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Green manure and long-term fertilization effects on soil zinc and cadmium availability and uptake by wheat (*Triticum aestivum* L.) at different growth stages

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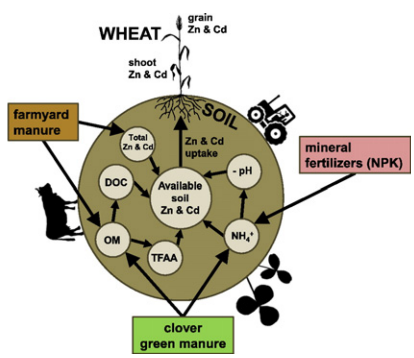
HIGHLIGHTS

- Long-term farmyard manure application dominated wheat grain Zn concentrations.
- Clover green manure increased wheat yields but not grain Zn concentrations.
- Organic matter application increased available soil Zn and Cd.
- DGT-available soil Zn and Cd are sensitive to short-term fertilizer application.
- DGT-available soil Cd positively correlated with wheat shoot and grain Cd.

GRAPHICAL ABSTRACT



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ABSTRACT

Zinc (Zn) deficiency in human populations depending on cereals as a main source of Zn is a global malnutrition problem. In this field study, we investigated the potential of green manure application to increase soil Zn availability and wheat grain Zn concentrations (biofortification) on a Luvisol with different long-term fertilizer management. We also studied cadmium (Cd), as wheat is a major contributor of this undesired non-essential element to human diets. Clover (*Trifolium alexandrinum* L.), mustard (*Sinapis alba* L.) or no green manure was grown on field plots which had been managed with farmyard manure or mineral fertilizers for 65 years in Switzerland. After green manure incorporation into the soil, spring wheat (*Triticum aestivum* L.) was grown on all plots. The "diffusive gradients in thin films" (DGT) method and DTPA extraction were used to compare soil Zn and Cd availability among the treatments. In contrast to mustard, clover increased soil mineral nitrogen concentrations and

Abbreviations: DOC, Dissolved organic carbon; DGT, Diffusive gradients in thin films; DTPA, Diethylene-triamine-pentaacetic acid; FLOW, Wheat flowering; FYM, Farmyard manure treatment of the ZOFE long-term field trial; GREEN, Split-plot factor with levels NGM, SIN and TRI; HARV, Wheat harvest; INIT, Initial soil sampling; NGM, Control treatment without green manure; NPK, Mineral fertilization treatment of the ZOFE long-term field trial; SIN, *Sinapis alba* L. (white mustard); FERTILIZER, Whole plot factor with levels NPK and FYM; SOW, Wheat sowing; TFAA, Total free amino acids; TILL, Wheat tillering; TRI, *Trifolium alexandrinum* L. (berseem clover); ZOFE, Zurich Organic Fertilization Experiment A493.

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Biofortification
DGT
Farmyard manure
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Non-legume

wheat biomass; however, neither increased grain Zn concentrations. DGT-available Zn and Cd increased temporarily after both farmyard manure and mineral nitrogen fertilizer application. Higher DTPA-extractable soil Zn and Cd, lower wheat grain yields, but higher grain Zn concentrations were obtained with farmyard manure compared to mineral fertilizers, independent of the green manure treatment. Farmyard manure added Zn, Cd and organic matter that increased the soil binding capacity for Zn and Cd. The decomposition of clover residues caused higher wheat grain yields, but only marginally lower grain Zn concentrations. The absence of a stronger dilution of grain Zn was probably due to organic acid and nitrogen release from decomposing clover, which facilitated Zn uptake by wheat. The study revealed that both long- and short-term field management with organic matter alters soil Zn and Cd concentrations but that the long-term effects dominate their uptake by wheat, in Zn sufficient soil.

1. Introduction

Zinc (Zn) deficiency is a global human malnutrition problem, often called "hidden hunger" (Stein, 2010). An estimated sixth of the world's population is at risk of inadequate zinc intake (Wessells and Brown, 2012), and an annual death toll of about 116,000 children under the age of five has been attributed to Zn deficiency (Black et al., 2013). Zinc malnutrition is generally due to insufficient dietary Zn intake, and is most prevalent in populations depending on staple foods based on cereals such as wheat, rice and maize as the primary source of this essential micronutrient (Pfeiffer and McClafferty, 2007; Welch and Graham, 2004). Although cereals, and especially modern high-yield cultivars, are poor sources of bioavailable Zn, they still represent a major dietary Zn source, especially in low-income countries (Cakmak, 2008). Next to rice, wheat is the most important cereal for human nutrition, and with a production of >740 Mt in 2016, global wheat production is still increasing (FAO, 2016).

The problem of low grain Zn concentrations is exacerbated by inadequate selection of wheat cultivars and by the fact that many soils on which wheat is grown are low in plant-available Zn. This can be due to low total soil Zn concentrations (e.g. in sandy and strongly leached tropical soils) or to factors that limit the availability for plant uptake (phytoavailability) of soil Zn which is actually present in sufficient amounts (e.g. in calcareous soils). The most important soil factors leading to low availability of Zn in soils are high pH, high contents of calcium carbonate, clay, iron and aluminium oxides and low moisture content (Alloway, 2009; Cakmak, 2008).

Given the important role of wheat-based staple foods in many regions with high prevalence of Zn deficiency, enhancing the density of bioavailable Zn in wheat grains is a major global strategy in fighting Zn malnutrition. One option in this strategy, known as biofortification, is genetic improvement of cultivars for increased accumulation of bioavailable grain Zn. A complementary option is agronomic biofortification through improved cultivation practises (Cakmak, 2008) such as soil and foliar Zn fertilization which were both shown to be effective for increasing wheat grain Zn concentrations (Cakmak et al., 2010; Zou et al., 2012). Nitrogen (N) application was also found to be beneficial for wheat Zn accumulation, and this effect was primarily attributed to the role of N as component of metal transport and storage proteins (Erenoglu et al., 2011; Kutman et al., 2011; Kutman et al., 2010) and to the acidification of the soil through nitrification if N fertilizer is added in the form of ammonium (Lorenz et al., 1994). The potential of organic matter application in enhancing Zn phytoavailability in soil and thereby its accumulation in crop plants has received little attention though.

Application of organic matter in the form of farmyard or green manures leads to the release of decomposition products such as water-soluble organic ligands (e.g. citrate, oxalate, malate) (Franchini et al., 2001; Jones et al., 2003) which can complex trace elements, such as Zn, bound to soil particles and thus increase their solubility (Evans, 1991). Organic matter also affects the activity and abundance of soil microorganisms such as arbuscular mycorrhizal fungi which can enhance plant Zn uptake (Altomare and Tringovska, 2011; Cavagnaro, 2008; Lehmann et al., 2014; Ryan and Angus, 2003). Furthermore, organic matter improves soil structure and thereby allows plant roots to explore a larger soil volume (Mäder et al., 2002), it increases soil moisture retention (Hudson, 1994), which facilitates nutrient transport to the plant roots and it can be a source of Zn.

Green manures are plant residues which are incorporated into soil while still green or at maturity (Fageria, 2007). Only a few studies have investigated their effects on Zn uptake by subsequent crops. In a pot experiment with a Zn-deficient soil from Iran, Aghili et al. (2014) found higher soil Zn availability, lower pH and increased Zn accumulation in wheat grains after application of red clover and sunflower green manure to the soil. They used ^{65}Zn-labelled green manure to quantify the amount of Zn taken up by wheat derived from the green manure and the soil. In a field study conducted by Soltani et al. (2014) on the same type of Zn-deficient soil, green manure treatments with sunflower, sudan grass, red clover and safflower increased dissolved organic carbon (DOC) and amino acid concentrations in rhizosphere soil solution samples as well as grain Zn concentrations in wheat. Similar results were also obtained in a pot experiment using the same soil and green manure species (Habiby et al., 2014).

Farmyard manures often contain considerable amounts of metals such as Zn and Cd (Nicholson et al., 1999), and many agricultural soils are actually at risk of pollution by these trace elements when excessive quantities of manure are applied long-term (Bolan et al., 2004; Luo et al., 2009; Nicholson et al., 2003). EDTA- and DTPA-extractable soil Zn and Cd increased upon long-term application of farmyard manure (Benke et al., 2008; Li et al., 2007; Lipoth and Schoenau, 2007; Nikoli and Matsi, 2011; Richards et al., 2011; Wang et al., 2016). Few studies however investigated the effect of farmyard manure application on Zn and Cd accumulation in wheat grains. In the famous Broadbalk Wheat Experiment at Rothamsted, higher wheat grain Zn (Fan et al., 2008) but lower Cd concentrations (Jones and Johnston, 1989) were found under long-term farmyard manure application compared to mineral fertilization. In field trials, long-term application of cattle manure increased wheat grain Zn concentrations depending on soil properties, but did not affect grain Cd (Hamner and Kirchmann, 2015). In another field study, Lipoth and Schoenau (2007) and Wang et al. (2016) found increased Zn and Cd concentrations in wheat straw and grains, depending on the soil and type of farmyard manure applied. However, Tlustoš et al. (2016) and Li et al. (2007) observed no increase in Zn and Cd accumulation in wheat straw and grains after manure or compost application. They attributed this partly to the "dilution" of both elements in a larger biomass, a concept described by Jarrell and Beverly (1981). These studies showed that the observed effects largely depended on the soil type, the composition of farmyard manure applied and the duration of application.

