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Bioavailability of Cu and Zn in raw and anaerobically digested pig slurry

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A B S T R A C T

The impact of anaerobic digestion on the bioavailability of copper and zinc from pig slurry was assessed. Both chemical and biological approaches were used independently on raw slurry (RS) and anaerobically digested pig slurry (DS). This work, using ultracentrifugation pellets from the same pig slurry before and after an anaerobic treatment, confirmed that Cu and Zn behave differently in terms of bioavailability, and contrasting results were obtained by chemical and biological assessments. A chemical approach combined a preliminary study of the pH effect on particulate/dissolved metal partitioning, sequential extraction, and biochemical fractionation. This approach tended to show a lower mobility of metals from digested slurry (DS). A biological approach was carried out with *Zea mays* and *Vicia faba* to study Cu and Zn uptake in soil amended with RS or DS. This assay could not differentiate the two slurries.

Keywords:

Anaerobic digestion
Maize
Broad bean
Availability
Risk assessment

1. Introduction

Owing to their value as fertilizers, pig slurries are generally spread on land (Sánchez and González, 2005). This is the most natural way to decompose slurries, at the same time avoiding heavy use of chemical fertilizers on agricultural soils. However, the levels of copper and zinc in pig slurries are elevated due to the use of animal feed supplements (Jondreville et al., 2003). In recent years, it has been reported that slurry-borne copper and zinc may constitute a long-term environmental risk when slurries are spread on arable land for decades, due to their accumulation in the topsoil (L'Herroux et al., 1997; Nicholson et al., 2003).

Bioavailability has been recognized for years as a promising tool in risk assessment of contaminated soil or soil-like materials. However, the term bioavailability long remained poorly defined even though the concept was widely used by scientists. Its definition depended on the design of the method to measure it and on the study organism considered, but ISO (2005) gave the following definition in ISO 11074: "bioavailability is the degree to which chemicals present in the soil may be adsorbed or metabolized by human or ecological receptors or are available for interaction with biological systems". More recently, the working group "Bioavailability" of ISO/TC190-Soil Quality developed a guidance document from the several existing methods and for selection and application of methods to assess the

bioavailability of contaminants in soil and soil materials (ISO, 2008). Harmsen (2007) reported that there are two methods to assess bioavailability: chemical measurements in the soil matrix and biological measurements using organisms exposed to soil or soil eluates. Bioavailability of slurry-borne metals in soils can then be examined using a chemical or biological approach to assess the environmental availability and the toxicological bioavailability, respectively.

Chemical approaches include, in particular, single extractions and sequential fractionations. Single extractions can be poorly correlated to metal bioavailability and plant uptake (Bolan et al., 2004). Sequential extraction procedures allow fractionation of the metals into various chemical forms generally covering soluble, exchangeable, precipitated, organic, and occluded forms. Despite the recognized non-specific nature of chemical sequential extraction methods, their analytical simplicity and rapidity have rendered them very useful and they are widely employed for metal form identification and estimation of the bioavailability of metals under field conditions.

On the other hand, biological approaches may involve plants, animals, or microorganisms, and different methods have been developed to assess the toxicological bioavailability of soil contaminants. Harmsen (2007) gave an overview of the international standards for the determination of the toxicity of chemicals with respect to soil organisms. As an example, ISO 11269-2 (1995) defines how to assess the effects of chemicals on the emergence and growth of higher plants. For such organisms, bioavailability of heavy metals has often been evaluated by measuring tissue levels (Pichtel and Anderson, 1997; Tao et al., 2003). Some authors have

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used these data to evaluate the fate of metals in the food chain and the associated risks (Cimino and Toscano, 1993; Jarausch-Wehrheim et al., 1996).

Recently, in a context of sustainable development, anaerobic digestion (AD) has been receiving attention since it produces renewable energy in the form of biogas, mainly composed of methane. The development of AD facilities particularly focuses on agricultural by-products like cattle or pig slurries, which already contain the optimal range of anaerobic microflora from the animals' intestinal tract and which also present a buffer capacity due to the presence of ammonium and bicarbonates. Moreover, AD is conservative for nitrogen; so digested slurry (DS) is spread on land in equivalent quantities to raw slurry (RS; Marcato et al., 2007). The treatment is also conservative for trace metals (Marcato et al., 2008) but does involve chemical changes. Several authors have described a shift of heavy metals away from mobile forms towards more stable and less bioavailable forms (Lake et al., 1985; Lavado et al., 2005) during AD. Most studies using a biological approach compared aerobic and anaerobic organic by-products of different origins (Alonso Alvarez et al., 2002; Lavado et al., 2005; Fuentes et al., 2006). Moreover, there is little information on the phytoavailability changes occurring in heavy metals in the same organic substrate before and after anaerobic treatment, in particular for agricultural by-products.

