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SHORT COMMUNICATION

# Root Elongation Method for the Quality Assessment of Metal-Polluted Soils: Whole Soil or Soil-Water Extract?

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**Abstract**

Root elongation method may be implemented using two internationally accepted protocols: exposing plants to either soil-water extract or whole soil. But which of the two protocols is more suitable for root elongation analysis undertaken for the quality assessment of metal-polluted soils? Soils were sampled at various distances from the site of the Middle Urals Copper Smelter located in Russia. White mustard was used as a bioindicator. We observed considerable differences in root elongation under the two protocols. In plants grown in whole soil, root length inversely correlated with pollution index, but in soil-water extract, metal concentrations had no effect on root length. Nutrient and metal concentrations in the soil-water extract were not buffered, due to the absence of the solid soil phase. It is for this reason that in highly polluted soils, root growth was greater in soil-water extracts rather than in whole soils, whereas in background soils (in the absence of toxicity), root growth was greater in whole soils compared with soil-water extracts. The quantity, intensity, and capacity factors are a plausible explanation for the differences in root length between the two protocols. The soil-water extract does not represent actual soil with respect to the desorption-dissolution reactions that take place between the soil solid phase and the soil solution. For this reason, whole soil protocol should be used for measuring root elongation given that only under this protocol, direct contact between metal-polluted soil and test organisms correctly replicates the risks inherent in the actual soil habitat.

**Keywords** Aqueous extracts · Inhibition · Phytotoxicity · Toxicity · Ecotoxicity · Middle Urals

## 1 Introduction

The ecotoxicity methods considered in published guidelines for the quality assessment of metal-polluted soils are based on responses (growth, reproduction, mortality, etc.) of various organisms (plants, earthworms, microorganisms, etc.) (ISO 17402 2008; ISO 17616 2008). Among these, the root elongation method (ISO 11269-1 2012; US EPA 1996) is widely

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used, due to its low cost, simplicity, and short duration (e.g., He et al. 2020). This method is based on measuring the protrusion of the radicle out of the seed coat and subsequent root growth. The variable that is measured is the root length after several days, while shoot growth is not taken into account in this test. The utility of this method for the quality assessment of metal-polluted soils is based on the radicle's ability to absorb metals (e.g., Zhang et al. 2020).

The root elongation method can be implemented using two internationally accepted protocols: exposing plants to either soil-water extract or whole soil. Specifically, the US EPA (1996) protocol for root elongation recommends using test solutions (e.g., soil-water extracts) and Petri dishes filled with inert substrate (such as sand or glass beads), explicitly stating that native soils should not be used as substrates. In contrast, the ISO 11269-1 (2012) protocol recommends direct contact between soil and tested plants.

Thus, the following question arises: which protocol—whole soil or soil-water extract—is preferable for root elongation studies for the purposes of quality assessment of metal-polluted soils? There is also an intermediate version of these two protocols that recommends using a soil-water suspension (Vorobeichik and Pozolotina 2003). However, we will not consider this intermediate version in the present study because it has not been recognized as part of any international protocol nor is it used in any other studies.

The study of Leitgib et al. (2007) has attempted to explicitly answer the above-mentioned question. This study compared several ecotoxicity evaluation methods, including the whole soil and soil-water extract protocols of the root elongation method. The study demonstrated considerable differences in root elongation under the two protocols in metal-contaminated soils. The study emphasized that whole soils should be used for ecotoxicity bioassays because only direct contact between metal-polluted soil and test organisms correctly replicates the risks inherent in the actual soil habitat. However, after the work of Leitgib et al. (2007), several studies performed root elongation assays using soil-water extracts rather than whole soils, despite known differences in plant responses between the protocols (Bagur-Gonzalez et al. 2011; Camarillo-Ravelo et al. 2015; Chan-Keb et al. 2018; Garcia-Carmona et al. 2019; Gonzalez et al. 2013; Romero-Freire et al. 2016; Romero-Freire et al. 2015; Tiquia 2010; Torri et al. 2009). Furthermore, the study of Leitgib et al. (2007) did not attempt to uncover the mechanism that governs the differences between the two protocols.

In the context of this paper, it is important to consider that while the intensity of toxicity is controlled by soluble metal concentrations, it also depends on the factors governing the buffering of metal concentrations in the soil solution. For instance, in the study of Sauvé et al. (1996), plant tissues accumulated an average of 2000 times the amount of total copper (Cu) dissolved in the solution. This is only possible if Cu in the soil solution is buffered by desorption-dissolution mechanisms (Sauvé 2002). Furthermore, Ginocchio et al. (2002) demonstrated that Cu concentration in vegetables depended on several soil Cu pools, such as total, extractable, and free ionic $Cu^{2+}$.

