Determination of EC_{50} values for Cu, Zn and Cr on Microorganisms Activity in a Mediterranean Sandy Soil

Vicenç Carabassa[1], Xavier Domene[1,2], Oriol Ortiz[1,3], Evan A. N. Marks[4], and Josep M. Alcañiz[1,2]

[1] CREAF, Cerdanyola del Vallès, Spain

[2] Univ Autònoma Barcelona, Cerdanyola del Vallès, Spain

[3] Escuela Politécnica Superior, Universidad de Zaragoza, Huesca, Spain

[4] Departamento de Química, Facultad de Ciencias, Universidad de Burgos, Burgos, Spain

Correspondence: V. Carabassa, CREAF, Cerdanyola del Vallès 08193, Spain
e-mail: v.carabassa@creaf.uab.cat

Abstract

Ecotoxicity of three potentially toxic metals (PTM) (Cu, Zn and Cr) in a slightly acidic sandy soil was tested using the soil respiration test (OECD-217) in order to determine EC_{50} values for the carbon transformation activity of microorganisms. Addition of an organic amendment of *Populus* leaves was also crossed with metal spiking in order to investigate possible interaction with metal toxicity. Soil respiration was measured at day 1 and day 28 after the soil spiking with the PTM to assess short-term effects on soil microbial activity. Of the three metals tested, Cu showed the highest toxicity at the longest exposure times (day 28) and Zn showed a strong inhibitory effect in the short-term (day 1), even though later toxicity diminished significantly. Cr was the least toxic studied PTM. Organic amendment outweighs any adverse effects of these metals, increasing soil respiration, even in the treatments with high doses of metals.

Abbreviations: CEC, cation-exchange capacity; **EC_{50}**, Median effective concentration; **NOEC**, no observed effect concentrations; **OECD**, Organization for Economic Co-operation and Development; **OM**, organic matter; **PTM**, potentially toxic metal; **WHC**, water holding capacity; **SIR**, substrate-induced respiration

Keywords: Effective concentrations, Potentially toxic metals, Metal toxicity, Soil microorganisms, Soil organic matter

1. Introduction

Diffuse contamination by potentially toxic metals (PTM) in soils is an increasing problem worldwide. Historically, metal pollution has caused adverse effects on the environment and human health, exemplified by disease outbreaks in Minamata due to Hg [1, 2] and in Itai-Itai due to Cd [3]. Currently, sources of diffuse soil contamination by trace metals are increasing in number, associated with inappropriate agricultural practices and repeated application of urban and industrial wastes. These sources have created major problems (inhibition of soil microorganism activity, inhibition of soil microbial growth and reproduction, inhibition of litter decomposition, reduced crop yields) in European soils [4].

PTM are well known to be toxic to most organisms when present in excessive concentrations [5], including to soil microorganisms which play an important role in soil functions and associated ecosystem services [6, 7]. Toxic effects of pollutants on soil organisms are difficult to estimate if only the total content in soils is known [8]. Ecotoxicological methods are required to reveal the actual toxicity of soil pollutants, associated with the actual bioavailable fraction which is modulated by interactions among toxins and between toxins and soil components [9]. Ecotoxicological tests provide a link between chemical monitoring and risk assessment of potential impacts on humans and biota [10], as well as datasets for the derivation of maximum safe environmental concentrations. In this context, the development of microbial populations under chemical stress conditions constitutes an important research topic in ecotoxicology. For this reason, there is an obvious interest in determining the relation between chemicals' concentrations and the effects on populations [11].

Stabilization or inactivation of PTM in soil can be achieved by adding amendments (lime, apatite, zeolites, organic materials or Mn and Fe oxides, etc.) that are able to absorb, complex or (co)precipitate trace elements. The application of organic by-products has been also demonstrated to be useful for contaminant immobilization [12], with some advantages over other methods such as low cost, its *in-situ* applicability and its low environmental impact [13]. Organic amendments are considered especially effective in Cr stabilization, while in the case of other trace elements (As, Cu, Zn, and Pb) they may have both positive and negative effects [14].

Ecotoxicity tests are conducted by exposing organisms to a dose gradient of toxic compounds under controlled experimental conditions [15]. An increasing amount of evidence demonstrates that soil microorganisms are more sensitive to PTM (causing biotic stress) as compared to soil fauna or plants [5]. However, sensitivity ranges widely between species and this imply changes in the microbial diversity promoted by ecotoxic effects. These changes in the microbial diversity imply changes in the structure of microbial ecosystems [16, 17]. Despite setbacks, including limited long-term ecological relevance [5], several methods based on microbial activity and measures of biomass enable the assessment of toxic effects on soil microorganisms; namely, these methods are microbial biomass by fumigation-extraction [18], microbial activity by the substrate induced respiration [19] and basal respiration [20], microbial enzymatic activities such as nitrification [21], dehydrogenase activity [22], glucosidase activity [23], phosphatase activity [24], catalase activity [25], arginine ammonification [26] and carbon transformation test [27] using Biolog® system.

Some older studies are of limited use in terms of comparability because they did not employ standard methods [7]. Despite the large body of research on metal toxicity to soil microorganisms and microbial

processes, the amount of available data is always insufficient and the interpretation too uncertain to establish risk-based thresholds [28]. In this context, the Organisation for Economic Co-operation and Development (OECD) defined a methodology to evaluate the effect of crop protection chemicals on the C-transforming activity carried out by soil microorganisms (OECD-217) [6], that has not been tested by PTM.

