**Proposed modification to avoidance test with *Eisenia fetida* to assess metal toxicity in agricultural soils affected by mining activities**

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**Abstract**

Use of avoidance tests is a quick and cost-effective method of assessing contaminants in soils. According to the standard protocol ISO 17512-1 (2008), one option for assessing earthworm avoidance behavior is a two-section test, which consists of earthworms being given the choice to move between a test soil and a control substrate. For ecological relevance, tested soils should be field-contaminated soils. For practical reasons, artificial soils are commonly used as the control substrate. Interpretation of the test results compromised when the test soil and the artificial substrate differ in their physico-chemical properties other than just contaminants. In this study we identified the physico-chemical properties that influence avoidance response and evaluated the usefulness of adjusting these in the control substrate in order to isolate metal-driven avoidance of field soils by earthworms. A standardized two-section avoidance test with *Eisenia fetida* (ISO 17512-1, 2008) was performed on 52 uncontaminated and contaminated (Cu >155 mg kg-1, As >19 mg kg-1) agricultural soils from the Aconcagua River basin and the Puchuncaví Valley in Chile. Regression analysis indicated that the avoidance response was determined by soil organic matter (OM), electrical conductivity (EC) and total soil Cu. Organic matter content of the artificial substrate was altered by peat additions and EC by NaCl so that these properties matched those of the field soils. The resultant EC80 for avoidance (indicative of soils of “limited habitat”) was 433 mg Cu kg-1 (339 – 528 mg kg-1 95% confidence intervals). The earthworm avoidance test can be used to assess metal toxicity in field-contaminated soils by adjusting physico-chemical properties (OM and EC) of the artificial control substrate in order to mimic those of the field-collected soil.

**Keywords:** Chile; Copper; Threshold values; Earthworm; Organic matter; Electrical conductivity

**1. Introduction**

Areas close to mining activities in central Chile have been contaminated with metals such as Cu, Zn, Cd, Pb, and metalloids, such as As (De Gregori et al., 2003; Ginocchio et al., 2006). In particular, soils with high metal concentrations can have negative effects on plants and edaphic organisms (Bustos et al., 2015; Ginocchio, 2000; Ginocchio et al., 2002; Verdejo et al., 2015). In response to the need to evaluate the toxicity of metals on different organisms, the use of bioassays has attracted much interest in recent years (Chapman et al., 2013; Garcia-Lorenzo et al., 2014; Hassan et al., 2016).

Of the organisms used to assess metal toxicity, the epigeic earthworm *Eisenia fetida* L. is highlighted for several reasons. Ecologically, *Eisenia fetida* is considered to be representative of soil fauna and earthworms in particular (OECD-222, 2016 and references therein). From a practical point of view, epigeic earthworms are much more preferable than any other lumbricid species for use in toxicity testing as they are easy to culture, reach sexual maturity quickly and reproduce rapidly in the laboratory (OECD-222, 2016; OECD 207, 1984). Consequently, *Eisenia fetida* is recognised as the standard earthworm for toxicity testing and has been widely used in many different studies (Sivakumar, 2015). In one study, *Eisenia fetida* has been found to be neither more nor less sensitive to chemicals than other earthworms (Laskowski et al., 1998). However, in other studies *Eisenia fetida* has been found to be less sensitive to Zn and Pb than other earthworms (Langdon et al., 2005; Spurgeon and Weeks, 1998).

Avoidance behavior by earthworms is a sensitive, non-lethal, easily measured toxicity endpoint that has been standardized in the ISO 17512-1 (2008) protocol. This standard details methods for both a two-section and a six-section avoidance test. The former has been employed by a number of researchers but the latter has not been widely adopted, probably because of practical considerations related to producing the circular, 6-chambered experimental vessels required (Lowe et al., 2016). Therefore in the present study, avoidance tests will refer to the two-section test. This avoidance test comprises earthworms being given the choice to move between a test soil and a control substrate. Thus, the avoidance test assesses the potential of a soil to be inhabited.