Thus, we assumed that the differences between the two protocols of the root elongation method are caused by desorption-dissolution reactions that take place between the soil solid phase and the soil solution under the whole soil protocol but are absent in the soil-water extract protocol. Therefore, in the present paper, we endeavored to reveal the mechanism that determines the differences between the two protocols—soil-water extract and whole soil—of the root elongation method.

## 2 Materials and Methods

### 2.1 Soil Sampling and Characterization

The study was carried out in the area that has long been exposed to air pollution (since 1940) from the Middle Urals Copper Smelter (N 56°51′0.8″ E 59°54′25.6″), located in the southern taiga subzone, near the town of Revda, 50 km from Ekaterinburg, Russia. The smelter's pollutants consist mostly of sulfur dioxide and polymetallic dust containing Cu, cadmium (Cd), lead (Pb), and zinc (Zn), among other elements. Total smelter emissions were 350,000 tons per year$^{-1}$ in 1975, dropping to 5 tons per year$^{-1}$ in 2010 (www.sumz.umn.ru). Despite the recent drop in emissions, there is no evidence of ecosystem recovery in the highly polluted areas (Vorobeichik et al. 2014) or of reduced metal content in the topsoil (Vorobeichik and Kaigorodova 2017).

Three zones can be distinguished along the contamination gradient, impact, buffer, and background zones (1–3 km, 4–7 km, and 20–30 km away from the smelter, respectively), representing successive stages of industrial degradation of native spruce-fir forest ecosystems (Smorkalov and Vorobeichik 2011). There is detailed information available on the study area with regard to the effect of metal pollution on soil properties (Kaigorodova and Vorobeichik 1996; Vorobeichik and Kaigorodova 2017), in particular with regard to the effect of metal pollution on the thickness of forest litter (Korkina and Vorobeichik 2018; Vorobeichik 1995) and the state of soil organic matter (Korkina and Vorobeichik 2018; Prokopovich and Kaigorodova 1999). Likewise, there is detailed information on the effect of metal pollution on tree stand (Usoltsev et al. 2012), herbaceous vegetation (Vorobeichik et al. 2014), soil fungi (Mikryukov et al. 2015), soil respiration (Smorkalov and Vorobeichik 2011; Smorkalov and Vorobeichik 2016), soil-dwelling macroinvertebrates (Vorobeichik 1998; Vorobeichik et al. 2019; Vorobeichik et al. 2012), soil microarthropods, and ground running macroinvertebrates (Ermakov 2004). Some of this information is summarized in Table 1. Importantly, soil and biota responses were negatively affected by metal concentrations along the contamination gradient.

Five sampling sites were selected along the contamination gradient at distances of 1, 2, 6, 30, and 33 km to the west of the smelter (Table 2). At each sampling site, soils were sampled with a shovel at three points, at a distance of 50–200 m from each other. The three soil samples were analyzed separately. Thus, 15 samples of the A horizon were available for analysis. However, on the O horizon, only 14 samples were extracted because forest litter was too shallow at one sampling point (R30W1), preventing the extraction (described in detail below). The volume of each soil sample was ~ 5 L. Soils were transported to the laboratory, air-dried, and homogenized. Samples of the A horizon were sieved through a 2-mm mesh. In samples of forest litter, coarse elements (e.g., conifer cones) were thoroughly removed by hand.

Silt loam Retisols were the common soils in the study area (Table 1) (IUSS Working Group WRB 2015). At each point, we sampled forest litter (O horizon) according to its depth and the mineral topsoil (A horizon) to a depth of ~ 10 cm. Consistent with previous reports (Korkina and Vorobeichik 2018; Vorobeichik 1995), the litter depth was 1–2 cm in the background zone, 5–7 cm in the buffer zone, and 10–15 cm in the impact zone, due to a reduction in soil macroinvertebrate detritivores (Vorobeichik et al. 2012) and a decrease in the activity of cellulose-decomposing microorganisms (Korkina and Vorobeichik 2018).

