



# Green manure effect on the ability of native and inoculated soil bacteria to mobilize zinc for wheat uptake (*Triticum aestivum* L.)

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## Abstract

**Purpose** Green manuring can increase the plant available fraction of zinc (Zn) in soil, making it a potential approach to increase wheat Zn concentrations and fight human Zn deficiency. We tested whether green manure increases the ability of both the native soil bacteria and inoculated Zn solubilizing bacteria (ZSB) to mobilize Zn.

**Methods** Wheat was grown in a pot experiment with the following three factors (with or without); (i) clover addition; (ii) soil x-ray irradiation (i.e. elimination of the whole soil biota followed by re-inoculation with the native soil bacteria); and (iii) ZSB inoculation. The incorporation of clover in both

the irradiated and the ZSB treatments allowed us to test green manure effects on the mobilization of Zn by indigenous soil bacteria as well as by inoculated strains.

**Results** Inoculation with ZSB did neither increase soil Zn availability nor wheat Zn uptake. The highest soil Zn availabilities were found when clover was incorporated, particularly in the irradiated soils (containing only soil bacteria). This was partly associated with the stimulation of bacterial activity during the decomposition of the incorporated green manure.

**Conclusion** The results support that the activity of soil bacteria is intimately involved in the mobilization of Zn following the incorporation of green manure.

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**Abbreviations**

ZSB	Zinc solubilizing bacteria
AM	Arbuscular mycorrhizae
HIGH Zn soil	Soil with sufficient plant Zn availability
LOW Zn soil	Soil with low plant Zn availability
DGT	Diffusive gradients in thin films method
NO GM	Treatments without green manure addition
+ GM	Treatments with green manure addition
NO IRR	Treatments with non-irradiated soil
X-RAY IRR	Treatments with x-ray irradiated soil
NO ZSB	Treatments without ZSB inoculation
+ ZSB	Treatment with ZSB inoculation
DTPA	Diethylene triamine penta-acetic acid
cfu	Colony forming unit
ANOVA	Analyses of variance

**Introduction**

Zinc (Zn) is an essential micronutrient as it contributes to protein structure and function in all living organisms (Broadley et al. 2007; Holt et al. 2012). Soil available Zn refers to the fraction present in soluble forms in the soil solution that can be directly absorbed by plant roots (Nolan et al. 2003). About 50% of the soils used for cereal cultivation are low in plant available Zn (Graham and Welch 1996). When the availability of Zn in soil is not sufficient to cover crop physiological requirements, cereal yield quantity and quality (i.e. Zn concentration in edible parts) are reduced (Alloway 2009; Frossard et al. 2000). The production of low-Zn cereal grains contributes largely to human Zn deficiency in low income countries, because, in such areas, diets are often based on cereal meals. Soil factors associated with low soil Zn availability include high pH, high clay, high calcium carbonate content and low total Zn content (Alloway 2009).

To fight Zn deficiency in humans, biofortification strategies aim at increasing the density of Zn in the edible parts of crops. These range from the application of inorganic Zn fertilizer to the development of new cereal cultivars more efficient in taking up Zn and transferring it to the grains (Cakmak 2008). Cadmium (Cd) is chemically similar to Zn

and has numerous adverse effects on human health when ingested chronically (Godt et al. 2006). Competition (Hart et al. 2002), but also synergy (Nan et al. 2002; Sarwar et al. 2014) have been reported between the uptake of Zn and Cd by wheat and thus, both metals should be considered while studying Zn biofortification.

An alternative (or complementary) strategy is to act on soil physicochemical (e.g. pH, organic matter content) and biological (e.g. microbial activity) parameters governing the availability of Zn in soil. For this, green manure (i.e. any plant material incorporated into the soil while green or soon after maturity primarily to improve soil fertility) has the advantage not only to release soluble Zn from decomposing biomass, but also and above all to promote the mobilization of Zn in soil (Aghili et al. 2014; Siddique et al. 2014). Zn mobilization in soil is defined as the transfer of Zn bound to soil particles (e.g. organic matter, iron (Fe) oxides) to more soluble Zn species in the soil solution (either as free Zn<sup>2+</sup> or as Zn<sup>2+</sup> bound to soluble organic compounds).

In a pot experiment performed with an Iranian soil low in plant available Zn, Aghili et al. (2014) observed an increase in wheat grain Zn concentrations from 20 to 31 mg Zn kg<sup>-1</sup> when sunflower green manure was incorporated compared to the non-amended control. Increased wheat Zn uptake upon green manure incorporation has been reported in a few other pot and field experiments (Grüter et al. 2017a; Habiby et al. 2014; Soltani et al. 2014). In these studies, increased Zn phytoavailability following the addition of green manure was mainly thought to be associated with (i) the mobilization of Zn through the release of small organic ligands (e.g. organic and amino acids) from decomposing green manure which can form soluble complexes with soil bound Zn (Gramlich et al. 2013); (ii) the addition of nitrogen (N) through biological fixation by Fabaceae that can stimulate the plant synthesis of Zn membrane transporters and organic ligands (e.g. amino acids, nicotianamine) involved in Zn uptake, transport and storage within plants (Palmer and Guerinot 2009) and (iii) the stimulation of soil microbial activity resulting in the mobilization of Zn, e.g. through the mineralization of soil organic matter and the release of organically bound Zn (Bingeman et al. 1953).

In addition to decomposing exogenous and soil organic matter, soil fungi and bacteria are involved

in a variety of soil chemical reactions controlling Zn phytoavailability. Furthermore, arbuscular mycorrhizae (AM) are known for their role in facilitating plant phosphorus (P) and Zn acquisition by enhancing root soil exploitation (Jansa et al. 2003; Kothari et al. 1991; Ortas 2012; Watts-Williams et al. 2013). On the other hand, ericoid and ectomycorrhiza are examples of soil fungi that, by the production of various metabolites such as protons and organic acids (e.g. malate, citrate), can mobilize Zn bound to soil particles and soil organic matter and increase the bioavailability of Zn in the rhizosphere (Fomina et al. 2005; Martino et al. 2003). Similar Zn mobilization mechanisms have been highlighted for specific heterotrophic bacterial strains, often referred to as Zinc Solubilizing Bacteria (ZSB) (Costerousse et al. 2018; Saravanan et al. 2007; Sunithakumari et al. 2016). Several soil bacterial isolates have been shown to obtain Zn from their environment by producing and taking up Zn-chelating siderophores (Bobrov et al. 2014; Leach et al. 2007). Such compounds are also suspected to facilitate the mobilization of Zn in the rhizosphere (Hafeez et al. 2013). Another microbial process controlling the availability of Zn corresponds to the release of ammonium ( $\text{NH}_4^+$ ) (e.g. during the decomposition of organic matter) which may exchange with soil bound Zn or promote the release of protons (i.e. through nitrification). Carbon dioxide ( $\text{CO}_2$ ) released from microbial respiration may also contribute to Zn mobilization through its dissolution in water and the following release of protons from the dissociation of carbonic acid ( $\text{H}_2\text{CO}_3$ ).

In a pot study performed with a Cd-contaminated soil, Muehe et al. (2015) reported that a strong alteration of native soil microbial communities (i.e. soil irradiation with  $\gamma$ -rays leading to a decrease in diversity) did not affect soil HCl-extractable Zn and Cd nor *Arabidopsis halleri* growth but reduced plant Zn and Cd uptake. Some other pot (Khande et al. 2017; Singh et al. 2017) and field studies (Abaid-Ullah et al. 2015; Sharma et al. 2012) found that inoculation with ZSB increased crop Zn uptake as a result of increased Zn phytoavailability. These experiments suggest that soil microorganisms can have a positive impact on the availability of metals in soil. Zn mobilization by the native soil bacteria and/or inoculated ZSB is likely stimulated by nutrients and energy supplied by

decomposing green manure. However, this remains to be tested.

To achieve this, we performed a factorial pot experiment including two green manure treatments, with or without the addition of clover (*Trifolium alexandrinum* L.). After green manure incorporation, half of the experimental soil was x-ray irradiated, which eliminated soil biota (including soil fungi), and re-inoculated exclusively with native soil bacteria. Additionally, a mix of six ZSB (their potential to increase Zn availability under in vitro conditions is described in Costerousse et al. (2018)), was inoculated to half of the pots. Spring wheat (*Triticum aestivum* L.) was grown to maturity in all these treatments and the experiment was conducted in two soils, one with sufficient (HIGH Zn soil) and one with low Zn availability (LOW Zn soil). The diffusive gradients in thin films (DGT) method were used as a sensitive proxy to monitor changes in plant available Zn throughout the pot experiment (Grüter et al. 2017a; Tandy et al. 2011). Treatment effects on Cd availability in soil and uptake by wheat was also monitored. We expected that Zn availability (and wheat Zn uptake) would be greater in the soils treated with clover, especially in the treatments simultaneously inoculated with ZSB. In other words, we hypothesized that the incorporation of clover would amplify Zn mobilization by native soil bacteria and the inoculated ZSB.

## Materials and methods

### Design and timeline of the pot experiment

A cross-factorial pot experiment was performed in which spring wheat (*Triticum aestivum* L., cv. Fiorina) was cultivated in two soils: one with sufficient (HIGH Zn soil) and one with non-sufficient plant available Zn (LOW Zn soil). The design of the pot experiment consisted of three factors with two levels each; (i) green manure addition (no green manure (NO GM), *Trifolium alexandrinum* (also designated as clover) (+GM)); (ii) soil x-ray irradiation (non-irradiated (NO IRR), x-ray irradiated (X-RAY IRR)); and (iii) ZSB inoculation ((NO ZSB), inoculated (+ZSB)). Destructive soil and plant sampling was done at two time points, i.e. wheat flowering (i.e. 60 days after wheat planting for HIGH Zn and 66 days

for LOW Zn) and wheat maturity (i.e. 123 days after wheat planting). For each time point, four experimental replicates were prepared. This resulted in 128 pots (4\*2(GM)\*2(IRR)\*2(ZSB)\*2(SOIL)\*2(Sampling points)), all arranged in a completely randomized design on the glasshouse bench with rearrangement of the pots every second week.

