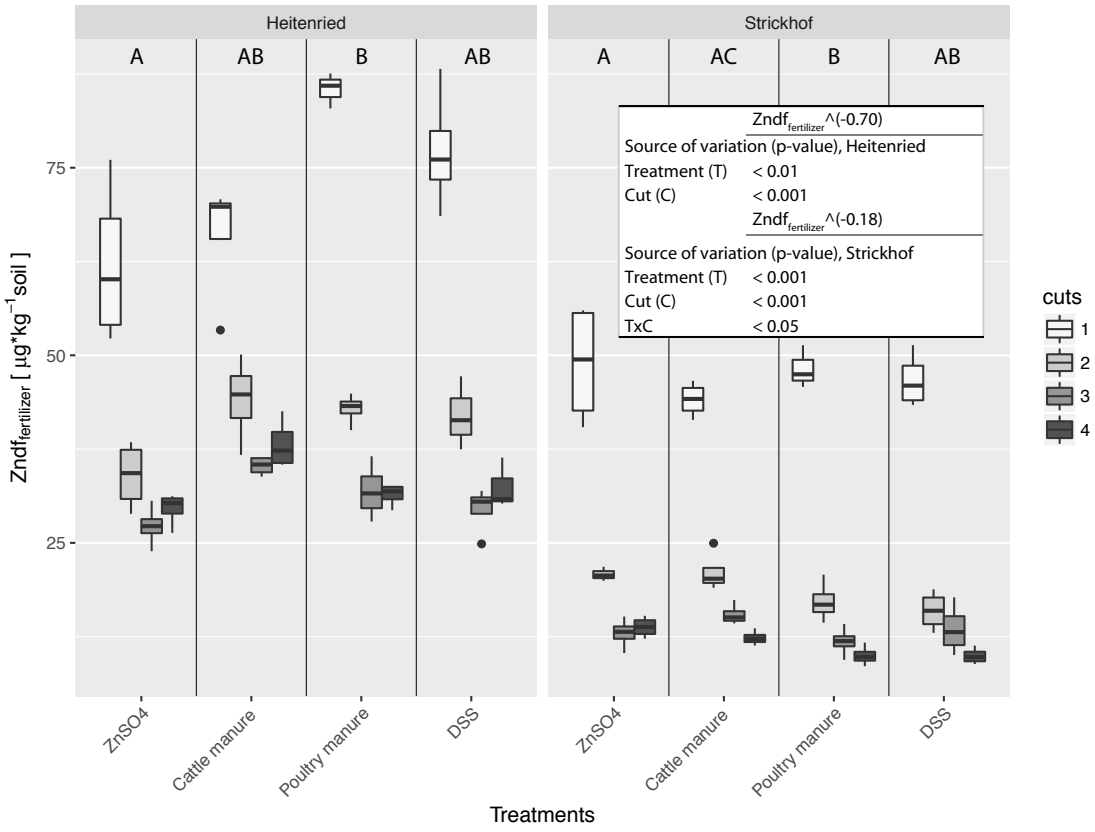

Figure 11: Zn derived from fertilizer ($Zndf_{fertilizer}$ in $\mu g*kg^{-1}$ soil) in plant shoots per treatment and per cut. Capital letters indicate the significant difference between the treatments within a soil (Tukey's HSD, $\alpha = 0.05$). Black dots (•) represent the outliers. Not significant (ns). Dried sewage sludge (DSS).

Percentage of Zn in plant shoots derived from the soil

Figure 12 illustrates the percentage of Zn derived from the soil in the shoot ($Zndf_{soil\%}$). The $Zndf_{soil\%}$ was significantly higher in the Strickhof soil than in the Heitenried soil (Kruskal Wallis test, $p < 0.001$). The overall mean of the respective soil types was 79.6 % for Heitenried and 87.6 % for Strickhof. With a $Zndf_{soil\%}$ of 81.8 % the "$ZnSO_4$" treatment of the Heitenried soil was significantly higher compared to the other treatments. For the Strickhof soil, there was no treatment effect. In all treatments, the $Zndf_{soil\%}$ of first cut was significantly lower than for the three following cuts (Kruskal-Wallis, $p < 0.001$).

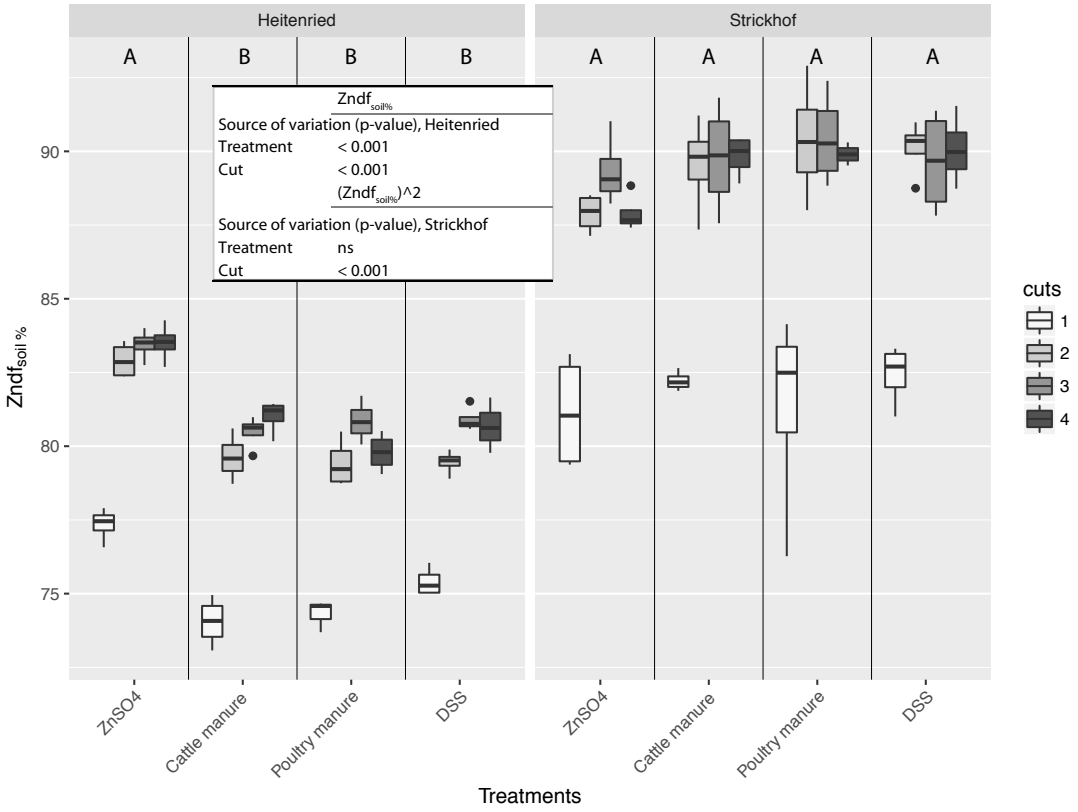

Figure 12: Fraction of Zn in the plant shoots derived from the soil ($Zndf_{soil\%}$) per treatment and per cut. Capital letters indicate the significant difference between the treatments within a soil (Tukey's HSD, α = 0.05). Black dots (•) represent the outliers. Not significant (ns). Dried sewage sludge (DSS).

Amounts of Zn taken up in plant shoots derived from the soil

The amount of Zn in shoots derived from the soil ($Zndf_{soil}$, μg kg^{-1} soil) is represented in Figure 13. The soil effect on $Zndf_{soil}$ was significant (Kruskal Wallis test, $p < 0.01$). The overall $Zndf_{soil}$ mean of the Heitenried soil calculated across the 4 cuts was 168.3 μg kg^{-1} soil and 149.6 μg kg^{-1} for Strickhof. For Heitenried, no treatment effect was observed. For Strickhof, the $Zndf_{soil}$ treatment means ranged from 141.8 μg kg^{-1} for the treatment $ZnSO_4$ to 158.3 μg kg^{-1} for the treatment cattle manure. In Heitenried, $Zndf_{soil}$ decreased from the first to the third cut and did not change afterwards. However, in Strickhof $Zndf_{soil}$ continued decreasing in the fourth cut.

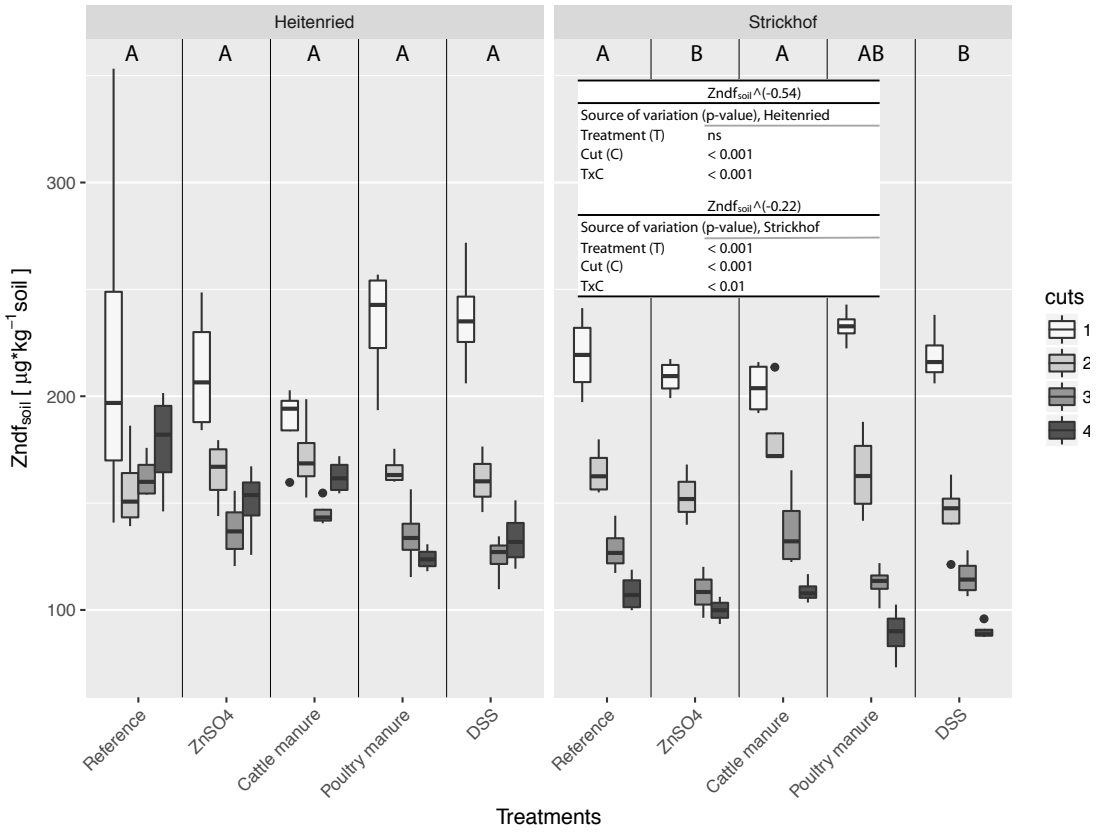

Figure 13: Zn in plant shoots derived from soil ($Zndf_{soil}$ in μg*kg^{-1} soil) per treatment and per cut and for both soils. Capital letters indicate the significant difference between the treatments within a soil (Tukey's HSD, $\alpha = 0.05$). Black dots (•) represent the outliers. Not significant (ns). Dried sewage sludge (DSS).