The aim of the present work was to assess the phytoavailability of Cu and Zn from raw and anaerobically digested pig slurry accumulated in the topsoil after decades of spreading of slurry on agricultural land. Both chemical and biological approaches were used. The chemical characterization involved three experiments: partitioning of Cu and Zn between the dissolved and the particulate fractions depending on the pH, and both a chemical and a biochemical sequential extraction procedure. The biological approach consisted of Cu and Zn uptake assessment in a growth chamber experiment with maize (*Zea mays*) and broad bean (*Vicia faba*). This approach was carried out using ultracentrifugation pellets instead of liquid slurries in order to model a long spreading period.

2. Materials and methods

2.1. Sample collection and preparation

Raw slurry and digested slurry were collected at the inlet and outlet, respectively, of the 150 m³ continuous stirred-tank anaerobic digester as described by Marcato et al. (2008). This reactor treated about 11 m³ d⁻¹ of pig slurry from a farrow-to-finish herd, with a retention time of about 15 d. Marcato et al. (2008) established the mass balance of the digestion plant. These authors showed that N was conserved throughout the treatment while part of the P, Ca, and Mg accumulated in the reactor as Ca and Mg phosphates (Marcato et al., 2007). Moreover, in the DS, Cu and Zn granulometric partitionings were linked to the dry matter (DM) pattern. As a consequence, the fate of both metals depends on the fate of the solid matter, and particularly on that of the organic matter (OM). To simulate spreading periods of several decades, a method was devised to concentrate the DM, Cu, and Zn, and eliminate most of the ammonium, which can be toxic to higher plants when applied at high levels (Mantovi et al., 2005). The RS and DS samples were centrifuged using a Beckam J2-21M/E centrifuge (14 000g, 30 min). The supernatants were discarded and the pellets were air dried at 40 °C to obtain a homogeneous powder. These solid fractions will be referred to hereinafter as raw slurry solids (RSS) and digested slurry solids (DSS).

2.2. Chemical approach—evaluation of environmental availability

2.2.1. Chemical analyses

The main physico-chemical characteristics of slurry and pellet were determined. The dry matter was measured following drying at 105 °C for 24 h. Extraction of metals from the slurry samples was undertaken using the modified strong acid digestion process described by Marcato et al. (2008), with a Gerhardt Kjeldatherm. An accurately weighed sample of 5 g (slurries) or 1 g (slurry solids) was digested with 20 ml 65% HNO₃ and 20 ml 37% HCl. The samples were kept overnight at 20 °C and then heated to 160 °C for 2 h. After cooling to room

temperature they were filtered at 0.45 µm and then adjusted to 100 ml with deionized (UHQ) water. Three extraction replicates were performed for each sample. Cu and Zn were determined by plasma optical emission spectrophotometry (ICP OES thermo-IRIS Intrepid II XDL Duo).

For DM, Cu and Zn, a removal percentage was calculated as described by Møller et al. (2002) to characterize the ultracentrifugation efficiency and validate the method used to model the original slurry compositions.

2.2.2. Effect of pH on particulate/dissolved partitioning

A preliminary experiment was performed on liquid slurries, before centrifugation, to determine the partitioning of Cu and Zn between dissolved and particulate fractions depending on pH. Slurry pH was adjusted from its initial value (i.e. 6.53 for RS and 7.95 for DS) to reach pH target values of 4.0 and 9.0 by adding 10 M HCl or 10 M NaOH dropwise, respectively, to an accurately weighed sample of slurry. This adjustment emphasized the buffer capacity of the DS due to the simultaneous presence of bicarbonates and ammonia resulting from the enzymatic conversion of urea (Vanotti et al., 2003). In DS, the bicarbonate content was assayed at 15 g L⁻¹ following the titrimetric method proposed by Anderson and Yang (1992). Then, concentrated HCl had to be added in a large quantity (about 4% of DS volume) to decrease the pH value, and the target value of 4.0 was overreached and the pH fell to 2.7.

Then, the solutions were stirred for 24 h at room temperature before being filtered at 0.45 µm (Millipore). The filter and the particulate fraction were dried at 105 °C and weighed. Cu and Zn concentrations in the particulate fraction were determined by ICP OES as described above. Dissolved Cu and Zn in the filtrate were calculated by mass balance from the composition and the weight of both the initial slurry and the particulate fraction.

2.2.3. Biochemical sequential extraction

In order to characterize the Cu and Zn patterns in the OM, pellets were fractionated following the method proposed by Linères and Djakovitch (1993) and recently normalized in France (AFNOR, 2005). Fractionation consisted of mixing 1 g of slurry pellet with 2 g of calcinated sand in a coarse-pored (40–90 µm) sintered glass crucible. The mixture was boiled in successive extractants using a Velp Scientifica FIWE extractor:

- the soluble and neutral detergent fibre (NDF) fractions were determined by extraction with neutral detergent (100 °C) for 60 min.
- the acid detergent fibre (ADF) fraction was determined by extraction with acid detergent (100 °C) for 60 min, and
- the acid detergent lignin (ADL) fraction was determined by room temperature extraction with sulphuric acid for 3 h.