Wheat-based foods also contribute substantially to human cadmium loads (EFSA, 2012). Cadmium is chemically similar to Zn, but has no biological functions and is toxic to humans under chronic cadmium exposure. High human Cd intake is primarily related to the consumption of food produced from crops grown on soils of high phytoavailable Cd(McLaughlin and Singh, 1999). Cadmium pollution of agricultural soils has primarily resulted from Cd-containing mineral P fertilizer applications (Smolders and Mertens, 2013). As both, antagonistic (Hart et al., 2002; Khan et al., 2014; Khoshgoftarmanesh and Chaney, 2007; Olsen and Palmgren, 2014) and synergistic (Köleli et al., 2004) effects between Zn and Cd have been reported in their uptake by plants, it is important to also examine Cd accumulation in studies on wheat Zn biofortification.

In this study, we performed a field experiment to investigate the potential of green manure application to increase soil Zn availability and wheat grain Zn concentrations on soil with different long-term fertilizer management.

The experiment was carried out on plots of a Swiss long-term field trial which had been fertilized with either mineral fertilizers or farmyard manure for the past 65 years (Oberholzer et al., 2014) on a loamy acidic soil under temperate climate. A non-leguminous and leguminous green manure plant was grown on different subplots of each plot, including subplots without green manure, followed by spring wheat as the main crop. The legume *Trifolium alexandrinum* L. (berseem clover) and a Brassicaceae, *Sinapis alba* L. (white mustard), were chosen for the experiment, as they are widely applied green manure plants in Switzerland. On plots treated with farmyard manure we expected i) wheat to produce less biomass because of lower soil N availability than on mineral fertilized plots, but also ii) higher concentrations of Zn and Cd in grains due long-term inputs of Zn, Cd and organic carbon and therefore more available soil Zn and Cd. After the application of clover green manure, we expected i) higher wheat yields compared to the control due to the N fertilization effect and ii) higher wheat grain Zn and Cd concentrations induced by the release of N and organic ligands from decomposing residues, increasing soil Zn and Cd availability.

2. Materials and methods

2.1. Experimental design

The study was conducted from August 2014 to July 2015 on plots of the long-term agricultural field trial "Zurich Organic Fertilization Experiment" (ZOFE) at Zürich-Reckenholz (47°25′37″N, 8°31′6″E, 440 m asl) in Switzerland. The trial, which was established in 1949, includes 12 treatments of different organic, inorganic and combined fertilization regimes, which are all replicated in 5 blocks on the same field (Oberholzer et al., 2014). Apart from fertilization management, the entire field has been managed conventionally in the same way with an 8-year crop rotation scheme (Table 1). The soil had been classified as a Luvisol and its texture as a sandy loam according to WRB soil classification (IUSS Working Group WRB, 2014; Oberholzer et al., 2014). It is acidic and free of carbonate with a pH($CaCl_2$) of 5.2 (Table 2).

For our experiment, we used the plots treated with farmyard manure (FYM) and mineral fertilizer (NPK). Over the period of the experiment (August 2014 – July 2015), the total precipitation was 1017 mm and the mean air temperature 10.7 °C (Source MeteoSwiss). The daily mean soil temperature at 10 cm depth reached a minimum of 0.4 °C in February 2015 and a maximum of 26.7 °C in July 2015.

Each of the 5 NPK and 5 FYM plots was subdivided into three subplots for the green manure treatments. The three green manure treatments, allocated to the subplots of each plot as indicated in Fig. 1, were the cultures of *Sinapis alba* L. (SIN) and *Trifolium alexandrinum* L. (TRI) and a control treatment with no green manure plant (NGM). Thus, the experiment had a split-plot design with the two levels NPK and FYM of the whole-plot factor FERTILIZER and the three levels NGM, SIN, and TRI of the split-plot factor GREEN.

Table 1
Crop rotation on the experimental plots from 2008 till 2015.

Year	Crop (long-term rotation)
2008	spring barley
2009	grass-clover ley
2010	grass-clover ley
2011	winter wheat + intercrop
2012	maize
2013	maize
2014	winter wheat + intercrop
2015	spring wheat

Table 2
Initial soil characteristics (0–20 cm) of the mineral fertilized (NPK) and farmyard manure treated (FYM) plots.

Parameter	NPK	FYM
pH ($CaCl_2$)	5.18a	5.27a
Clay (g kg^{-1})	130a	108a
Texture[a]	Sandy loam	Sandy loam
Total C (g kg^{-1})	8.52b	9.68a
Total Cd (μg kg^{-1})	227b	315a
Total Cu (mg kg^{-1})	16.7b	21.2a
Total Fe (g kg^{-1})	15.3a	15.1a
Total K (g kg^{-1})	1.07a	0.95a
Total Mn (g kg^{-1})	0.648a	0.529b
Total N (g kg^{-1})	0.889a	0.967a
Total P (g kg^{-1})	0.729a	0.697a
Total S (g kg_{-1})	0.142a	0.155a
Total Zn (mg kg^{-1})	58.6b	77.9a
DTPA-extractable Cd (μg kg^{-1})	67.3b	115.3a
DTPA-extractable Cu (mg kg^{-1})	3.11b	4.82a
DTPA-extractable Fe (mg kg^{-1})	86.8a	100.8a
DTPA-extractable Mn (mg kg^{-1})	37.1a	37.9a
DTPA-extractable Zn (mg kg^{-1})	1.06b	2.28a
DGT-available Cd (μg L^{-1})	0.194a	0.272a
DGT-available Cu (μg L^{-1})	1.65b	3.08a
DGT-available Mn (μg L^{-1})	47.0a	49.3a
DGT-available Zn (μg L^{-1})	7.06a	13.74a

Values shown are means of 5 plot replicates. Means with same letter are not significantly different at $p \leq 0.05$ according to Student's *t*-test.
[a] (IUSS Working Group WRB, 2014).

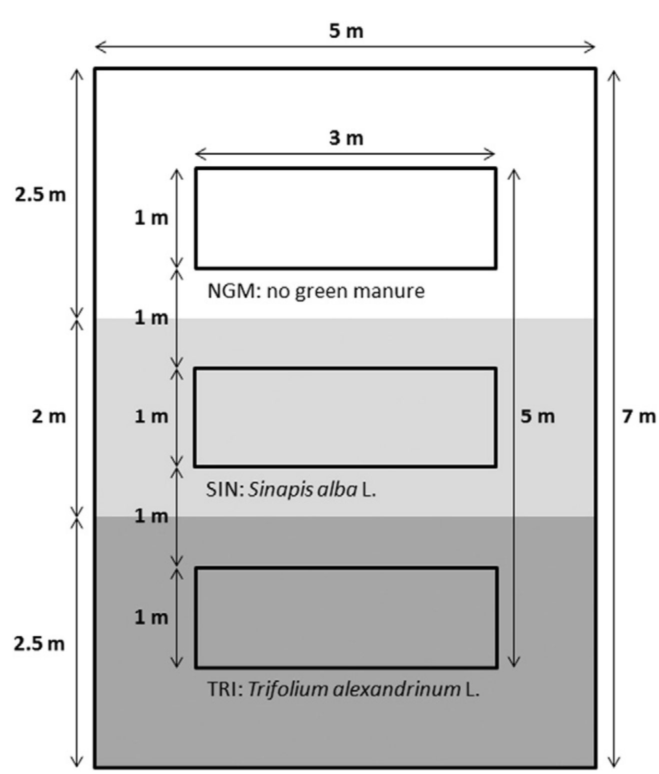

Fig. 1. Scheme of experimental plot (35 m^2) divided into three subplots for green manure treatments (NGM: no green manure (control), SIN: *Sinapis alba* L., TRI: *Trifolium alexandrinum* L.), indicated by different shades of grey. Samples were taken from the central 3 m^2 of each subplot only.

The green manure plants were sown in August 2014 after ploughing and harrowing of all plots, following a culture of winter wheat (Table 1). Sowing density was 23 kg ha^{-1} for *Sinapis alba* L. (cv. Albatros) and 25 kg ha^{-1} for *Trifolium alexandrinum* L. (cv. Elite II). The green manure plants died during the winter due to frost and were ploughed into the soil (20 cm depth) in March 2015. Following seedbed preparation by harrowing, spring wheat (*Triticum aestivum* L., cv. Fiorina) was sown on the whole plot at a density of 425 grains m^{-2} and harvested in July 2015 after 127 days of growth.