Avoidance tests with earthworms using metal-spiked soils have suggested that the avoidance response is primarily dependent on metal concentrations (Lukkari and Haimi, 2005; Scheffczyk et al., 2014). However, this does not necessarily apply to field conditions, because it is well known that the availability of metals in metal-spiked soils is greater than in field-collected soils{Ginocchio, 2006 #1} resulting in over-estimates of toxicity (Davies et al., 2003). For this reason, Nahmani et al. (2007b) highlighted the importance of using field-contaminated soils in bioassays to assess metal toxicity instead of metal-spiked soils.

The ISO-17512 (2008) proposes three alternatives for use as a control substrate in earthworm avoidance tests: (a) a control soil as similar as possible to the test soil in all characteristics other than the presence of contaminants, (b) a soil with the characteristics described in ISO-11269-2 (2012) (*i.e.* organic carbon content ≤ 1.5 %, sand content of 50% to 75%, < 20% of particles less than 0.02 mm, and pH of 5 to 7.5), or (c) an artificial substrate as described in ISO-11268-2 (2012) (*i.e.*, 10% sphagnum peat, 20% kaolinite clay, and 70% quartz sand). Due to the necessity of standardizing bioassays, the commonest choice is probably the use of an artificial control substrate. Moreover, uncontaminated soils with similar properties to contaminated soils may not be available.

However, testing earthworm avoidance behavior of field-contaminated soil with an artificial control substrate as the control may be a problem. The soil and control substrate may differ in several physico-chemical properties, rather than just the contaminants. Specifically, several researchers have reported that avoidance response can be affected by organic matter content (OM) (Amorim et al., 2008; Natal-Da-Luz et al., 2008), pH (Amorim et al., 2008; Chelinho et al., 2011; Ma and Bonten, 2011), electrical conductivity (EC) (Owojori and Reinecke, 2009) and texture (Amorim et al., 2008; Chelinho et al., 2011; Natal-Da-Luz et al., 2008). As a result, the effect of metals on the earthworm avoidance response of field-contaminated soils may be masked by the differences in the physico-chemical properties of the field-contaminated test soil and the artificial control substrate.

Our study concerns the impact of the artificial substrate recommended for use in avoidance tests (ISO 17512-1, 2008). We hypothesize that it is possible to suppress the effect of the physico-chemical properties of this test substrate on avoidance response by modifying the substrate. Any avoidance, subsequent to such an adjustment will reveal the effect of the metals on the earthworm avoidance response. Therefore, the objectives of the present study were to determine which physico-chemical properties drive earthworm avoidance, to modify the artificial substrate to remove the effect of these properties and therefore to detect the effect of metals on earthworm avoidance response in field-collected soils.

**2. Materials and methods**

**2.1. Soil collection**

In order to obtain a wide range of total metal concentrations and physico-chemical properties, 52 agricultural soils were collected based on prior knowledge of the spatial distribution of Cu in the Aconcagua River basin (Aguilar et al., 2011) and the Puchuncaví Valley (Gonzalez et al., 2015) (Supplementary Figure 1, Supplementary Table 1). These soils were exposed to historical contamination by mining and/or smelting activities, as described in detail in Aguilar et al. (2011) and González et al. (2015). For instance, the Chagres and Ventanas smelters have been in operation since 1927 and 1964, respectively, and have emitted significant loads of metals to the atmosphere, especially before 1991, after which time emissions mitigation measures were put in place at the smelters. Another example, is the destruction of a tailings dam during an earthquake in 1965, in the El Melón área, resulting in signfiicant metal additions to down stream soils. Thus, metals have been aged in the studied soils for several decades.

Soils were classified as uncontaminated or contaminated on the basis of background metal concentrations in central Chile. Soils were classified as contaminated if they contained either total Cu >155 mg kg-1 (Aguilar et al., 2011) or total As >19 mg kg-1 (Poblete et al., 2015). Samples were taken from a 2 m2 area to a depth of 20 cm, collecting approximately 40 kg of soil. The samples were dried in an oven at a temperature of 40 °C for 48 h, sieved though a 2 mm mesh and homogenized in a mixer with an inner plastic cover. The mixer was washed beforehand to avoid cross contamination. In order to verify the homogeneity of the soils, 2 subsamples were obtained and analyzed from each sample. Each subsample was dried in an oven at a temperature of 40 °C for 48 h prior to laboratory analyses.