The Wende cellulose fraction (CEW) was determined by successively boiling the same sand and slurry mixture for 30 min in sulphuric acid (0.26 M) and potassium hydroxide (0.23 M).

Liquid fractions obtained from the following biochemical extractions were collected to analyse the Cu and Zn associated with each kind of compound: (i) NDF fraction: soluble carbohydrates, most proteins, lipids and soluble mineral substances; (ii) ADF fraction: hemicelluloses; (iii) ADL fraction: lignin; and (iv) CEW fraction: celluloses. The resulting fractions were diluted to obtain acid and salt concentrations suitable for ICP OES and Cu and Zn concentrations were determined.

2.2.4. Chemical sequential extraction

During the last decades, several extraction schemes have been proposed to assess metal fractionation; Stover et al. (1976) proposed a method to fractionate organic substrates, which was later modified by Sposito et al. (1982) and then by McGrath and Cegarra (1992). This latest procedure is quite simple to carry out and less time consuming than several other methods since it involves only four steps. The first uses CaCl₂ to extract water-soluble and exchangeable forms, and heavy metals extracted with this saline solution are well correlated with uptake by plants (Canet et al., 1998). Moreover, pig slurry is from an anaerobic ecosystem (intestinal tract) and Theis and Hayes (1978) showed that ZnCO₃ may form in the 6.0 to 8.0 pH range for a redox potential of about -300 mV. The procedure proposed by McGrath and Cegarra (1992) avoided the use of any acidic reagents before the extraction of the carbonate fraction thus avoiding partial extraction of carbonate-bound Zn.

Then, the chemical fractionation of Cu and Zn from RSS and DSS was carried out according to the procedure of McGrath and Cegarra (1992). The procedure involved the following steps corresponding to different forms of Cu and Zn: (1) for determination of water-soluble and exchangeable metals, 5 g of slurry solid fraction was stirred with 50 mL 0.1 M CaCl₂ for 16 h, centrifuged (30 000g), and the supernatant was collected; (2) to determine organically bound metals, the residue from the previous extraction was dried and stirred with 50 mL 0.5 M NaOH for 16 h and then centrifuged (30 000g); the supernatant was collected; (3) metals associated with carbonates were extracted by stirring the dry residue from the previous step with 50 mL 0.05 M for 6 h and then centrifuging (30 000g); the

supernatant was collected; and (4) residual metals were determined by digesting the previous residue with aqua regia (AR).

The supernatants collected from the second and the third steps were digested in aqua regia (HCl:HNO₃ 1:3) to decompose the OM in the extract. Cu and Zn concentrations were determined in supernatants by ICP OES.

As mass losses occurred through the sequential extraction, samples were dried between two steps to determine the mass of both remaining solid and supernatant. The amount of metal in each extract was then calculated as described by McGrath and Cegarra (1992):

$$\begin{aligned} \text{Step (1): } & c_1 v_1 \\ \text{Step (2): } & c_2(v_2 + s_1) - c_1 s_1 \\ \text{Step (3): } & c_3(v_3 + s_2) - c_2 s_2 \\ \text{Step (4): } & c_4 v_4 - c_3 s_3 \end{aligned}$$

where c_i = concentration in the supernatant i (mgL⁻¹); v_i = volume (cm³) of supernatant i ; s_i = volume (cm³) of supernatant entrained in the pellet from extract i .

2.3. Biological approach—Evaluation of the toxicological bioavailability

A growth chamber experiment was conducted using soil amended with slurry solids to evaluate the bioavailability of Cu and Zn from raw or digested pig slurry. Soil was sampled from the surface layer (0–20 cm), air dried, sieved (<4 mm), and stored at room temperature before use. It was a Neoluvisol made up of 35% clay, 41% silt, 24% sand, with 1.33% organic C and a pH of 6.4. ISO (1995) recommends using both mono- and dicotyledonous plants to determine the effects of pollutants on soil flora and recommends several plant species. *Z. mays* is one of them and was chosen for our biological approach. Moreover, to better evaluate the toxicological bioavailability of pollutants, Harmsen (2007) recalls that different test methods should be combined. *V. faba* is a model plant used to assess the genotoxic potential of contaminants in soil (White and Claxton, 2004). It was used in our laboratory to evaluate micronucleus induction in root cells by copper and zinc (Marcato-Romain et al., 2009). Thus, *V. faba* was used as the dicotyledonous species to assess the bioavailability of slurry-borne Cu and Zn, despite the fact that this species is not one of the species directly recommended by ISO (1995).