Cattle farmyard manure had been applied to the FYM plots every two years at a rate of 5000 kg dry organic matter ha^{-1}, which is determined by loss on ignition. During our experiment, it was applied 19 days before wheat sowing (with a nutrient input of 64 kg P ha^{-1}, 257 kg K ha^{-1}, 26 kg ha^{-1} mineral N, 219 kg ha^{-1} organic N, see Table S.1). Mineral fertilizer was applied annually. The NPK plots received mineral phosphorus (P) mainly in the form of superphosphate (22 kg P ha^{-1}, Landor P26, Switzerland) and potassium (K) in the form of K_2SO_4 (71 kg K ha^{-1}) 12 days before wheat sowing. Until 2010, mineral P was added as basic slag, a by-product of steel production, which is no longer available. Mineral nitrogen was applied in three doses: 50 kg N ha^{-1} in the form of $(NH_4)_2SO_4$ at the three-leaf-stage and two times 40 kg N ha^{-1} in the form of NH_4NO_3 at the first-node-stage and at the beginning of flowering.

2.2. Soil and plant sampling

Initial (INIT) soil samples were taken in August 2014 by an Edelman auger (0–20 cm), 13 days after harvest of the previous crop (winter wheat). For determination of soil pH, texture, total and available element concentrations, 12 samples per plot were taken on a grid, mixed and subdivided into two subsamples. One subsample was kept at 4 °C for later analysis, the other one was immediately dried at 40 °C, sieved to 2 mm and finely ground with a vibratory disc mill RS 1 (Retsch, Germany). For the determination of dissolved organic carbon (DOC), total free amino acids (TFAA) and mineral nitrogen, five samples were taken from each subplot, mixed and extracted on the same day.

Samples of the green manure plants were collected after 104 days of growth for yield estimation and element analysis. Six times 0.2 m of mustard rows, representing 0.24 m^2, and three squares of 0.1 m^2 of clover were harvested from each subplot with green manure plants. The samples were dried at 60 °C until constant weight and milled (RS 1, Retsch, Germany).

The soils were sampled again at wheat sowing (SOW), tillering (TILL, 52 days after sowing) and flowering (FLOW, 93 days after sowing), taking 5 samples with an Edelman auger (0–20 cm) from each subplot, which were bulked, mixed, and split into 3 replicate subsamples per composite sample. One subsample was extracted on the day of sampling, one kept at 4 °C and one immediately dried at 40 °C, sieved to 2 mm and ground (RS 1, Retsch, Germany).

Shoot samples of the wheat plants were collected at tillering, flowering and harvest (HARV, 127 days after sowing). The term shoot is used here to denote the entirety of above-ground plant parts at tillering and flowering but excluding ears at harvest, while the term chaff denotes all parts of the ears including rachis, glumes and awns but not the grains. At tillering and flowering, all wheat plants on a 1 m long section of a row were cut 1 cm above the ground in the middle of each subplot, dried at 60 °C, weighed and milled. We also recorded the number of tillers at the first two samplings. At harvest, the same sampling procedures were applied, but three sections of 1 m length instead of only one were sampled per subplot and the samples were divided into shoots, grains and chaff. The thousand-kernel-weight (TKW) was determined from two sets of 500 grains per sample. Shoot and grain samples were milled for analysis using a vibratory disc mill RS 1 (Retsch, Germany) for the shoots and a mixer mill MM 200 (Retsch, Germany) for the grains.

2.3. Soil analysis

Total soil N and C concentrations of ground initial soil were analysed with an NCS analyser (FlashEA 1112 Series, Thermo Fisher Scientific Inc., Waltham, MA USA). Soil texture was determined using the sedimentation method with 2 mm sieved initial soil as described by FAL et al. (2008). Soil pH was measured in 0.01 *M* $CaCl_2$ on ground soil (INIT, SOW, TILL and FLOW) with a soil-to-solution ratio of 1:2.5 (FAL et al., 1999).

To determine pseudo-total element concentrations of other elements in initial soil samples, aliquots of 1 g of ground soil were digested in 2 mL of nanopure water and 8 mL of aqua regia (2 mL of 70% HNO_3 and 6 mL of 37% HCl) according to FAL et al. (2015b) at 120 °C for 90 min in a DigiPREP MS digestion system (SCP Science, Quebec, Canada). International Soil-Analytical Exchange reference soils 921, 951 and 997 were used to validate the results (http://www.wepal.nl/).

Extraction with diethylene-triamine-pentaacetic acid (DTPA) was used to characterize the fractions of soil Cd, Cu, Fe, Mn and Zn available for plant uptake. DTPA extraction is commonly used to assess the phytoavailability of trace metals in agricultural soils. Extracts were obtained by suspending ground soil (INIT, SOW, TILL and FLOW) in 0.005 *M* DTPA, 0.01 *M* $CaCl_2$ and 0.1 *M* triethanolamine (TEA) at pH 7.3 at a soil-to-solution ratio of 1:2 and shaking it for 2 h according to Lindsay and Norvell (1978). International Soil-Analytical Exchange soils 921 and 930 were used as references.

As a second method to characterize the phytoavailability of soil Cd, Cu, Mn and Zn we applied the "diffusive gradients in thin films" (DGT) method developed by Davison and Zhang (1994), using DGT soil samplers (DGT Research Ltd., Lancaster, UK) with 0.92 mm diffusive layer thickness and a chelex-100 resin gel. Field-moist samples (INIT, SOW, TILL and FLOW samples stored at 4 °C) with a dry weight of approximately 50 g were sieved to 2 mm, adjusted to 100% water holding capacity and equilibrated for 24 h at 25 °C, before the DGT device was deployed for 72 h at 25 °C following the procedure of Tandy et al. (2011). Immediately after deployment, the resin was removed and extracted with 1 mL of supra pure 1 *M* HNO_3 for 24 h. After 5-fold dilution with nanopure water, the extracts were analysed by means of ICP-MS for metals. The amount of a metal, M, accumulated by the DGT resin was calculated following Zhang et al. (2004) as:

$$\boldsymbol{M} = \boldsymbol{C}(\boldsymbol{V}_{acid} + \boldsymbol{V}_{gel})/\boldsymbol{f}_e \tag{1}$$

where C is the metal concentration measured in the extract, V_{acid} is the volume of nitric acid used for elution (1 mL), V_{gel} is the volume of the resin gel (0.15 mL), and f_e is the elution factor (0.8). The time average concentration of Cd, Cu, Mn and Zn at the interface of the DGT and the soil (C_{DGT}) was then calculated as:

$$\boldsymbol{C}_{DGT} = \boldsymbol{M}\Delta\boldsymbol{g}/(\boldsymbol{DAt}) \tag{2}$$

where A is the surface area of the DGT sampling device in contact with the soil (2.54 cm^2), t is the deployment time (3 days = 3 × 86,400 s), Δg is the diffusive layer thickness (0.092 cm), and D is the diffusion coefficient of the metal in the diffusive gel (cm^2 s^{-1}).

Mineral N, DOC and TFAA were extracted from field-moist soil samples (INIT, SOW, TILL and FLOW samples) with 2 *M* KCl at a soil-to-solution ratio of 1:5 for 1 h at room temperature on the day of soil sampling following Jones and Willett (2006). The extracts were filtered through Whatman No 42 filter papers, subdivided into aliquots for the analysis of the various parameters and frozen until analysis. Spectrophotometry (Cary 50 UV–Visible Spectrophotometer, Varian Inc., Palo Alto, CA USA) was used for the analysis of ammonium, following Mulvaney (1996), and nitrate, following Miranda et al. (2001). DOC was analysed using a total organic carbon analyser after (TOC-L, Shimadzu Corp., Kyoto, Japan) after 5-fold dilution of extract subsamples with nanopure water. TFAA were determined fluorometrically (Fluorometer LS-50B, PerkinElmer, Waltham, MA USA) using the o-phthaldialdehyde β-mercaptoethanol (OPAME) method described by Jones et al. (2002).

2.4. Plant analysis and calculations

For the elemental analysis of plant shoots, 200 mg aliquots of milled sample material were digested with 15 mL of 65% HNO_3 at 120 °C for 90 min and with 3 mL of 30% H_2O_2 at 120 °C for another 90 min in a DigiPREP MS digestion system (SCP Science, Quebec, Canada). For the analysis of grain samples, 100 mg aliquots of milled grains were digested with 1 mL of 65% HNO_3 and 2 mL of 30% H_2O_2 for 30 min at a pressure of 40 bar and a temperature of 240 °C in a microwave digestion system (turboWAVE, MLS GmbH, Leutkirch, Germany). International Plant-Analytical Exchange references 186, 783, 203, 124 and 148 (http://www.wepal.nl/) were included as reference standards in the analysis of each batch of the respective plant samples (wheat shoots, grains, mustard and clover green manure).

Nitrogen and C concentrations of plant samples were determined by analysis of milled sample material with an NCS analyser (FlashEA 1112 Series, Thermo Fisher Scientific Inc., Waltham, MA USA).

The amounts of metals accumulated in wheat shoots and grains were calculated by multiplication of harvested biomass with the respective tissue metal concentrations. Chaff biomass was included in the shoot biomass for this calculation, assuming that average metal concentrations were the same in chaff and shoot tissues. Grain Zn and Cd derived from post-anthesis relocation of metals accumulated in the shoots were estimated from the difference between shoot (including chaff) metal contents at harvest and at flowering. This was based on findings from Pearson and Rengel (1994) who showed Zn remobilization from the leaves, especially the flag leaf, to the grains during grain development and Harris and Taylor (2013) who found net remobilization of Zn from shoot tissues to the grains with a 60–70% contribution from the stems, but no net remobilization for Cd. However, in our study, leaf and stem tissues were not analysed separately and were treated as a homogenous unit.