**Table 1** Characteristics of the background, buffer, and impact zones, based on previous studies

| Characteristic | Background zone | Buffer zone | Impact zone |
|---|---|---|---|
| Landscape description | Spruce-fir forest on the watershed between rivers Bol'shaya Talitsa and Belyi Atig<br>346–404 m a.s.l.<br>30 km (N 56.801° E 59.425) and 33 km (N 56.808° E 59.361) from the smelter | Spruce-fir forest on foothills of the Belaya mount<br>390–412 m a.s.l.<br>6 km (N 56.857° E 59.801) from the smelter | Spruce-fir forest on the eastern midslope of Shaitan Ridge<br>380–419 m a.s.l.<br>1 km (N 56.848° E 59.863) and 2 km (N 56.844° E 59.878) from the smelter |
| Soil description, WRB[1] | Albic Retisol (cutanic) | Leptic Retisol (toxic) | Stagnic Retisol (cutanic, toxic) |
| Humus forms[2] | Oligomull-Dysmull | Dysmull-Humimor | Eumor |
| Tree stand composition[3] | 60–80% of *Abies sibirica*, 20–40% of *Picea obovata* with up to 20% of *Betula* spp. | 30–60% of *Abies sibirica*, 30–40% of *Picea obovata* with up to 20% of *Betula* spp., and 10% of *Larix sibirica* | 20–70% of *Abies sibirica*, 20–70% of *Picea obovata* with up to 40% of *Betula* spp., and 20% of *Populus tremula* |
| Tree stand age[3] | 75–90 | 85 | 98–102 |
| Tree stand volume ($m^3$ $ha^{-1}$)[3] | 395 | 408 | 252 |
| Dominants of herbaceous vegetation[4] | *Oxalis acetosella*, *Dryopteris* spp., *Calamagrostis arundinacea*, *Aegopodium podagraria*, *Ajuga reptans* | *Calamagrostis arundinacea*, *Oxalis acetosella* | *Agrostis capillaris* |
| Number of herbaceous species (per 625 $m^2$)[4] | 61 | 28–41 | 6.8–13 |
| Earthworms abundance (individual $m^2$)[5] | 238 | 93 | 1.0 |
| Litter specific respiration rate (mg $CO_2$ $g^{-1}$ $h^{-1}$)[6] | 0.20 | 0.10 | 0.05 |

[1] Vorobeichik and Kaigorodova (2017)

[2] Korkina and Vorobeichik (2018)

[3] Usoltsev et al. (2012)

[4] Vorobeichik et al. (2014)

[5] Vorobeichik et al. (2019)

[6] Smorkalov and Vorobeichik (2016)

**Table 2** Chemical properties of the studied soils

| Sample | Distance, km | pH | Total metal content, $mg\ kg^{-1}$ | | | | Exchangeable metal content, $mg\ kg^{-1}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cu | Cd | Pb | Zn | Cu | Cd | Pb | Zn |
| Organic horizon (O) | | | | | | | | | | |
| R1W1 L | 1 | 4.4 | 2798 | 14 | 1768 | 1005 | 2.6 | 0.14 | 0.14 | 10 |
| R1W2 L | 1 | 4.2 | 3790 | 18 | 2401 | 1248 | 15 | 0.79 | 0.92 | 59 |
| R1W3 L | 1 | 4.4 | 3507 | 38 | 3195 | 2153 | 4.6 | 0.34 | 0.91 | 33 |
| R2W1 L | 2 | 4.4 | 4313 | 17 | 2534 | 1387 | 15 | 0.22 | 0.51 | 25 |
| R2W2 L | 2 | 4.1 | 4935 | 32 | 5100 | 1799 | 22 | 0.86 | 1.4 | 64 |
| R2W3 L | 2 | 4.7 | 4423 | 72 | 5255 | 3046 | 9.6 | 0.38 | 0.85 | 46 |
| R6W1 L | 6 | 4.7 | 533 | 10 | 514 | 1134 | 2.9 | 0.09 | 0.23 | 19 |
| R6W2 L | 6 | 4.9 | 2199 | 19 | 1191 | 1326 | 4.4 | 0.07 | 0.36 | 12 |
| R6W3 L | 6 | 4.7 | 1956 | 19 | 928 | 993 | 12 | 0.19 | 1.4 | 23 |
| R30W2 L | 30 | 4.7 | 82 | 2.2 | 84 | 173 | 0.69 | 0.03 | 0.09 | 5.4 |
| R30W3 L | 30 | 4.5 | 61 | 2.7 | 78 | 206 | 0.23 | 0.02 | 0.06 | 4.0 |
| R33W1 L | 33 | 4.6 | 53 | 1.6 | 47 | 136 | 0.24 | 0.02 | 0.06 | 3.6 |
| R33W2 L | 33 | 4.7 | 64 | 1.9 | 89 | 275 | 0.44 | 0.02 | 0.20 | 6.2 |
| R33W3 L | 33 | 4.3 | 56 | 1.5 | 72 | 182 | 0.39 | 0.02 | 0.06 | 3.8 |
| Mineral horizon (A) | | | | | | | | | | |
| R1W1 S | 1 | 4.3 | 645 | 3.1 | 76 | 207 | 1.6 | 0.11 | 0.03 | 8.8 |
| R1W2 S | 1 | 3.9 | 798 | 3.3 | 227 | 245 | 21 | 0.47 | 0.20 | 31 |
| R1W3 S | 1 | 4.5 | 1009 | 5.1 | 376 | 363 | 3.3 | 0.20 | 0.05 | 15 |
| R2W1 S | 2 | 4.1 | 776 | 4.9 | 35 | 212 | 21 | 0.50 | 0.05 | 34 |
| R2W2 S | 2 | 4.2 | 1364 | 7.7 | 164 | 323 | 21 | 0.61 | 0.04 | 42 |
| R2W3 S | 2 | 4.2 | 1434 | 5.5 | 143 | 388 | 14 | 0.33 | 0.04 | 37 |
| R6W1 S | 6 | 4.3 | 192 | 2.6 | 17 | 198 | 0.52 | 0.07 | 0.03 | 14 |
| R6W2 S | 6 | 4.2 | 150 | 2.2 | 34 | 259 | 0.27 | 0.05 | 0.03 | 11 |
| R6W3 S | 6 | 4.3 | 305 | 4.0 | 77 | 403 | 0.78 | 0.09 | 0.03 | 15 |
| R30W1 S | 30 | 4.3 | 48 | 0.73 | 28 | 110 | 0.05 | 0.01 | 0.03 | 1.0 |
| R30W2 S | 30 | 4.3 | 32 | 0.45 | 23 | 86 | 0.07 | 0.01 | 0.03 | 1.6 |
| R30W3 S | 30 | 4.2 | 36 | 0.64 | 24 | 77 | 0.05 | 0.01 | 0.03 | 1.2 |
| R33W1 S | 33 | 4.0 | 34 | 0.47 | 25 | 99 | 0.09 | 0.02 | 0.03 | 1.4 |
| R33W2 S | 33 | 4.1 | 30 | 0.92 | 20 | 120 | 0.04 | 0.01 | 0.03 | 2.0 |
| R33W3 S | 33 | 4.0 | 34 | 0.46 | 20 | 73 | 0.07 | 0.02 | 0.03 | 1.2 |