Shoot Zn uptake

The cumulated shoot Zn uptake over the four cuts is represented in Figure 14. The total Zn uptake was significantly higher (Kruskal Wallis test, $p < 0.001$) for the ryegrass growing on the Heitenried soil compared to the Strickhof soil. The overall mean Zn uptake was 815 $\mu g\ kg^{-1}$ soil for Heitenried and 671 $\mu g\ kg^{-1}$ soil for Strickhof. With the Heitenried soil, the Zn uptake of the Zn fertilized treatment tended to be higher than the reference treatment. However, this effect is not statically supported because of the high variability of the yield in this particular reference treatment (Figure 7). In the Strickhof soil, the highest Zn uptake was observed with the cattle manure, which was significantly higher than the reference, the $ZnSO_4$ and the dried sewage sludge (DSS) treatments. Regarding the contribution from the two sources, the plant took up the highest amount of Zn from the soil. Except for the cattle manure treatment of the Strickhof soil, the Zn from the fertilizer always replaced a portion of the Zn derived from the soil, when compared to the reference treatments. Figure 15 shows the correlation between the total Zn uptake (Figure 14) and the additional total carbon (C_{input}) and total nitrogen (N_{input}) introduced with the organic fertilizers. In Heitenried the response of Zn shoot uptake to the carbon input was significant but weak (Figure 15, a and c) with a correlation coefficient (r^2) of 0.23. No correlation between uptake and nitrogen was found. However, for Strickhof (Figure 15, b and d) the correlation coefficients were higher ($r^2 = 0.58$) for the carbon and ($r^2 = 0.53$) nitrogen input, which indicates that both significantly ($p < 0.001$) increased the Zn shoot uptake.

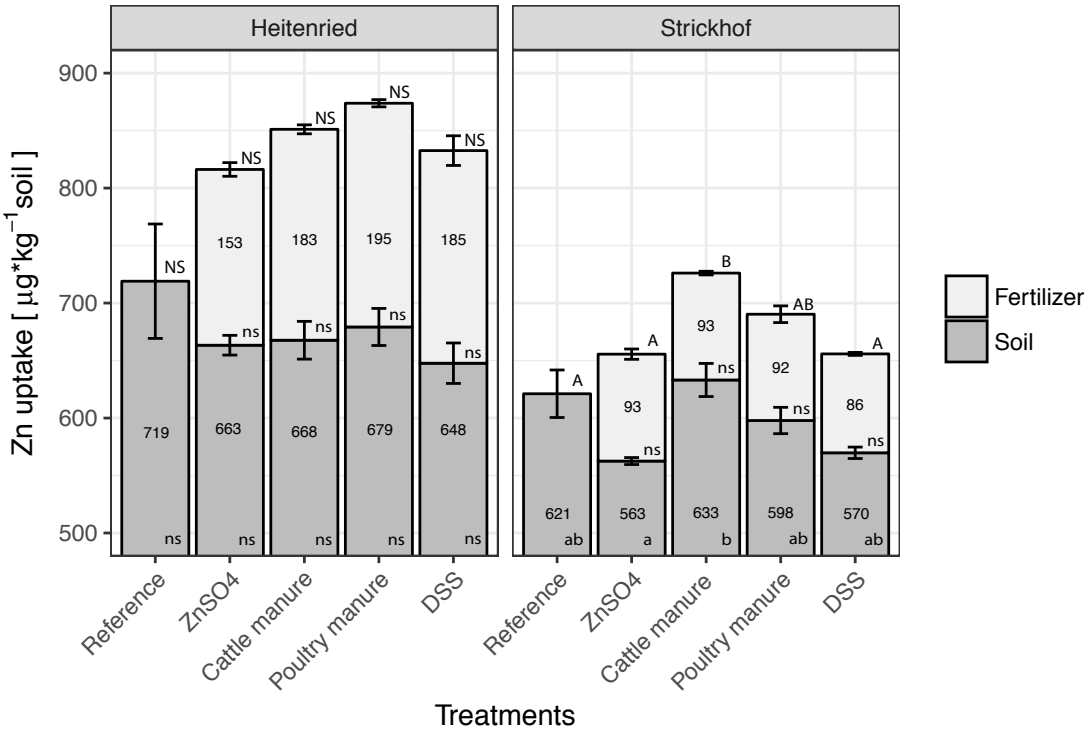

Figure 14: Cumulative shoot Zn uptake (in µg kg^{-1} soil) from the four ryegrass cuts, split into the fractions derived fertilizer and soil. Capital letters indicate the significant differences of the cumulated Zn uptake between the treatments within a soil (Kruskal Wallis test, α = 0.05). Lower case letters indicate the significant difference within the Zn uptake from the fertilizer respectively from the soil. Dried sewage sludge (DSS).

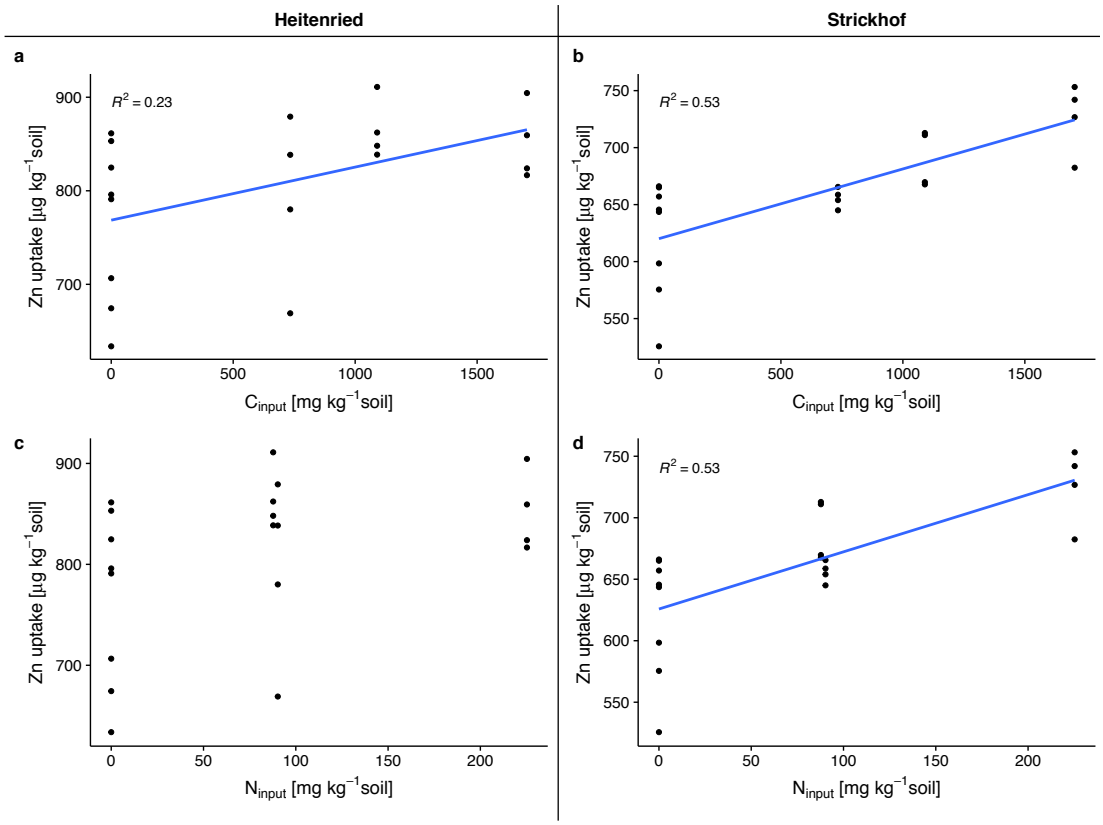

Figure 15: Correlation of total shoot Zn uptake with **additional** carbon (C_{input}) and nitrogen (N_{input}) input introduced with the organic fertilizers (Table 7) for Heitenried a), c) and for Strickhof b), d). The reference and the $ZnSO_4$ treatments are represented with a C_{input} and N_{input} of zero.

Zn fertilizer recovery and apparent use efficiency

The Zn fertilizer recovery (ZnRec, %) determined with the isotopic approach (Eq. 7) was approximatively twice as high for the Heitenried soil than for Strickhof (Figure 16). The $Zn_{fertilizer}$ recovery ranged from 10.2 % ("$ZnSO_4$") to 12.0 % ("poultry manure") for the Heitenried soil and from 5.1 % ("DSS") to 6.2 % ("$ZnSO_4$") for the Strickhof soil.

The apparent use efficiency (AUE) calculated with Eq. 8 was approximately 3 units lower than the ZnRec on the Heitenried soil. Here, the AUE was significatively correlated with ZnRec (r^2 = 0.92), whereas AUE and ZnRec were not correlated for Strickhof.

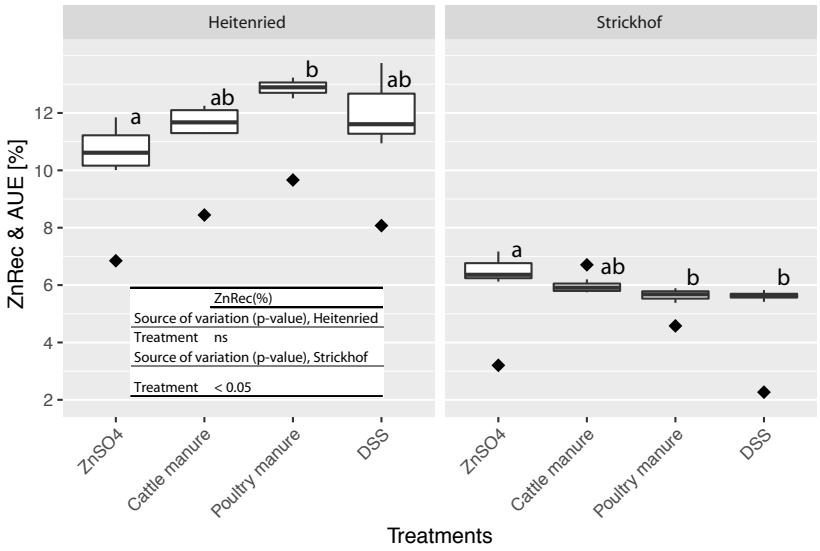

Figure 16: Cumulative Zn fertilizer recovery assessed by the labeling approach (ZnRec), and the apparent use efficiency (AUE) for the four ryegrass cuts per treatment and for both soils. Letters indicate the significant differences between the treatments within a soil (Tukey's HSD, α = 0.05) for the Zn fertilizer recovery. Diamonds (◆) represent the apparent use efficiency (AUE) calculated with Eq. 9. Dried sewage sludge (DSS).

Incubation experiment

Two proxies were used to assess the plant available Zn in the incubated soils: DTPA-extractable Zn and the free Zn concentration of the soil solution (C_{DGT}) extracted by diffusive gradients in thin films (DGT) (Figure 17). The mean DTPA-extractable Zn was 5 mg kg^{-1} for the Heitenried soil and 5.75 mg kg^{-1} for the Strickhof soil. These values are higher than the threshold for soil Zn-deficiency of 0.5 mg kg^{-1} suggested by Cakmak (2007). The comparison of the absolute values is not possible between the two soil types, as the amount of Zn extracted with DTPA is affected by the soil pH (Menzies et al., 2007). However, this comparison is possible for C_{DGT} (Tandy et al., 2011), which was significantly lower in the Strickhof soil compared to Heitenried (Kruskal Walllis test, $p < 0.001$).