2.5. Fertilizer analysis

The same DigiPREP MS digestion system (SCP Science, Quebec, Canada) as used for plant sample digestion was also used to digest fertilizer samples. For metal analysis of mineral fertilizers, aliquots of 500 mg fertilizer were digested in 23 mL of nanopure water and 2 mL of 37% HCl at 120 °C for 90 min in the digestion system.

Farmyard manure samples were first dried at 105 °C for 24 h and ashed at 500 °C for 4 h, then 1 g of ash was digested in 2 mL of nanopure water and 8 mL of aqua regia (2 mL of 70% HNO_3 and 6 mL of 37% HCl) at 120 °C for 90 min in the digestion system, following the procedure described by FAL et al. (2015b). Total N in the farmyard manure samples were determined by means of the Kjeldahl method (FAL et al., 2015a), ammonium-N by means of the steam distillation method as described by Stevenson (1996). The organic matter content of the farmyard manure samples was determined by the difference between total dry matter and ash content according to FAL et al. (1998a, 1998b).

2.6. Chemical analysis of extracts and digests

Element concentrations in aqua regia digests (Ca, Cu, Fe, K, Mg, Mn, P, S and Zn), DTPA extracts (Cd, Cu, Fe, Mn and Zn), wheat shoot and grain digests (Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn), mineral fertilizer digests (Ca, Cd, Cu, Fe, K, Mg, Mn, Na and Zn) and farmyard manure digests (Cd, Cu, Fe, K, Mn, P and Zn) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES 5100, Agilent Technologies, Santa Clara, CA USA). Inductively coupled plasma mass spectrometry (ICP-MS 7500ce, Agilent Technologies, Santa Clara, CA USA) was used to measure Cd in aqua regia extracts, wheat shoot and grain digests and Cd, Cu, Mn and Zn in DGT extracts. Green manure digests were analysed by ICP-OES (Vista-MPX CCD Simultaneous, Varian Inc., Palo Alto, CA USA) for Ca, Fe, K, Mg, P and S and by ICP-MS (810, Varian Inc., Palo Alto, CA USA) for Cd, Cu, Mn and Zn.

2.7. Statistical analysis

All statistical analyses and figures were made by means of R version 3.0.2 (R Core Team, 2013). Analysis of variance (ANOVA) was performed using linear mixed effects models in combination with the function lmer of the R package lme4 (Bates et al., 2014). 3-way ANOVA models were used to analyse the three fixed effects FERTILIZER (2 levels: NPK, FYM), GREEN (2 levels: NGM, SIN, TRI) and TIME (4 levels: INIT, SOW, TILL, FLOW) with all interactions and random effects for main and split-plots:

$$\boldsymbol{lmer}(\boldsymbol{y} \sim \boldsymbol{FERTILIZER}^{*}\boldsymbol{GREEN}^{*}\boldsymbol{TIME} + (1|\boldsymbol{plot}) + (1|\boldsymbol{subplot}), \boldsymbol{data} = \boldsymbol{d}) \tag{3}$$

To analyse effects at specific growth stages (variable TIME), 2-way ANOVA models were used:

$$\boldsymbol{lmer}(\boldsymbol{y} \sim \boldsymbol{FERTILIZER}^{*}\boldsymbol{GREEN} + (1|\boldsymbol{plot}), \boldsymbol{data} = \boldsymbol{d}) \tag{4}$$

The function glht from the package multcomp was used for Tukey's HSD pairwise comparisons and for the analysis of specific contrasts in treatment means (Hothorn et al., 2008). Student's *t*-test was applied to determine initial differences between the NPK and FYM soils. For a robust check for correlations between the residuals of the 2-way ANOVA and plant or soil covariates, we used the function covMcd of the R robustbase package (Rousseeuw et al., 2016). Data were transformed as necessary to meet the assumption of normally distributed residual errors and random effects. Differences with $p \leq 0.05$ were considered significant.

3. Results

3.1. Initial soil characteristics

The long-term management with farmyard manure did not only lead to significantly higher total soil C contents, but also to higher total and DTPA-extractable soil Zn, Cd and Cu concentrations as compared to long-term fertilization with NPK (Table 2). The size of the DTPA-extractable metal pools relative to the total amounts were higher in FYM (2.9, 37 and 23% for Zn, Cd and Cu, respectively) than in NPK treated soil (1.8, 30 and 19%). The DGT-available soil Cu concentrations were also higher in the FYM soil, while there was only a tendency for higher concentrations of DGT-available Zn and Cd. All total element concentrations lay within the range of values typically found in uncontaminated agricultural soils (Blume et al., 2016). DTPA extractable Zn was clearly above 0.5 mg kg^{-1}, a threshold below which soils are potentially Zn-deficient (Mertens and Smolders, 2013).

Inputs of Zn with the application of farmyard manure and mineral N, P and K fertilizers were 1847, 4.21, 88.3 and 0.58 g Zn ha^{-1}, respectively (Table S.1). Cadmium inputs with fertilizer were only measurable in farmyard manure (0.82 g ha^{-1}) and mineral P fertilizer (2.67 g ha^{-1}). Macro- and micronutrient concentrations and organic and dry matter contents of the applied cattle farmyard manure were in the range of those found for cattle manure originating from six farms by Levi-Minzi et al. (1986).

3.2. Green manure growth and accumulation of elements

Clover produced more above-ground biomass than mustard, and due to its higher N content this biomass also had a lower C/N ratio (Table 3). Shoot Zn and Cd concentrations of clover were higher on

Table 3
Shoot biomass and element concentrations of green manure plants grown on the two fertilization treatments, NPK and FYM.

Green manure	Soil	Biomass	Zn	Cd	N	C/N	Zn uptake	Cd uptake
		($t\ ha^{-1}$)	($mg\ kg^{-1}$)	($\mu g\ kg^{-1}$)	($g\ kg^{-1}$)		($g\ ha^{-1}$)	($mg\ ha^{-1}$)
Sinapis alba L.	NPK	1.26b	34.9b	249ab	14.8b	31.5a	45.0b	314b
	FYM	1.35b	56.4b	271ab	13.7b	33.6a	73.6b	364b
Trifolium	NPK	1.94a	48.3b	208b	32.6a	13.6b	91.8b	389b
alexandrinum L.	FYM	2.29a	93.3a	477a	30.8a	14.7b	211.2a	1090a

Values shown are means of 5 plot replicates. Means with same letter are not significantly different at $p \leq 0.05$ according to Tukey multiple comparisons.

the FYM than on the NPK soil, while for mustard they were not significantly different. The treatment effects on green manure yield and metal accumulation resulted in around three times higher total Zn and Cd found in the clover shoots on the FYM soil compared to the other three treatment combinations.

3.3. Soil Zn and Cd availability

DTPA-extractable soil Zn and Cd concentrations were not affected by the green manure plants, but were on average higher in the FYM than in the NPK soil (Fig. 2a and b). Both concentrations were higher during the wheat cultivation than at the beginning of the experiment. The increase occurred between sowing and tillering for Zn, but already before sowing for Cd. DGT-available Zn and Cd concentrations were neither significantly affected by the fertilization, nor by the green manure treatments, although they tended to be higher after treatment with green manure plants, most clearly at the time of wheat sowing in the FYM soil (Fig. 2c and d). However, Zn and Cd DGT concentrations significantly changed over time with FYM and NPK having maximum concentrations at sowing and tillering, respectively.

Mineral soil N concentrations (sum of nitrate-N and ammonium-N) followed a temporal pattern similar to the DGT Zn and Cd (Fig. 3a). They peaked at wheat sowing in the FYM soil and at tillering in the NPK soil. At wheat sowing, mineral N was highest in the clover treatment in both soils. At tillering this clover effect had disappeared. The effect was paralleled by nitrate as the dominant N-form at sowing in the clover treatments, while ammonium became the dominant N-form at tillering and flowering in the NPK soil (Fig. S.1).