Clover was grown and incorporated in half of the HIGH Zn and the LOW Zn soils before wheat was planted to all the treatments. There were therefore two successive plant cultures on the same soils in the +GM treatments, whereas only wheat was grown in the NO GM treatments. After green manure growth and incorporation (but before wheat planting), half of the experiment soils (with and without green manure) were x-ray irradiated and re-inoculated with the native soil bacteria. ZSB were inoculated to the wheat seedlings and to the soil at wheat planting and only to the soil at wheat flowering.

#### Origins, characteristics and initial fertilization of the two soils

The soil with sufficient Zn plant availability (HIGH Zn soil) was sampled from the long-term field trial “Zurich Organic Fertilizer Experiment” (ZOFE) conducted at the research institute for sustainability sciences (ISS) (Agroscope, Zurich Reckenholz, Switzerland) (Oberholzer et al. 2014). This old field experiment, established in 1949, aims at comparing the effects of different organic and inorganic fertilization regimes on crop yield and soil chemical and biological properties. The HIGH Zn soil was sampled from the farmyard manure treatment (FYM) mainly because it had the highest diethylene triamine penta-acetic acid (DTPA) extractable Zn (4.22 mg kg<sup>-1</sup>) compared to other treatments of the ZOFE trial. The soil with non-sufficient plant Zn availability (LOW Zn soil) was imported from Turkey, Central Anatolia. The LOW Zn soil had DTPA extractable Zn concentrations below 0.5 mg kg<sup>-1</sup> (0.23 mg kg<sup>-1</sup>), and can therefore be considered as low in plant available Zn (Cakmak et al. 1996).

Initial soil characteristics are described in Table 1. The HIGH Zn soil was a carbonate free and acidic (pH 5.5) while the LOW Zn soil contained carbonate and was slightly alkaline (pH 7.3). The LOW Zn soil had a higher clay content and cation exchange capacity compared to the HIGH Zn soil. Total Cd, N and

Zn contents were higher in the HIGH Zn soil as compared to the LOW Zn. Likewise, DTPA extractable trace elements, Olsen P, microbial C and N biomasses and organic C were higher in the HIGH Zn than in the LOW Zn soil.

All essential macro- and micronutrients (except Zn) were added to the two soils at green manure sowing and N was added again at wheat planting, wheat tillering and wheat flowering resulting in total in 100 mg N added per kg<sup>-1</sup> soil (Table 2).

#### Green manure growth and incorporation

The green manure used in this study was the Fabaceae *Trifolium alexandrinum* L. cv. Elite II. Green manure seeds were purchased from Fenaco (UFA Samen, Winthertur, Switzerland). For each soil, four boxes (36 cm W \*35 cm D \*15 cm H) were filled with 14 kg of soil (dry weight equivalent). The initial fertilization was then applied to the eight boxes (Table 2), and soils were mixed carefully to ensure homogenous distribution of the nutrients. Half of the boxes, labelled as +GM, were used for growing *T. alexandrinum* while the other half, labelled as NO GM, were used as control treatment with no green manure amendment. Clover was sown directly on the soil surface at a rate of 0.08 g per kg<sup>-1</sup> soil (i.e. equivalent seeding density of 90 kg ha<sup>-1</sup>). Soil moisture in the boxes was maintained around 60% water holding capacity by adding water frequently. All boxes (with and without green manure) were transferred to the glasshouse with climatic conditions set to a photoperiod of 16 h with a light intensity of 50 kLux, a relative humidity of 65–70% and day/night temperature of 22 °C/16 °C.

Two weeks after green manure sowing, the LOW Zn soil was inoculated with *Rhizobium leguminosarum* (strain reference: DSMZ No. 30139) to ensure effective nodulation of the clover. This rhizobium strain was obtained from the German Collection of Microorganisms and Cell Cultures (<https://www.dsmz.de/>). For this purpose, *R. leguminosarum* was incubated and multiplied for 4 days on a rotary shaker at 28 °C in Yeast Extract Mannitol (YEM) broth prepared with yeast extract 1 g l<sup>-1</sup>, mannitol 10 g l<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 0.5 g l<sup>-1</sup>, MgSO<sub>4</sub> 0.2 g l<sup>-1</sup> and NaCl 0.1 g l<sup>-1</sup>. To inoculate LOW Zn, 20 ml of this *R. leguminosarum* culture was dispensed per kg<sup>-1</sup> dry soil. *R. leguminosarum* was also inoculated in LOW Zn where no green manure was grown. The HIGH Zn

**Table 1** Physicochemical and biological properties of the two soils used in this study

Parameter <sup>a</sup>	HIGH Zn	LOW Zn
pH (CaCl <sub>2</sub> )	5.47	7.34
Clay (g kg <sup>-1</sup> )	139	233
Texture <sup>b</sup>	sandy loam	sandy clay loam
Cation exchange capacity (cmol(+) kg <sup>-1</sup> )	5.66	34.76
Maximum water holding capacity (H <sub>2</sub> O (ml) per kg soil DW <sup>-1</sup> )	300	494
Total inorganic C (g kg <sup>-1</sup> )	<LOD <sup>c</sup>	14.90
Total organic C (g kg <sup>-1</sup> )	10.93	5.10
Total Cd (μg kg <sup>-1</sup> )	352.7	67.8
Total N (g kg <sup>-1</sup> )	1.04	0.38
Total Zn (mg kg <sup>-1</sup> )	76.6	26.6
DGT extractable Cd (μg l <sup>-1</sup> )	0.427	<LOD <sup>c</sup>
DGT extractable Zn (μg l <sup>-1</sup> )	15.6	<LOD <sup>c</sup>
DTPA extractable Cd (μg kg <sup>-1</sup> )	125.7	10.0
DTPA extractable Zn (mg kg <sup>-1</sup> )	4.22	0.23 <sup>d</sup>
Olsen P (mg kg <sup>-1</sup> )	36.27	2.12
Basal soil respiration (mg CO <sub>2</sub> -C kg <sup>-1</sup> h <sup>-1</sup> )	0.231	0.274
Microbial C (mg C kg <sup>-1</sup> )	83.3	26.1
Microbial N (mg N kg <sup>-1</sup> )	15.7	3.09

Soil analyses were performed before green manure sowing as an initial soil characterization

<sup>a</sup>Apart from total soil N, DGT extractable Zn and Cd, basal soil respiration and microbial C and N biomasses, the rest of the soil parameters given in this table was obtained from Grüter et al. (2017b)

<sup>b</sup>IUSS Working Group WRB (2014)

<sup>c</sup>Below limit of detection (<LOD)

<sup>d</sup>For LOW Zn, the DTPA extractable Zn was below 0.5 mg kg<sup>-1</sup>, commonly considered as the Zn deficiency threshold (Cakmak et al. 1996)

**Table 2** Nutrients added before green manure sowing and N application throughout wheat growth

Time of application	Element	Compound	Fertilization (mg kg soil <sup>-1</sup> )
Before green manure sowing			
	N	Mg(NO <sub>3</sub> ) <sub>2</sub> . KNO <sub>3</sub> . Cu(NO <sub>3</sub> ) <sub>2</sub>	25
	P	KH <sub>2</sub> PO <sub>4</sub>	60
	K	KH <sub>2</sub> PO <sub>4</sub> . K <sub>2</sub> SO <sub>4</sub> . KNO <sub>3</sub>	150
	S	K <sub>2</sub> SO <sub>4</sub> . MnSO <sub>4</sub>	25
	Fe	FeNaEDTA	10
	B	H <sub>3</sub> BO <sub>3</sub>	0.691
	Mn	MnSO <sub>4</sub>	0.703
	Mg	Mg(NO <sub>3</sub> ) <sub>2</sub>	17.2
	Cu	Cu(NO <sub>3</sub> ) <sub>2</sub>	0.081
	Mo	Na <sub>2</sub> MoO <sub>4</sub>	0.061
At wheat planting, tillering and flowering (applied three times)			
	N	NH <sub>4</sub> NO <sub>3</sub>	25 at each time point

soil was not inoculated with this rhizobium as preliminary tests highlighted infectious potential of this soil. Nodules were found on clover roots in both soils after 6 weeks of growth.

At this time, the green manure shoots were cut 1 cm above soil surface, pooled separately for each soil to a single green manure sample, dried at 60 °C, weighed for biomass estimation and milled homogeneously. From each of the two green manure samples, ca. 3 g of dry shoot matter were taken for total C, N, Zn and Cd element analyses (cf. “Plant analyses”). Soils were left for 3 weeks under the same conditions in the glasshouse to allow for green manure root decomposition. Only then, milled green manure shoots were transferred to their respective +GM box and soils were mixed again homogeneously. After an additional week of equilibration in the glasshouse, soils were transferred to 4 °C until further use.