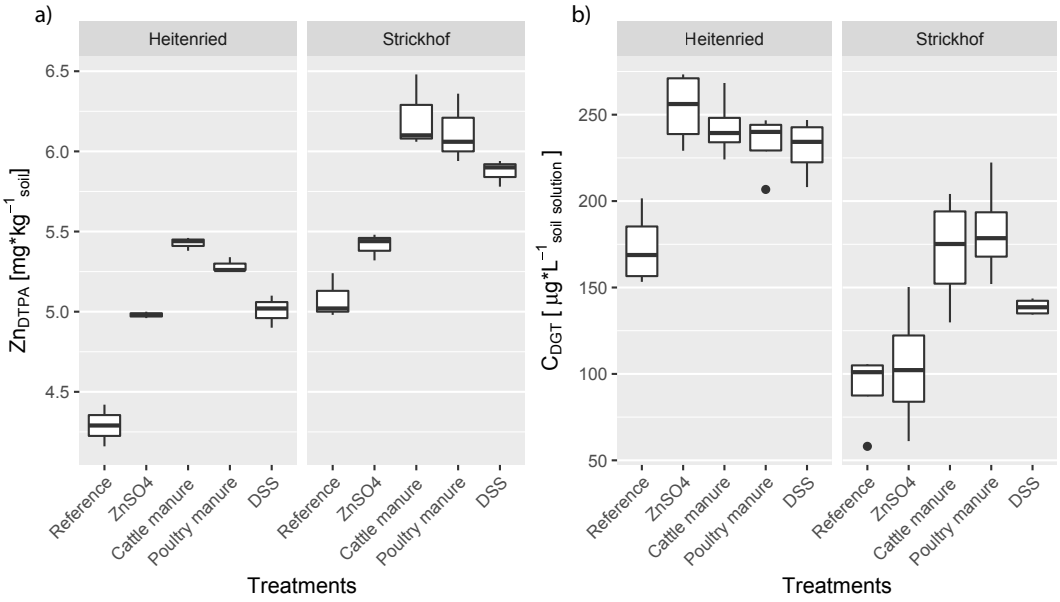

Figure 17: Incubation experiment Zn soil extractions: a) DTPA extractable Zn (in mg*kg^{-1}_{soil}), b) free Zn concentration of the soil solution (C_{dgt}) extracted by diffusive gradients in thin films (DGT). The boxplot shows the analytical reproducibility, where DTPA was extracted in three replicates (n=3) and DGT in four replicates (n=4). Black dots (•) represent the outliers. Dried sewage sludge (DSS).

Discussion

Zn source tracing with the indirect labeling method

In the present conditions, the indirect method with stable Zn isotopes provided realistic results to trace Zn in a soil-plant system fertilized with mineral as well as organic fertilizers. The mean Zn fraction in plant shoots derived from the Zn fertilizer ($Zndf_{fertilizer\%}$) ranged from 12.2 % on the Strickhof soil to 20.4 % on the Heitenried soil (Figure 10). The ryegrass recovered about 11 % and 5.5 % of the Zn added with fertilizers on Heitenried and Strickhof, respectively (Figure 16). The order of magnitude of these results are consistent with the findings of similar source tracing studies investigating the fate of Zn from complex fertilizers with the radionuclide ^{65}Zn. Nanzer (2012) applied the indirect method to study the Zn transfer from sewage sludge ashes to shoots of ryegrass and obtained a mean $Zndf_{fertilizer\%}$ of 14.5 % and a fertilizer recovery ranging from 0.1 to 7.3 % at a fertilization rate of 3.5 mg Zn kg^{-1} soil. Aghili et al. (2014a) used a direct approach with ^{65}Zn-labeled green manure and found a mean $Zndf_{fertilizer\%}$ of 7.2 % and a fertilizer recovery between 1.4 and 4.1 % at a fertilization rate of 0.183 and 0.418 mg Zn kg^{-1} soil.

The source quantification with isotopes delivered more accurate data than the apparent use efficiency (AUE, Figure 16). By using the AUE, the assumption is made that the amount of zinc derived from the soil is constant. However, the results from the isotopic approach showed that the Zn derived from the fertilizer can replace part of the Zn derived from the soil (Figure 14). This effect, which cannot be revealed with a difference method, results in an underestimation of the AUE, as we could observe in Figure 16. On the contrary, if the input leads to a solubilisation of the isotopically non-exchangeable Zn pool of the soil or if the plant develops strategies to access this pool, the AUE will be overestimated. This effect was observed by Aghili et al. (2014a) where sunflower green manure inputs induced a mobilization of the non-exchangeable Zn pool of the soil. A trend of such an effect was suggested with the cattle manure treatment of the Strickhof soil. Here the $Zndf_{soil}$ was slightly (but not significantly) higher than the reference treatment (Figure 14). For this reason, the AUE of this treatment was found to be higher than the ZnRec obtained with the isotopic approach (Figure 16).

In the present study, only two sources were considered for Zn uptake in the shoots: the fertilizer and the soil. However, the Zn derived from the seeds ($Zndf_{seeds}$) should also be considered in source tracing experiments with the indirect labeling method (Brookes, 1982; Pypers et al., 2006). As the Zn isotope composition of the seeds is the same than the fertilizer (natural abundance), the $Zndf_{seeds}$ induces an overestimation of the $Zndf_{fertilizer}$. The results from the ^{67}Zn:^{66}Zn ratios of the first cut of all treatments (Figure 9) showed a significant isotope dilution effect, which could definitively be attributed to the $Zndf_{seeds}$. This effect did explain the higher $Zndf_{fertilizer}$ and $Zndf_{fertilizer\ \%}$ in the first cut of all treatments (figures 4 and 5). The total

amount of Zn added with the seeds accounted for 44 μg*kg^{-1} soil. Considering that the difference in $Zndf_{fertilizer}$ between the first and the second cuts is the amount of Zn actually derived from the seeds, which is a conservative estimate as it is known that smaller amounts of P are still translocated from the seed to the shoot after the first cut (Brookes, 1982), provides a first estimation of the amounts of Zn derived from the seeds ($Zndf_{seeds}$). $Zndf_{seeds}$ would be in average 34 μg kg^{-1} soil with a standard deviation of 10 μg kg^{-1} soil in Heitenreid and 31 μg kg^{-1} soil with a standard deviation of 6 μg kg^{-1} soil in Strickhof. Altogether, this estimation of $Zndf_{seeds}$ makes up a small portion of the overall mean Zn uptake for Heitenried (4.2 %) and for Strickhof (4.6 %). However, this leads to a decrease in the recovery rate of the $Zndf_{fertilizer}$ by ryegrass. If we remove the amount of $Zndf_{seeds}$ from the overall $Zndf_{fertilizer}$ shown in Figure 14 for each treatment, we reach recovery rates of Zn derived from the fertilizer over the 4 cuts ranging between 8.8 to 10.3 % in Heitenried and between 3.6 to 4.5% in Strickhof. This is a very preliminary estimation of $Zndf_{seeds}$ which should be improved especially for soils that contain very little available Zn. Methods for estimating the fraction of P derived from the seeds have been described by Brookes (1982) and Pypers et al. (2006) and could be adapted to Zn in the future.

Soil effect

All results showed a significant soil effect which can most probably be explained by soil pH. The dry matter production (Figure 7) was slightly lower for the ryegrass growing on the Heitenried soil than on the Strickhof soil. These results are consistent with similar studies using ryegrass where the biomass production was lower at low pH (Nanzer, 2012; Smith, 1994) as the growth condition might be more favorable at neutral and alkaline soil pH.
The Zn concentration of the shoots (Figure 8) and total Zn uptake (Figure 14) were significantly lower for all treatments grown on the Strickhof soil compared when compared to Heitenried. This suggests that even tough, the total Zn content of the Strickhof soil was twice as high as the Heitenried soil, the plant-available pool of the former was smaller. This is in accordance with the higher soil pH of Strickhof: the higher the soil pH, the larger the Zn fraction adsorbed to oxides and organic compounds and the lower the plant availability (Mertens and Smolders, 2013). This outcome was also confirmed by the free Zn concentration in soil solution extracted with the DGT technique (Figure 17) which was lower in Strickhof compared to Heitenried.

Fertilizer effect

A fertilizer effect on element transfer can be direct, i.e. this is the transfer of element measured from the fertilizer to the plant, or indirect as the fertilizer can modify soil properties affecting element availability and plant uptake. This indirect effect is measured by a change of element

derived from the soil taken up by the plant in the presence of fertilizer (Sinaj et al., 2002; Morel and Fardeau, 1989; Aghili et al., 2014).

In the Heitenreid soil, the concentration of Zn extracted by DGT (C_{DGT}) was similar across the fertilized treatments, the recovery of fertilizer Zn in plant shoots was also similar, and the uptake of Zn derived from the soil in the presence of fertilizers decreased a little in comparison to what was observed in the treatment without zinc addition. Finally, no relation was observed between the C and N added with the organic fertilizers and Zn uptake. Altogether, these results suggest that Zn derived from the organic fertilizers was as available as Zn from ZnSO4, and that the total uptake of Zn was additive with the amount of Zn derived from the soil being constant.

The Strickhof soil illustrates another situation. The concentration of Zn extracted by DGT (C_{DGT}) was was higher in the organically fertilized treatments than in the ZnSO4 treatment, which suggests either that Zn added as ZnSO4 was sorbed on this soil (the level of C_{DGT} $ZnSO_4$ was identical to the level of C_{DGT} in the non-fertilized treatment) and/or that the addition of organic fertilizers increased Zn availability e.g. through the build-up of complexes between Zn and dissolved organic carbon (Castilho et al., 1993; Bolan et al., 2004) slowing down the sorption of Zn on soil surfaces. The significant positive correlation between total Zn uptake by ryegrass with C inputs added with organic fertilizers in Strickhof reinforces the hypothesis that DOC-Zn were built and increased Zn availability and use in this soil. The increased N inputs by the organic fertilizers had probably little effect on plant growth due to the extremely high mineral N inputs added in all treatments. Finally, the addition of $ZnSO_4$ led to a decrease of $Zndf_{soil}$ while organic fertilizers did not affect soil Zn uptake. The replacement of nutrients taken up from the soil in a fertilized soil by nutrients added in a water-soluble form has been already observed for P in ryegrass growing on a soil with a high level of available P (Morel and Fardeau, 1989).

Conclusions

This is the first study quantifying the transfer of Zn from organic fertilizers and from the soil to plants on soils labeled with stable Zn isotope. The uptake of Zn present in the diverse organic fertilizers used in this work was similar to uptake of Zn added in a water-soluble form ($ZnSO_4$). The results confirm the important role of soil pH on soil and fertilizer Zn availability and use by plant. In addition, the results suggest that organic carbon added with the organic fertilizers also affects availability and use of Zn by plants under alkaline conditions.

From a methodological point of view the results of the present study showed that:

1. The indirect labeling method with stable isotopes was successfully used to determine the Zn contribution from mineral and organic fertilizers to the plant.

2. The isotope technique is needed when a soil amendment affects the Zn derived from the soil. The difference method (e.g. AUE) does not take into account variation of the soil source and might lead to an under- or overestimations of fertilizer use efficiency.
3. The indirect labeling method used in combination with a plant which can be cut several times, provides a qualitative evaluation of the Zn derived from the seeds.

Compared to radioisotopes, the use of stable isotopes is not subject to safety issues or legal regulations. It might be of interest to carry out this type of source tracing experiments also in the field. Moreover, the indirect labeling method with Zn stable isotopes would be useful to investigate the long-term effects of organic soil inputs. Finally, isotope enriched crops could directly be used to study the Zn bioavailability of food and feed.