For each plant, three treatments were performed: control soil (without any fertilization), soil amended with RSS, and soil amended with DSS (Table 1). Amendment was calculated considering that annual spreading of RS and DS is about 60 m³ ha⁻¹ (Marcato et al., 2007), corresponding to annual copper and zinc application rates of 1 kg Cu and 2.5 kg Zn per hectare. The maize assay simulated slurry loads equivalent to a long spreading period and the bean assay represented a shorter period, with application rates corresponding to just a few decades of spreading (Table 1). In the RS treatment of maize, the % OM in the culture substrate was about three-fold higher than in the control soil. To avoid toxic effects on plants, a preliminary incubation of both RSS and DSS was carried out to allow mineralization of the organic carbon: the pots were placed in the dark at 28 °C for 50 d and the moisture content was maintained at 60% of the water-holding capacity.

Plants were sown at four seeds per pot and thinned after a week to one plant per pot. Then, the pots were placed for 7 weeks at 24 °C day, 20 °C night, with a 16-h photoperiod. Relative humidity was kept at 70% day, 75% night. The plants were watered with tap water to maintain the culture substrate moisture at 60% of the water-holding capacity. At the end of the growing period, plants were harvested and washed with deionized water. Aerial parts and roots were immediately separated and dry weights were determined (40 °C). The dry samples were mineralized separately in a 1:1 mixture of HNO₃ and H₂O₂ at 80 °C for 4 h. After filtration, Cu and Zn content were determined with an IRIS Intrepid II XDL ICP OES spectrophotometer.

2.4. Statistical analysis

In both chemical and biochemical fractionations, comparisons of treatments were made by using the Student's *t*-test. In the biological approach, the data were analysed using a one-way ANOVA, and the Newman-Keuls test was used to determine the significance level between treatments ($p < 0.05$).

Table 1
Levels of Cu, Zn, and organic matter (OM) in the culture substrates.

	Control soil	Long term/maize		Middle term/bean	
		RS	DS	RS	DS
Cu (mg kg ⁻¹)	20.0 ± 0.2	48.9 ± 0.3	42.8 ± 0.1	27.8 ± 0.1	26.1 ± 0.1
Zn (mg kg ⁻¹)	80.0 ± 0.3	171.7 ± 1.0	132.4 ± 0.3	100.9 ± 0.2	94.4 ± 0.2
OM (g kg ⁻¹)	24.0	70.2 ± 0.6	51.6 ± 0.2	33.8 ± 0.2	28.4 ± 0.1

3. Results

3.1. Chemical availability

3.1.1. Effect of pH on particulate/dissolved partitioning

The behaviour of Cu and Zn partitioning between particulate and dissolved forms as a function of pH was not significantly different in RS and DS (Fig. 1). For neutral to alkaline pH values, Cu and Zn were exclusively linked to solids. For lower pH values (from about 6.0 to acidic conditions), Zn passed gradually into solution until dissolved Zn represented about 40% of the total Zn at pH 2.7. In contrast, Cu remained associated to the particles whatever the pH, over a range of 2.7–9.0.

3.1.2. Compositions of ultracentrifugation pellets and removal efficiencies

The removal efficiencies (Table 2) confirmed the relevance of using the ultracentrifugation solid fractions to represent the original slurries with regard to DM, Cu, and Zn. Removal of Cu was higher than 80%, and reached 90% for Zn. Removal efficiency was lower for DM probably due to the loss of soluble mineral and organic compounds that cannot be separated even with 0.45 μm filtration (Japenga and Harmsen, 1990).

From the data reported by Marcato et al. (2008), a relationship can be established between particle size and removal efficiency. With the removal efficiency found here for DM, Cu, and Zn, three size thresholds of particles separated by ultracentrifugation were determined. The highest threshold (1.7 μm) was obtained with DM, while the removal efficiency of Cu and Zn led to particle sizes of 1.1 and 1.0 μm, respectively. Thus, the ultracentrifugation pellet contained the particles the most concentrated in Cu and Zn present in pig slurries. Metal losses during this treatment corresponded to the fraction associated with particles of a diameter lower than about 1 μm.

The discarded fraction might contain the most chemically mobile forms of Cu and Zn. Moreover, ultracentrifugation can lead to granulometric changes and might modify the association between metals and OM. Finally, the use of large quantities of slurry solids to model decades of spreading leads to large inputs of OM, which is normally mineralized in the soil over the years. However, this appeared to be the most suitable approach to preserve the chemical fractionation of Cu and Zn in RS and DS and was preferred to spiking directly with the metals.

3.1.3. Biochemical sequential extraction

The extracts obtained from biochemical fractionation showed that most of the Cu and Zn from slurry solids was recovered in the

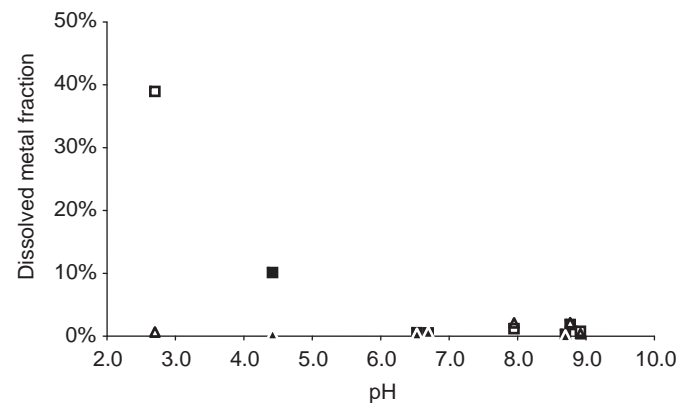


Fig. 1. Dissolved fraction (<0.45 μm) of Cu and Zn in raw or digested slurry depending on pH (▲: Cu in RS; △: Cu in DS; ■: Zn in RS; □: Zn in DS).