#### Soil x-ray irradiation and re-inoculation with native bacteria

The goal of soil x-ray irradiation and re-inoculation was to obtain soils containing only bacterial communities. In this context, half the volume of NO GM and +GM soils (i.e. containing green manure residues) were transported to Synergyhealth (Däniken, Switzerland, <http://www.synergyhealthplc.com/us>) where they were x-ray irradiated with a dose range of 25 to 75 kGy. Such levels of irradiation have been reported to kill soil bacteria, fungi and invertebrates (McNamara et al. 2003). Soil sterility was checked after irradiation by inoculating Nutrient Agar (NA) with tenfold serial dilutions according to Trevors (1996) (data not shown). In order to re-introduce native soil bacterial communities in x-ray irradiated soils, 6.25 mL of filtered soil suspensions (25% (w:v) in 0.3 g L<sup>-1</sup> NaCl) were added per kg<sup>-1</sup> dry soil (the same volume of sterile NaCl 0.3 g L<sup>-1</sup> was added to each NO-IRR treatment). This was done following the procedure described by Aghili et al. (2014), except that we used two additional filtration steps, one through Whatman No. 42 filter paper (i.e. 2.5 µm pore size) in order to exclude fungi mycelia and spores, and a final filtration step through 0.22 µm pore size membranes to concentrate tenfold the obtained soil filtrates so as to reduce their volume. The 3-step filtration procedure used to prepare each soil filtrate (referred later as “bacterial wash”) is described in

details in the supplementary material (Sect. 1). Each bacterial wash was prepared from the corresponding NO IRR soil.

#### ZSB inoculation

Half of the pots were inoculated with a mix consortium of ZSB including three strains of *Pseudomonas* (*P. chlororaphis*, *P. moraviensis*, *P. syringae*; accession numbers from 16S rRNA gene sequences blasted against the NCBI nr database: FJ652611.1, HG000013.1, AY574913.1), two strains of *Plantibacter flavus* (both HE716918.1) and one strain of *Streptomyces narbonensis* (KU324447). Two of the inoculated strains (*S. narbonensis* and *P. moraviensis*) were isolated from the HIGH Zn soil itself, and the four others from a soil with very similar chemical properties as reported in Costerousse et al. (2018). In the same study, the six inoculated ZSB were selected out of 115 Zn solubilizing isolates based on their abilities to produce organic acids, release siderophores and dissolve scarcely soluble Zn salts (Zn oxide and Zn carbonate) in plate and broth assays. To multiply ZSB for wheat seedlings and soil inoculation, ZSB were grown separately overnight in Nutrient Broth (NB) (peptone 5 g l<sup>-1</sup> (BD Difco, Allschwil, Switzerland), meat extract 3 g l<sup>-1</sup> (Sigma-Aldrich, Buchs, Switzerland)) with shaking at 28 °C. To quantify viable bacterial cells in NB, relation between optical density (OD) at 600 nm with the number of Colony Forming Units (CFU) per mL was used.

In the +ZSB treatments, the 6 strains were inoculated to the wheat seedlings (i.e. prior to planting) and twice to the soil (i.e. at wheat planting and flowering). This was done to promote survival and persistence of the inoculated strains in soil and at the interface of the wheat roots throughout the pot experiment. Indeed, previous soil inoculation studies with phosphorus solubilizing bacteria have shown that density of inoculated strains may decrease within one month after inoculation (Meyer et al. 2017a; Mosimann et al. 2017).

For seedling inoculation, wheat seeds were germinated (cf. next section of the materials and methods, “Pot experiment”) and four days old seedlings were dipped for 2 h in a 2-L bacterial suspension containing the equivalent of 2 × 10<sup>7</sup> CFU mL<sup>-1</sup> of each ZSB. For soil inoculation, NB aliquots containing 8 × 10<sup>9</sup> CFU from each ZSB were pooled and

centrifuged at 3000 g for 10 min. Using NaCl 0.3 g L<sup>-1</sup>, bacterial cells were washed twice and cell concentration adjusted to 8 × 10<sup>6</sup> CFU mL<sup>-1</sup> of each ZSB. At wheat planting and flowering, 12.5 mL from this mixed ZSB suspension was added per kg of dry soil and hence, the equivalent of 10<sup>5</sup> CFU of each ZSB was added per g of soil at both time points (or 7.5 × 10<sup>7</sup> CFU per pot<sup>-1</sup>).

#### Pot experiment

The pots were previously washed with hot water, sprayed with ethanol 70% and air dried. The following solutions were applied and mixed to the soils prior to potting (i.e. per kg dry weight); (i) 12.5 mL of initial N fertilization solution (containing 25 mg of N in the form of NH<sub>4</sub>NO<sub>3</sub>; Table 2), (ii) 6.25 mL of filtered soil suspension (bacterial wash) in X-RAY IRR treatments and (iii) 12.5 mL of mixed ZSB suspension in +ZSB treatments (the same volumes of sterile NaCl 0.3 g L<sup>-1</sup> was added to NO IRR and NO ZSB pots). After mixing the soils thoroughly, samples were taken for soil analyses at wheat planting and 750 g of soil (dry weight equivalent) were placed into each pot.

When setting up the pot experiment, all wheat seeds (*Triticum aestivum* L., cv. Fiorina) were surface sterilized with 10% H<sub>2</sub>O<sub>2</sub> for 30 min and 0.3% previcur fungicide (propamocarb and fosetyl, Bayer) for 20 min. After rinsing with autoclaved nanopure water, the seeds were placed on autoclaved sand for 4 days during which germination and primary growth of the seedlings occurred. Half of the seedlings were inoculated with ZSB. The pots were transferred to a glasshouse bench and two wheat seedlings were planted per pot (thinned to only one plant per pot one week later). Climatic conditions were set to a photoperiod of 14 h (light intensity of 12 kLux), a relative humidity of 40–45% and day/night temperature of 20 °C/14 °C. Soil moisture in the pots was raised on a balance to ca. 60% water holding capacity by watering every second day.

#### Soil analyses

Soil analyses were performed at three time points: before green manure sowing (i.e. for the initial soil characterization; Table 1), at wheat planting (i.e. on soil samples taken after the addition of N and the

bacterial wash (in X-RAY IRR treatments) or the NaCl solution (in NO IRR treatments)) and at wheat flowering. At this time point, half of the pots were sampled destructively for soil and wheat shoot analyses. The remaining half of the pots were maintained until wheat maturity where no soil but only wheat roots, shoots and grains were sampled and analyzed. No soil analyses were performed at wheat planting on the +ZSB soil treatments as ZSB consortium had just been inoculated and had had no time to take action.

Table 3 summarizes the various soil analyses performed at each time point and how the soil samples were prepared for analysis. Clay content, soil texture, cation exchange capacity, maximum water holding capacity, soil organic and inorganic C content, total element concentrations, DTPA extractable Zn and Cd and Olsen P data were obtained from Grüter et al. (2017b), where the analytical methods can also be found. The methods used for the remaining soil analyses (soil pH, total soil N, DGT extractable Zn and Cd, soil respiration, microbial C and N contents and soil mineral N concentrations) can be found in the supplementary material (Sect. 2).

#### Plant analyses

Milled and oven dried green manure shoot samples (60 °C) were taken before it was incorporated to the corresponding soil.

At wheat flowering, half of the pots were removed from the glasshouse for wheat root and shoots sampling. We used the root staining procedure described by Phillips and Hayman (1970) to look for mycorrhizal structures and compare root mycorrhizal colonization among the treatments at wheat flowering. Wheat shoots were cut 1 cm above the ground, dried for 5 days at 40 °C, weighed and milled.

At wheat maturity, the plants were harvested and processed following the same procedure. The wheat shoots (i.e. including chaff) were separated from the wheat grains. At wheat maturity only, the roots were washed with tap water, and dried at 40 °C for wheat root biomass determination. Number of ears, grains per ear and grains per pots were also reported at wheat maturity.

Plant Zn and Cd concentrations were measured by digesting 200 mg of dried plant material in 2 mL 11 M HNO<sub>3</sub> for 30 min at ca. 250 °C and a pressure of 40 bar in the high pressure single reaction change

**Table 3** List of soil analyses performed at the different time points

Parameters	Time point			Soil preparation
	Before green manure sowing	Wheat planting	Wheat flowering	
pH	x	x	x	b
Clay	x			b
Texture	x			b
Cation exchange capacity	x			a
Maximum water holding capacity	x			a
Soil organic and inorganic C content	x			b
Total element concentrations	x			b
Total soil N	x	x	x	b
DGT extractable Zn and Cd	x	x	x	a
DTPA extractable Zn and Cd	x			b
Olsen P	x			b
Soil respiration	x		x	a
Microbial C	x		x	a
Microbial N	x			a
Soil mineral N (NO <sub>3</sub> <sup>-</sup> and NH <sub>4</sub> <sup>+</sup> )		x	x	a

<sup>a</sup>parameters measured on moist soil. Fresh soil samples were always kept at 4 degrees between their collection and their analyses

<sup>b</sup>analyses performed on oven dried soil (40 °C for 48 h), sieved 2 mm and ground with a vibratory disc mill. For texture analysis, the soil was not ground

microwave system (turboWave, MWS microwave systems, Leutkirch, Germany). Zn and Cd concentrations in the extracts were measured by IPC-MS (7500ce, Agilent Technologies, Santa Clara, CA USA). Total N concentrations in green manure and wheat shoots and grains were determined with an NCS analyser (FlashEA 1112 Series, Thermo Fisher Scientific Inc., Waltham, MA USA).

### Statistical analyses

Statistical analyses were performed using the JMP® software, Version 12.2.0 (SAS Institute Inc., Cary, NC, USA). Graphics were constructed also with JMP® or using GraphPad Prism, Version 7.0a (GraphPad Software, La Jolla, CA). Two-way and three-way analyses of variance (ANOVA) were used to test the effects of the factors (green manure addition, soil x-ray irradiation, ZSB inoculation) and their interactions. Statistical analyses were performed separately for the two soils. Tukey's HSD post hoc test was performed to further assess significances of the differences between the means. The "Fit Y by X" function of JMP® was used for Pearson linear

regressions. When needed, data were log, square root or Box-Cox transformed to fit normal distribution of their residuals which was verified using the Shapiro–Wilk W test.