The detection limits were as follows: 0.02 $mg\ kg^{-1}$ for Cd and 0.06 $mg\ kg^{-1}$ for Pb in organic horizons and 0.01 $mg\ kg^{-1}$ for Cd and 0.03 $mg\ kg^{-1}$ for Pb in mineral horizons

Exchangeable metal contents and pH were determined in the 0.01 M $KNO_3$ extract, at soil/solution ratio of 1/8 (for O horizon) or 1/4 (for A horizon)

Total concentrations of Cu, Cd, Pb, and Zn were determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Agilent 5110, USA). For samples of the A horizon, microwave digestion with a mixture of concentrated $HCl + HNO_3$ was carried out, according to the Russian Federal Register FR 1.31.2009.06787. For samples of the O horizon, microwave digestion with a mixture of concentrated $H_2O_2 + HNO_3$ was carried out, according to the procedure of the microwave manufacturer (Milestone Inc., USA).

Exchangeable concentrations of Cu, Cd, Pb, and Zn were also determined by ICP-OES, using a solution of 0.01 M $KNO_3$ as the extractant, at a soil/solution ratio of 1/4 (for the A horizon) or 1/8 (for the O horizon). The final suspension was shaken for 120 min and then filtered through an ashless filter paper. Supplementary Table 1 shows exchangeable fraction in total metal content.

It might be argued that the most appropriate agent for exchangeable metal extraction from metal-polluted soils is 0.01 M $CaCl_2$ (e.g., review of Kim et al. 2015). However, divalent cations (such as $Ca^{2+}$) promote flocculation of dissolved organic carbon in the soil solution (Sauvé 2002). On account of this mechanism, the use of 0.01 M $CaCl_2$ may underestimate the exchangeable metal fraction due to the precipitation of metals with a strong affinity for dissolved organic carbon (Mondaca et al. 2015; Neaman et al. 2009). It is for this reason that we prefer to work with 0.01 M $KNO_3$, which is also widely used for exchangeable metal extraction from metal-polluted soils (e.g., Almas et al. 2000; Luo et al. 2006; Moreno-Caselles et al. 2000; Perez-Esteban et al. 2013).

Total and exchangeable metal analyses were carried out in duplicate samples; average values are shown (Table 2). Duplicates for the analysis were prepared separately. Certified reference samples were also digested in duplicate, in order to assure quality. Values obtained were within 10% of the certified values.

The soil pH was measured in the same 0.01 M $KNO_3$ extract. It is well-known that soil pH in salt extracts tends to be lower in comparison with water extracts due to displacement of Al ions from the soil exchangeable complex by the salt cation (McBride 1994).