Table 2
Compositions of pellets from raw and digested slurries and associated ultracentrifugation removal efficiencies.

	RSS	DSS
OM (% DM)	72.4 ± 1.1	63.9 ± 0.2
Cu (mg kg ⁻¹ DM)	353 ± 18	558 ± 26
Zn (mg kg ⁻¹ DM)	1053 ± 34	1872 ± 80
Removal efficiency (%)		
DM	72.3 ± 12.3	79.6 ± 29.6
Cu	80.8 ± 3.7	84.8 ± 9.9
Zn	93.2 ± 10.3	93.9 ± 15.9

Table 3
Biochemical fractionation (%) of DM, Cu, and Zn from raw and digested slurry pellets.

Fraction	Dry matter		Cu		Zn	
	RSS	DSS	RSS	DSS	RSS	DSS
NDF	91.2 ± 1.9	82.6 ± 1.2	77.9 ± 8.5	97.6 ± 15.3	78.6 ± 5.6	100.0 ± 4.1
ADF	5.4 ± 1.0	11.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	13.1 ± 14.0	0.0 ± 0.0
ADL	2.3 ± 1.4	5.3 ± 0.3	22.1 ± 10.1	2.4 ± 3.4	8.3 ± 0.8	0.0 ± 0.0

NDF fraction, that is to say associated with carbohydrates, proteins, lipids, or soluble mineral substances (Table 3). In DSS, the quantities of Cu and Zn recovered in that first fraction increased to reach about 100% of the total content.

3.1.4. Sequential chemical extraction

The sequential extraction scheme applied to RSS and DSS indicated that the behaviour of the two metals contrasted strongly. The general trends in metal distribution among the various fractions however were fairly similar in the raw and digested slurries (Fig. 2) although the actual levels of the metals in each fraction differed significantly before and after AD. Exchangeable forms extracted with CaCl₂ represented the smallest fraction, with no zinc extracted from either of the slurry solids.

Cu in exchangeable form represented less than 2% of the total metal in both RSS and DSS, although the levels were slightly higher in DSS. This is consistent with the absence of dissolved Cu and Zn in RS or DS described previously (Fig. 1). The great majority of the Cu was linked to OM and extracted with NaOH: 75% of the Cu was recovered in the NaOH-extractable fraction in RSS, but significantly less, about 50%, in DSS. In DSS, Cu was more abundant in the two most stable fractions, that is to say carbonate-bound and residual fractions. Residual forms of Cu, extracted with AR, were two-fold more abundant in DSS than in RSS.

Zinc, on the other hand, was principally recovered in the EDTA-extractable fraction. The decrease noted in NaOH-extractable Zn between RS and DS was greater than that of NaOH-extractable Cu; Zn bound to OM showed a three-fold decrease in the treated slurry. In contrast, EDTA- and AR-extractable fractions increased and represented respectively, 64% and 27% of the total zinc.

3.2. Phytoavailability

DM yields and Cu and Zn levels in aerial parts and roots of maize and bean plants are reported in Fig. 3. Both RSS and DSS treatments led to an increase of the maize plant biomass, but this increase was not statistically significant (Fig. 3a). Moreover, one bean plant receiving RSS treatment did not grow and only four

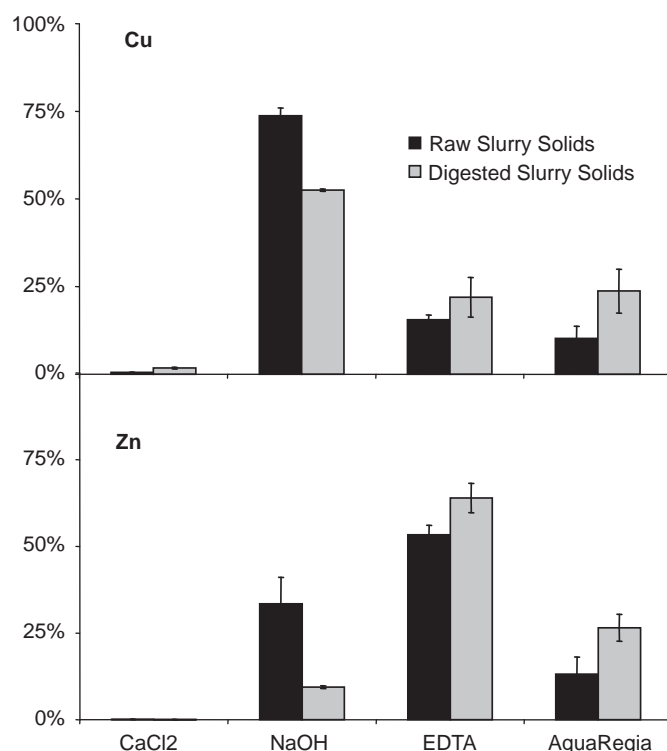


Fig. 2. Chemical fractionation of Cu and Zn in RSS and DSS following the extraction scheme of McGrath and Cegarra (1992).