The main aim of this study was to validate the OECD-217 standardized protocol for testing PTM toxicity. The short-term toxicity of Cu, Zn and Cr (with and without organic amendment) was assessed by the OECD-217 standardized protocol, aiming to provide additional short-term ecotoxicological data on soil microbial activity of three PTM which is useful for the derivation of thresholds for their content in soils.

2. Material and methods

The ecotoxic effects of Zn, Cr and Cu were assessed as effects on glucose-induced respiration rates according to the OECD Guideline 217 [6], based on measurements of CO_2 evolution following the addition of glucose. This was assessed in a natural soil relatively poor in organic matter. The activating or inhibiting effect of the addition of a natural organic matter source (fresh plant leaves) on the toxicity of these three metals was also taken into consideration.

2.1 Test soil

The soil corresponded to the fine fraction (<2 mm) of the A horizon (0--20 cm) of a Eutric arenosol developed derived from weathering of granodiorite in the Prades mountains (Tarragona, NE Spain). The topsoil layer (20 cm) was collected for the preparation of the tests. Soil was analysed by current standard methods: particle size was determined by sedimentation (Robinson pipette), organic carbon content by wet oxidation in acid dichromate, total nitrogen using the Kjeldahl method, microbial-C by the fumigation-extraction method, cation-exchange capacity (CEC) after ammonium acetate cation displacement, electrical conductivity in an extract, 1:5, w/v, and pH in a water suspension, 1:2.5, w/v. A summary of the main analytical characteristics of soil is shown in Table 1. The soil had a sand fraction of about 70%, ensuring a homogeneous spiking with metals and mixing of the organic matter source. The high C/N ratio indicates a low degree of native organic matter transformation due to the presence of *Cistus laurifolius* L. which has high leaf phenol content [29]. The soil was moderately acidic but pH values and low electrical conductivity are unlikely to limit microbial activity. Due to the low CEC, high bioavailability of metals might be expected in this soil. Overall, the soil properties were in agreement with the requirements of the OECD-217 procedure, except by the organic carbon content (2.6 g kg^{-1}), which is lower than the recommended value (5 g kg^{-1}).

After collection, soil was sieved to 2 mm and stored at 4°C. Before starting microbial tests soil was moistened to 10% of water holding capacity (WHC) and kept in dark at 21°C for three days.

2.2 Treatments: PTM spiking and organic amendment

PTM were added to soil individually in a water solution prepared to provide 50% of the soil's maximum WHC, applied as dissolved salts of $ZnCl_2$, $CuSO_4 \cdot 5\ H_2O$, and $CrCl_3 \cdot 6\ H_2O$.

Following the protocol, preliminary assays were conducted in which each metal was incorporated at

seven logarithmically-increasing concentrations, between 0.001 and 1000 mg kg^{-1} in the case of Zn, and between 4.5×10^{-4} and 450 mg kg^{-1} for Cu and Cr. In the definitive test, the PTM were incorporated within the range of concentrations where respiration inhibition was observed in the preliminary assays.

For the treatments with organic amendment PTM were spiked after adding fresh organic matter to soil at a rate of 350 g kg^{-1}. This rate is very high in order to ensure a clear effect of the amendment. Fresh organic matter consisted of green *Populus nigra* L. leaves, washed with distilled water and dried at 40°C until constant weight. Once dried, leaves were ground and sieved to <200 µm.

A summary of the treatments is provided in Table 2.

2.3 Soil incubation and CO_2 measurements

Three replicates were prepared per metal concentration and treatment (OM+, with addition of fresh organic matter; OM--, without addition of fresh organic matter), each consisting of a 1.2 L plastic container filled with 300 g of soil. All the replicates were incubated in the dark at 21°C for 28 days. To prevent anoxic conditions, the containers were periodically aerated three times weekly. Soil moisture was checked weekly gravimetrically and replaced as necessary with deionized water.

The CO_2 produced by glucose-induced respiratory activity was measured by utilizing NaOH traps [30] for 12 consecutive hours after glucose addition, according to the OECD-217 procedure. The amount of glucose required to achieve a maximum respiratory response in this soil had been previously determined using a series of concentrations of glucose (2000, 5000 and 8000 mg kg^{-1}). At the beginning of the test glucose was homogeneously mixed with the soil and respiration measurements were carried out at the beginning of the incubation time (day 1) and the end (day 28). At the end of the test, electrical conductivity and pH were measured.

2.4 Effective concentrations estimation

A concentration-response curve was constructed using the response values expressed as percent of the response in controls (soils without addition of PTM). The concentration–response curves were adjusted to a regression model (logistic or Gompertz models), chosen according to best fit with the available data. These were then used for the estimation of effective concentrations for each metal and incubation time (days 1 and 28). The non-linear estimation module of Statistica 6.0 software (StatSoft ®) was used for this purpose.

3. Results

3.1 Preliminary assays

As expected, the addition of fresh organic matter (OM+) increased glucose-induced soil respiration. Treatments without organic matter addition (OM--) presented a maximum respiration rate slightly lower than 9 mg C-CO_2 kg^{-1} per hour at day 1 (Cu OM-- day 1), whereas the maximum for OM+ soil was >130 mg C-$CO_2 \cdot kg^{-1}$ per hour for the same day (Cu OM+ day 1) (Fig. 1).