## 2.2 Root Elongation Method

The choice of white mustard (*Sinapis alba* L., Brassicaceae, cultivar Raduga, www.gavrish.ru) for soil quality assessments was dictated by the guidelines of the Russian Federal Register FR 1.31.2012.11560, as described in detail in Nikolaeva and Terekhova (2017). The germination rate of white mustard seeds was determined according to the soil-water protocol as described below, except that distilled water was used in place of soil extract.

Specially designed boxes were used (Fig. 1). Each box had two compartments with dimensions of 13.5 × 8.5 × 0.8 cm each (length × width × height, respectively); i.e., the volume of each compartment was 92 $cm^3$. The lower compartment was for root growth, whereas the upper one was for shoot growth.

For the whole soil protocol, the lower box compartment was filled with soil (volume of ~ 92 $cm^3$), which was moistened with deionized water. Then, ten seeds were placed on the soil surface and covered with filter paper. For the soil-water protocol, aqueous extracts were prepared immediately before the bioassay using a soil/water ratio of 1/4 (for the A horizon) or 1/8 (for the O horizon). The final suspension was shaken for 120 min and then filtered through an ashless filter paper. Then, the filter paper was placed in the lower box compartment and homogeneously moistened with 10 mL of the soil-water extract. Afterwards, ten seeds were placed on the filter paper.

In both protocols—soil-water extract and whole soil—the root elongation values were measured after incubation at 22.5 ± 0.2 °C for 96 h (4 days) in the dark, inside of an incubation chamber. Three replicated boxes were used for each sample; thus, there were 30 measurements for each sample.

Below, we will discuss the difference between "root length in soil-water extract" and "root length in whole soil" as a numerical result of subtraction of the latter from the former, referred to as the "difference in root length" for simplicity.

## 2.3 Data Analysis

Simple linear regressions between the variables were carried out using the Minitab Express 1.5.2 software. For each sampling point, we calculated the pollution index as follows (Vorobeichik and Pozolotina 2003):

$$\mathrm{PI_i} = \frac{1}{n}\sum_{j=1}^{n}\left(\frac{C_{\mathrm{ij}}}{C_{\mathrm{jb}}}\right)$$

where $PI_i$ is the pollution index of the $i$th point, $C_{ij}$ is the concentration of the $j$th element in the $i$th point, $C_{jb}$ is the average concentration of the $j$th element at a distance of 33 km from the smelter, and $n$ is the number of elements analyzed (Cu, Cd, Pb, and Zn, i.e., $n = 4$ in the present study). Metal concentrations at a distance of 33 km from the smelter (Table 2) can be considered to represent background concentrations, as evidenced by our multiple previous studies (Table 1).

The index is the factor by which the contamination is increased on average for all metals, compared with the background level. This index was calculated for both total and exchangeable concentrations, referred to as "pollution index (total)" and "pollution index (exchangeable)", respectively (Fig. 2).

# 3 Results and Discussion

## 3.1 Chemical Properties of Soils Under Study

Metal concentrations—both total and exchangeable—decreased with distance from the smelter, in both the O and A horizons (Table 2). These results are consistent with previous reports for the study area (Korkina and Vorobeichik 2018; Smorkalov and Vorobeichik 2011). In contrast, soil pH in the salt extracts did not change with distance, in either the O or A horizons (Table 2). Furthermore, total concentrations of Cu, Cd, Pb, and Zn were strongly intercorrelated ($p < 0.005$), in both the O and A horizons. Likewise, exchangeable concentrations of Cu, Cd, Pb, and Zn strongly correlated with each other ($p < 0.005$), in both the O and A horizons. Thus, it was not possible to discern the effect of any specific metal on plant responses in the present study. For this reason, in further discussions, we will use the pollution index and will refer to "metals" in general rather than to a particular metal.