replicates were harvested. These plants presented a slight decrease in the DM yield compared to the control soil. A similar effect was observed for the bean plants of the DSS treatment. Elemental analysis carried out on the plants did not show any reduction in N levels.

Cu and Zn levels always increased when the plants were grown in slurry-amended soil (Figs. 3b and c). For both species of plant and both types of slurry, the levels of the metals in the aerial parts were in the optimal range estimated at 5–20 mg Cu kg⁻¹ DM and 25–150 mg Zn kg⁻¹ DM (Marschner, 1995).

Fig. 3b shows that Cu was poorly mobile in maize and bean plants, root levels being about five-fold higher than levels in aerial parts. Cu accumulated in the roots of maize and bean plants but, as previously reported by Jarausch-Wehrheim et al. (1996), root levels were not correlated to the soil concentration.

In contrast, Zn was mobilized to aerial parts—their Zn levels were only 2.5-fold lower than those of the roots (Fig. 3c). Moreover, the levels of zinc in above-ground biomass increased in a dose-dependent manner with the quantity of slurry supplied (Fig. 4).

4. Discussion

The preliminary experiment confirmed that Zn is much more sensitive to pH changes than Cu, as described by different authors considering Zn in soil or in organic substrates such as sludge (Alloway, 1995; Artola et al., 2000). Moreover, the results indicate that there is no clear difference between RS and DS concerning dissolved metals, in line with the findings of Artola and Rigola (1992), who found the same correlation between Zn adsorption on different types of sludge (aerobic, anaerobically digested, thickened, and dewatered sludge) and over a range of pH from 5.8 to 8.0.