In OM-- treatments microbial glucose-induced soil respiration was effected by added Zn, Cu and Cr in the range of studied concentrations, the responses following a lognormal distribution.

According to the protocol, when results from tests are evaluated and the difference in respiration rates

between the treatment and control is equal to or less than 25% at any sampling time after day 28, the tested product can be evaluated as having no long-term influence on carbon transformation in soils. Little negative effect was observed on soil respiration at low levels of pollution in the different preliminary assays for all the studied metals. In addition, stimulating effects at low concentrations of Zn were observed. However, this effect was not observed in OM+ treatments.

In the range of studied concentrations inhibitory effects were observed at days 1 and 28 in the OM-- soil polluted with Cu or Zn. Regarding the OM+ treatments toxicity was not observed even though respiration was lower at high doses. In the case of the soil polluted with Cr (OM+ and OM--), no significant toxicity was detected in the range of the studied doses, and in the OM-- soil practically all Cr doses had soil respiration higher than the control, statistically significant at day 28 measurements.

In light of the above, preliminary assays showed the existence of relevant toxic effects only in the absence of organic matter amendment. Particularly, inhibition was detected in the case of Zn OM-- (day 1), Cu OM-- (day 1) and Cu OM-- (day 28). In the preliminary assays carried out with soil polluted with Zn, an inhibitory effect was observed at day 28 when the reference soil had not been amended with organic matter. However, the large variability obtained after 28 days of incubation suggested the need to undertake definitive assays to confirm these results. In these cases the definitive assays were carried out using the same range of concentrations from which toxicity was detected in the preliminary assays. The assays corresponding to treatments polluted with Cr (Cr OM+, Cr OM--), soil polluted with Cu or Zn and amended with organic matter (Cu OM+, Zn OM+) did not show toxicity and therefore the definitive assays were not carried out.

3.2 Definitive assays

According to the results obtained in the preliminary assays, the ranges of doses used in the definitive series are indicated in Table 3. Note that the doses range of toxicity used in the preliminary assays was different for Cu and Zn.

Definitive assays showed that soil respiration decreased as metal dose increased in all assays. The Zn series showed a pronounced toxicity at days 1 and 28 (Fig. 2), whereas the effect at day 28 was more pronounced. The same pattern was found for Cu series (Fig. 2).

3.3 Effective concentrations

Regression models were applied to calculate effective concentrations. The models with the best fits were the Gompertz model for Zn treatments, and the logistic model for Cu treatments. The significance was high for the two models ($p < 0.001$). The median effective concentration (EC_{50}) values obtained with the regression models presented different exposure time patterns for Cu and Zn (Table 4), specifically that Zn toxicity was lower with increased exposure time (lower EC_{50} at day 28) whereas Cu toxicity had the opposite trend (higher EC_{50} at day 28).

4. Discussion

When metal toxicity data for soil microbial processes and populations cited in the literature is summarized, an enormous variability becomes apparent [5, 14, 30]. When representing acute and

chronic effects, we can classify the curves obtained into different typologies of typical toxicology functions [11]. Therefore, we can identify curves of type I or "rank without effects series of inhibition", type II or "stimulation followed by inhibition", type III or "stimulation" and type IV or "no change". Bååth [31] published ranges of no observed effect concentrations (NOEC) for Cd, Cu, Zn and Pb; in forest soils with a low organic matter content, the NOEC for Cu is distributed in a range from 2 to 1600 $\mu g\ g^{-1}$; for the case of Zn, the NOEC were between 32 and 1000 $\mu g\ g^{-1}$. Saviozzi et al. [32] and Renella et al. [33] established a rank order of metal toxicity depending on the metal's inhibition power on soil respiration; in this ranking, Cu is above Zn, therefore being the most toxic for the CO_2 production. Many authors who in these tests have applied organic amendments or used soils with high organic matter contents have not detected toxic effects even at high metal concentrations [12, 34]. However, there exist totally contradictory results, with significant inhibitions of heterotrophic respiration due to the presence of one of the studied metals, despite the addition of organic matter [35].

There are only two groups of factors that can contribute to this variability among the results reported in different previous studies: (1) factors which modify the toxicity of the metals and, (2) differences in the sensitivity of soil microorganisms or in microbial processes [7]. It is extremely difficult to sort out these factors when metal toxicity is studied in soils due to difficulties in assessing (or measuring) the bioavailability of the metals [36, 37], and also due to the complexity of soil microbial communities [5]. In fact, bioavailability can only be estimated since it only can be measured by the growth of the organism of interest and by the absorption or the toxicity of the metal [38]. However, over the last ten years there has been considerable progress in defining bioavailability and in taking account of differences in bioavailability and sensitivity of soil organisms in EU risk assessment research [39]. In any case, the metals used in the present work were incorporated into the soil in a soluble form that is considered to have high bioavailability, independent of subsequent chemical reactions or changes in the soil.