III. STABLE ISOTOPE COMPOSITION OF ZINC IN DIFFUSIVE GEL GRADIENTS IN THIN FILMS (DGT) EXTRACTS: A METHOD TO DETERMINE ZN DERIVED FROM FERTILIZERS IN THE PLANT AVAILABLE POOL OF SOILS.

Abstract

The determination and the prediction of nutrient plant availability is important to optimize crop production. In previous studies, the amount of trace elements assessed with “diffusive gradients in thin films” (DGT) samplers was found to correlate with plant availability, independently of soil properties. In the present study, we applied an isotope dilution technique with ^{67}Zn indirect soil labeling to verify the ability of DGT extracts to assess soil Zn availability to plants. An acidic (Heitenried, pH_{H2O} 4.9) and an alkaline (Strickhof, pH_{H2O} 7.7) soil were labeled with ^{67}Zn-labeled and fertilized with cattle manure, poultry manure, dried sewage sludge (DSS) and water-soluble Zn ($ZnSO_4$). The DGT extracts were sampled on incubation soil, whereas for the ryegrass a pot experiment was set up. We hypothesized, that the DGT extracts and the ryegrass shoot biomass should have the same isotope composition, if both access the same isotopically exchangeable, plant available pool of soil Zn. The results from all treatments were pooled using the Bland-Altman approach, in order to determine the mean difference (± uncertainty) between the ^{67}Zn:^{66}Zn ratio measured in the DGT and the plant extracts. We found a mean difference of the ^{67}Zn:^{66}Zn ratio of 0.0018 (± 0.0018), which indicated that there was no significant difference between the isotope ratio of the DGT and the plant extract. These results suggest that the passive DGT samplers extracted the same Zn pool than ryegrass. In addition, the isotope composition of the DGT extract and the plant shoots was used to calculate the fraction of Zn derived from the fertilizer ($Zndf_{fertilizer\%}$) in both extracts. The mean difference between the $Zndf_{fertilizer\%}$ determined with the DGT and the plant extract was -0.64 (± 2.29, $Zndf_{fertilizer\%}$). The high uncertainty reflected the low resolution of DGT to measure the Zn derived from the fertilizer. However, the $Zndf_{fertilizer\%}$ of the DGT extract allowed to identify the same trends, regarding the soil and the fertilizer effects on $Zndf_{fertilizer\%}$.

Introduction

Zinc (Zn) is an essential micronutrient for plants. An accurate determination and reliable prediction of soil Zn availability for plants is thus crucial to implement fertilization strategies in crop production, in order to prevent Zn deficiency. In the past, several methods have been proposed to predict Zn plant tissue concentration or Zn plant uptake with the aim to determine if yield will be limited by Zn deficiency or if the plant will respond to a fertilizer input (Reuter and Robinson, 1997; Römheld, 2012; Sinclair and Edwards, 2008). Most of these methods are based on chemical soil extractions with neutral salt solutions (e.g. KCl, $MgCl_2$, $CaCl_2$, $NaNO_3$, NH_4NO_3) acids (e.g. oxalic acid, acetic acid, HCl) or chelating agents (e.g. DTPA, EDTA) (Beckett, 1989; Davis et al., 1995; Fujii and Corey, 1986; Lebourg et al., 1996; Lindsay and Norvell, 1978; Menzies et al., 2005). The Zn fractions and the amount of Zn extracted with such methods can significantly vary among different soil types (Marzouk et al., 2013; Young et al., 2000). The validity of soil extracts to assess plant Zn availability, is therefore limited to specific conditions for which they were calibrated for (Degryse et al., 2009). In contrast to conventional soil extractions, the "diffusive gradients in thin films" (DGT) method appeared to be a more reliable technique to determine plant availability of trace metals in soils, as the amount of Zn extracted with this technique generally correlates well with plant Zn tissue concentrations among different soil types (Degryse et al., 2009; Nolan et al., 2005; Tandy et al., 2011; Zhang et al., 2014; Zhang et al., 2001). The DGT sampler is made of a filter membrane, followed by a diffusion gel and Chelex resin layer (Zhang and Davison, 1995). The device can be placed directly onto moist soil to sample free- or ligand-bound ions from the soil solution (Gramlich et al., 2014). As the diffusion gel separates the binding sites of the resin layer from the soil, only diffusive Zn supply is captured (Degryse et al., 2009). Unlike chemical extractions, the DGT technique does not change the soil solution equilibrium. Therefore, the Zn fraction extracted with the DGT method is almost unaffected by the soil properties, and can be generalized to a wide range of soil types. However, the DGT sampler only explores a limited and unknown soil volume. DGT provides the Zn concentration of the soil volume surrounding the exposure window and cannot be extrapolated to the soil volume. During the deployment time (24 – 72 hours), DGT samplers function like plant roots and do not only access Zn in the soil solution but also takes into account the Zn resupply from the solid phase of the soil (Degryse et al., 2009; Tandy et al., 2011). Tandy et al. (2011) successfully used DGT to predict the Zn concentration in barley grown on a wide range of agricultural soils varying in pH, soil organic matter and soil structure. However, the suitability of the DGT method has only been verified by correlating the amount of Zn bound in the DGT sampler with Zn tissue concentrations.

For ryegrass, the isotopically exchangeable pool of soil Zn was found to be the main Zn source (Sinaj et al., 2004). Isotope dilution techniques can be used to assess the ability of methods

to extract plant available soil nutrients, provided that plant uses only isotopically exchangeable nutrient pool (Demaria et al., 2005; Fujii and Corey, 1986; Mason et al., 2013; Six et al., 2012). A plant growing on a soil labeled with Zn isotopes will have the isotope composition of the soil solution. If a chemical extraction method solubilizes only plant available Zn, the isotope composition of the soil extract and of the plant will be identical. However, if the extraction method also mobilizes Zn from the unavailable pool, the isotope composition of the extract will be diluted by the unlabeled Zn fraction. In other words, if a soil extract (chemical or resin extraction) has the same isotope composition as the plant grown in the same soil, it would be a proof that the extraction method and the plant take up Zn from the same soil pool. This "performance test" has already been realized with success by Six et al. (2012) for phosphorus on highly weathered P-deficient tropical soils. In this study, the specific activity (SA) of phosphorus ($^{33}P/^{31}P$) determined using several chemical extraction methods, ion exchange membranes and DGT, was compared with the SA of maize. Among those methods, DGT was found to match the best and the most consistently with the SA of the plant tissues. The same observation was made by Mason et al. (Mason et al., 2013), who compared the SA of wheat and three phosphorus soil tests (Colwell, resin and DGT) on fourteen Australian soils. To our knowledge, there is no comparable study for Zn. The first studies in which coupled DGT and stable Zn isotopes were combined, were published by Malinovsky et al. (2005) followed by Desaulty et al. (2017). Their aim was to use DGT in combination with stable Zn isotopes (natural abundance) to study geochemical processes and to trace anthropogenic pollution in aquatic systems. In both studies the diffusional isotope fractionation of Zn produced in the DGT diffusion gel was investigated and quantified (about −0.06‰ for $\delta^{66}Zn/^{64}Zn$) in Desaulty et al. (2017).

Isotope dilution techniques in labeled soil systems can be used to quantify the contribution of a fertilizer to the plant available pool of a nutrient (Frossard et al., 2011; McBeath and McLaughlin, 2013; Morel and Fardeau, 1991; Nanzer et al., 2014). Such approaches are of interest because they allow to evaluate the efficiency of fertilizers in the absence of a plant. With the indirect soil labeling method, the application of unlabeled fertilizers cause an isotope dilution of the isotopically exchangeable pool of the soil. If the latter is homogenously labeled, it is possible to quantify the amount of an element derived from the fertilization by mass balance (McBeath et al., 2013). For a detailed description of the stable isotope source tracing methodology, readers are referred to chapters 1 and 2 of the present thesis. The DGT extraction technique in combination with stable Zn isotopes presents a great potential to develop a laboratory method to evaluate the efficiency of fertilizers without the presence of a plant.

For the present study, we hypothesized that, as for phosphorus, the DGT sampler accesses the same Zn soil pool than the plant. This hypothesis is based on two assumptions: i) the diffusional Zn supply is the dominant process for plant uptake ii) the plant does not limit Zn

uptake (Desaulty et al., 2017). Therefore, we selected two agricultural soils with an average Zn status. The aim was to avoid extreme soil conditions such as Zn deficiency or contamination. Furthermore, Italian Ryegrass (*Lolium multiflorum*) was used as model plant, because this grass is known to access only the isotopically exchangeable Zn pool of the soil under Zn sufficient conditions (Sinaj et al., 2004). We compared the ^{67}Zn:^{66}Zn ratio of the DGT extract from an ^{67}Zn labeled soil incubation experiment with the ^{67}Zn:^{66}Zn ratio of Italian Ryegrass from an analogue pot experiment. For both approaches, an acidic (Heitenried, pH_{H2O} 4.9) and an alkaline (Strickhof, pH_{H2O} 7.7) soil were fertilized with water soluble Zn ($ZnSO_4$), cattle manure, poultry manure and dried sewage sludge (DSS). Furthermore, we investigated the possibility of using the isotope composition of the DGT extracts to calculate the contribution of the fertilizer to the plant available pool of the soil.

Material and Methods

For the detailed description of the soils, the pot experiment (growth trial), the sample processing and isotope analysis readers are referred to the second chapter of this thesis. Only the essential elements of the experiment are summarized below:

Pot experiment with Italian ryegrass

For the pot experiment two arable soils with different pH and total Zn content were selected. The soils are designated as "Heitenried" for the low pH ([d]pH 4.9, [e]Zn_{total} 54.1 mg kg^{-1}soil) respectively "Strickhof" for the high pH ([d]pH 7.7, [e]Zn_{total} 101.1 mg kg^{-1} soil). Both soils were labeled with ^{67}Zn and stored during two years before the experiment. Each pot was filled with 400 g of soil (DW equivalent). Three organic fertilizers (cattle manure, poultry manure, dried sewage sludge) and a water soluble ($ZnSO_4$) fertilizer were applied at a rate of approximately 1.5 mg Zn kg^{-1} soil. An unfertilized reference treatment was also prepared. Four replicates, here called "treatment replicates", were provided for each treatment (Figure 19). Italian ryegrass (*Lolium multiflorum,* var. Gemini*)* was sown and adequate amounts of macro- and micronutrients (except Zn) were supplied after germination and after each cut. The ryegrass was cut four times. For the purpose of this study, only the samples from the fourth cut were used, as the seed nutrient contribution to the plant becomes negligible after the third cut (Brookes, 1982; Bühler et al., 2003). The plant samples were mineralized by acid digestion. The Zn concentration the resulting extracts were analyzed by ICP-OES. The remaining volume of the plant extracts was processed by ion exchange chromatography to separate the Zn from the matrix, in order to reduce spectral interferences on Q-ICPMS during the isotope measurements.

Soil incubation experiment

"Incubation pots" were prepared in the same manner as the pots of the growth trial. However, only one pot per treatment was set up (Figure 19) and no ryegrass was sown. The incubated soil received only the first load of macro- and micronutrients added to the pot experiment because of the absence of nutrient export through biomass removal. The incubation experiment was performed under the same environmental conditions as the growth trial: a climate chamber with a daily photoperiod of 14 hours at 25 klx. Temperature and relative humidity (RH) were set to 24 °C and 60% during daytime and to 18 °C and 65% RH during

[d] pH in H_2O with 1:2.5 solid:liquid ratio
[e] HNO_3 microwave digested Zn fraction of the soil

night time, respectively. The water content was weekly adjusted to 80 % of the maximal water holding capacity of the soil (WHC_{max}: Heitenried = 386 g H_2O kg^{-1} soil, Strickhof = 446 g H_2O kg-^{1} soil). The incubation experiment was ended simultaneously with the growth trial after the fourth ryegrass cut, 48 days after sowing.