## Results

### Characteristics of the incorporated green manure

Green manure shoots grown on and incorporated to the LOW Zn soil had a lower N concentration (25.7 g kg<sup>-1</sup>) compared to those incorporated in the HIGH Zn soil (32.3 g kg<sup>-1</sup>) (Table 4). Consequently, the C/N ratio of the green manure incorporated in the LOW Zn soil was higher and less N was incorporated per kg<sup>-1</sup> soil in the LOW Zn (67.1 mg N) than in the HIGH Zn soil (95.8 mg N) (Table 4). Clover Zn and Cd concentrations were tenfold lower in the LOW Zn than in the HIGH Zn soil. Thus, far less Zn and Cd were reincorporated per kg<sup>-1</sup> soil in the LOW Zn (20.3 µg of Zn and 0.121 µg of Cd) compared to the HIGH Zn soil (265.9 µg of Zn and 1.46 µg of Cd)



**Table 4** Characteristics of the green manure (*Trifolium alexandrinum* L.) grown in situ for 6 weeks in the HIGH Zn and the LOW Zn soils

Soil	Green manure element concentration <sup>a</sup>				Incorporated with green manure shoots kg <sup>-1</sup> soil			
	C/N ratio	N (g kg <sup>-1</sup> )	Zn (mg kg <sup>-1</sup> )	Cd (µg kg <sup>-1</sup> )	Biomass (g)	N (mg)	Zn (µg)	Cd (µg)
HIGH Zn	12.7 ± 0.1	32.3 ± 0.4	93.1 ± 2.1	490.2	2.97	95.8	276.5	1.46
LOW Zn	15.6 ± 0.2	25.7 ± 0.4	8.0	46.2	2.61	67.1	21.6	0.121

Before incorporation, clover shoots were harvested and pooled in two green manure samples (one produced in the HIGH Zn and one in the LOW Zn soil). Total C, N, Zn and Cd analyses were performed on these two representative samples. Values after the means within a column correspond to the standard errors of the analytical replicates

<sup>a</sup>C/N ratio and N values are means of five analytical replicates. Zn and Cd values are means of two to four analytical replicates (except for Cd concentration in LOW Zn where only one value was available). No standard deviation was calculated when two analytical replicates were analyzed

(Table 4). These amounts do not include the N, Zn and Cd incorporated with the green manure roots.

#### Soil N and biological parameters

ANOVA tests revealed significant green manure and soil x-ray irradiation effects on soil pH at wheat planting in the HIGH Zn soil (Table 5) and in both soils at wheat flowering (Table 6). However, differences in soil pH means were small (i.e. 0.1 to 0.3 pH units) and were therefore considered as irrelevant. Total soil N was significantly increased by the incorporation of green manure in both soils at wheat planting (Table 5) and wheat flowering (Table 6). At wheat planting, this increase of ca. 80 mg N corresponded nearly to the amount of N added with the green manure (95.8 mg N). In both soils, soil x-ray irradiation did not affect total soil N at wheat planting (Table 5) but decreased it at wheat flowering (Table 6).

At wheat planting, soil NO<sub>3</sub><sup>-</sup> concentrations (but not NH<sub>4</sub><sup>+</sup>) were lower in the +GM than in the NO GM treatments (Table 5). This resulted in lower total soil mineral N concentrations in the +GM compared to the NO GM treatments in both the HIGH Zn and the LOW Zn soils at wheat planting. This was also the case at wheat flowering in the LOW Zn (Fig. 1B) but not in the HIGH Zn soil (Fig. 1A) (see Table S1 for three-way ANOVA of mineral N concentrations at wheat flowering). Mineral N concentrations were lower in the LOW Zn compared to those found in the HIGH Zn soil at wheat flowering (Fig. 1).

In the HIGH Zn soil, lower NO<sub>3</sub><sup>-</sup> and higher NH<sub>4</sub><sup>+</sup> concentrations were found in the X-RAY IRR compared to the NO IRR treatments at wheat planting (Table 5) and wheat flowering (Fig. 1). In this soil, this was accompanied by higher mineral N concentrations in the X-RAY IRR compared to the NO IRR treatments at both of these time points. On the other hand, in the LOW Zn soil, there were similar levels of NO<sub>3</sub><sup>-</sup> in the X-RAY IRR and the NO IRR treatments and NH<sub>4</sub><sup>+</sup> concentrations were only increased by soil x-ray irradiation in the +GM treatments at wheat planting (Table 5). As for the HIGH Zn soil, lower NO<sub>3</sub><sup>-</sup> concentrations were found in the X-RAY IRR compared to the NO IRR treatments of the LOW Zn soil at wheat flowering (Fig. 1) and at this time point NH<sub>4</sub><sup>+</sup> was either entirely depleted (in NO IRR) or very low (in X-RAY IRR).

Soil respiration and microbial C biomass were increased by the incorporation of green manure in both the HIGH Zn and the LOW Zn soils (Table 6). Soil respiration was also increased by soil x-ray irradiation while microbial C biomass was lower in the X-RAY IRR compared to the NO IRR treatments in both soils (Table 6). ZSB inoculation had no significant effects on soil pH, total soil N, microbial C biomass and soil respiration (except in the HIGH Zn soil where ZSB inoculation significantly decreased soil respiration in +GM\_X-RAY IRR) (Table 6). Likewise, ZSB inoculation did not affect soil mineral N concentrations at wheat flowering (Fig. 1).

**Table 5** Soil parameters measured at wheat planting

Green manure	Soil x-ray irradiation	Soil pH		Total soil N (g kg <sup>-1</sup> )		Mineral NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )		Mineral NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )		DGT Zn (µg L <sup>-1</sup> )		DGT Cd (µg L <sup>-1</sup> )	
		HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn
NO GM	NO IRR	5.8 <sup>c</sup>	7.7 <sup>a</sup>	1.03 <sup>b</sup>	0.340 <sup>b</sup>	26.6 <sup>a</sup>	32.1 <sup>a</sup>	5.30 <sup>b</sup>	5.25 <sup>b</sup>	57.8 <sup>d</sup>	NA	1.48 <sup>d</sup>	NA
	X-RAY IRR	5.9 <sup>b</sup>	7.8 <sup>a</sup>	1.02 <sup>b</sup>	0.342 <sup>b</sup>	20.4 <sup>ab</sup>	25.2 <sup>a</sup>	15.6 <sup>a</sup>	9.06 <sup>b</sup>	82.9 <sup>c</sup>	10.7	2.56 <sup>c</sup>	0.334
+GM	NO IRR	5.8 <sup>c</sup>	7.7 <sup>a</sup>	1.11 <sup>a</sup>	0.395 <sup>a</sup>	16.5 <sup>b</sup>	11.0 <sup>b</sup>	4.25 <sup>b</sup>	7.09 <sup>b</sup>	127.4 <sup>a</sup>	33.6	4.42 <sup>a</sup>	0.524
	X-RAY IRR	6.1 <sup>a</sup>	7.7 <sup>a</sup>	1.10 <sup>a</sup>	0.401 <sup>a</sup>	8.43 <sup>c</sup>	11.2 <sup>b</sup>	20.4 <sup>a</sup>	18.8 <sup>a</sup>	110.6 <sup>b</sup>	9.84	3.60 <sup>b</sup>	0.509
Two-way ANOVA(F ratio)													
Green manure (A)		41.4***	3.15	116.4***	135.7***	51.6***	88.2***	0.733	27.6***	210.0***		859.4***	
Soil x-ray irradiation (B)		306.1***	4.04	2.77	0.731	21.2***	1.28	36.9***	49.4***	1.49		3.06	
A*B		43.1***	3.58	0.269	0.175	0.366	1.60	1.78	12.9**	38.8***		193.0***	

Means with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test. Two-way ANOVA results show F ratio and the asterisks represent level of significance (\*\*\*)  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ )

Soil samples for the analyses at wheat planting were taken before potting (after the addition of N and the bacterial wash (in X-RAY IRR treatments) or the NaCl solution (in NO IRR treatments)). At this time point, ZSB consortium had just been inoculated and thus, no soil samples were taken from the +ZSB treatments

NA—DGT Zn and Cd values not available. This also prevented any statistical analyses of the DGT Zn and Cd results for the LOW Zn soil at this time point