For the present work, in the case of chromium, no evidence of inhibition was observed. The beginnings of a hormetic effect were detected at lower doses, and no inhibition was found at higher doses. This might be due to the fact that chromium was added in the trivalent form, which is less toxic than the hexavalent form [40]. Moreover, the trivalent cations bind strongly to clay particles [41], reducing the bioavailability of chromium. However, the experimental soil had a low proportion of clay and low organic matter contents, and consequently a reduced CEC. For this reason it is expected that metal adsorption by these components would be minimal. As for the possibility of the preliminary test concentrations having been excessively low, several authors [35, 42] have found inhibitory effects for Cr at lower concentrations than the preliminary ones in this work. The stimulating effect observed for Cr might be attributable to the fact that this element is essential for many organisms, whereas it is in relatively high concentrations in RNA. Moreover, the cations of PTM can induce the liberation of nutrients adsorbed on the soil, causing indirect positive effects [11] in closed experimental systems.

Regarding the changes of toxicity with exposure time, in the case of the soil polluted with Zn, toxicity was lower after 28 days. This effect, reflected in a relative increase in respiration at the same dose rank, may be attributable to physiological changes or adaptations to metal pollution leading to a recovery or selective effect on the community [43].

The addition of fresh organic matter led to significant increases in substrate-induced respiration (SIR) (Fig. 1). Other studies have shown that microbial biomass increases with the addition of fresh leaves, given the availability of easily-degradable substrate [44--46]. This increase in microbial biomass is associated with an increase in soil respiration [47]. The addition of organic matter masked any effect of the rank of doses studied. This masking of the toxicity may therefore be due to an increase in the microbial biomass produced by the organic matter addition [37], to the adsorption and immobilization of metals on the added organic matter [48], or a combination of these factors. It has been reported that a broad variety of organic components increase the adsorption of Zn [49], and that the mineralization of the organic matter favours the precipitation of inorganic metallic compounds [50]. On the other hand, it is known that Cu associates easily with humified or stable organic matter [51, 52] to which it can be strongly adsorbed [53, 54]. It is for this reason that the addition of organic amendments is a common practice for the immobilization of PTM, improving the quality of the soil and facilitating revegetation of polluted soils [50, 53]. In this manner, the obtained results contribute to the validation of this practice.

When measuring toxicity at day 1 in the OM+ treatments, taking into account a short period for interaction of the metal, the main cause of the observed lower toxic effect is probably the availability of labile substrate which favoured the growth of the bacterial biomass and increased its mineralisation activity, concealing any decrease in microbial populations. In this way, the inhibitory effect of the metal would be offset by the activating effect of the organic matter. This decrease of the toxic effect is observed in day 1 as well as day 28 measures, even though after 28 days the amount of emitted CO_2 diminishes significantly.

The decrease of SIR observed in OM+ at day 28 followed the same pattern than the decrease observed for day 28 measures Cu OM-- treatment. Stenström et al. [55] demonstrated that SIR measured in soils where glucose was added at different pre-incubation times showed a transition of K-strategists towards r-strategists. R-strategist species were responsible of 63% of SIR after four days of incubation, but later this trend reversed and the contribution of r-strategist microorganisms was reduced (at 46 days of incubation the r-strategy group was responsible for only 16% of the SIR). The results are in agreement with the observations of Ritz et al. [56], who detected that after the addition of an easily decomposable substrate to soil the SIR increased significantly for only 25 days.

No inhibitory effects were observed in OM+ treatment SIR measurements (days 1 and 28). The fact that relative differences (to the control) between the measures in OM+ treatments at days 1 and 28 were not observed can be due to the fact that organic matter, as a source of mineralisable carbon (independent of its recalcitrance), had not run out completely. Only in the case of the soil polluted with Cu and amended with organic matter a slight decrease of the relative respiration was observed at day 28. Therefore, in agreement with the results of the OM-- soils, Cu proved to be the most toxic metal. Moreover, some authors [57] have demonstrated that in presence of soluble organic matter, Cu is the more available of the studied metals.

In light of the results, the protocol OECD-217 is a valid methodology for the detection of adverse effects of the three metals studied on substrate-induced soil respiration. Cr, Zn, and Cu toxicities, evaluated by the OECD-217 soil respiration test, were low in the slightly acidic sandy test soil chosen for its potential high bioavailability of toxic metals. The EC_{50} values obtained in the present work are comparable to

those presented by other authors who have worked with soils of similar characteristics [48, 56]. The magnitude of the inhibitory effects that have been observed for the three metals studied (Cu > Zn > Cr) is also coherent with the consulted references, Cu being the most toxic metal after 28 days of exposure [31, 32]. However, the microbial responses produced in the short-term assays were unpredictable and bore little resemblance to the long-term effects observed in the field in other studies [7].

Acknowledgements

This study was a part of the project AGL2002-03297 that has been founded by the Ministry of Science and Technology of Spanish Government.

The authors have declared no conflicts of interest.

6. References

[1] S. Takaoka, Y. Kawakami, T. Fujino, F. Oh-ishi, F. Motokura, Y. Kumagai, T. Miyaoka, Somatosensory disturbance by methylmercury exposure, *Environ. Res.* **2008**, *107 (1)*, 6--19.

[2] S. Ekino, M. Susa, Minamata disease revisited: An update on the acute and chronic manifestations of methyl mercury poisoning, *J. Neurol. Sci.* **2007**, *262 (1--2)*, 131--144.

[3] R. Shuto, in *Encyclopedia of Toxicology* (Ed.: P. Wexler), Elsevier, New York **2005**, pp. 655--656.

[4] D. Stanners, P. Bourdeau, *Europe's Environment. The Dobrís Assessment*, European Environment Agency, Copenhagen **1995**.