DGT soil extraction

DGT samplers were deployed was realized following the protocol described by Hooda et al. (1999). First 50 g (dry weight equivalent) of moist incubated soil were filled into a 50 ml beaker and watered to field capacity (100 % WHC). Four replicates per treatment, here called "processing replicates", were prepared (Figure 19). The containers were sealed with parafilm and placed into and pre-incubated at 24°C during 24 hours. The DGT samplers (Figure 18) were provided by DGT Research Ltd from Lancaster (UK). Prior deploying the DGT sampler, the soil slurry was gently stirred. Then the exposure area of the DGT sampler was rinsed with water and carefully placed on the soil surface, by assuring an optimal contact between the DGT filter and the soil. The beakers were sealed with parafilm and placed into an incubator at 24°C during 72 hours. Four separate DGT samplers (no soil contact) were kept in the same conditions, as blanks. After this incubation period, the DGT samplers were thoroughly rinsed to remove the soil particles. Finally, the DGT device was dismantled (Figure 18) and the resin layer was transferred into micro-centrifuged tubes filled with 1 ml HNO_3 1M to elute the Zn during 24h. The DGT extracts were purified by ion exchange chromatograph. Preliminary tests (data not shown) did not reveal significant spectral interferences during the mass spectroscopy measurements of DGT soil extracts. For this purpose, the soil from two additional incubation pots with unlabeled Heitenried, and Strickhof soil, repectively was used to extract Zn with DGT samples at natural abundance. The isotope composition of these extracts were compared to natural Zn abundances reported by Berglund et al. (2011) and did not show significant differences. The Zn concentration and the Zn isotope composition of the eluates were independently determined using Q-ICPMS.

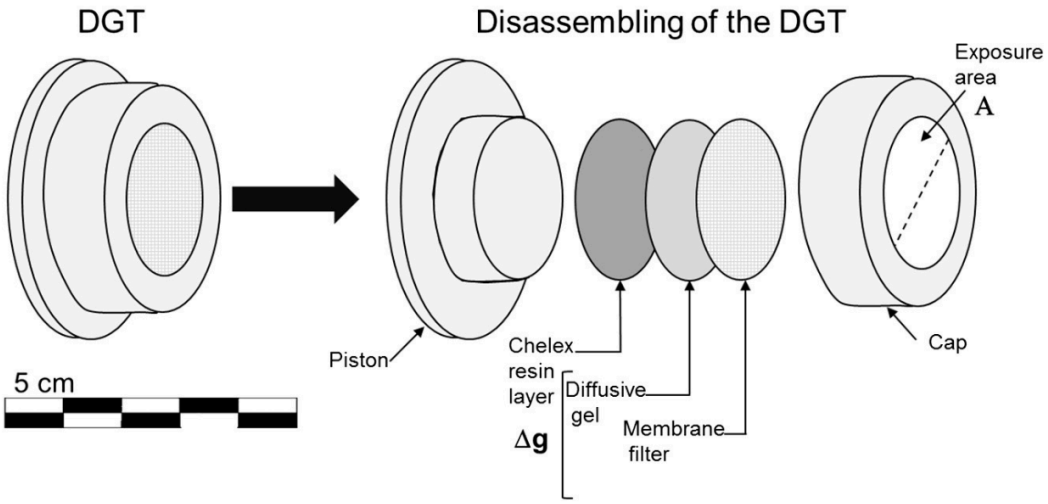

Figure 18: Disassembling of the diffusive gradients in thin films (Desaulty et al., 2017)

Calculations

The mass (*M*) of Zn bound in the resin gel was calculated following the equations from Zhang et al. (Zhang et al., 2004):

$$M = \frac{C * \left(V_{acid} + V_{gel}\right)}{f_e} \qquad \text{Eq. (1)}$$

where *C* is the Zn concentration (µg L^{-1}) in the DGT resin eluate, V_{acid} volume of HNO_3 used for the elution, V_{gel} the volume of the resin gel layer, and f_e the elution factor (0.8) for Zn. This elution factor takes into account that only 80 % of the Zn is eluted from the resin (Zhang and Davison, 1995). From the mass (*M*) of Zn bound in the resin gel, the time averaged Zn concentration (C_{DGT}) at the interface of the DGT exposure area and the soil can be calculated:

$$C_{DGT} = \frac{M * \Delta g}{D * t * A} \qquad \text{Eq. (2)}$$

Where Δg is the thickness of the diffusive gel plus the thickness of the filter (cm), D the diffusion coefficient of Zn in the gel (5.92 E^{-06} cm^2 s^{-1} at 24 °C), t the deployment time (s) and *A* is the exposure area of the DGT (cm^2).

Isotope analysis

The ^{67}Zn:^{66}Zn ratios of the DGT and the plant extracts were determined with a Q-ICPMS. For technical information about the instrument and a detailed description of the isotope analysis, the readers are referred to the second chapter of the thesis.

Zn derived from fertilizer

The percentage of Zn derived from the fertilizer in the DGT extract - and in the plant shoots was calculated by mass balance using Eq. 3. The terms of the equation described in Table 9. For the detailed derivation of the formula, readers are kindly referred to the first chapter of this thesis.

$$Zndf_{fertilizer\%} = \frac{{}^{66}Zn_{soil} * \left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink} - {}^{67}Zn_{soil}}{\left({}^{67}Zn_{fert} - {}^{67}Zn_{soil}\right) - \left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink} * \left({}^{66}Zn_{fert} - {}^{66}Zn_{soil}\right)} * 100\ (\%) \qquad \text{Eq. (3)}$$

Table 9: Terminology for Eq. 3.

Term	**Definition**
${}^{66}Zn_{fert}$	^{66}Zn fractional abundance (mol/mol) in the fertilizer
${}^{67}Zn_{fert}$	^{67}Zn fractional abundance (mol/mol) in the fertilizer
${}^{66}Zn_{soil}$	^{66}Zn fractional abundance (mol/mol) of the soil given by the reference treatment (plant or DGT extract)
${}^{67}Zn_{soil}$	^{67}Zn fractional abundance (mol/mol) of the soil given by the reference treatment (plant or DGT extract)
$\left(\frac{{}^{67}Zn}{{}^{66}Zn}\right)_{sink}$	^{67}Zn:^{66}Zn ratio of the fertilized treatment (plant or DGT extract)

Statistics

The experimental treatment structure is illustrated in Figure 19: the growth trial was made of four treatment replicates, whereas the DGT extracts where realized with four processing replicates from a single incubation pot per treatment. This design did not allow to perform conventional statistical analyses. In order to compare the ^{67}Zn:^{66}Zn ratios from the DGT- and the plant extracts, the data from all treatments was pooled and analyzed with a Bland-Altman approach (Altman and Bland, 1983; Giavarina, 2015). The Bland-Altman method allows to perform inter-method-comparisons illustrated by a Bland-Altman plot (Figure 22) which provides a mean difference (bias) between two methods and its limits of agreement (95 % confidence interval). These plots were computed using the software package R (Version 3.3.2) and the package "BlandAltmanLeh". Data distributions were checked for normality using Bland-Altman plots in combination with the Shapiro-Wilk test.

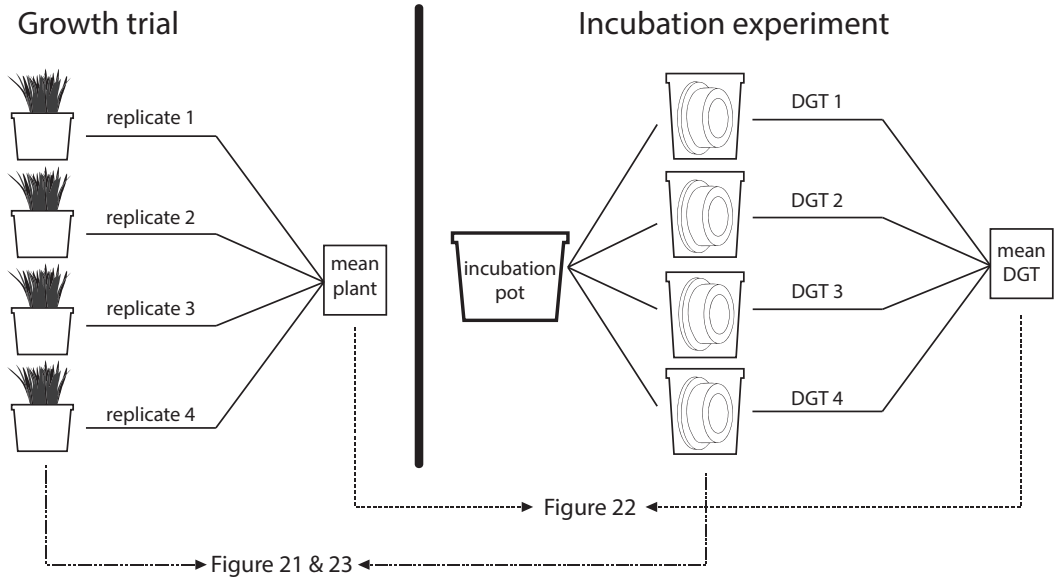

Figure 19: Experimental treatment structure for statistics: the growth trial (left) is made of four treatment replicates, whereas the DGT extracts were realized with four processing replicates from a single incubation pot for each treatment. The arrows indicate the link of the results with the corresponding Figure (Figure 21, 22 and 23) in the section "*Results and discussion*".

Results and discussion

DGT as predictor of Zn plant availability:

First of all, the total Zn plant uptake of the four cuts was positively correlated (r^2 = 0.87) with C_{DGT} (Figure 20). The Zn total uptake was between 621 µg kg^{-1} soil (Strickhof, reference treatment) and 865 µg kg^{-1} soil (Heitenried, poultry manure treatment), which indicates a sufficient Zn supply for plant growth, when compared to previous studies using ryegrass as model plant (Nanzer, 2012). Also, in the second chapter, we demonstrated that Zn fertilization did not lead to an increase in yield, which indicated that plant growth was not limited by Zn availability. Here, the uptake response showed that the plant was able to assimilate more Zn than the minimal amount required for an efficient plant growth. Our results were in accordance with previous studies, which found a positive and significant correlation between Zn concentration in plants and Zn measured by DGT (Degryse et al., 2009; Koster et al., 2005; Tandy et al., 2011; Zhang et al., 2001). However, the direct comparisons with existing literature is difficult, as past studies always considered plant Zn tissue concentrations instead of total plant uptake.