**2.2. Physico-chemical characterization of the soils**

The physico-chemical characteristics of the soils were determined using standard methods. Soil texture was determined using the simplified hydrometer method (Sheldrick and Wang (1993). Water holding capacity (WHC) was determined by the saturation and gravity drainage method (ISO 11269-1, 1993). Organic carbon content was determined by wet combustion with Na-dichromate and sulfuric acid without heat application, and OM content was estimated using the conversion factor of 1.724 (Sadzawka et al., 2006). Soil pH and EC were measured in an aqueous suspension at a soil:water ratio of 1:5 (Sadzawka et al., 2006).

In order to determine total Cu, Cd, Pb, Zn and As, the samples were digested in boiling nitric acid followed by the addition of perchloric acid. Approximately 1 g of soil was finely ground in an agate mortar and weighed in an Erlenmeyer flask. Concentrated nitric acid (25 mL) was then added, and a Teflon stopper with a 30 cm long glass reflux tube was used to prevent the volatilization of As during the digestion process (adapted from Verlinden (1982)). The sample was digested at 60 °C overnight and then at 120 °C for 1 h. The stopper was removed, and the nitric acid was evaporated to obtain a volume of approximately 5 mL. The sample was cooled, and 5 mL of concentrated perchloric acid was added. Again, a Teflon stopper with a 30 cm long glass reflux tube was used, and the sample was digested at 220 °C for 30 min; the nitric acid was volatilized during this stage. The sample was then cooled and filtered into a 100 mL volumetric flask.

Total concentrations of Cu, Pb, Zn, and As were determined by atomic absorption spectroscopy. Quality was assured by similarly digesting in duplicate the following certified reference samples: PACS-2 obtained from the National Research Council Canada, and GRX-2 obtained from the United States Geological Survey. The obtained values were within 10% of the certified value. Spikes of Cu, Pb, Zn and As were performed on every 10th sample and recovery was 100% ± 7%.

**2.3. Avoidance test procedure**

The test was based on the standardized protocol of ISO 17512-1 (2008). Firstly, *Eisenia fetida* individuals were conditioned by approximately one month in an environmental chamber at 20 ± 2 °C with a constant light intensity of 400-800 lx and a photoperiod of 12 h/12 h day/night. The breeding media during this period was a mixture of one volume of horse manure and one volume of peat. For the test, 1 L black plastic pots were used (11 cm lower diameter x 12 cm in height x 15 cm upper diameter) along with adult earthworms with a visible clitellum and a wet weight in the range of 0.3-0.5 g. One day before beginning the bioassay, the field soil and the artificial substrate were moistened with deionized water to 60% of their WHC and structured by mechanical mixing of the moistened materials, after which they were left to rest for one day.

At the beginning of the test, the pots were divided into two equal sections by the introduction of a vertical separator. One half of the pot was filled with a field soil and the other half was filled with the artificial substrate. The pots were filled to a height of 5-6 cm (250 g of dry soil in each half). The separator was then removed and 10 earthworms were placed on the separating line of each test pot. Next, each pot was covered with a mesh cover and the pots returned to the environmental chamber for 48 hours. This procedure was performed with five replicates for each of the 52 field-collected soils. At the end of the test period, the field-collected soil and artificial substrate were separated again by inserting the separator. The number of earthworms in each section of the pots was counted. The earthworms that were divided by the insertion of the separator were counted as 0.5, irrespective of the length of the earthworm.

**2.4. Expression of the results**

Avoidance response was expressed as the percentage of live earthworms in each field-collected soil (Supplementary Table 2), as shown below:

$EFS =\left(\frac{N}{N\_{T}}\right)\*100$ (eq. 1)

where, EFS is the percentage of earthworms in the field-collected soil, N the number of earthworms in the field-collected soil, and NT the total number of earthworms in the pot. Based on ISO 17512-1 (2008), an EFS <20% means that the test soil is classified as a limited habitat. In ecotoxicological terms the concentration at which 80% of the earthworms avoid a soil is termed an EC80.