[5] K. E. Giller, E. Witter, S. P. McGrath, Toxicity of PTM to microorganisms and microbial processes in agricultural soils: a review, *Soil Biol. Biochem.* **1998**, *30 (10--11)*, 1389--1414.

[6] OECD, *Guidelines for testing of chemicals 217, Soil microorganisms: Carbon Transformation Test*, OECD, Paris **2000**.

[7] K. E. Giller, E. Witter, S. P. McGrath, PTM and soil microbes, *Soil Biol. Biochem.* **2009**, *41*, 2031--2037.

[8] V. Gyuricza, F. Fodor, Z. Szigeti, Phytotoxic effects of heavy metal contaminated soil reveal limitations of extract-based ecotoxicological tests, *Water Air Soil Pollut.* **2010**, *210*, 113--122.

[9] Y. J. An, Assessment of comparative toxicities of lead and copper using plant assay, *Chemosphere* **2006**, *62*, 1359–1365.

[10] S. M. Steinberg, E. J. Pozomiek, W. H. Engelmann, K. R. Rogers, A review of environmental applications of bioluminescence measurements, *Chemosphere* **1995**, *30*, 2155–2197.

[11] G. Welp, G. W. Brümmer, Toxicity of Increased Amounts of Chemicals and the Dose-Response Curves for Heterogeneous Microbial Populations in Soil, *Ecotoxicol. Environ. Saf.* **1997**, *37(1)*, 37--44.

[12] D. Wasilkowski, A. Mrozik, Z. Piotrowska-Seget, J. Krzyzak, M. Pogrzeba, G. Płaza, Changes in Enzyme Activities and Microbial Community Structure in Heavy Metal-Contaminated Soil Under *in Situ* Aided Phytostabilization, *Water Air Soil Pollut.* **2014**, 42 (11), 1618–1625.

[13] P. Burgos, P. Madejón, F. Cabrera, E. Madejón, By-products as amendment to improve biochemical properties of trace element contaminated soils: Effects in time, *Int. Biodeterior. Biodegrad.* **2010**, *64 (6)*, 481--488.

[14] J Kumpiene, A Lagerkvist, C Maurice, Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments: A review, *Waste Manage.* **2008**, 28, 215–225.

[15] B. Brohon, R. Gourdon, Influence of soil microbial activity level on the determination of contaminated soil toxicity using Lumistox and MetPlate bioassay, *Soil Biol. Biochem.* **2000**, *32*, 853–857.

[16] A. M. Chaudri, S. P. McGrath, P. Gibbs, B. C. Chambers, C. Carlton-Smith, J. Bacon, C.

Campbell et al., Population size of indigenous *Rhizobium leguminosarum* biovar. *trifolii* in long-term field experiments with sewage sludge cake, metal-amended liquid sludge or metal salts: effects of zinc, copper and cadmium, *Soil Biol. Biochem.* **2008**, *40*, 1670–1680.

[17] J. Mertens, D. Springael, I. De Troyer, K. Cheyns, P. Wattiau, E. Smolders, Long-term exposure to elevated zinc concentrations induced structural changes and zinc tolerance of the nitrifying community in soil, *Environ. Microbiol.* **2006** *8*, 2170–2178.

[18] E. D. Vance, P. C. Brookes, D. S. Jenkinson, An extraction method for measuring microbial biomass C, *Soil Biol. Biochem.* **1987**, *19*, 703–707.

[19] J. P. E. Anderson, K. H. Domsch, A physiological method for the quantitative measurement of microbial biomass in soils, *Soil Biol. Biochem.* **1978**, *10*, 215–221.

[20] A. Cornfield, Carbon Dioxide Production During Incubation of Soils Treated with Cellulose as a Possible Index of Nitrogen Status of Soils, *J. Sci. Food Agric.* **1961**, *12 (11)*, 763-765.

[21] H. Lees, The Effects of Various Organic Materials on Soil Nitrification, *Biochem. J.* **1948**, *42 (4)*, 528--531.

[22] A. Thalmann, A Contribution to the Determination of the Dehydrogenase Activity in Soils Using Tri Phenyl Tetrazolium Chloride, *Landwirtschaft Forsch.* **1968**, *21 (3--4)*, 249--258.

[23] M. A. Tabatabai, in *Agronomy: A Series of Monographs, Vol. 9. Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties* (Ed.: A. L. Page), Soil Science Society of America, Madison **1982**.

[24] M. A. Tabatabai, J. M. Bremner, Use of *p*-Nitrophenyl phosphate for assay of soil phosphatase activity, *Soil Biol. Biochem.* **1969**, *1*, 301--307.

[25] T. Beck, Die Messung der Katalaseaktivität von Böden, *J. Plant Nutr. Soil Sci.* **1971**, *130*, 61--68.

[26] K. Alef, D. Kleiner, Arginine ammonification, a simple method to estimate microbial activity potentials in soils, *Soil Biol. Biochem.* **1986**, *20*, 561–565.

[27] H. Y. Yao, J. M. Xu, Substrate utilization pattern, biomass and activity of microbial communities in a sequence of heavy metal-polluted paddy soils, *Geoderma* **2003**, *115 (1--2)*, 139--148.

[28] US EPA, *Guidance for Developing Ecological Soil Screening Levels (Eco-SSLs)*, US Environmental Protection Agency, Washington, DC **2003**.

[29] T. Vogt, P. G. Gülz, Accumulation of leaf flavonoids on leaf development in *Cistus laurifolius*, *Phytochemistry* **1994**, *36 (3)*, 591--597.