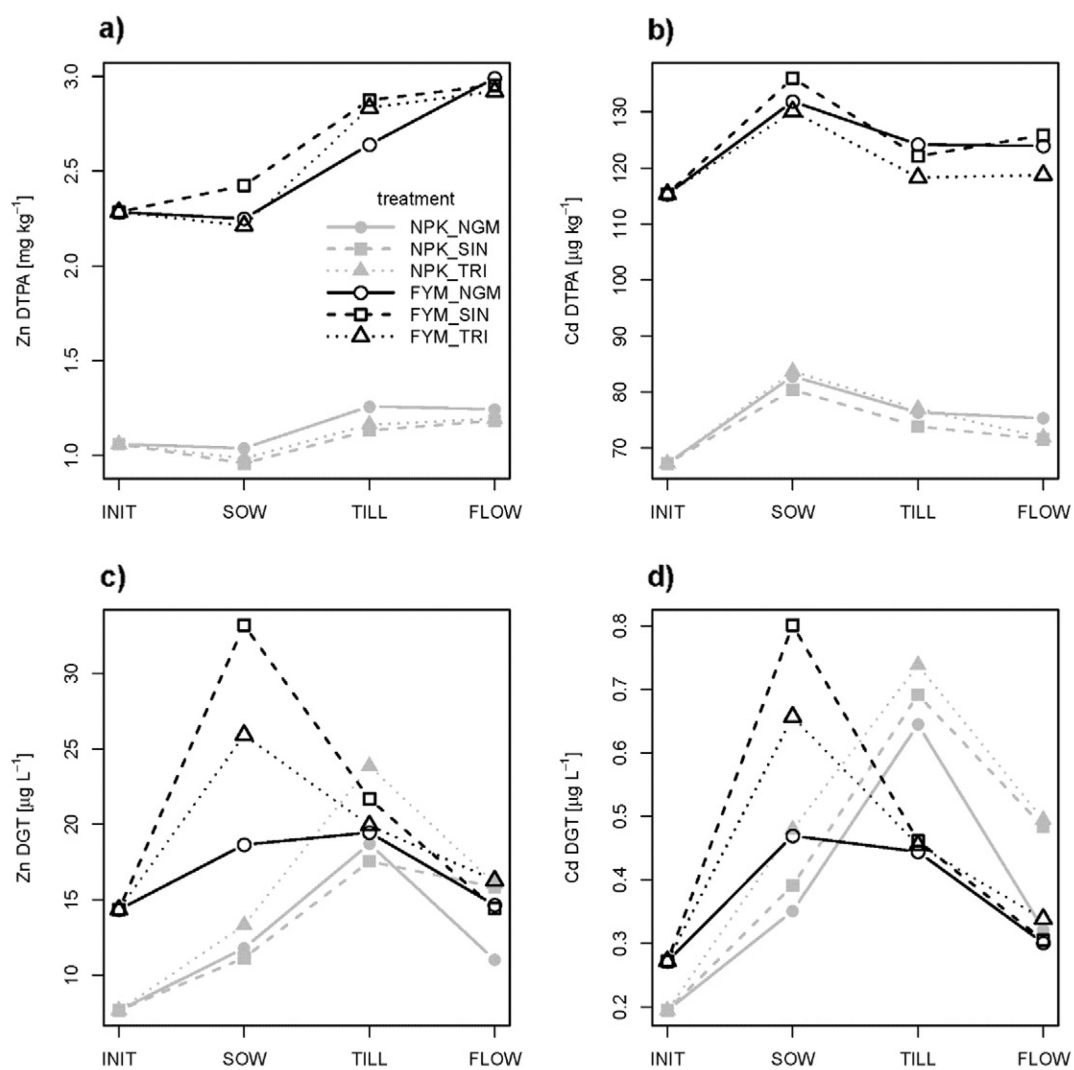

Fig. 2. DTPA extractable soil Zn (a) and Cd (b) and DGT available soil Zn (c) and Cd (d) in the FYM and NPK soil at the beginning of the experiment before the growth of green manure plants (INIT) and at sowing (SOW), tillering (TILL) and flowering (FLOW) of the subsequent wheat cultivation.

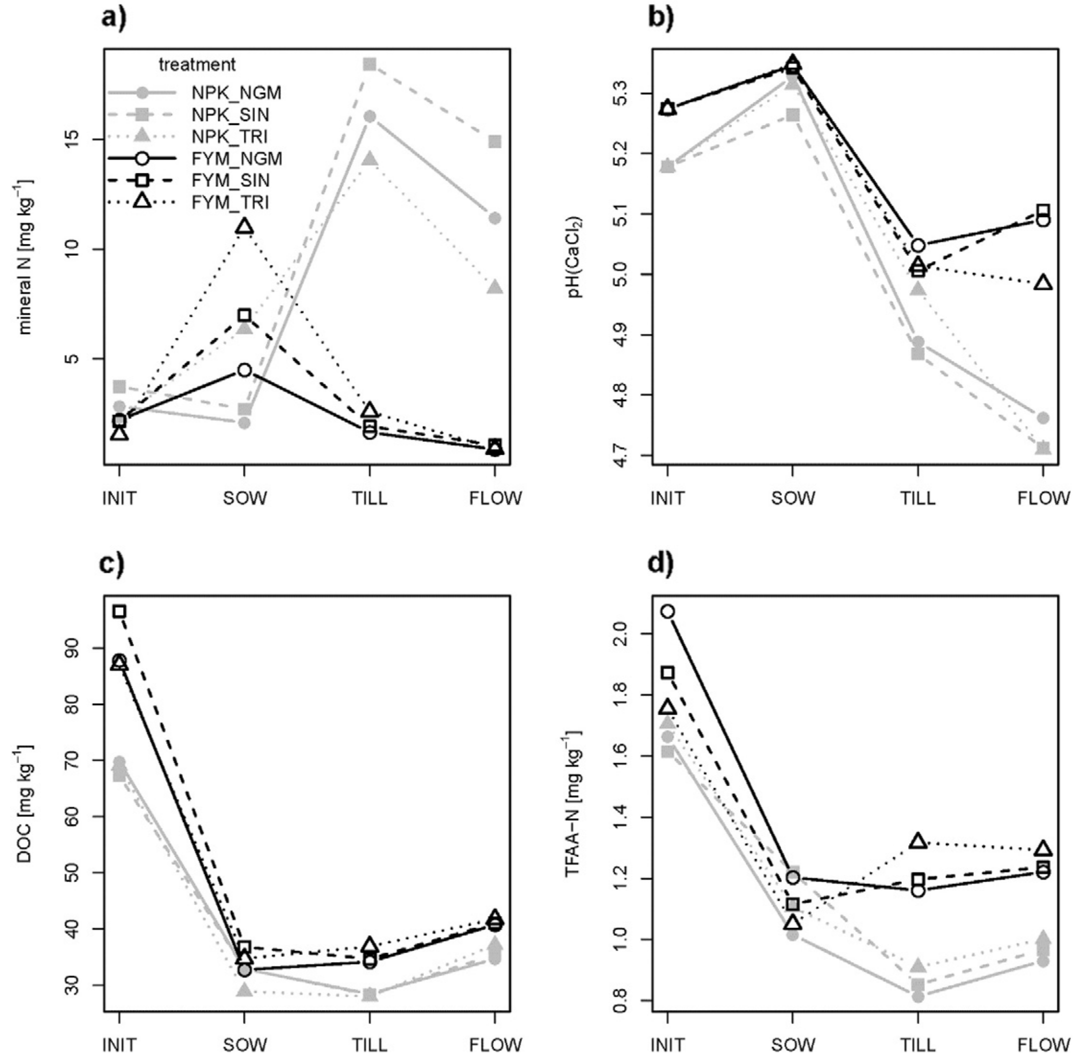

Fig. 3. Mineral N (sum of nitrate and ammonium, see Fig. S.1) (a), soil pH($CaCl_2$) (b), dissolved organic carbon (DOC) (c) and total free amino acids-nitrogen (TFAA-N) (d) in the FYM and NPK soil at the beginning of the experiment before the growth of green manure plants (INIT) and at sowing (SOW), tillering (TILL) and flowering (FLOW) of the subsequent wheat cultivation. Mineral N, DOC and TFAA-N were measured in 2 *M* KCl extracts.

Soil pH, DOC and TFAA concentrations were all much lower towards the end than at the beginning of the experiment (Fig. 3b-d). Soil pH decreased after wheat sowing. While initially it was similar in both soils, it became significantly lower in the NPK than in the FYM soil at wheat flowering. Similarly, DOC and TFAA were lower in NPK than in FYM soil at tillering ($p = 0.036$ and $p = 0.026$) and flowering ($p = 0.082$ and $p = 0.046$), but in contrast to pH they dropped already before wheat sowing. Log(DOC) and TFAA were positively correlated ($R = 0.85$, $p < 0.001$). Neither pH, nor DOC and TFAA were significantly affected by the green manure treatments. When investigating the residuals of the linear mixed effects models for DGT-available Zn and Cd including treatment effects, they correlated negatively with soil pH and positively with DOC and TFAA at all three dates when soil samples were analysed for these parameters during wheat cultivation (Table S.2). This means that the green manure and long-term fertilization treatments do not fully account for the differences in DGT-available Zn and Cd concentrations, but that these differences can be further explained by soil pH and organic ligand concentrations.