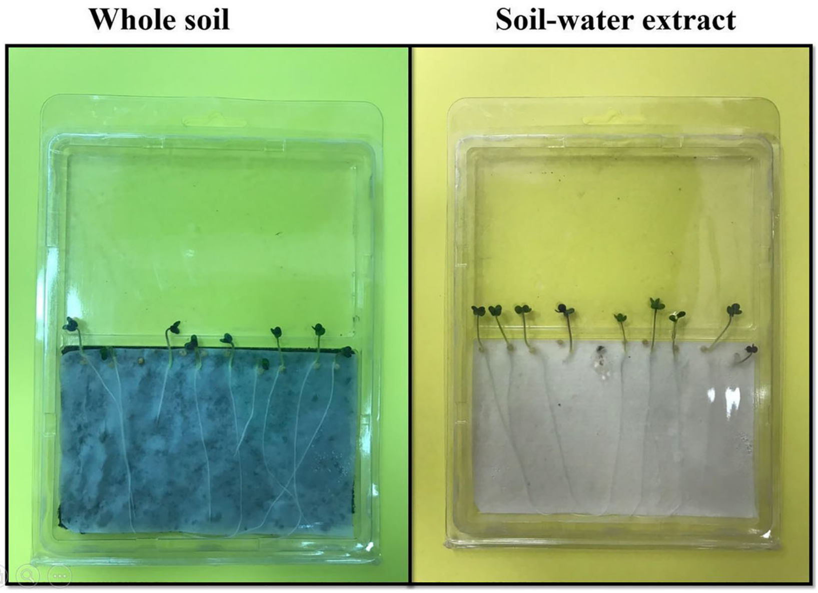


**Fig. 1** Experimental boxes used for root elongation bioassay

## 3.2 Root Elongation Method

The seed germination rate of white mustard was 98.6 ± 0.7%, underscoring excellent seed viability. However, subsequent root elongation exhibited high variability. The variation coefficient (standard deviation 100/average) was less than 50% in most cases and up to 74% in 2 cases in the A horizon of sample R2W (Supplementary Table 2).

Here and below, we discuss the results for the A horizon (Fig. 2), whereas the results for the O horizon presented the same tendencies, exhibiting slightly weaker regression coefficients (Supplementary Fig. 1). Importantly, the root length in the whole soil treatment was inversely correlated with pollution indexes (both exchangeable and total, Fig. 2a and c), underscoring the inhibiting effect of metal toxicity on root growth in the soils under study, similar to our earlier studies

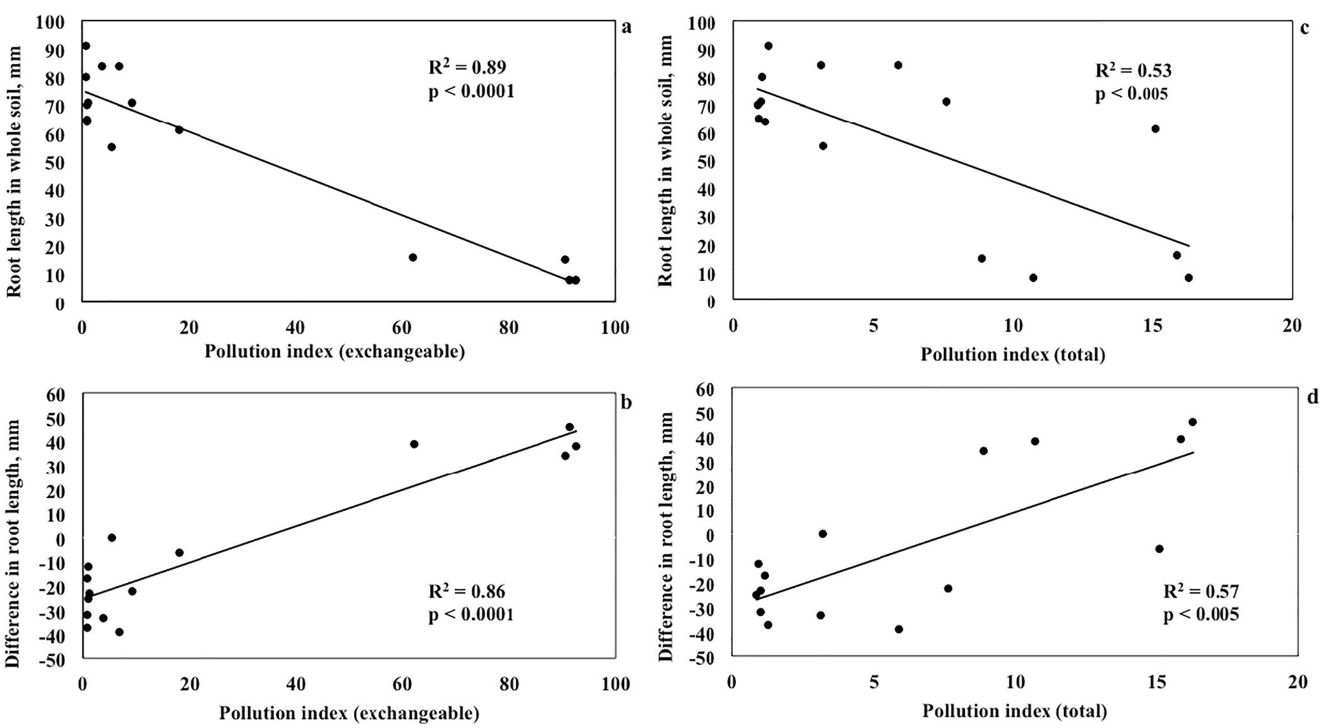


**Fig. 2** The relationships between **a** root length in whole soil and pollution index (exchangeable), **b** difference in root length (i.e., difference between “root length in soil-water extract” and “root length in whole soil” as a numerical result of subtraction of the latter from the former) and pollution index (exchangeable), **c** root length in whole soil and pollution index (total), and **d** difference in root length and pollution index (total). Data for the A horizon was used ($n = 15$). Similar relationships for the O horizon are shown in Supplementary Fig. 1