[30] J. P. E. Anderson, *Soil Respiration*, Soil Science Society of America, Madison **1982**.

[31] E. Baath, Effects of Heavy-Metals in Soil on Microbial Processes and Populations (a Review), *Water Air Soil Pollut.* **1989**, *47 (3--4)*, 335--379.

[32] A. Saviozzi, R. Levi-Minzi, R. Cardelli, R. Riffaldi, The influence of PTM on carbon dioxide evolution from a typic xerochrept soil, *Water Air Soil Pollut.* **1997**, *93 (1--4)*, 409--417.

[33] G. Renella, M. Mench, A. Gelsomino, L. Landi, P. Nannipieri, Functional activity and microbial community structure in soils amended with bimetallic sludges, *Soil Biol. Biochem.* **2005**, *37(8)*, 1498--1506.

[34] W. R. Chaney, J. M. Kelly, R. C. Strickland, Influence of Cadmium and Zinc on Carbon-

Dioxide Evolution from Litter and Soil from a Black Oak Forest, *J. Environ. Qual.* **1978**, *7 (1)*, 115--119.

[35] F. H. Chang, F. E. Broadbent, Influence of Trace-Metals on Carbon-Dioxide Evolution from a Yolo Soil, *Soil Sci.* **1981**, *132 (6)*, 416--421.

[36] R. Clemente, D. J. Walker, M. P. Bernal, Uptake of PTM and As by *Brassica juncea* grown in a contaminated soil in Aznalcóllar (Spain): The effect of soil amendments, *Environ. Pollut.* **2005**, *138 (1)*, 46--58.

[37] E. Filcheva, M. V. Cheshire, C. D. Campbell, D. B. McPhail, Effect of heavy metal contamination on the rate of decomposition of sewage sludge and microbial activity, *Appl. Geochem.* **1996**, *11 (1--2)*, 331--333.

[38] J. D. Wolt, *Dynamic Expression of Concurrent Sorption-Degradation of Complex Organic-Molecules in Soil, Sediment and Sludges*, Division of Environmental Chemistry Extended Abstracts, ACS National Meeting. American Chemical Society, Washington, DC **1994.**

[39] E. Smolders, K. Oorts, P. van Sprang, I. Schoeters, C. R. Janssen, S. P. McGrath, M. J. McLaughlin, The toxicity of trace metals in soil as affected by soil type and ageing after contamination: using calibrated bioavailability models to set ecological soil standards, *Environ. Toxicol. Chem.* **2009**, *28*, 1633–1642.

[40] D. Eastmond, J. MacGregor, R. Slesinski, Trivalent Chromium: Assessing the Genotoxic Risk of an Essential Trace Element and Widely Used Human and Animal Nutritional Supplement, *Crit. Rev. Toxicol.* **2008**, *38*, 173–190.

[41] L. Haanstra, P. Doelman, Glutamic-Acid Decomposition as a Sensitive Measure of Heavy-Metal Pollution in Soil, *Soil Biol. Biochem.* **1984**, *16 (6)*, 595--600.

[42] P. Doelman, L. Haanstra, Short-Term and Long-Term Effects of Cadmium, Chromium, Copper, Nickel, Lead and Zinc on Soil Microbial Respiration in Relation to Abiotic Soil Factors, *Plant Soil* **1984**, *79 (3)*, 317--327.

[43] G. Renella, M. Chaudri, P. C. Brookes, Fresh additions of PTM do not model long-term effects on microbial biomass and activity, *Soil Biol. Biochem.* **2002**, *34*, 121--124.

[44] E. A. Paul, F. E. Clark, *Soil Microbiology and Biochemistry*, Academic Press, San Diego **1989**.

[45] S. Fontaine, A. Mariotti, L. Abbadie The priming effect of organic matter: a question of microbial competition?, *Soil Biol. Biochem.* **2003**, *35 (6)*, 837--843.

[46] D. G. Zvyagintsev, in *Beyond the Biomass* (Eds.: H. Ritz, J. Dighton, K. E. Giller), Wiley, Chichester **1994**, pp. 29–37.

[47] A. S. Mamilov, O. M. Dilly, Microbial characteristics during the initial stages of litter decomposition in forest and adjacent cropland soil, *Ecol. Eng.*. **2007** *31 (3)*, 147--153.

[48] M. R. H. Bhuiya, A. H. Cornfield, Effects of addition of 1000 ppm Cu, Ni, Pb and Zn on carbon dioxide release during incubation of soil alone and after treatment with straw, *Environ. Pollut.* **1972**, *3*, 173--177.

[49] L. M. Shuman, Effect of organic waste amendments on zinc adsorption by two soils, *Soil Sci.* **1999**, *164 (3)*, 197--205.

[50] D. J. Walker, R. Clemente, A. Roig, M. P. Bernal, The effects of soil amendments on heavy metal bioavailability in two contaminated Mediterranean soils, *Environ. Pollut.* **2003**, *122 (2)*, 303--312.

[51] R. G. McLaren, R. S. Swift, J. G. Williams, The Adsorption of Copper by Soil Materials at Low Equilibrium Solution Concentrations, *J. Soil Sci.* **1981**, *32 (2)*, 247--256.