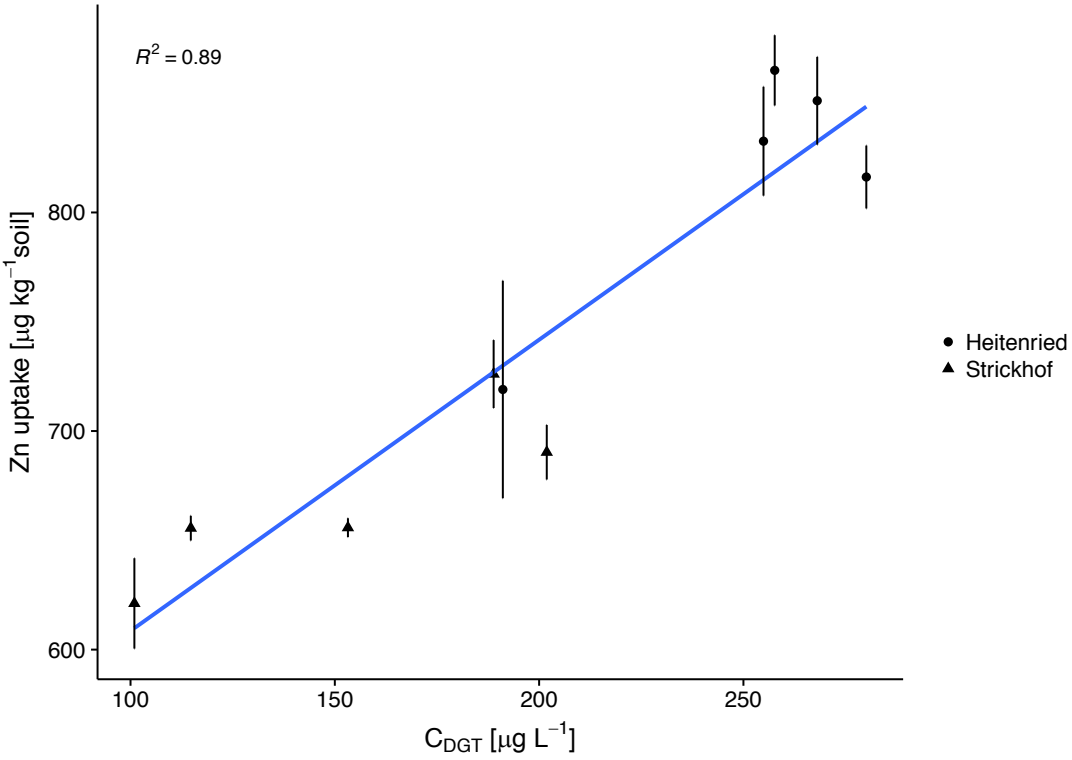

Figure 20: Correlation (n=10) between the total Zn uptake (*µg* kg^{-1} soil) of ryegrass shoots over the four cuts and C_{DGT} (µg L^{-1}), which represents the Zn concentration at the interface of the filter membrane of the DGT sampler and the soil. The total Zn uptake results from the mean (n = 4, ± standard error) from the treatment replicates, whereas the C_{DGT} shows the mean from the processing replicates (n = 4).

Figure 21 shows the ^{67}Zn:^{66}Zn-ratios measured in the DGT sampler and plant extracts from the incubation and the pot experiment, respectively. Here, the ^{67}Zn:^{66}Zn ratios of the reference treatments represent the Zn isotope composition of the ^{67}Zn labeled plant available soil pool. The addition of the unlabeled Zn fertilizers ($ZnSO_4$, cattle manure, poultry manure and dried sewage sludge) induced an isotope dilution of the Zn derived from the labeled soil in the ryegrass- (fourth cut) as well as in the DGT extract. The experimental design (Figure 19) did not allow to perform statistical analysis to compare the results of the individual treatments.

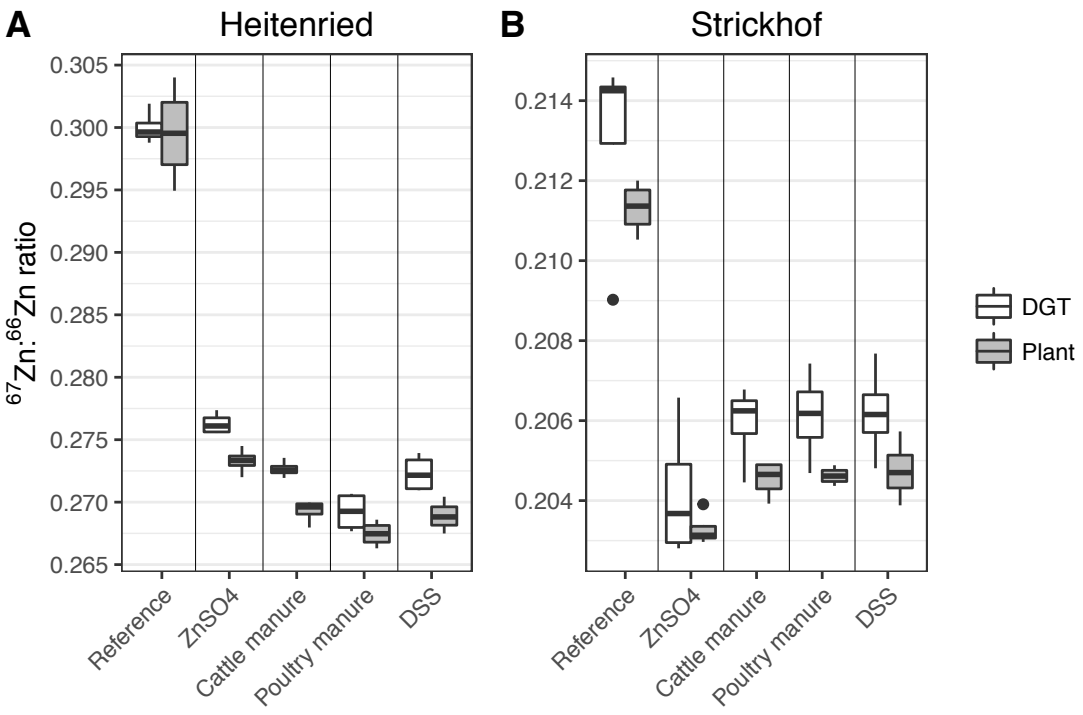

Figure 21: Boxplot representing the ^{67}Zn:^{66}Zn ratio in the DGT- versus ryegrass-extract of the fouth cut A) from Heitenried soil and B) from Strickhof soil. Dried sewage sludge (DSS).

All the data were pooled using the Bland-Altmann approach (Figure 22) in order to highlight the difference of the ^{67}Zn:^{66}Zn ratios between DGT and plant extracts. As illustrated in Figure 22, the Bland-Altmann plot (Figure 5) was built using the mean ^{67}Zn:^{66}Zn ratio from the plant extracts with four treatment replicates and the mean ^{67}Zn:^{66}Zn ratio from the DGT extracts with four processing replicates. For each treatment, these two means were averaged (Figure 22, x-axis) and plotted against their difference (Figure 22, y-axis). This method of comparison showed a positive bias of +0.0018 (± 0.0018) of the ^{67}Zn:^{66}Zn ratio, indicating that the DGT ^{67}Zn:^{66}Zn ratio measured with the DGT sampler was slightly higher than ^{67}Zn:^{66}Zn ratio found in the plant. A positive bias, in other words, a lower ^{67}Zn:^{66}Zn ratio in the plant, would suggest an isotope dilution in the plant extracts. On the one hand, it is possible that the plant extracts were slightly contaminated by Zn impurities contained in the chemicals used during the sample processing by ion exchange chromatography, as already mentioned in the first chapter of this thesis. On the other hand, an isotope dilution could also occur if the plant accesses soil Zn of the non-exchangeable pool through root exudates such as phytosiderophores. However, Sinaj et al. (2004) demonstrated that ryegrass (*Lolium multiflorum*) grown in Zn polluted acid soils did not access non-exchangeable Zn. Moreover, also Zn derived from the seeds could in

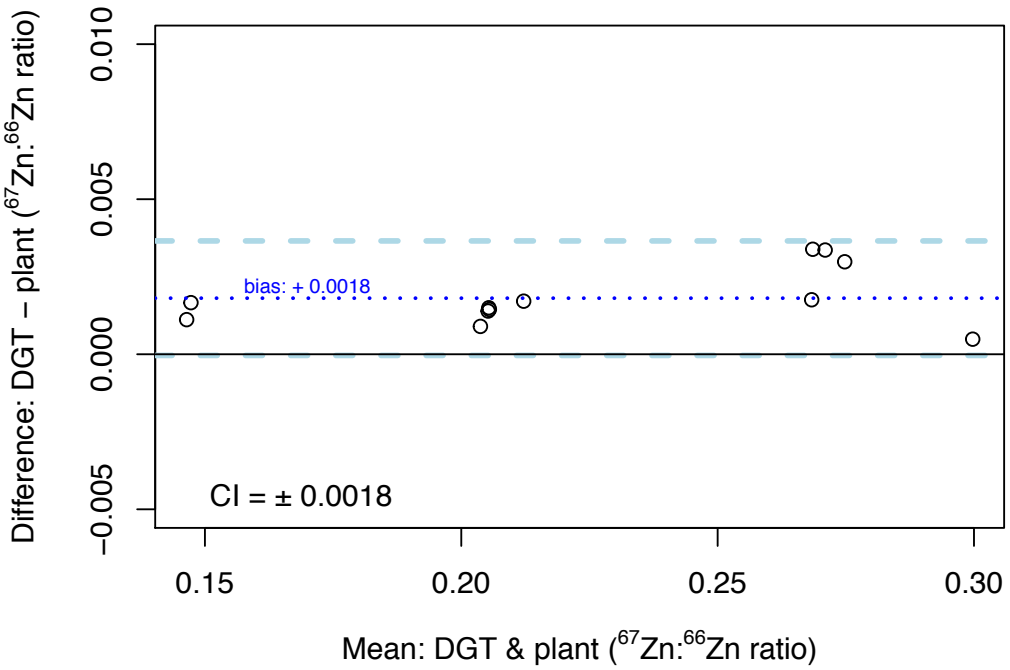

Figure 22: Bland-Altman plot for ^{67}Zn:^{66}Zn ratios measured in the DGT- and plant extracts. For each treatment, the ^{67}Zn:^{66}Zn ratio measured in the plant shoots and the DGT extracts was averaged (x-axis) and plotted against their difference (y-axis). The dotted line represents the bias (+0.0018) and the dashed lines the 95% confidence interval (CI = ±0.0018) of the bias.

principle result in isotope dilution in the plant. However, here we can reject this hypothesis, as only samples from the fourth cut were used.

It should be noted that, the bias observed in Figure 22 was close to the analytical detection limit of the Q-ICPMS: In the first chapter of the present thesis, the performance of the Q-ICPMS was evaluated by comparing isotope measurements of plant extracts with a MC-ICPMS, known to have a 100-fold higher precision than the Q-ICPMS. The instrumental comparison was also analyzed using the Bland-Altmann approach, which showed a limit of agreement of ± 0.0014 (^{67}Zn:^{66}Zn ratio). This range can be considered as the detection limit of the Q-ICPMS, below which differences in the ^{67}Zn:^{66}Zn ratios cannot be statistically. Considering, the instrumental uncertainty and the present limit of agreement of ± 0.0018 of the bias (Figure 22), it was not possible to confirm a significant difference between the DGT extracts and the plant extract regarding the ^{67}Zn:^{66}Zn ratios. Consequently, these results indicated that the plant and the DGT sampler had extracted from the same Zn soil pool. As a complement to Sinaj et al. (2004), our findings indicate that even in alkaline soil conditions, ryegrass does not seem to employ specific uptake strategies to access the non-exchangeable Zn pool of the soil.