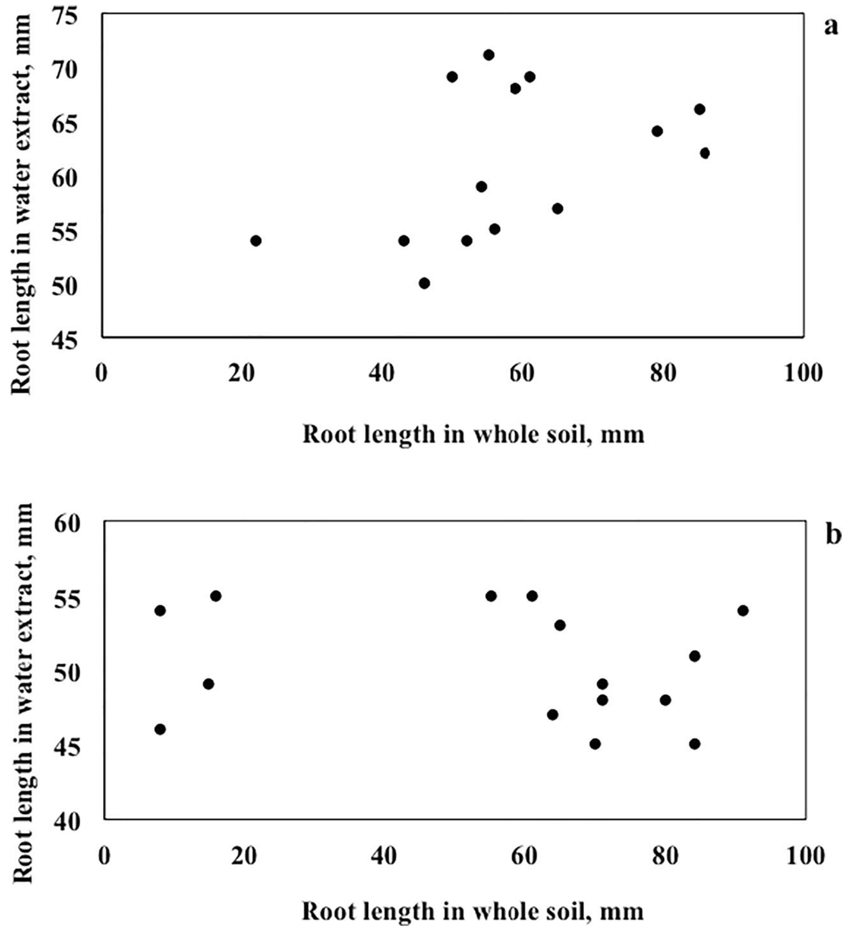


**Fig. 3** Relationship between root length in whole soil and root length in soil-water extract in **a** O horizon and **b** in A horizon. The relationship was not significant ($p > 0.05$)

carried out under field conditions in the study area (Table 1). From this point of view, the results of the whole soil protocol correctly replicate the risks inherent in the actual soil habitat.

### 3.3 Differences in Root Elongation Results Under the Two Protocols

We observed considerable differences in root elongation results under the two protocols—whole soil and soil-water extract—for both the O and A horizons (Supplementary Table 2). The correlation between the results of both protocols was not statistically significant for either the O or A horizons ($p > 0.05$) (Fig. 3). Importantly, metal concentrations had no effect on the root length of plants exposed to soil-water extract. From this perspective, the results of the soil-water extract were not useful for the quality assessment of metal-polluted soils.

Specifically, the difference in root length was positively correlated with pollution indexes (both exchangeable and total) (Fig. 2b and d). The difference in root length was positive for high metal concentrations and negative for low metal concentrations. In other words, in highly polluted soils, root growth was greater in the soil-water extracts than in whole soils. In the case of low metal concentrations (i.e., in the absence of toxicity), root growth was higher in whole soil than in the soil-water extracts.

In the context of this paper, it is important to consider the quantity, intensity, and capacity factors because they are known to govern the phytoavailability of metals in soil (e.g., Echevarria et al. 1998; Song et al. 2004). Specifically, the quantity factor refers to the total metal quantity or content in soil. The intensity factor indicates the pool of metal in soil that is immediately available to plant roots at any given time. Finally, the capacity factor denotes the buffering capacity of soil to supply metal ions to the soil solution.