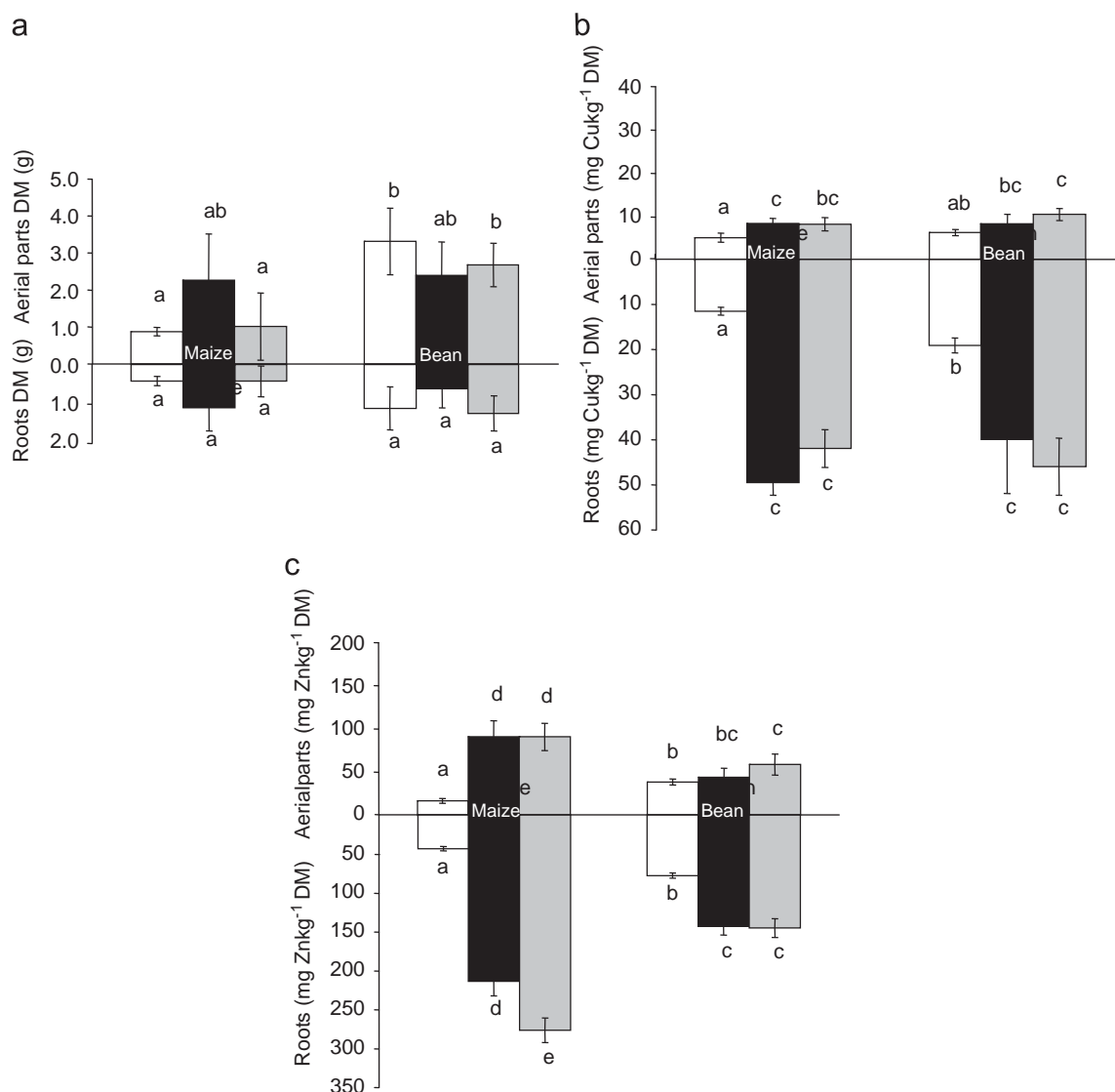


Fig. 3. Dry matter yields (a), Cu levels (b), and Zn levels (c) in maize and bean plants (empty symbols: control soil; black symbols: RSS-amended soil; grey symbols: DSS-amended soil). Mean values with different letters are significantly different ($p < 0.05$) as measured by the Newman-Keuls test.

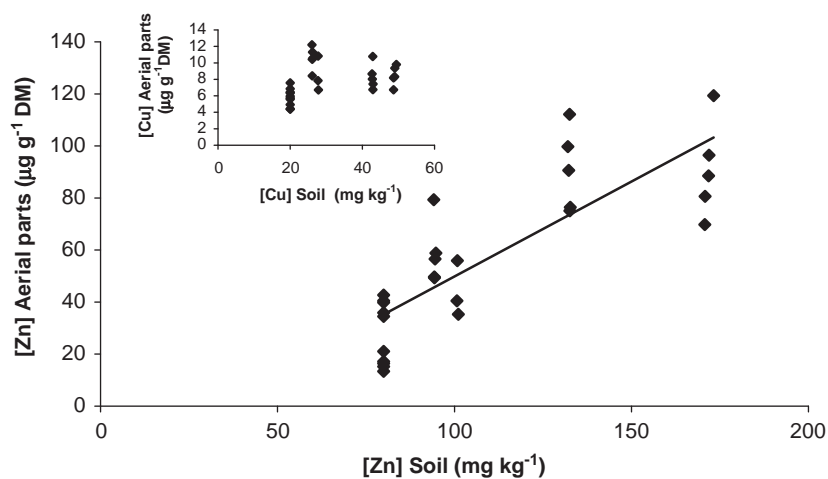


Fig. 4. Cu and Zn levels in plants depending on the culture substrate concentrations.

Moreover, the biochemical fractionation showed that Cu and Zn were mainly associated with the soluble neutral detergent compounds, particularly in DSS. Two hypotheses can be put forward to explain this shift towards the NDF fraction:

- The anaerobic microbial biomass had a strong affinity for the metals, and particularly for Cu, as reported by Artola et al. (2000). These authors showed that 1 g of anaerobically digested sludge total solids could bind 1.4 mmol Cu and 0.6 mmol Zn, representing metal concentrations in DM 100-fold higher than those measured in RS or DS; and
- Anaerobic conditions (Eh–300 mV, neutral pH) favoured the formation of precipitated sulphides and carbonates (Theis and Hayes, 1978) which may find its way into the NDF extract due to the large pores of the sintered glass crucible.

The patterns of the chemical sequential extraction are in line with the results of this biochemical fractionation. Cu was mainly recovered in the NaOH-extractable fraction while Zn was recovered in the EDTA-extractable fraction in both RSS and DSS. The presence of Zn in this carbonate-containing fraction may account for the increase observed in the level of dissolved Zn on lowering the pH, due to the dissolution of carbonates (Fig. 1). In DSS, the AR-extractable fractions increased. These results support the hypothesis of Cu and Zn recovery in NDF due to both biosorption (especially Cu, but also Zn) and the presence of Zn precipitates (e.g. carbonates, sulphides). Moreover, these results corroborate those of different extraction schemes applied to sewage sludge, composts, or amended soils, which found that Cu was mainly organically bound or present in the residual fraction, while Zn was less specifically extracted but generally occurred in acid-soluble (or carbonate), organic and residual fractions (Stover et al., 1976; Vieira e Silva et al., 1992; Pichtel and Anderson, 1997; Planquart et al., 1999; Wong et al., 2001; Van Hullebusch et al., 2005). Sequential extraction procedures have often been used to estimate the availability of heavy metals to plants. The results presented here show that the most available forms, i.e. extractable with CaCl_2 , are almost absent. Moreover, during AD, the Cu and Zn fractions associated with the OM shifted towards more stable fractions, suggesting that these metals would be less available to plants. However, the main Cu- and Zn-containing fractions remained similar before and after AD. This implies that availability of Cu and Zn from RSS and DSS is governed by the dominant fraction rather than by the entire distribution pattern.