Assessing the fraction of Zn derived from the fertilizer in the DGT extract

As the isotopic composition of Zn were shown to be identical in the plants shoots and in the DGT extracts, the fractional Zn contribution of the fertilizer ($Zndf_{fertilizer\%,}$ Eq. 3) within the DGT extract were calculated. These values were compared with the $Zndf_{fertilizer\%}$ calculated from the isotope composition of the plant extracts from the fourth ryegrass cut (see Chapter 2 of the present thesis). Both $Zndf_{fertilizer\%}$ calculated either by the plant shoots or the DGT extracts are shown in Figure 23. The differences between the ^{67}Zn:^{66}Zn ratios of the plant shoots and the DGT extracts (Figure 21) were propagated in the mass balance formula (Eq. 3) used to calculate the $Zndf_{fertilizer\%}$. The terms of this equation (Eq. 3) are composed of the ^{66}Zn and ^{67}Zn isotope abundances (mole mole^{-1}) of the two sources (fertilizer and soil) and the ^{67}Zn:^{66}Zn ratio of the Zn fertilized treatments. As the ^{66}Zn and ^{67}Zn isotope abundances of the fertilizer were constant, the resulting $Zndf_{fertilizer\%}$ could either be affected i) by the isotope composition of the soil (given by the reference treatments) or by ii) the ^{67}Zn:^{66}Zn ratio of the Zn fertilized treatments in plants shoots or DGT extracts (sink). For the Heitenried soil, the differences between the $Zndf_{fertilizer\%}$ in the DGT extracts and the plant shoots was driven by the difference in the ^{67}Zn:^{66}Zn ratios of the fertilized treatments, because the isotope composition of the reference treatment was similar in both extracts (Figure 21A). Here, the higher ^{67}Zn:^{66}Zn ratios of the DGT extracts seen in Figure 21A resulted in a lower $Zndf_{fertilizer\%}$ for all treatments. The highest divergence between the $Zndf_{fertilizer\%}$ in the plants shoots and DGT extracts was found in the dried sewage sludge (DSS) treatment, where the medians differed by 2.57 units. For the Strickhof soil, the ^{67}Zn:^{66}Zn ratios of both, the reference and the Zn fertilized treatments,

tended to be lower in the plants shoots, when compared to the DGT extracts (Figure 21B). Therefore, the median of the $Zndf_{fertilizer\%}$ in the DGT extracts and the plant shoots grown on the Strickhof soil were almost identical, except for the $ZnSO_4$ treatment. The experimental design did not allow to perform exhaustive statistical analyses. The variability of ^{67}Zn:^{66}Zn ratios in the DGT extracts of the Strickof treatments (Figure 21) was higher than that of the plant extracts. Neither the data of the present study, nor previous studies could be used to provide a reasonable explanation for this higher variability in the alkaline soil. As the mass balance formula is composed of several terms, the impact of such high uncertainties is even more important as they propagated within the calculation of the $Zndf_{fertilizer\%}$. By comparing $Zndf_{fertilizer\%}$ from the DGT extract with the $Zndf_{fertilizer\%}$ from the plant extract using the Bland-Altmann approach, we obtained a negative bias of -0.64 (DGT $Zndf_{fertilizer\%}$- Plant $Zndf_{fertilizer\%}$) and a limit of agreement of ± 2.29 ($Zndf_{fertilizer\%}$). This resolution is about five times weaker than the one described as acceptable for agronomic purposes (± 0.49, $Zndf_{fertilizer\%}$). This threshold value was defined in the first chapter of this thesis, by pooling the $Zndf_{fertilizer\%}$ from known Zn source tracing studies. The resolution of DGT to determine Zn derived from the fertilizer is low. However, the $Zndf_{fertilizer\%}$ in the DGT extracts revealed distinct fertilizer and soil effects on the Zn fraction derived from the fertilizer in the plant available Zn pool of the soil, following the same pattern as the $Zndf_{fertilizer\%}$ in plant shoots. The $Zndf_{fertilizer\%}$ in the passive DGT sampler confirmed that in high pH soil conditions (Strickhof) the Zn from the organic fertilizers was less available than from the mineral fertilizer ($ZnSO_4$). For the low pH soil (Heitenried) the opposite trend could be observed, as the $Zndf_{fertilizer\%}$ from the poultry manure was approximately 4 units higher than the water-soluble Zn fertilizer. One could speculate that soluble organic Zn complexes were highly available/extractible at low pH (Heitenried) and immobilized at high pH (Strickhof). The effect of the interaction between soil DOC (dissolved organic carbon) content and soil pH on the Zn concentration of the soil solution was reported by Castilho et al. (1993), who showed that the positive effect of DOC on Zn solubility was higher at low soil pH.

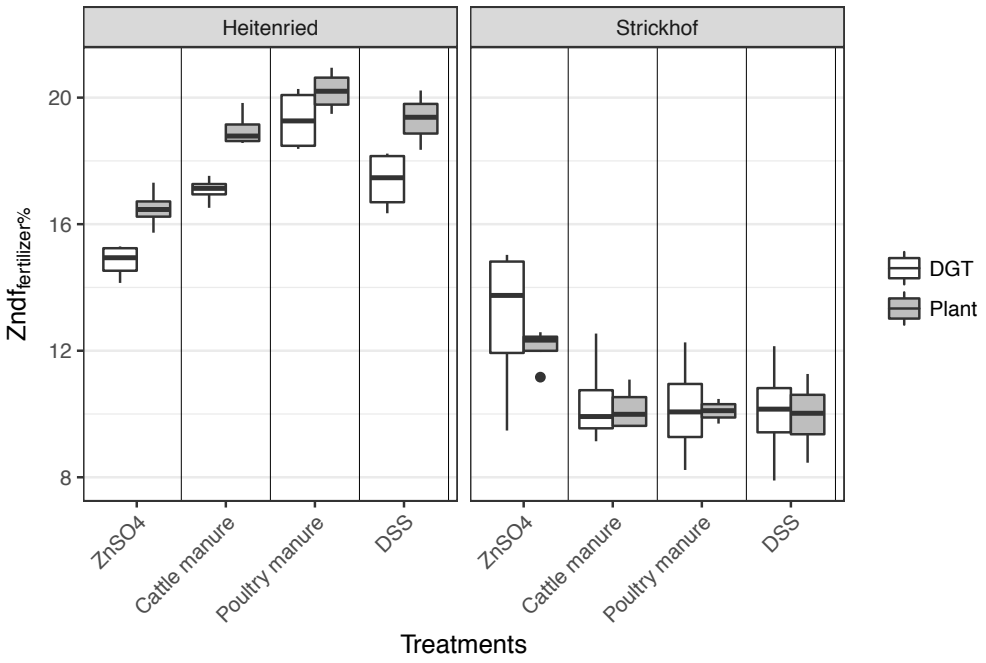

Figure 23: Percentage of Zn derived from the fertilizer (*Zndf*$_{fertilizer\%}$) calculated with the isotope composition of the DGT extracts (white boxes) and the isotope composition the plant extracts (grey boxes). Dried sewage sludge (DSS).

Conclusions

The results indicate that the DGT samplers accessed the same Zn soil pool as the ryegrass. The combination of DGT and isotope dilution techniques creates opportunities to further applications in soil/plant systems. On the one hand, the comparison of the isotope ratio of DGT extracts and plant tissues from isotope labeled soil systems might provide a tool to detect and to quantify the benefit of plant strategies as root exudation, which allow to access the non-exchangeable nutrient pools in the soil. For instance, it would be possible to use the indirect labeling method to compare the isotope composition of Zn efficient and Zn inefficient cultivars with the DGT extracts and quantify the amount of Zn derived from the non-isotopically exchangeable pool of the soil. This approach would provide an alternative method to the comparison of E- and L-values (Sinaj et al., 2004; Smolders et al., 1999), especially in cases where the concentration of water-extractable Zn, which is required for the determination of the E-value, is difficult to measure. DGT sampler might also be a promising technique for isotope source tracing, e.g. to evaluate or to compare fertilizer Zn contribution to the plant available pool in soils. Nevertheless, the experiment design of this study contained certain limitations, which did not allow to provide solid statistical statements. Even though these weaknesses did not put into question the validity of our results, there is still great potential to further develop DGT extractions in isotope enriched soil systems.

GENERAL DISCUSSION & OUTLOOK

The general discussion is divided into two sections. The first part contextualizes the importance of isotope tracers to investigate the fate of Zn in soil-plant systems and why isotope techniques should be implemented in agronomic research. The second section discusses the main outcomes of the thesis as follows:

i) Methodological adjustment of Zn isotope analysis in this specific field of research,
ii) Indirect soil labeling method for Zn source tracing in soil-plant systems,
iii) Diffusive gel in thin film extraction combined with the isotope dilution technique,
iv) The benefits of organic fertilizers for Zn plant-availability.

Research context

The importance of tracers to study Zn dynamics in the plant-soil system

Zinc "plant availability" has to be considered as a dynamic concept and as an integral part of the whole Zn cycle in a soil-plant system. Three parameters characterize Zn plant availability (Brümmer et al., 1986):

1. The quantity factor, which consists of the Zn pools potentially available for the plant. In agroecosystems, this factor is mainly influenced by system inputs (e.g. fertilization and atmospheric deposition) outputs (e.g. harvest, leaching, runoff), and the physicochemical properties of the soil, which control the Zn storage.
2. The intensity factor, representing the concentration of Zn ions in the soil solution, which can be taken up by the plant. Here, soil pH, which regulates the charge density of the soil particles is the most relevant factor.
3. and, the rate (reaction kinetics) of Zn transfer between the solid and liquid phases as well as to plant roots. Abiotic factors as temperature and soil water content but also biologic activity and plant physiology do regulate the fluxes between the different Zn pools.

It is important to mention that the total Zn content of a soil is at least two orders of magnitude larger than the amount of Zn in the soil solution (Diesing et al., 2008). Isotope dilution techniques with tracers are convenient to quantify small quantities of Zn circulating within a large pool.

Chemical or resin membrane soil extractions, also combined with speciation techniques (e.g. EXAFS: extended X-ray absorption fin structure spectroscopy) can be used to describe Zn soil

pools (Diesing et al., 2008; Sarret et al., 2004). However, chemical extractions disturb the soil/solution equilibrium and therefore cannot deliver a reliable information on the rate of Zn release to the soil solution. Furthermore, it is challenging to perform soil extractions directly in a soil-plant system as it is difficult to physically separate rhizosphere soil from the roots. Therefore, plant uptake is measured independently from soil extractions, which are generally carried out using bare soil. On the other hand, the factor "plant" alone does not necessarily reflect the plant availability but rather plant uptake, which can lead to experimental artifacts or data misinterpretations. Since plants can develop acquisition strategies, they can take up more Zn than available in the soil (Marschner et al., 1986). Furthermore, root growth and Zn uptake are affected by many other parameters, such as light, nitrogen, precipitation and temperature. For instance, increased plant Zn uptake as induced by organic soil fertilization, might not only be related to the additional Zn supply with the fertilizer, but also to significant changes of the physicochemical properties of the soil (decrease in soil pH) or to physiological modifications of the plant (increased biomass production) (Grüter et al., 2017). Isotopes have the advantage of being traceable in the entire system. Isotope dilution techniques associated with conventional soil extractions, speciation techniques or plant uptake studies allow to investigate Zn pools, measure Zn fluxes between pools and evaluate biotic or abiotic processes involved in those Zn fluxes. These approaches have been used to analyze phosphorus cycling in the soil-plant system (Bunemann, 2015; Fardeau, 1995; Frossard et al., 2011). The basic methodological approaches developed for phosphorus (e.g. the direct and indirect labeling the isotope exchange kinetics) can be transferred to zinc. Moreover, Zn offers multiple advantages for isotope dilution techniques: Zn possesses five stable isotopes, one long-life radioisotope and is not redox-sensitive. However, the radioisotope (^{65}Zn) is a gamma (2.22 Mev) and beta (0.33 Mev) emitter with a half-life of 244 days. These characteristics imply legal limitations for isotope tracing studies in the field as well as safety and waste disposal issues. On the other hand, isotope dilution techniques with stable Zn isotopes are exposed to several challenges. First, the risk of sample contamination is important because Zn is omnipresent in the environment. Second, stable Zn isotope analyses of environmental samples require significant resources, in terms of equipment and time. This can be a limiting factor, especially when large sample sets need to be analyzed.