[52] J. R. Sanders, T. M. Adams, B. T. Christensen, Extractability and Bioavailability of Zinc, Nickel, Cadmium and Copper in 3 Danish Soils Sampled 5 Years after Application of Sewage-Sludge, *J. Sci. Food Agric.* **1986**, *37 (12)*, 1155--1164.

[53] D. J. Ross, D. J. McQueen, H. A. Kettles, Land Rehabilitation under Pasture on Volcanic Parent Materials - Changes in Soil Microbial Biomass and C-Metabolism and N-Metabolism, *Aust. J. Soil Res.* **1994**, *32 (6)*, 1321--1337.

[54] V. Hornburg, G. W. Brümmer, Behavior of Heavy-Metals in Soils. 1. Heavy-Metal Mobility, *Z. Pflanzenernähr. Bodenkunde* **1993**, *156 (6)*, 467--477.

[55] J. Stenstrom, K. Svensson, M. Johansson, Reversible transition between active and dormant microbial states in soil, *FEMS Microbiol. Ecol.* **2001**, *36 (2--3)*, 93--104.

[56] K. B. Ritz, S. Griffiths, R. E. Wheathley, Soil Microbial Biomass and Activity under a Potato Crop Fertilized with N with and without C, *Biol. Fertil. Soils* **1992**, *12 (4)*, 265--271.

[57] M. C. Hernandez-Soriano, J. C. Jimenez-Lopez, Effects of soil water content and organic matter addition on the speciation and bioavailability of PTM, *Sci. Total Environ.* **2012**, 423, 55–61.

Figures

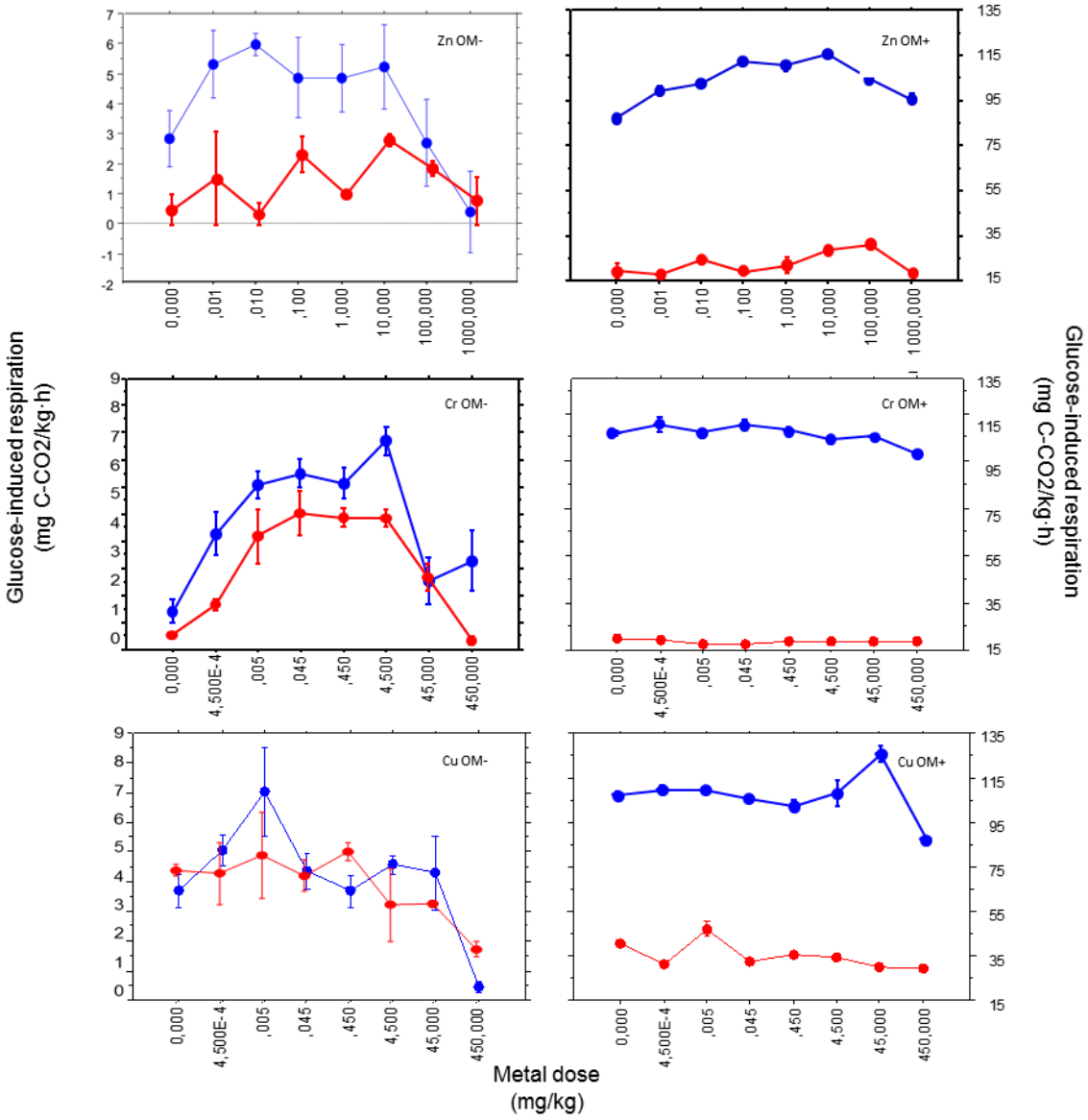

Figure 1. Changes of relative SIR (Substrate-induced Respiration) on the preliminary assay on the soil polluted with increasing concentrations of Zn, Cu and Cr, with (OM+) and without (OM--) organic amendment. Measures relative to day 1 are represented in blue, and to day 28 in red. Bars show standard error. Dose is presented in logarithmic scale.