3.4. Wheat growth and accumulation of Zn and Cd

At tillering, wheat biomass was higher on FYM than on NPK soil, but at flowering more biomass had been produced on the NPK than on the FYM soil, although at harvest, the differences in grain and shoot biomass were still only marginally significant ($p = 0.051$ and $p = 0.076$, respectively) (Fig. S.2, Table S.3). The green manure treatment with clover resulted in a significantly increased wheat biomass at all growth stages, while the mustard had no effect on wheat biomass. The clover effect was in line with the production of more tillers and grains, while grain size was not affected by the green manure treatments (Fig. S.2, Table S.3). Similarly, the fertilizer effect on grain biomass corresponded to a higher number of grains on the NPK than on the FYM soil; however, in contrast to the clover manure treatment, the fertilization had no effect on tillering. The larger number of grains more than compensated for a slightly smaller grain size on the NPK soil. This effect on grain size was probably due to a better N supply with mineral fertilizer application in the later growth stages (Delogu et al., 1998). Generally, grain yields of both fertilization treatments were low compared to reference yields for spring wheat under mineral fertilization (5 t ha^{-1}) or under organic farming (4 t ha^{-1}) in Switzerland (Flisch et al., 2009; Rudmann and Willer, 2005).

Shoot Zn concentrations generally increased between tillering and flowering and then decreased again to reach the lowest values at harvest. At tillering, they were significantly higher where clover was applied, but this effect disappeared at later growth stages (Fig. 4a). Grain Zn concentrations were closely correlated with shoot Zn concentrations

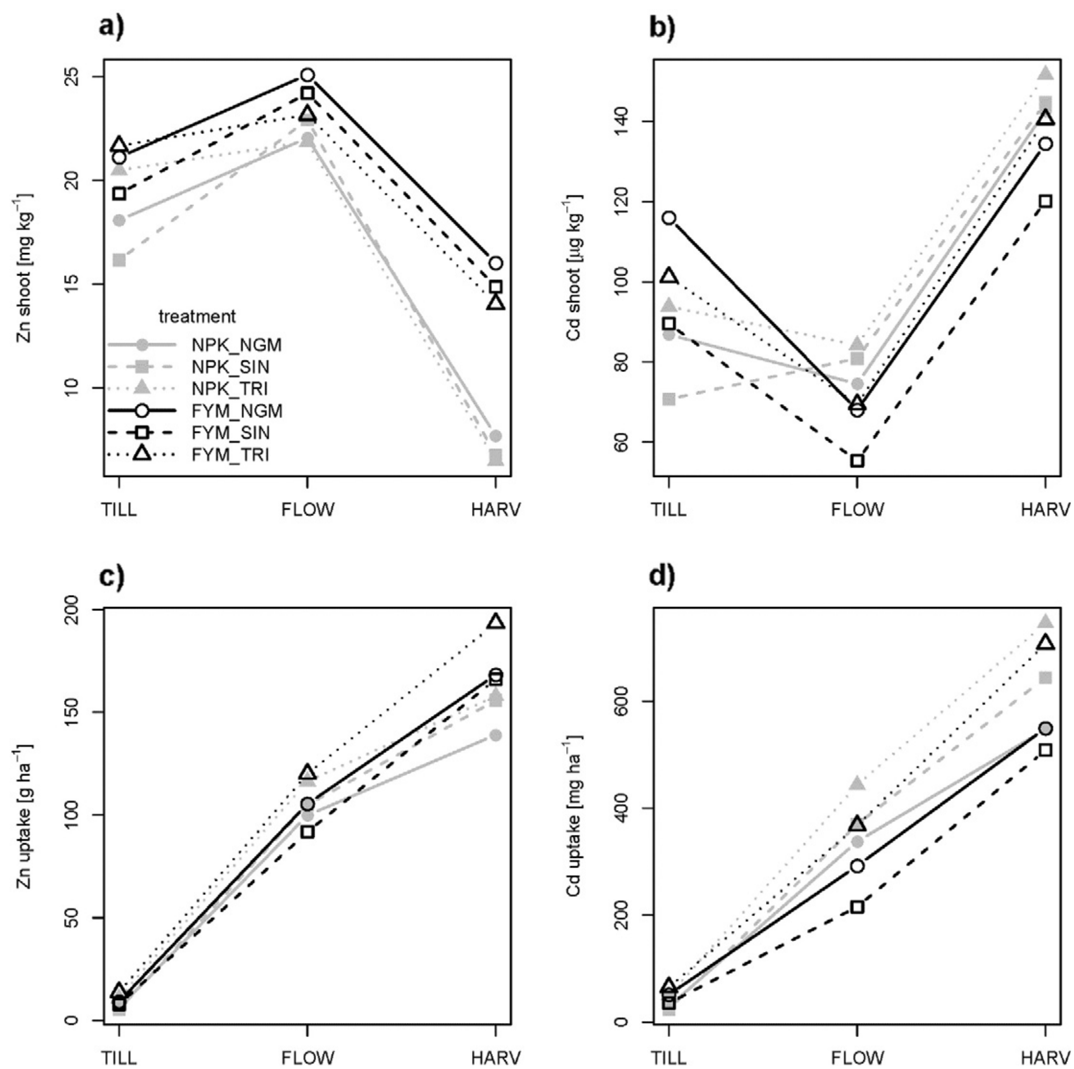

Fig. 4. Wheat shoot Zn (a) and Cd (b) concentrations and cumulative wheat Zn (c) and Cd (d) uptake at tillering (TILL), flowering (FLOW) and harvest (HARV). Zn and Cd uptake at HARV are the sum of Zn and Cd accumulated in wheat shoots, grains and chaff.

at harvest (Table S.2). Both were lower on the NPK than on the FYM soil, but only the grain Zn concentrations responded to the green manure treatments ($p = 0.038$), indicating that clover green manure decreased grain Zn concentrations (Figs. 4a and 5a). However, this effect was only significant for the single contrast between NGM and TRI in the FYM ($p = 0.023$), but not the NPK treatment ($p = 0.37$). In contrast to

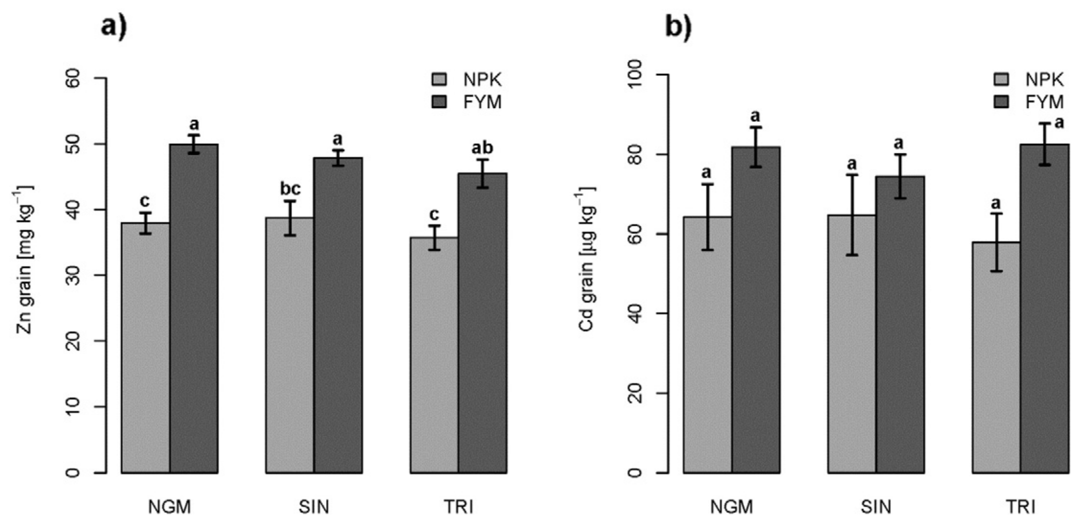

Fig. 5. Wheat grain Zn (a) and Cd (b) concentrations as affected by the experimental treatments (Mean ± SE, n = 5). Means with same letter are not significantly different at $p \leq 0.05$ according to Tukey multiple comparisons.

shoot Zn, shoot Cd concentrations increased between flowering and harvest (Fig. 4b). The correlation between grain and shoot Cd concentrations at harvest was even stronger than that between grain and shoot Zn. The green manure treatments had no effect on shoot and grain Cd concentrations (Figs. 4b and 5b). There was also no significant difference in shoot and grain Cd concentrations between the two fertilizations, although they tended to be systematically higher on the FYM than on the NPK soil, paralleling the corresponding effect on shoot and grain Zn. When comparing grain Zn and Cd concentrations with shoots at harvest, grain Zn concentrations were 3–6 times higher and grain Cd concentrations about 2 times lower than shoot concentrations. The concentrations of both metals in shoots and grains were positively correlated with the concentrations of the respective DGT-available soil pools of these metals at tillering and flowering (Table S.2) and the correlation was strong for Cd (Pearson r between 0.59 and 0.83), but only moderate for Zn (Pearson r between 0.32 and 0.51). And only for Zn, a positive correlation between DTPA-extractable Zn concentrations at flowering and shoot (Pearson $r = 0.63$) and grain Zn concentrations (Pearson $r = 0.52$) was found.