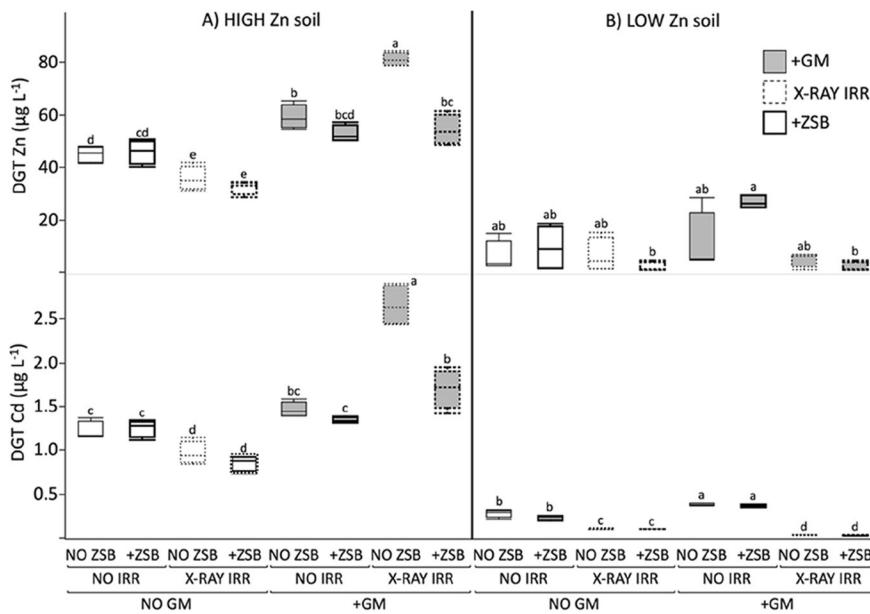
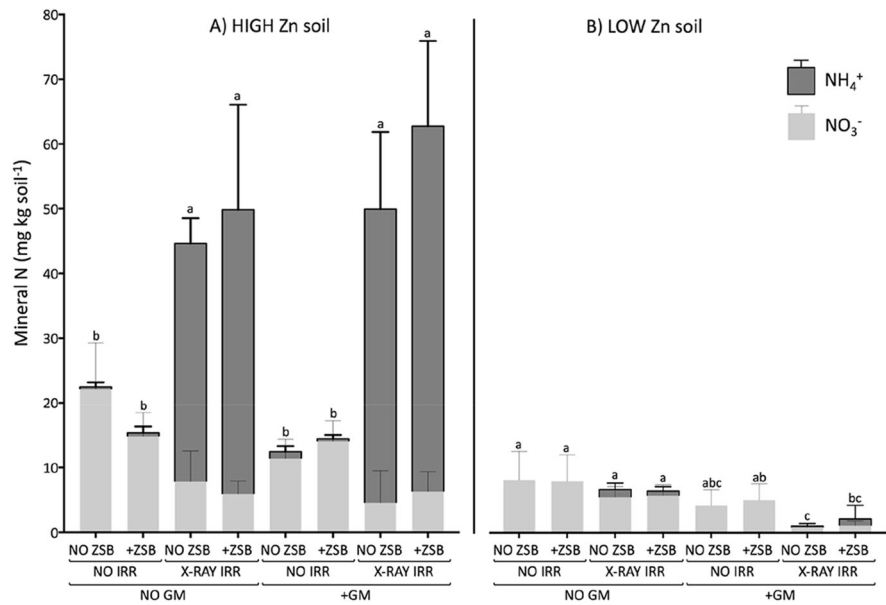
**Table 6** Selected soil parameters measured at wheat flowering

Green manure	Soil x-ray irradiation	ZSB inoculation	Soil pH		Total soil N		Soil respiration		Microbial C	
			HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn
NO GM	NO IRR	NO ZSB	5.8 <sup>c</sup>	7.8 <sup>ab</sup>	1.02 <sup>b</sup>	0.339 <sup>c</sup>	0.175 <sup>e</sup>	0.289 <sup>de</sup>	59.4 <sup>ab</sup>	38.1 <sup>b</sup>
		+ZSB	5.8 <sup>bc</sup>	7.8 <sup>ab</sup>	1.02 <sup>b</sup>	0.334 <sup>c</sup>	0.166 <sup>e</sup>	0.277 <sup>e</sup>	54.3 <sup>abc</sup>	39.7 <sup>b</sup>
	X-RAY IRR	NO ZSB	5.9 <sup>ab</sup>	7.9 <sup>a</sup>	0.955 <sup>c</sup>	0.324 <sup>c</sup>	0.247 <sup>bc</sup>	0.326 <sup>c</sup>	28.5 <sup>de</sup>	17.8 <sup>c</sup>
		+ZSB	5.9 <sup>ab</sup>	7.8 <sup>ab</sup>	0.966 <sup>c</sup>	0.330 <sup>c</sup>	0.224 <sup>cd</sup>	0.313 <sup>cd</sup>	15.7 <sup>e</sup>	17.7 <sup>c</sup>
+GM	NO IRR	NO ZSB	5.8 <sup>c</sup>	7.7 <sup>b</sup>	1.09 <sup>a</sup>	0.436 <sup>a</sup>	0.213 <sup>d</sup>	0.342 <sup>bc</sup>	63.4 <sup>ab</sup>	62.1 <sup>a</sup>
		+ZSB	5.8 <sup>c</sup>	7.8 <sup>ab</sup>	1.09 <sup>a</sup>	0.428 <sup>a</sup>	0.226 <sup>cd</sup>	0.373 <sup>ab</sup>	72.1 <sup>a</sup>	70.6 <sup>a</sup>
	X-RAY IRR	NO ZSB	6.0 <sup>a</sup>	7.8 <sup>ab</sup>	1.06 <sup>ab</sup>	0.382 <sup>b</sup>	0.319 <sup>a</sup>	0.442 <sup>a</sup>	46.6 <sup>bc</sup>	39.2 <sup>b</sup>
		+ZSB	6.0 <sup>a</sup>	7.8 <sup>ab</sup>	1.03 <sup>b</sup>	0.385 <sup>b</sup>	0.278 <sup>b</sup>	0.408 <sup>a</sup>	37.9 <sup>cd</sup>	31.6 <sup>b</sup>
Three-way ANOVA (F ratio)										
Green manure (A)			0.281	8.35 <sup>**</sup>	138.6 <sup>***</sup>	341.6 <sup>***</sup>	131.9 <sup>***</sup>	195.3 <sup>***</sup>	32.6 <sup>***</sup>	211.4 <sup>***</sup>
Soil x-ray irradiation (B)			88.4 <sup>***</sup>	11.2 <sup>**</sup>	62.2 <sup>***</sup>	50.0 <sup>***</sup>	221.2 <sup>***</sup>	58.0 <sup>***</sup>	122.4 <sup>***</sup>	282.7 <sup>***</sup>
ZSB inoculation (C)			0.082	1.06	0.394	0.042	9.18 <sup>**</sup>	0.975	2.68	0.157
A *B			6.71 <sup>*</sup>	0.156	0.505	22.0 <sup>***</sup>	2.16	0.389	2.91	10.1 <sup>**</sup>
A *C			0.880	0.072	2.99	0.103	0.046	3.56	2.65	0.010
B *C			0.400	1.06	0.162	1.62	11.9 <sup>**</sup>	2.16	5.32 <sup>*</sup>	8.39 <sup>**</sup>
A *B *C			0.220	2.45	1.12	0.00	4.28 <sup>*</sup>	3.84	0.780	5.33 <sup>*</sup>

Means with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test. Three-way ANOVA results show F ratio and the asterisks represent level of significance (\*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ )

Soil mineral N concentrations (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) as well DGT extractable Zn and Cd were also measured at this time point and these results are shown in Figs. 1 and 2, respectively

**Fig. 1** Mineral N ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) at wheat flowering in the HIGH Zn (A) and the LOW Zn (B) soil. Bar plots represent stacked  $\text{NH}_4^+$  and  $\text{NO}_3^-$  means with their associated standard errors ( $n=4$ ). Plots with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test (performed on the total mineral N data separately for HIGH Zn and LOW Zn)



**Fig. 2** DGT extractable soil Zn and Cd at wheat flowering in the HIGH Zn (A) and the LOW Zn (B) soil ( $n=4$ ). Box plots with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test (performed four times separately on DGT Zn and Cd data for HIGH Zn and LOW Zn). Boxes filled with grey represent treatments where

green manure had been incorporated before wheat planting (+GM). Dashed boxes represent treatments where soil was x-ray irradiated and re-inoculated with native soil bacteria exclusively (X-RAY IRR). Thick black boxes represent pots where ZSB were inoculated (+ZSB)

No mycorrhizal structures were found on wheat roots sampled at wheat flowering in any of the treatments (data not shown).

Soil Zn and Cd extractable with diffusive gradients in thin films (DGT)

In the HIGH Zn soil, DGT extractable Zn and Cd were the highest in the +GM treatments at wheat planting (Table 5) and flowering (Fig. 2A) (see Table S1 for three-way ANOVA of DGT Zn and Cd at wheat flowering). At wheat planting, HIGH Zn soil x-ray irradiation increased DGT Zn and Cd in the NO GM but decreased these soil parameters in the +GM treatments (Table 5). Soil x-ray irradiation had the opposite effect at wheat flowering in the HIGH Zn soil as it decreased DGT Zn and Cd in the NO GM but increased these same parameters in the +GM treatments (Fig. 2A). Consequently, in this soil, DGT Zn and Cd levels were much higher in the +GM\_X-RAY IRR compared to what was found in any of the other treatments at wheat flowering. In the HIGH Zn soil, while ZSB inoculation did not affect DGT Zn and Cd in the NO GM, it decreased these parameters in the +GM treatments (Fig. 2A). This was the most striking in +GM\_X-RAY IRR, where ZSB inoculation decreased DGT Zn and Cd by up to 35% (Fig. 2A).

In the LOW Zn soil, DGT Zn and Cd values were much lower compared to those observed in the HIGH Zn soil and these were even lower in the X-RAY IRR treatments at wheat flowering (Fig. 2B). The incorporation of green manure to the LOW Zn soil increased DGT Zn and Cd at wheat flowering in the NO IRR treatments, although this increase was significant only for DGT Cd (Fig. 2B). In contrast to what was observed in the HIGH Zn soil at wheat flowering, the lowest DGT Cd values in the LOW Zn soil were found in the +GM\_X-RAY IRR treatments and ZSB inoculation had no effect on DGT extractable Zn and Cd (Fig. 2B).

Pearson correlations were further investigated between DGT Zn or Cd and soil pH, total soil N and mineral N concentrations, soil respiration and microbial C biomass at wheat flowering (Table S2). For the X-RAY IRR treatments of the HIGH Zn soil, strong positive relationships were found between DGT extractable Zn and total soil N ( $r=0.88$ ;  $p<0.001$ ), soil respiration ( $r=0.89$ ;  $p<0.001$ ) and microbial C

biomass ( $r=0.80$ ;  $p<0.001$ ) (Table S2 and Fig S1). For the NO IRR treatments in the HIGH Zn soil, positive but weaker correlations were found between DGT extractable Zn and total soil N ( $r=0.78$ ;  $p<0.001$ ) and soil respiration ( $r=0.65$ ;  $p<0.01$ ). Correlation results obtained between DGT extractable Cd and the above-mentioned soil biochemical parameters were very similar to the once observed for DGT extractable Zn in the X-RAY IRR and the NO IRR treatments in the HIGH Zn soil. In the non-irradiated LOW Zn soil, DGT extractable Cd (but not Zn) was significantly positively correlated with total soil N, soil respiration and microbial C biomass (Table S2). Opposite results were found for the DGT extractable Cd in the x-ray irradiated LOW Zn, because there was far less DGT Cd in the +GM compared to the NO GM treatments of the x-ray irradiated LOW Zn (Fig. 2B).