The importance of Zn tracers in agronomic research

Biofortification research programs (e.g. HarvestPlus) but also organizations such as The International Rice Research Institute (IRRI) have the mission to develop agronomic or genetic strategies to i) improve crop tolerance to Zn deficiency and ii) to increase the mineral content and bioavailability in the edible part of crops (IRRI, 2006). These goals can be achieved either by increasing Zn uptake or through improving Zn translocation into the grain (Frossard et al.,

2000; Grusak and Cakmak, 2009; Hirschi, 2008; Palmgren et al., 2008). A better understanding of the Zn cycling in agricultural systems would allow to identify the Zn-pools, -fluxes, and related processes with the highest potential to contribute to crop biofortification. For instance, we observed a significant depletion of the DTPA and DGT extractable Zn pool of the top soil in treatments from long-term field trials fertilized only with nitrogen, phosphorus and potassium (Dürr-Auster, unpublished). These results could be explained by a negative Zn balance (Zn export > input) but also by the low buffer capacity of the soil to replenish the soil solution with Zn from the solid phase. Similar observations were made by Grüter et al. (2017) who suggested that long-term effects of Zn management practices do significantly influence the Zn plant availability. These findings show the importance of considering each factor as a component of the entire and dynamic Zn cycling system.

Isotope tracer techniques with so called “non-traditional” stable isotopes, such as Zn, were essentially developed in the geochemistry to study isotope fractionation processes (Hoefs, 1997). Even though the basic principles of isotope systems are common to all disciplines, isotope enriched source tracing in a soil plant system for agronomic purposes is different in many perspectives. First, fractionation processes can be completely ignored (Fry, 2007). Second, Zn source tracing in pot or field experiments are affected by contamination and high soil heterogeneity, which affects the experimental uncertainty. The samples (soil and biomass) are by nature incompatible with clean laboratories, which are commonly used for isotope analyses. Finally, pot and field experiments often generate large sample sets, which require significant resources for their analysis. Consequently, the analytical methods have to be adjusted for isotope enriched source tracing in soil-plant systems. To achieve this goal, interdisciplinary exchanges are required in order to integrate stable isotope analytic in this specific field of agronomic research.

What are the main outcomes of this thesis?

Isotope analysis: how to make it easy?

Source tracing can only succeed if the isotope composition of the two sources (plant available soil pool and fertilizer) and the sink can be distinguished from each other. In this thesis, we showed that in a ^{67}Zn enriched soil plant system, the Q-ICPMS (equipped with a helium reaction cell) was precise enough to fulfill this condition. Furthermore, the experimental variability of the isotope ratios from the ^{67}Zn-enriched treatments (RSD of approximately 0.5 %) was higher than the analytical precision of the MC-ICPMS (RSD of approximately 0.01%). In these conditions, we did not only conclude that the Q-ICPMS was precise enough, but also

that the benefit of the MC-ICPMS, in terms of analytical precision, was not relevant. In the present thesis, aspects such as analytical costs, ease of operation were not explicitly examined for the instruments. However, from our experience it was evident the Q-ICPMS analyses were faster and cheaper than those with the MC-ICPMS. Finally, MC-ICPMS instruments are most probably rarer than Q-ICPMS, the latter can also be of advantage in terms of accessibility.

In the first chapter, we showed that the experimental variability of the Zn isotope ratios significantly impacted the quality of the results. The growth trials and the sample processing were performed in ordinary facilities, which were not specifically designed for stable isotope experiments. Only, the ion exchange chromatography was realized in a room, that was thoroughly cleaned and where most of the objects made of metal were either removed or wrapped into polymer bags. These precautions were not only taken to prevent sample Zn contamination, but also to protect the infrastructure from acid vapors, which occur during the sample-extract evaporation. We highly recommend performing a risk assessment, as part of the planning of source tracing experiments with stable Zn isotopes, in order to identify the major sources of contamination. Special attention has to be paid to irrigation water, laboratory water and commercial acids and chemicals, as the Zn contents can significantly vary over time and from one batch to another.

We also showed that using ion exchange chromatography to separate the Zn from the sample-matrix significantly increased the quality of Zn isotope measurements on the Q-ICPMS. This procedure is essential to reduce spectral and matrix interferences occurring with the mass spectroscopy. For chromatographic separation we applied the protocol of Pinna et al. (2001), which was originally developed to measure ^{70}Zn. We applied this method systematically for all samples we processed. However, we believe that there is a potential to adapt the separation protocol specifically for ^{67}Zn enriched plant samples in order to reduce the number of elution steps. This simplification was already suggested by Araújo et al. (2017) and would considerably increase the sample throughput and save consumables. In the third chapter of this thesis, the Zn from the DGT extracts was not separated from the matrix prior the isotope measurements. This omission did not have any impact on the quality of the results. However, the DGT does not only extract Zn, but also several other cations and even ligands (Gramlich et al., 2014). In future studies, we recommend to always evaluate the matrix effect on the spectral interferences during isotope measurement of DGT ^{67}Zn-enriched soil extracts.

The fact that:

a) ^{67}Zn-enriched plant and soil extracts can be analyzed on widely-used instruments such as an Q-ICPMS,
b) non-specific infrastructure is required for the ^{67}Zn-enriched sample processing,

c) the simplification of the sample processing allows a comparatively high sample throughput with limited resources,

might allow to democratize the use of stable Zn tracers for the investigation of the Zn-cycling in soil plant systems.

The indirect labeling method with stable Zn isotopes

The isotope technique allowed us to highlight effects of the fertilizers on the Zn derived from the calcareous soil, probably induced by the addition organic matter. Aghili et al. (2014a) used ^{65}Zn as tracer to quantify the positive effect of green manure soil inputs on the Zn derived from soil in Zn deficient conditions. Similar studies pointed out the importance of organic carbon inputs on the transfer of Zn from the solid to the liquid phase of the soil (Grüter et al., 2017; Habiby et al., 2014; Soltani et al., 2014). However, in these latter studies the contribution of the Zn derived from the soil was not quantified because of the absence of isotope tracers. In the second chapter of this thesis, we showed that the conventional difference method (AUE), which compares plant Zn uptake of a fertilized treatment with a control treatment, can lead to under- or overestimations of the fertilizer use efficiency and cannot, by definition, reveal any changes of the Zn derived from the soil. As mentioned in the previous section, the technical and analytical simplification of the stable Zn isotope tracing techniques, might allow their use as a complement in traditional Zn studies. In the past, tracer techniques were already applied to investigate the fate of Zn in polluted soils (Ayoub et al., 2003; Diesing et al., 2008; Sarret et al., 2004). However, similar studies in unpolluted or Zn deficient soils are scarce. As for phosphorus, the indirect labeling method with stable Zn isotopes can be applied to measure E- and L-values (Frossard et al., 2011). Izquierdo et al. (2016) used ^{67}Zn to measured isotopically-exchangeable Zn in Zn deficient paddy soil, and found that the E-value of Zn decreases under anaerobic conditions but also that isotope dilution techniques are particularly challenging during the aerobic-anaerobic transition, as during flooding the phase transfer of Zn is faster than the isotope exchange. The measurement of L- and E-values with stable Zn isotopes could also be used to evaluate the effect of the soil Zn-solubilizing bacteria by incubating the soil with different strains or to test ability of different crop cultivars to increase Zn plant uptake with the help of specific mechanisms, such as the exudation of organic acids or phytosiderophores.

The indirect labeling technique with stable Zn isotopes also has drawbacks. Special attention has to be paid to the mass of isotopes added to the soil during the labeling. On the one hand, the label needs to be intense enough to be distinctively measurable in the sources and in the sink, on the other hand the isotope mass should not significantly impact the steady-state of the soil phases. The ^{67}Zn soil labeling approach used in the present study did not allow drawing conclusions about the Zn-status of the original soils. The large volume of nutrient solution

(several hundred liters) used to label our soil with ^{67}Zn might have affected many soil characteristics. Even though a large fraction of the added Zn was probably lost with the drainage, we estimated a potential increase in 30 % DTPA-extractable Zn due to the labeling procedure. Naturally, such an increase would strongly affect the steady-state of the exchangeable Zn fraction of the soil. However, these conditions do not invalidate of our results. The purpose of this study was mainly methodological. In further studies, we recommend to spike the soil with low amounts of highly enriched ^{67}Zn spikes in order to minimize the disturbances of exchangeable Zn pool of the soil. Prior to soil labeling, it is essential to establish a mathematical model with the estimated source contributions and the achievable analytical precision in order to determine the minimum amount of spike required and to evaluate the impact of the spike on the Zn status of the soil. Finally, the indirect labeling method requires a sufficient incubation time to allow the isotope-label to distribute homogenously within the exchangeable Zn pool of the soil.

DGT, the future of soil extractions?

In the third chapter of this thesis we used an isotope dilution approach to assess the ability of the DGT technique to extract the isotopically exchangeable Zn pool of soils, which is the main Zn source for ryegrass in non-deficient Zn conditions (Sinaj et al., 2004). The ^{67}Zn:^{66}Zn ratio of the ryegrass shoots and the DGT extract corresponded, which indicated that the plant and the DGT device extracted the same isotopically exchangeable Zn pool. Even though, previous studies already demonstrated the suitability of DGT techniques to predict Zn plant availability (Degryse et al., 2009; Tandy et al., 2011) the isotope dilution technique represents the ultimate quality check for soil extraction methods (Demaria et al., 2005). The combination of DGT and isotope dilution techniques also open the door to new applications. In this work, we demonstrated that DGT can be used to determine the Zn fertilizer contribution in the soil solution and that this fraction corresponded to the percentage of Zn derived from the fertilizer in ryegrass. Applying DGT in incubation experiments is less demanding in terms of resources, than a growth trial. Therefore, one could imagine the use of DGT devices for routine laboratory tests, not only to predict nutrient plant availability, but also assess fertilizer use efficiency. Furthermore, the DGT technique could be applied to quantify the ability of different crop cultivars to increase Zn uptake using strategies such as root exudation of organic acids or phytosiderophores (Marschner et al., 1986). In this case, DGT would be an alternative method to the determination of to E- and L-values (Sinaj et al., 2004). A similar approach could be used also to investigate the effect of biotic or abiotic changes on the isotopically exchangeable pool, as for instance the effect of soil inoculation with Zn solubilizing bacteria.

The benefits of organic fertilizers for Zn plant availability

In the second chapter of this thesis, we showed that Zn present in different organic fertilizers was as available for ryegrass as mineral Zn added in water-soluble form ($ZnSO_4$). The results are furthermore suggesting that in alkaline soil conditions, the addition of organic carbon also affects plant uptake of Zn derived from the soil. We hypothesized that the addition of organic fertilizers favor the build-up of dissolved organic carbon (DOC) – Zn complexes, slowing down the Zn sorption on soil surfaces rendering the Zn more easily available for plant uptake. The significance of DOC-Zn complexes on Zn plant uptake was already observed for green manure soil amendments (Aghili et al., 2014a; Habiby et al., 2014; Soltani et al., 2014). Hence, organic fertilizers might a great potential for agronomic biofortification in areas with low Zn plant availability. One the one hand, organic resources such as animal manures, household compost, crop residues or cover crops can be more available in many rural areas than commercial fertilizers. On the other hand, soil amendments with organic fertilizers might also favor phase transfer between soil matrix and solution and increase the amount of plant available soil Zn.

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