Macronutrient concentrations (Fig. S.3a–c) in wheat shoots were affected by both fertilizer and green manure treatment. Shoot N and S concentrations were higher on NPK than FYM soil at all wheat growth stages, whereas shoot P concentrations were lower on NPK than on FYM soil. These findings reflect the different forms of fertilizer inputs, with N and S added in a more available form with mineral fertilization than farmyard manure. However, the higher organic P input through farmyard manure translated into higher wheat P accumulation (Table S.1). The green manure treatment with clover significantly increased N, P and S concentrations in wheat at tillering. As in the shoots, wheat grain N and S concentrations were higher and P concentrations were lower on NPK compared to FYM soil (Fig. S.3d–f). The green manure treatment with clover increased grain S concentrations and tended to increase also the concentrations of grain N. Neither grain N nor grain S concentrations positively correlated with grain Zn or Cd concentrations.

The total amounts of Zn and Cd accumulated over time in the above-ground parts of the wheat crops primarily reflect the increase in biomass (Fig. 4c and d, Fig. 6). Total Zn uptake was significantly influenced by the green manure treatment at tillering and harvest. It was highest on clover-treated FYM soil. At harvest, more Zn was found in the above-ground plant material grown on FYM than on NPK soil. Total Cd uptake was increased in the green manure treatment with clover at all wheat growth stages. Similar amounts of Cd were taken up before and after flowering, while more Zn was taken up before than after flowering.

The total Zn contents in the wheat shoots at flowering were higher than at harvest (Fig. 6a). Therefore, the fraction of grain Zn derived from shoot Zn that had been accumulated before grain development started (net remobilization) was estimated to be 68% on NPK and 49% on FYM soil. In other words, more grain Zn derived from soil Zn taken up during grain-filling on the FYM (51%) than on the NPK soil (32%), in line with the higher total Zn uptake from FYM soil. On the contrary, total Cd contents in wheat shoots at harvest were higher than at flowering which did not indicate similar shoot-to-grain relocation of Cd that had been accumulated in the shoots before grain-filling (Fig. 6b). Finally, the amount of Zn in the grains related to total wheat Zn uptake was 77% on FYM and 85% on NPK soil, but only about 36% of total Cd taken up was found in the grains.

4. Discussion

4.1. Soil Zn and Cd availability

The different long-term management of the NPK and FYM plots (65 years) had strong impacts on total and available soil Zn and Cd concentrations and soil organic carbon content, which can be explained by the differences in the composition of the fertilizers applied. The FYM soil not only had higher absolute concentrations of total and DTPA-extractable Zn and Cd than the NPK soil, but also the size of the DTPA extractable metal pool relative to the total amount was higher for both metals in the FYM soil. Farmyard manure obviously was not only a major source of soil Zn and Cd, but apparently also supplied or rendered them in an available form. The increased adsorption capacity for soil metals due to higher soil organic matter content (Blume et al., 2016) and the metal-mobilizing effects of organic ligands entering the soil with the application of farmyard manure or generated by its degradation, explain this effect. Our findings are in line with a previous study by Benke et al. (2008) in which long-term cattle manure application increased EDTA-extractable soil Zn and Cd. Several studies indicated that DTPA-extractable soil Zn and Cd concentrations increase with organic matter application (Chaudhary and Narwal, 2005; He and Singh, 1993). However, since DTPA is a strong extractant, this does not mean that all the DTPA-extractable organically-bound Zn and Cd is phytoavailable.

In both soils, the DGT-available soil Zn and Cd concentrations were at a maximum at the same time as mineral nitrogen concentrations peaked. As described above, the application of farmyard manure itself may have added available Zn and Cd along with organic N, leading to the highest concentrations of both DGT-available metal concentrations and mineral soil N at wheat sowing in that treatment. Delcastilho et al. (1993) also reported peak concentrations of Zn and Cd in soil solution after cattle manure slurry application. Under mineral fertilization (NPK) however, the peak in Zn and Cd concentrations is more likely due to cation exchange with ammonium which was only added during wheat growth. But also the generation of soil acidity through nitrification and plant uptake of NH_4^+ with compensatory proton release for electrical charge balance, are factors that would have added to this metal-mobilizing ammonium effect (Grant et al., 1999; Lorenz et al., 1994). Increases in DGT-available Zn and Cd with decreased pH were also reported by Muhammad et al. (2012). Supporting these findings, DGT-available metals correlated positively with dissolved organic carbon and free amino acids but negatively with soil pH which were all generally higher in FYM than NPK soil at the different time points. In agreement with our study, Delcastilho et al. (1993) also found positive correlations of soil solution Zn and Cd concentrations with DOC and negative correlations with soil pH for measurements taken before and after cattle manure slurry application.

For the same reasons, ammonium release from decomposing plant residues (Cookson et al., 2002) could also explain the Zn- and Cd-mobilizing effect observed after the incorporation of the green manures. The C/N ratio of clover residues is sufficiently low to result in net N mineralization when they decompose in aerobic soils (Fageria, 2007). In fact, soil mineral N concentrations were drastically increased by the application of clover at the beginning of wheat growth, which can be attributed to the release of biologically fixed N from the decomposing residues. Some of the temporary increase in DGT-available Zn and Cd concentrations after green manure incorporation into the soils could have been due to the release of soluble Zn and Cd species from the decomposing biomass. However, Aghili et al. (2014) observed that the fraction of Zn in the above-ground parts of wheat that was recovered from decomposing green manure was small compared to Zn uptake from native soil Zn pools.

Another more plausible explanation for the finding that green manure application increased DGT-available Zn and Cd concentrations is that compounds such as amino and other low-molecular-weight organic acids were released with decomposition which formed soluble complexes with the two metals (Jones, 1998; Jones et al., 2003; Jones et al., 2005). Increased amino acid concentrations after legume (cluster bean and mung bean) cultivation and incorporation into soil were also observed by Praveen-Kumar et al. (2002). Furthermore, both farmyard and green manure incorporation could have stimulated the decomposition of soil organic matter and the release of organically bound Zn, Cd and N through a priming effect (Kuzyakov, 2010).

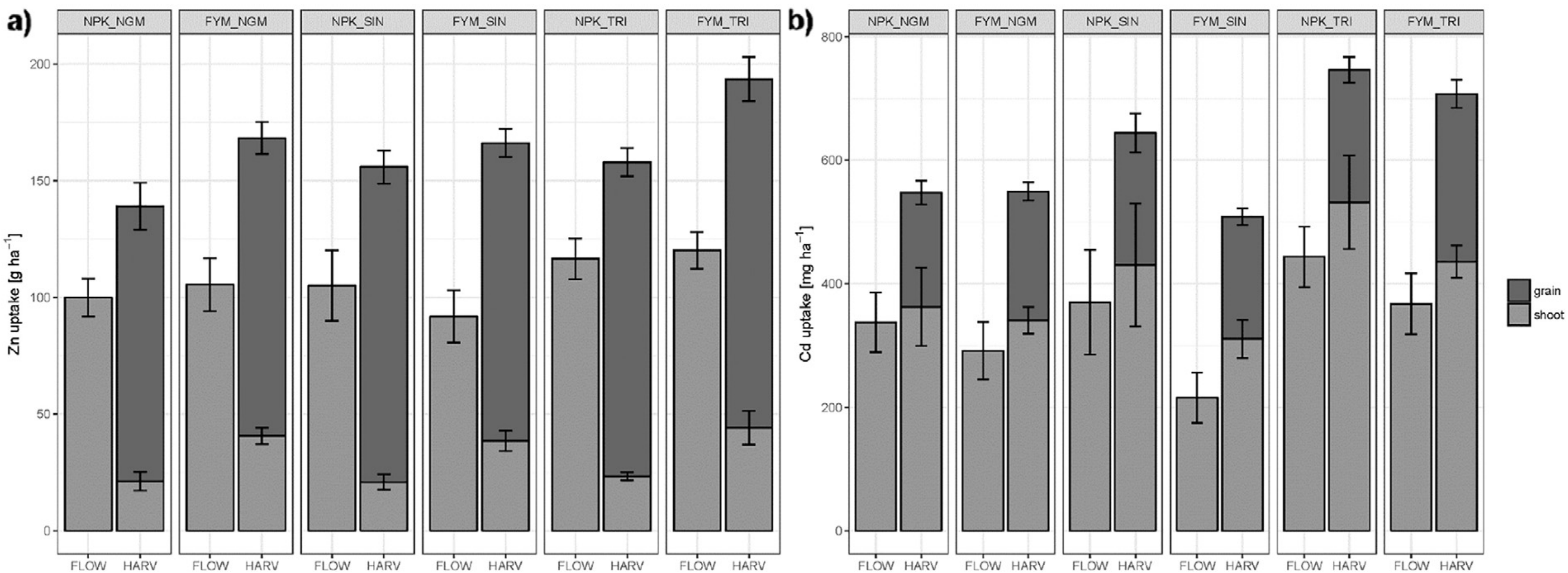

Fig. 6. Wheat shoot and grain total Zn (a) and Cd (b) uptake (Mean ± SE, n = 5) at flowering (FLOW) and harvest (HARV). The six treatment combinations between the fertilizer (NPK, FYM) and green manure treatments (NGM, SIN, TRI) are indicated at the top of each figure.