#### Wheat growth and element accumulation

There was a trend toward lower wheat shoot biomass in the +GM compared to the NO GM treatments in the HIGH Zn (at wheat maturity (Table 7)) and in the LOW Zn soil (both at wheat flowering (Table S3) and wheat maturity (Table 7)). Similarly, in the HIGH Zn soil, grain biomass (Table 7) and the number of grains produced per kg soil<sup>-1</sup> (Table S4) tended to be lower with green manure incorporation. On the other hand, in the LOW Zn soil, the highest grain biomass (Table 7) and number of grains produced per kg soil<sup>-1</sup> (Table S4) were observed in the +GM\_X-RAY IRR treatments. In none of the two soils, the incorporation of green manure affected wheat root biomass at wheat maturity (Table 7).

Unlike green manure incorporation, soil x-ray irradiation had strong effects on wheat biomass production. In the HIGH Zn and the LOW Zn soil, soil x-ray irradiation increased wheat shoot biomass at flowering (Table S3) and also wheat shoot, grain and root biomass at maturity (Table 7). Moreover, at wheat maturity, a higher number of ears per plant and grains produced per kg soil<sup>-1</sup> was observed in the X-RAY IRR compared to the NO IRR treatments (Table S4).

In both soils, wheat Zn concentrations were not (or marginally) increased by the incorporation of green manure at wheat flowering (Fig S2) and wheat maturity (Figs. 3 and 4) (see Table S5 for three-way ANOVA of shoot and grain Zn, Cd and N concentrations at maturity). In contrast, wheat shoot and grain

**Table 7** Wheat shoot, grain and root biomass at maturity

Green manure	Soil x-ray irradiation	ZSB inoculation	Shoot biomass		Grain biomass		Root biomass	
			(g kg soil <sup>-1</sup> )		(g kg soil <sup>-1</sup> )		(g kg soil <sup>-1</sup> )	
			HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn
NO GM	NO IRR	NO ZSB	1.63 <sup>ab</sup>	1.87 <sup>abc</sup>	2.24 <sup>ab</sup>	1.56 <sup>abc</sup>	0.214 <sup>abc</sup>	0.349 <sup>ab</sup>
		+ ZSB	1.44 <sup>b</sup>	1.66 <sup>bc</sup>	2.13 <sup>ab</sup>	1.16 <sup>c</sup>	0.167 <sup>bc</sup>	0.267 <sup>b</sup>
	X-RAY IRR	NO ZSB	1.83 <sup>a</sup>	2.17 <sup>a</sup>	2.45 <sup>a</sup>	1.52 <sup>abc</sup>	0.272 <sup>ab</sup>	0.394 <sup>ab</sup>
		+ ZSB	1.82 <sup>a</sup>	1.95 <sup>ab</sup>	2.50 <sup>a</sup>	1.60 <sup>abc</sup>	0.289 <sup>a</sup>	0.522 <sup>a</sup>
+ GM	NO IRR	NO ZSB	1.41 <sup>b</sup>	1.54 <sup>c</sup>	2.10 <sup>ab</sup>	1.54 <sup>abc</sup>	0.154 <sup>c</sup>	0.314 <sup>ab</sup>
		+ ZSB	1.43 <sup>b</sup>	1.52 <sup>c</sup>	1.67 <sup>b</sup>	1.42 <sup>bc</sup>	0.170 <sup>abc</sup>	0.303 <sup>ab</sup>
	X-RAY IRR	NO ZSB	1.63 <sup>ab</sup>	1.95 <sup>ab</sup>	2.26 <sup>a</sup>	1.93 <sup>a</sup>	0.254 <sup>abc</sup>	0.403 <sup>ab</sup>
		+ ZSB	1.63 <sup>ab</sup>	1.91 <sup>abc</sup>	2.34 <sup>a</sup>	1.89 <sup>ab</sup>	0.218 <sup>abc</sup>	0.396 <sup>ab</sup>
Three-way ANOVA (F ratio)								
Green manure (A)			9.72 <sup>**</sup>	8.98 <sup>**</sup>	6.28 <sup>*</sup>	10.2 <sup>**</sup>	3.80	0.641
Soil x-ray irradiation (B)			25.2 <sup>***</sup>	32.1 <sup>***</sup>	15.1 <sup>***</sup>	18.5 <sup>***</sup>	24.1 <sup>***</sup>	11.1 <sup>**</sup>
ZSB inoculation (C)			0.709	4.00	0.583	2.50	0.723	0.038
A*B			0.591	0.832	0.050	2.38	0.00	0.656
A*C			1.27	2.28	0.134	0.278	0.308	0.198
B*C			0.809	0.020	2.51	3.73	0.033	2.19
A*B*C			1.00	0.008	0.324	1.68	2.94	2.03

Means with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test. Three-way ANOVA results show F ratio and the asterisks represent level of significance (\*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ )

Cd concentrations was decreased by the incorporation of green manure in the HIGH Zn (only in X-RAY IRR) (Fig. 3) and in the LOW Zn soil in all the treatments (Fig. 4). In the LOW Zn soil, green manure addition tended to decrease shoot and grain N concentrations (Fig. 4).

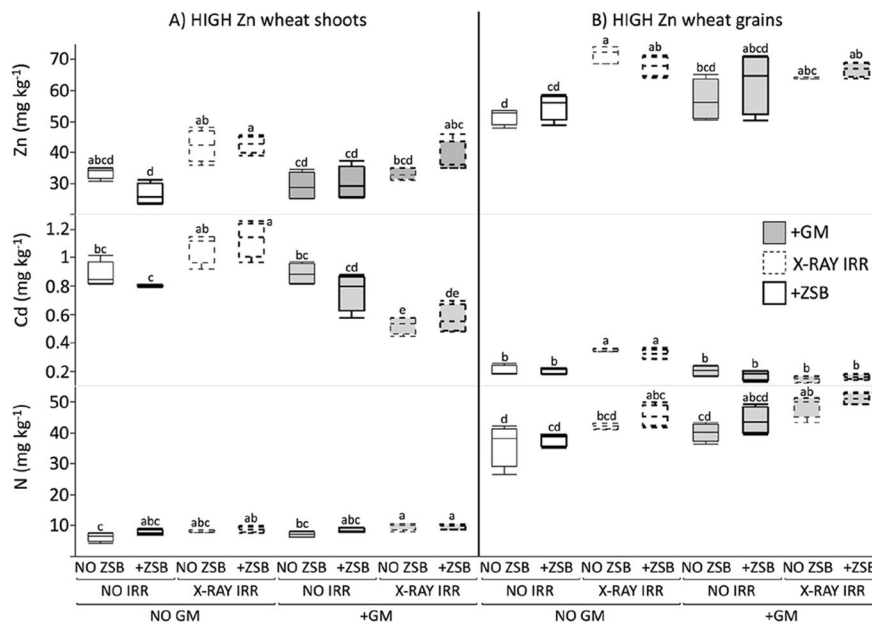
In the HIGH Zn soil, shoots and grain Zn, Cd and N concentrations were generally higher in the X-RAY IRR than in the NO IRR treatments (except for Cd concentrations, which decreased with the incorporation of green manure in the X-RAY IRR treatments) (Fig. 3). Consequently, in this soil, wheat took up more Zn, Cd and N in the X-RAY IRR than in the NO IRR treatments (see also Zn, Cd and N uptake in Table S6). In the LOW Zn soil, element concentrations were either unaffected (for Zn) or slightly increased by soil x-ray irradiation (Cd and N concentrations) (Fig. 4). Thus, as in the HIGH Zn soil, uptakes of Zn, Cd and N from irradiated LOW Zn were generally higher than in the NO IRR treatments (Table S6). In both soils, ZSB inoculation did neither affect wheat growth, nor element concentrations (Figs. 3 and 4).

For the HIGH Zn soil, positive correlations were found between concentrations of N and Zn in the wheat shoots at flowering ( $r = 0.70$ ;  $p < 0.001$ ) and in the wheat grains at maturity ( $r = 0.62$ ;  $p < 0.001$ ) (Table 8). No correlations between shoot and grain N and Cd concentrations were found for the wheat grown in this soil due to the lower wheat Cd concentrations observed in the +GM\_X-RAY IRR treatments (Fig. 3). For the LOW Zn soil, except for the shoot Zn concentrations at wheat flowering, shoot and grain N and Zn and Cd concentrations were positively correlated at wheat flowering and maturity (Table 8).