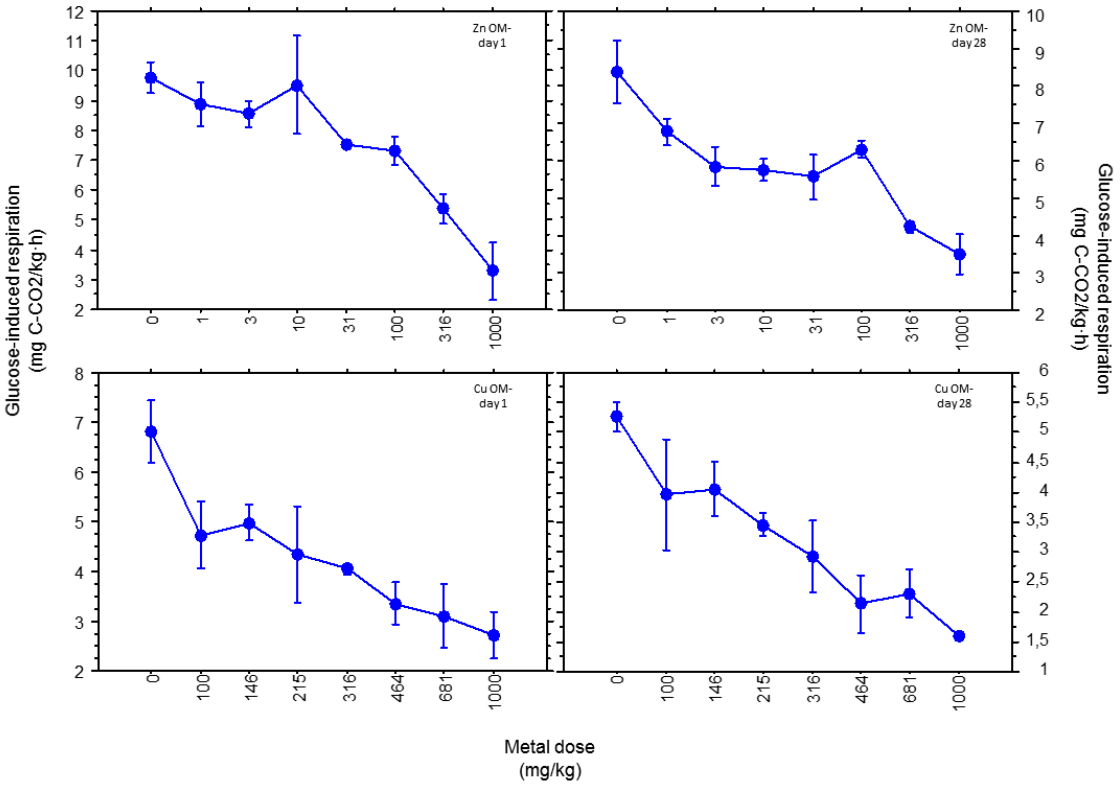

Figure 2. Changes of relative SIR on definitive assay for the soil polluted with increasing concentrations of Zn and Cu. Bars show standard error. Dose is presented in logarithmic scale.

Tables

Table 1. Main soil properties.

Parameter	Unit	Value
Coarse sand (2000--200 µm)	%	60.7
Fine sand (200--20 µm)	%	7.4
Silt (20--2 µm)	%	22.2
Clay (<2 µm)	%	9.7
$CaCO_3$	%	0
Organic carbon	%	0.26
Microbial carbon	mg kg^{-1}	108.2
C microbial / C organic	%	4.16
N Kjeldahl	g kg^{-1}	0.11
C/N	-	23.6
CEC	cmol kg^{-1}	7.3
Electrical conductivity (1:5, w/v)	dS m^{-1}	0.26
pH-water (1:2.5, w/v)	-	6.3

Table 2. Abbreviations and description of treatments.

Acronym	Treatment
Zn OM--	Addition of $ZnCl_2$
Zn OM+	Addition of $ZnCl_2$ and *Populus* leaves
Cu OM--	Addition of $CuSO_4 \cdot 5\ H_2O$
Cu OM+	Addition of $CuSO_4 \cdot 5\ H_2O$ and *Populus* leaves
Cr OM--	Addition of $CrCl_3 \cdot 6\ H_2O$
Cr OM+	Addition of $CrCl_3 \cdot 6\ H_2O$ and *Populus* leaves

Table 3. Range of concentrations used in the definitive series for the Zn OM-- and Cu OM-- treatments.

Zn OM-- (mg kg^{--1})	**Cu OM-- (mg kg^{--1})**
0	0
1	100
3	147
10	215
32	316
100	464
316	681
1000	1000

See abbreviations meaning in Table 2.

Table 4. EC_{50} values and 95% confidence interval (in brackets) for incubations (day 1) and (day 28).

Metal	**EC_{50} (mg kg^{-1})**	**EC_{50} (mg kg^{-1})**
	Day 1	Day 28
Zn	529 (257, 1089)	1285 (552, 2993)
Cu	958 (301, 3051)	517 (115, 2325)