The comparison of available soil Zn and Cd measurement by means of DGT and DTPA extraction reveals that the DTPA method clearly reflected the differences between the two long-term treatments of the soil while the DGT technique proved to be highly sensitive to short-term organic and mineral fertilizer inputs and was suitable for detecting temporal dynamics in soil Zn and Cd availabilities over a wheat growth period. DTPA probably extracted organically bound Zn and Cd which remained unavailable for the DGT device. Therefore, the two methods should be used complementarily rather than as alternative measures for the same property.

4.2. Wheat Zn and Cd uptake

To understand treatment effects on metal concentrations in wheat we need to understand how the treatments affect plant metal uptake and within-plant translocation, but also the production of biomass in which the metals are allocated. The main factor determining wheat biomass production was N supply. When more mineral soil N was available, more biomass was produced, indicating that N was the limiting nutrient. For this reason, higher wheat yields were obtained in the NPK soil with a higher N availability during wheat development than in the FYM soil, and in the TRI green manure treatment compared to NGM and SIN. The role of biological nitrogen fixation as an important N source for sustainable agricultural production is well known (Peoples et al., 1995) and was shown for example by Janzen et al. (1990) for two different green manures and a subsequent wheat crop.

Due to a combination of both lower yields at flowering and harvest and higher concentrations of phytoavailable soil Zn as indicated by higher DTPA-extractable soil Zn concentrations, Zn concentrations in wheat shoots and grains were higher in the FYM than in the NPK soil. A third important aspect which also explains this effect, were higher DOC and TFAA concentrations in the FYM than in the NPK soil at tillering and flowering, indicating a soil metal mobilization potential through organic and amino acids which form soluble complexes with Zn. Similarly, Gramlich et al. (2013) found increased Zn uptake by wheat in the presence of histidine which was partly due to the uptake of undissociated Zn-histidine complexes. In contrast to the NPK treatment, increased biomass production with clover green manure did not lead to lower wheat shoot Zn concentrations, even though this dilution of nutrients other than the limiting one has been seen before (Jarrell and Beverly, 1981). At the tillering stage, shoot Zn concentrations were even higher than in the control, along with higher shoot N, P and S concentrations. Only the grains had marginally lower Zn concentrations in the clover treatment, but the extent to which these concentrations were decreased was much lower than the increase in biomass production. This indicates that clover residues increased the phytoavailability of soil Zn, probably due to local acidification effects (Oliver et al., 1993) or increased available N, which also led to improved N nutrition of wheat. Improved N availability has been shown to increase shoot and grain Zn concentrations in wheat. This effect was attributed to higher abundances of N-containing ligands such as amino acids and nicotianamine, which are involved in within-plant Zn transport, transporter proteins such as ZIPs, YSL or HMA proteins involved in cellular trans-membrane transfer of Zn, and Zn-binding proteins acting as storage compounds for shoot and grain Zn (Erenoglu et al., 2011; Kutman et al., 2011). Nicotianamine is also a precursor of mugineic acid-family phytosiderophores (Mori and Nishizawa, 1987; Shojima et al., 1990) being released by graminaceous plants mainly for Fe acquisition, but also other micronutrients such as Zn (Cakmak et al., 1994; Oburger et al., 2014; Zhang et al., 1991). However, only small amounts of phytosiderophores are released by wheat roots if there is no Fe or Zn deficiency (Zhang et al., 1991).

Even though N might have played an important role in compensating for biomass effects on wheat Zn concentrations in the clover treatment, grain N concentrations did not correlate positively with grain Zn. This is in contrast with previous studies which found strong positive correlations between grain Zn and grain N concentrations (Cakmak et al., 2010; Kutman et al., 2010; Xue et al., 2012) or protein concentrations (Morgounov et al., 2007; Peleg et al., 2008; Zhao et al., 2009). In our study however, grain Zn concentrations were determined mainly by the different soil Zn availabilities in the NPK and FYM soils, rather than by the sink effect of grain proteins. Additionally, they might have been controlled by plant Zn homeostasis. On the FYM soil, wheat grain Zn concentrations (45–50 mg kg^{-1}) were above the target levels for biofortification of 37 mg kg^{-1} (Bouis and Saltzman, 2017) while on the NPK soil they were very close to targeted levels (35–39 mg kg^{-1}).

There was evidence for Zn accumulation in wheat shoots and net remobilization of Zn from pre-anthesis stores to the developing grains. The total Zn content of wheat shoots decreased between flowering and harvest due to a combination of decreasing shoot Zn concentrations and biomass, indicating that Zn was transported from these tissues to the developing grains. Several studies found pre-anthesis stores to be important sources of micronutrients such as Zn, Cu and Fe for grains (Harris and Taylor, 2013; Kutman et al., 2011; Xue et al., 2012). Total Zn uptake by above-ground wheat parts was highest at harvest, indicating that uptake of soil Zn must have occurred during grain-filling, in addition to within-plant Zn translocation. While translocation of Zn to the grains was estimated to be higher on NPK compared to FYM soil, more Zn from the soil was taken up post-anthesis in the case of FYM. Higher wheat root activity and exudation of organic acids (Bertin et al., 2003) or phytosiderophores (Zhang et al., 1991) in the second half of wheat growth might have led to mobilization of Zn more strongly bound to soil particles or soil organic matter (DTPA Zn pool) which was higher in FYM compared to NPK soil. Additionally, in NPK treatments there was a higher N availability during late wheat growth and it has been shown before that increased N supply increased translocation of Zn from the flag leaves to the grains (Erenoglu et al., 2011).

Grain Cd concentrations followed the same trend as grain Zn and tended to be higher on FYM than NPK plots probably due to the same reasons: lower yields, higher phytoavailable soil Cd and higher organic and amino acid concentrations. They were all below the food threshold of 0.2 mg kg^{-1} for the maximum permitted concentration of Cd in wheat grains set by the Swiss law (EDI, 1995) and the UN Codex Alimentarius Commission (FAO/WHO, 1995). In contrast to Zn, no net remobilization of Cd from vegetative parts to grains was observed, shoot Cd concentrations increased during grain-filling and concentrations in grains were lower compared to shoots. Our findings agree with those of Harris and Taylor (2013) and Wiggenhauser et al. (2016) who also found no net remobilization of Cd in wheat from leaves during grain-filling. Harris and Taylor (2013) suggested a direct pathway of Cd uptake from roots to grain via xylem-to-phloem transfer in the stem based on the absence of net remobilization and continued uptake of Cd by roots during grain filling.

4.3. Evaluation of soil Zn and Cd phytoavailability

The close correlations between the concentrations of DGT-available soil Cd and the accumulation of this metal in wheat shoots and grains highlights the suitability of the DGT method to predict Cd availability for plant uptake from soil. However, the correlation between DGT-available Zn and plant concentrations was much weaker and DTPA-extractable Zn correlated equally well with shoot and grain Zn concentrations. The differences in predictability of plant Zn and Cd concentrations by the two soil metal phytoavailability indicators is probably due to the differences in within-plant translocation of both metals or because of the two to three orders of magnitude difference in their absolute concentrations in the soil and the different plant tissues. The DGT method has been used to assess soil Zn and Cd bioavailability to plants in various other pot and field studies, as reviewed by Zhang and Davison (2015). However, only a few studies included non-contaminated soil or studied metal uptake by cereals (Nolan et al., 2005; Perez and Anderson, 2009; Tandy et al., 2011; Tian et al., 2008;

Williams et al., 2012; Yao et al., 2015). Tandy et al. (2011) showed that DGT Zn predicted Zn concentrations in barley leaves better than conventional extraction methods and soil solution analyses. Tian et al. (2008) found close relationships between DGT Cd and Zn and their concentrations in rice roots and grains and concluded that DGT accounts for the main factors affecting trace metal bioavailability to plants. Perez and Anderson (2009) found that DGT-available Cd increased with the application of Cd-rich phosphate fertilizer and that this effect was closely related to increased Cd concentrations in wheat grains. However, the DGT technique has not been used before to measure changes in trace metal availabilities during wheat growth in the field as affected by fertilization practices and to predict wheat grain Zn accumulation.

4.4. Conclusions

Even though the application of mineral fertilizers provided more available N to the developing wheat plants, uptake of available soil Zn and Cd by wheat were still higher on the FYM than the NPK soil. We therefore conclude that Zn and Cd availability was governed more by Zn, Cd and organic compounds added to the soil via long-term farmyard manure inputs than by mineral soil N. As hypothesized, the application of *Trifolium alexandrinum* L. and *Sinapis alba* L. as green manure had different impacts on soil Zn and Cd and their accumulation by wheat. While clover as a leguminous plant with its low C/N ratio increased soil mineral N and wheat biomass production, the non-legume plant mustard did not have such a fertilizer effect. Clover green manure could compensate for the potential decrease of wheat grain Zn concentrations due to increased biomass production with an increase in soil Zn availability which was attributed to soil acidification effects, the inputs of low-molecular-weight organic acids and improved N nutrition of wheat through clover residues. Additionally, DGT-available Cd concentrations measured from field soils were confirmed to be excellent predictors of Cd concentrations in wheat shoots and grains. The study revealed that both long- and short-term field management with organic matter alters soil Zn and Cd concentrations but that the long-term effects are dominating their uptake by wheat, in Zn sufficient soil.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.05.070.

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