## Discussion

Soil x-ray irradiation increased soil mineral N, wheat growth and element accumulation

In the HIGH Zn soil, x-ray irradiation increased soil  $\text{NH}_4^+$  concentrations considerably, which resulted in higher total mineral soil N concentrations in the X-RAY IRR compared to the NO IRR treatments



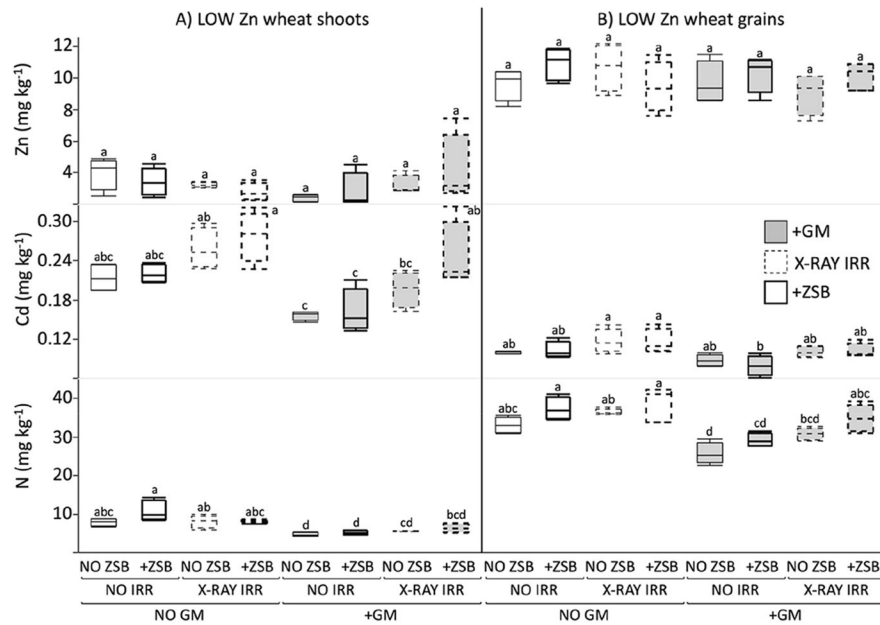
**Fig. 3** Wheat shoot (A) and grain (B) Zn, Cd and N concentrations at maturity in the HIGH Zn soil. Box plots with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test (performed six times separately on Zn, Cd and N data for HIGH Zn wheat shoots and grains). Boxes filled with grey represent treatments where green

manure had been incorporated before wheat planting (+GM). Dashed boxes represent treatments where soil was x-ray irradiated and re-inoculated with native soil bacteria exclusively (X-RAY IRR). Thick black boxes represent pots where ZSB were inoculated (+ZSB)

at wheat planting (Table 5) and wheat flowering (Fig. 1). Wheat grown in the irradiated HIGH Zn soil benefited from the increased N availability, by producing more biomass (including root) and accumulating more N in their tissues. This might have also been the case for the wheat grown in the irradiated LOW Zn soil, although differences in mineral N concentrations were no longer visible at wheat flowering. These irradiation effects can potentially explain the higher N, Zn and Cd uptake in the X-RAY IRR compared to the NO IRR treatments (Table S6). Indeed, increased root biomass (i.e. more roots and root hairs) might have enhanced wheat access to Zn and Cd in soil and higher wheat N concentrations could have promoted the synthesis of N rich compounds involved in trace element uptake and translocation within the plant (Palmer and Guerinot 2009). Soil irradiation was also reported to increase the availability of other macronutrients (e.g. P and sulfur) (McNamara et al. 2003) and to eliminate phytopathogens (Thompson 1990), which could contribute to higher biomass production and element uptake in the X-RAY IRR compared to the NO IRR treatments.

In previous studies, increased mineral N upon soil irradiation was attributed to microbial lysis and the subsequent release of N and N mineralizing enzymes (e.g. protease and deaminase) (Lensi et al. 1991; Salonius et al. 1967). As observed in our experiment, decrease in  $\text{NO}_3^-$  upon soil irradiation has often been reported and the underlying biochemical mechanisms are not fully understood (McNamara et al. 2003), although this may partly be attributed to the elimination of the nitrifiers.

One might have expected the wheat grown in NO IRR to establish symbiosis with AM fungi (but not in the X-RAY IRR soils, free of fungi). However, no mycorrhizal structures were found on wheat roots sampled at wheat flowering in any of the treatments, probably because of the initial soil P addition which might have inhibited root-mycorrhizal associations (Nouri et al. 2014). Alternatively, extensive soil storage over several years coupled with high disturbance such as drying and sieving may also have diminished the infective potential of the soils.



**Fig. 4** Wheat shoot (A) and grain (B) Zn, Cd and N concentrations at maturity in the LOW Zn soil. Box plots with identical letters are not significantly different at  $p < 0.05$  according to the Tukey HSD post hoc test (performed six times separately on Zn, Cd and N data for LOW Zn wheat shoots and grains). Boxes filled with grey represent treatments where green

manure had been incorporated before wheat planting (+GM). Dashed boxes represent treatments where soil was x-ray irradiated and re-inoculated with native soil bacteria exclusively (X-RAY IRR). Thick black boxes represent pots where ZSB were inoculated (+ZSB)

**Table 8** Pearson correlation coefficient ( $r$ ) between N, Zn and Cd in wheat shoots and grains considering all the treatments for both soils separately

		Wheat flowering		Wheat maturity			
		Shoot N		Shoot N		Grain N	
		HIGH Zn	LOW Zn	HIGH Zn	LOW Zn	HIGH Zn	LOW Zn
Wheat flowering	Shoot Zn	0.70***	0.29				
	Shoot Cd	-0.23	0.44*				
Wheat maturity	Shoot Zn			0.27	0.42*		
	Shoot Cd			-0.33	0.62***		
	Grain Zn					0.62***	0.43*
	Grain Cd					-0.34	0.42*

Asterisks in superscript indicate correlation probability with \*  $p < 0.05$ ; \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$

The incorporation of green manure increased soil Zn and Cd availability

Lower DGT extractable Zn and Cd, as well as reduced total soil N concentrations at wheat flowering in the X-RAY IRR soils might be due to the higher wheat uptake of Zn, Cd and N in these treatments

compared to those found in the NO IRR soils. In the HIGH Zn soil, there was however one exception to this, in the irradiated treatments incorporated with green manure (+GM\_X-RAY IRR), where the highest DGT extractable Zn and Cd were measured. For Zn, this cannot be explained by lower wheat uptake, as the uptake of Zn was similar or higher in



the +GM\_X-RAY IRR than in the other treatments (Table S6). Green manure residue decomposition provided soil bacteria (presumably dominant in X-RAY IRR, since these treatments corresponded to sterilized soil re-inoculated with a bacterial wash) with N and C, thereby stimulating their metabolic activity (Marschner and Bredow 2002). Increased bacterial activity due to the incorporation of green manure might have promoted the mobilization of Zn and Cd through the production of various metabolic compounds involved in trace element mobilization such as organic acids (e.g. gluconic, citric, malic and oxalic acid) (Costerousse et al. 2018; Fasim et al. 2002; Li et al. 2010) and metal chelating siderophores (Brandel et al. 2012). The strong and positive correlation observed between DGT Zn and Cd and soil bacterial activity (i.e. soil respiration and microbial C) in irradiated HIGH Zn supports the concept that bacterial activity was intimately associated with Zn and Cd mobilization in this soil (Fig S1). Furthermore, the absence of fungi and soil fauna in the X-RAY IRR treatments (which probably explains lower microbial biomass in these soils), might also have benefited to soil bacterial activity through reduced trophic competition. Accordingly, soil respiration levels in these treatments were higher compared to the NO IRR treatments.

The important amount of DGT extractable Zn released in the +GM\_X-RAY IRR treatments of the HIGH Zn soil (measured only in NO ZSB) may account for the decrease in Cd accumulation in wheat shoots and grains in these treatments (Fig. 3), due to competition for the same root and phloem cell membrane transporters (Hart et al. 2002). This was not observed in the LOW Zn soil, probably because the amount of Zn released in the +GM treatments of this soil was not sufficient to cause the same effect on wheat Cd uptake. Zn is known to be more efficiently transported towards the above ground biomass compared to Cd which tends to accumulate in the roots (Wiggenhauser et al. 2016). Similarly, Cd concentrations in wheat shoots and grains was shown to be reduced by mineral Zn application to soil (Khoshgofar et al. 2004; Oliver et al. 1994) and leaves (Javed et al. 2016; Sarwar et al. 2015).

Although no decrease in soil pH was observed in response to clover incorporation, soil microbes might have indirectly contributed to increase metal availability by releasing  $\text{NH}_4^+$  (as a result of green manure decomposition) and protons (e.g. generated by  $\text{NH}_4^+$

nitrification). These could exchange with soil bound Zn and Cd, thereby increasing metal availabilities in the treatments with green manure. Increases in soil Zn and Cd availability in the +GM\_X-RAY IRR treatments of the HIGH Zn soil, as well as in all the treatments where clover had been incorporated, cannot be attributed solely to changes in soil microbial activity. For instance, in the X-RAY IRR treatments, the radiations might have (at least partly) released N immobilized by soil microbes in the presence of green manure (estimated around 15 to 20 mg N per kg soil<sup>-1</sup> at wheat planting, considering a microbial C/N ratio of five and multiplying microbial N by a factor of two to account for non-extractable microbial N (Jenkinson et al. 2004)), thereby amplifying Zn and Cd mobilization by exchange mechanisms with  $\text{NH}_4^+$ . Microbial lysis in the irradiated treatments might have also released N mineralizing enzymes (e.g. protease and deaminase) that remain active after the irradiation treatment (Lensi et al. 1991), which could explain further release of  $\text{NH}_4^+$  and thus higher Zn and Cd availabilities in the +GM\_X-RAY IRR treatments. The increase in Zn and Cd availability following the incorporation of green manure could also originate from the release of Zn mobilizing ligands from decomposing green manure residues such as dissolved organic C and amino acids (Gramlich et al. 2013; Soltani et al. 2014). Positive correlations were indeed previously reported between dissolved organic soil C, total concentrations of free amino acids and DGT extractable Zn and Cd (Grüter et al. 2017a). Finally, the release of Zn and Cd from decomposing green manure could also explain some of the increase. However, in the pot experiment conducted by Aghili et al. (2014), only a small fraction of Zn taken up by wheat originated from decomposing green manure and the rest was derived from the resident soil Zn pool.