In the growth chamber experiment, in the plants grown in control soil, bean roots showed significantly higher Cu and Zn levels than maize roots. This difference might be due to a higher cationic exchange capacity in dicots than in monocots (Cathala and Salsac, 1975). Moreover, Cu and Zn levels in aerial parts of maize were low (Figs. 3b and c) and near the deficiency limits evaluated at between 1 and 5 mg Cu kg^{-1} DM and 25 mg Zn kg^{-1} DM (Marschner, 1995). The soil used in our study contained about 35% clay, which might have reduced the availability of Cu and Zn in the control treatments (Alloway, 1995; Brun et al., 2001). When the plants were grown in slurry-amended soils, Cu and Zn levels always increased and were in the optimal range. These results indicate that the decrease in the bean DM yield in the RS treatment might be due to instability of the abundant OM, as previously described by Walter et al. (2006), rather than to metal toxicity. The increase in Cu and Zn levels in plants grown on a slurry-amended soil is in line with the results of other works, which evaluated the Cu and Zn phytoavailability from organic substrates by a chemical and/or a biological approach (Pichtel and Anderson, 1997; Canet et al., 1998; Planquart et al., 1999). No correlation was found between the Cu concentration in the soil

Table 4

Regressions between the amounts of Cu and Zn taken up by the whole plants and the dry matter yields.

Regression equation	r^2
$\text{Cu}_{\text{control}} = 10.381\text{DM} - 4.827$	0.93
$\text{Cu}_{\text{slurry, maize \& bean}} = 24.217\text{DM} - 10.607$	0.90
$\text{Zn}_{\text{control}} = 54.601\text{DM} - 37.327$	0.95
$\text{Zn}_{\text{slurry \& maize}} = 122.39\text{DM} + 18.094$	0.95
$\text{Zn}_{\text{slurry \& bean}} = 126.13\text{DM} - 165.89$	0.92

and the levels in the roots or the leaves, as previously described in the literature (Pichtel and Anderson, 1997; Tao et al., 2003) showing that, for the same soil, the Cu that accumulated in maize plants was biomass dependent, i.e. that the level of Cu was constant (Fig. 4). In contrast, the Zn levels of the aerial parts were dependent on the total Zn concentration in the culture substrate but not on the type of slurry or the plant (Fig. 4), in accordance with previous findings (Pichtel and Anderson, 1997; Jarauschehrheim et al., 1999).

Apart from Cu and Zn levels in plant organs, quantities of metals taken up can be analysed using the biomass yields of whole plants. When plotting all the results of Cu uptake against the DM yields, two groups of plants can be identified: the control treatment and the slurry-supplied plants. Table 4 indicates the regression equations and coefficients characterizing the relationships between Cu uptake and the DM yield for both plants cultivated on a slurry-amended or a control soil. Likewise, considering Zn uptake, there was still a difference between the control plants and the slurry-amended plants. However, as amended culture substrates were much more concentrated in the maize assay than in the bean assay (Table 1), two groups of plants corresponding to the maize and the bean assays can be distinguished when Zn uptake is plotted against DM yield (Table 4).

This study indicated that AD does not reduce the bioavailability of metals with respect to higher plants, which is in contrast with the generally (but chemically) accepted shift towards more stable forms. The similar behaviour of Cu and Zn in RS and DS might be explained by the fact that the slurry is originally produced in an anaerobic environment (pig digestive tract). On the other hand, the use of sludge solids separated by ultracentrifugation to model the original slurries might have led to an underestimation of the most labile fraction, particularly for Cu. However, the recovery efficiencies were quite similar for RS and DS and for both metals; this is the reason why the underestimation would have been comparable for RS and DS. In contrast, the NH_4^+ elimination by ultracentrifugation might have led to a reduction of the chemical reactions in the soil after amending, and in particular of nitrification, which can slightly acidify the soil. This might have led to an underestimation of Zn uptake by the plants, in particular with DS, which contained higher amounts of ammonium due to N mineralization during the anaerobic treatment.

Moreover, this work showed that a single chemical approach to evaluate environmental availability is not representative of the bioavailability of pollutants from complex matrices like pig slurries. In particular, to take the role of the OM into consideration, such an approach should be combined with a biological study of toxicology and bioavailability. Further studies combining both a chemical and a biological approach should now be performed to assess the environmental risks of pollutants associated with organic amendments, and to evaluate the benefits or disadvantages of OM treatment processes. This is particularly true considering the current increase in the tonnage of sewage sludge produced and the development of AD to reduce the

quantities requiring disposal and to produce a renewable form of energy.

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