In recent decades, several studies attempted to predict the so-called phytoavailable metal fraction by correlating plant responses with various soil metal pools. It is generally thought that metal soluble fractions—extracted by chemically nonaggressive neutral salts—are useful for assessing metal phytotoxicity in contaminated soils (Kabata-Pendias 2004; McBride et al. 2009). Indeed, our recent study with soils near a Cu smelter in central Chile (Lillo-Robles et al. 2020) suggests that salt-extractable (i.e., exchangeable) Cu was the best indicator of metal phytotoxicity in soil, whereas total soil Cu was not a good predictor of plant responses. Consistent with this argument, the pollution index (exchangeable) was a stronger predictor of root length, compared with the pollution index (total) (Fig. 2a and c).

Thus, the results observed in the present study can be explained by the aforementioned quantity, intensity, and capacity factors. Specifically, in highly polluted soils, metal concentrations in the soil solution of the whole soil were buffered by desorption-dissolution reactions from the soil solid phase (Lillo-Robles et al. 2020; Sauvé 2002), causing high phytotoxicity and, therefore, poor root growth. Importantly, the radicle's ability to absorb metals is confirmed in other studies (e.g., Zhang et al. 2020). In contrast, metal concentrations in the soil-water extract were not buffered, due to the absence of the solid soil phase, limiting the expression of phytotoxicity. It is for this reason that in highly polluted soils, root growth was greater in the soil-water extracts than in whole soils.

On the other hand, in background soils (i.e., in the absence of toxicity), root growth was greater in whole soil than in soil-water extracts. In this case, root development was more intense in the whole soil, because the soil solid phase was capable of supplying nutrients to the soil solution at the same time as plant roots depleted nutrient ions from the soil solution through active uptake. In other words, the results observed in the present study in background soils can also be explained by the aforementioned quantity, intensity, and capacity factors. These factors are known to govern the phytoavailability of nutrients in soils, as discussed in detail elsewhere (Shirvani et al. 2005; Taiwo et al. 2010).

Indeed, after sowing, the radicle protrudes out of the seed coat and then explores deeper layers for nutrient absorption (Benoit et al. 2015; Durr and Mary 1998; Li et al. 2019). In Brassicaceae species, radicle protrusion occurs approximately 2 days after sowing (Dell'Aquila et al. 2000; Russo et al. 2010). Radicle growth depends on both mineral seed reserves and nutrient uptake from the substrate (Brunel-Muguet et al. 2015). This is especially true in small seeds (Peñaloza and Durán 2015), such as mustard seeds. From this perspective, the nutrient supply was limited to soluble concentrations in the soil-water extract due to the absence of a solid soil phase capable of supplying additional nutrients, which would explain why root development was more intense in the whole soil.

The results of white mustard root growth in the present study are consistent with those in the study of Vorobeichik and Pozolotina (2003), which used soils from the same study area. Specifically, the latter study used a soil-water suspension from the O horizon (at a soil/water ratio of 1:10) and common dandelion (*Taraxacum officinale* (L.) Weber ex F.H. Wigg). Similar to the present study, the inhibited root growth of the common dandelion was attributed to the pollution index. In this respect, the soil-water suspension protocol is closer to the one using whole soil than to that of soil-water extract, due to the presence of the soil solid phase. However, we argue that the whole soil protocol is preferable because it conforms with both its international counterpart (ISO 11269-1 2012) and the Russian national protocol (Russian Federal Register FR 1.31.2012.11560).

## 4 Conclusion

The novelty of this study has to do with the attempt to uncover the mechanism that governs the differences between the two protocols—whole soil and soil-water extract—of the root elongation method used in the quality assessment of metal-polluted soils.

Our results support the hypothesis that the differences between the two protocols of the root elongation method are associated with the presence of desorption-dissolution reactions between the soil solid phase and the soil solution under the whole soil protocol and their absence in the soil-water extract protocol. In other words, the results observed in the present study can be explained by the quantity, intensity, and capacity factors that govern the phytoavailability of metals in the polluted soils and the phytoavailability of nutrients in unpolluted background soils.

The results of the present study in the whole soil treatment were consistent with previous studies carried out under field conditions in the study area. Specifically, soil and biota responses in other studies were also negatively affected by metal concentrations along the contamination gradient. For this reason, in measuring root elongation, preference should be given to the whole soil protocol, since it allows for direct contact between metal-polluted soil and test organisms and thus correctly replicates the risks inherent in the actual soil habitat.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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