In the LOW Zn soil, irrespectively of the treatment, DGT extractable Zn and Cd values were generally close to zero at wheat flowering (Fig. 1B). Consequently, it is hard to appreciate the effects of our factors, especially on DGT extractable Zn, earlier measurements (e.g. at wheat tillering) would have probably shown larger differences between the treatments. The absence of correlations between DGT Zn measured in the LOW Zn soil at wheat flowering and the other soil parameters was due to the variability and low DGT Zn values observed in this soil. Higher

uptake of Zn and Cd by the wheat plants in x-ray irradiated LOW Zn might explain why no increased DGT Zn and Cd were found in these treatments in response to green manure addition. It is also possible that Zn and Cd released after clover incorporation precipitated with carbonates, which were abundant in the LOW Zn soil (Table 1). Moreover, Zn concentration of the clover grown in the LOW Zn soil were close to critical values for plant growth (White 1993). This could decrease its N fixation capabilities explaining lower N and clover inputs in this soil, and thus less effects of the green manure incorporation on the availability of Zn and Cd in the LOW Zn than in the HIGH Zn soil.

Surprisingly, DGT extractable Cd levels were decreased by green manure incorporation in the x-ray irradiated LOW Zn treatments at wheat flowering (Fig. 1B). This was probably due to higher wheat root Cd uptake in the X-RAY IRR soils, as the wheat grown in these treatments may have benefited from N released by irradiated microbial biomass. This was supported by the fact that we found the highest  $\text{NH}_4^+$  concentrations (Table 5), grain biomass (Table 7) and number of grains produced per  $\text{kg soil}^{-1}$  (Table S4) in the +GM\_X-RAY IRR treatments.

The wheat plants did not benefit from the increased soil Zn availabilities

Higher Zn availabilities in the +GM treatments were not associated with more wheat biomass nor with higher wheat Zn uptake. In the LOW Zn soil, this was in spite of clear wheat Zn deficiency symptoms (chlorosis and necrotic spots on the leaves) and very low shoot and grain Zn concentrations (ca. 3 and 10  $\text{mg Zn kg}^{-1}$ , respectively), considered as critical for wheat growth (White 1993). Contrasting results were obtained in a recent pot experiment conducted by Grüter et al. (2017b) using the same two soils, as well as the same clover and wheat variety, but under different growing conditions (climate chamber with higher air temperature and a different lighting system). In their study, the incorporation of clover green manure increased aboveground wheat Zn uptake by almost a factor of two in the LOW Zn and of five in the HIGH Zn soil. These different results might partly be explained by the higher amounts of clover incorporated in the study of Grüter et al. (2017b) (9.57 g and 5.21 g clover shoots per kg of HIGH Zn

and LOW Zn soil, respectively, in comparison to the amounts of 2.97 and 2.61 g in our experiment (see Table 3)). In this way, three to four times more N was incorporated with the clover shoots of Grüter et al. (2017b) leading to a strong increase in soil mineral N concentrations which was not the case in our experiment. Higher N input through green manure addition in that study might have increased the acquisition of Zn by the plant through the mechanisms mentioned above (i.e. higher root growth and soil exploitation, stimulation of root root-to-shoot transfer and remobilization of metals to the grain). In line with this and in agreement with several other studies (Cakmak et al. 2010; Kutman et al. 2010), we found positive correlations between wheat shoot and grains N and Zn and Cd concentrations (Table 7). Similarly as in Grüter et al. (2017b), we found more wheat root and shoot (excluding grains) biomass production in the LOW Zn soil than in the HIGH Zn soil, which may partly be attributed to the poor soil structure of the HIGH Zn soil (observed by high soil density and distribution of the roots along the pot walls). Low aggregate stability was confirmed by a low Ca content (1.68  $\text{g kg}^{-1}$ ), which is known to improve aggregate stability through building Ca bonds between clay and humus particles (Blume et al. 2016). Less grain biomass production was nevertheless found in the LOW Zn soil, probably because of wheat N limitations in this soil (see below).

In order to study specifically the effect of fresh green manure input on bacterial activity and its ability to mobilize Zn and Cd, we incorporated the clover shoot one week before the start of the wheat growth experiment. Even with a C/N ratio below 20, it generally takes a few weeks before mineral N gets released from decomposing green manure (Doran and Smith 1991; Green and Blackmer 1995). Thus, we found lower mineral N concentrations in the +GM compared to the NO GM treatments at wheat planting in both soils, which probably explains why wheat growth generally tended to be negatively affected by green manure incorporation (i.e. decreased aboveground biomass and lower number of grains produced per  $\text{kg soil}^{-1}$ ). This was more observed in the non-irradiated than in the irradiated treatments since, as mentioned above, the radiation might have contributed to the release of N immobilized by soil microbes in the presence of green manure. In the LOW Zn soil,

this release of N might have slightly favored wheat growth as indicated by the highest grain biomass in the +GM\_X-RAY IRR treatments (Table 7). Although in the HIGH Zn soil, microbial death and green manure N mineralization could bring the NO GM and the +GM treatments to similar wheat N concentrations, this was not the case in the LOW Zn soil where wheat treated with green manure still had lower shoot and grain N concentrations at wheat maturity (Fig. 4). Consequently, wheat N status was either not affected (in the HIGH Zn soil) or decreased (in the LOW Zn soil) by the incorporation of green manure. In the LOW Zn soil, no matter the treatment, N concentrations at wheat flowering in the shoots were either close or below critical values for wheat growth (ca. 21 mg N kg<sup>-1</sup>, Fig S2) (Reuter and Robinson 1997). The incorporation of green manure therefore further exacerbated wheat N limitations in this soil, although this was mostly observed in the NO IRR treatments. The uptake of N by wheat may have also been limited by high soil moisture content, poor solar radiations on the glass-house bench and the small pot sizes leading to high root density, which are all common phenomena in pot experiments (Meyer et al. 2017b; Passioura 2006).

Considering the importance of N in the processes leading to metal uptake and translocation within plants, it is likely that N limitations in the +GM treatments of the LOW Zn soil prevented the wheat plants to benefit from higher Zn availabilities. The other factors mentioned above may have additionally limited wheat plant growth and metal uptake in both soils. The very high shoot and grain Zn concentrations encountered in the HIGH Zn soil (ca. 30 and 60 mg Zn kg<sup>-1</sup>, respectively) may have also prevented any further increase in wheat Zn uptake in the treatments where green manure had been incorporated. However, using the same soil and wheat variety, Grüter et al. (2017b) observed an increase in wheat grain Zn concentrations from ca. 60 mg Zn kg<sup>-1</sup> in the control without green manure to 95 mg Zn kg<sup>-1</sup>, when the same clover was incorporated. In the latter experiment, the incorporation of clover decreased soil pH by nearly one pH unit, which might have indirectly increased wheat Zn uptake due to increased Zn availability on their HIGH Zn soil. Finally, the fact that in the LOW Zn soil, the incorporation of green manure lowered wheat shoot

and grain Cd, but not Zn concentrations, suggests that the plants controlled the uptake of Zn homeostatically, for instance through the production of Zn specific phytosiderophores (Puschenreiter et al. 2017).

ZSB inoculation did not affect Zn and Cd availability to wheat

Two of the inoculated strains (*S. narbonensis* and *P. moraviensis*) were isolated from the HIGH Zn soil itself, and the four others from a soil with very similar chemical properties (Costerousse et al. 2018). Thus, the inoculated bacteria were most probably capable to grow in this soil, although we have no experimental data supporting this. For the inoculated strains, mechanisms of Zn solubilization are thought to include various metabolic processes, indirectly involved in the mobilization of Zn such as organic acid production (i.e. from the extracellular oxidation of glucose or from the tricarboxylic acid cycle) and proton extrusion for bacterial respiration and charge balance maintenance (Gadd 2004; Glasauer et al. 2004). In Costerousse et al. (2018), the magnitude of these metabolic processes was highly dependent on the availability of nutrients, since the mere presence/absence of glucose was sufficient to inhibit Zn oxide solubilization for most of the inoculated ZSB. It is likely that glucose availability in the HIGH Zn soil was inadequate for the bacteria to reiterate Zn mobilization mechanisms highlighted under in vitro conditions. Reasons explaining that there was no increase in Zn and Cd mobilization following the inoculation with ZSB might be soil specific. Differences in pH, soil organic matter content and nutrient availabilities between ZSB's soils of origin and the LOW Zn soil are among the numerous factors which might have further negatively affected the survival of the inoculated strains and their capacities to solubilize Zn in this soil (Gyaneshwar et al. 2002; Mosimann et al. 2017).

## Conclusions

In this experiment, no biofortification effects of green manure incorporation in soils with sufficient and non-sufficient Zn availability (respectively referred to as HIGH Zn and LOW Zn) were found. This happened

despite the fact that clover incorporation increased soil Zn availability, but wheat Zn uptake might have been restricted by N limitations (in the LOW Zn soil), plant Zn homeostasis (in the HIGH Zn soil) and other factors inherent to pot experiments (e.g. high soil moisture content and root density in the pots). These findings therefore suggest the importance to provide crops with sufficient phytoavailable N so that it can benefit from higher Zn supply in soils where available Zn is too low to cover crop needs, be it through the addition of mineral or organic fertilizers. The results confirm that green manure incorporation can stimulate soil bacterial activity and that this increase is positively related to a mobilization of Zn in soil. We found no increasing effect of pure strains inoculation (i.e. ZSB consortium) on Zn availability in soil and its uptake by wheat. Thus, our study does not support the use of ZSB inoculum for increasing grain Zn concentrations. Instead, our results suggest that the native soil bacteria can mobilize soil Zn and that this may be enhanced via the incorporation of green manure, although it may not necessarily translate into higher wheat grain Zn concentrations due to the factors mentioned above.

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#### Declarations

Not applicable.

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