**2.5. Experiment 1: Bioassay ISO 11268-2 (ISOC)**

The avoidance test was performed using an artificial control substrate in accordance with ISO 11268-2 (2012). One half of the pot contained the tested field-collected soil and the other half contained the artificial control substrate, which was the same in all cases. The artificial control substrate comprised 10% peat, 20% kaolinite and 70% quartz sand (see Table 1 for the physico-chemical properties).

**2.6. Experiment 2: Modified bioassay**

The artificial control substrate was adjusted to match the OM content and the EC of the corresponding field-collected soil, since these properties were found to be confounding factors in Experiment 1.

**2.6.1. Bioassay ISOOM**

The artificial control substrate was amended with peat in order to match the OM content of the paired field-collected soil. The mass of peat required (PW, in grams) was determined using the following formula:

$P\_{W}=\frac{OM\_{P}xW\_{S}}{100x OM\_{S} x (1-C\_{W})}$ (eq. 2)

where OMP is the percentage of OM in the peat, according to a laboratory analysis (90% in the present study), WS the dry weight in grams of the artificial substrate required for each repetition (250 g in the present study), Cw the peat’s water content as a percentage (57% in the present study) and OMS the percentage of OM in the field-collected soil. The kaolinite and quartz sand contents used per repetition were adjusted with respect to the difference between WS and PW, maintaining the 20/70 ratio for kaolinite and quartz sand, respectively.

**2.6.2. Bioassay ISOOM-EC**

Prior to adjusting the EC, it was necessary to evaluate the effect of the adjustment of OM on the EC of the artificial substrate. Therefore, artificial substrates with different OM contents were analyzed for EC. The results indicate that OM does not affect EC (ANOVA, p >0.05), since EC remained constant at a value of 0.3±0.02 dS m-1 (n=3) in each treatment. As a result, the OM content was made equal to the level indicated in ISOOM.

In order to match the EC of the artificial substrate to that of the test soil, a solution of 10 g L-1 NaCl was used. The volume of solution (V, in mL) needed to be added to the artificial substrate was determined using the following formula:

$NaCl\_{S} =\frac{15EC\_{S}-5.4}{EC\_{M} } x WHC\_{A}xW\_{S}$ (eq. 3)

where ECS is the electrical conductivity of the test soil, ECM the EC of the solution (17.5 dS m-1), WHCA the percentage water retention capacity of the artificial substrate and WS the mass of artificial substrate in grams needed per repetition (250 g). Once the NaCl had been added to the artificial substrate, deionized water was added to reach a WHC of 60%.

The artificial substrate was dried in an oven at 40 °C for 24 hours. It was then remoistened with deionized water up to 60% of WHC and was stored for 4 days in an environmental chamber at a constant temperature (20 ± 2 °C). Analysis of EC was carried out at the end of the bioassay for the purpose of verification.

**2.6. Analysis of the results**

The association between the avoidance response and the physico-chemical characteristics of the field soils was determined in the three avoidance tests (ISOC, ISOOM, and ISOOM-EC). Simple and multiple regressions (Tables 2 and 3) were carried out between the percentage of earthworms in the field-collected soil and the following independent variables: total Cu concentration, pH, OM content, EC, and soil texture (expressed as the percentage of sand and clay). The normal distribution and homogeneity of the residues were verified (Kutner et al., 2004). Statistical analyses were carried out using Minitab 17. Effective concentrations (ECX) were determined using the Toxicity Relationship Analysis Program (TRAP) version 1.22 (US EPA, 2013).

**3. Results and discussion**

**3.1. Physico-chemical properties of the soils**

The physico-chemical characteristics of the soils are presented in Table 1 and Supplementary Table 3. In total 30 of the soils were classified as uncontaminated and 22 as contaminated (total Cu >155 mg kg-1 and/or total As >19 mg kg-1).

**3.2 Effect of** **physico-chemical properties on avoidance response in uncontaminated soils (ISOC)**

Results of all the avoidance tests are presented in Supplementary Table 2. Simple and multiple regression analyses with the uncontaminated soils (n=30) were carried out to assess the effect of the physico-chemical properties on avoidance response in the absence of elevated concentrations of metals. Table 2 shows the best regression models for the avoidance response versus the physico-chemical properties for each bioassay.

The best model obtained from ISOC explained 26% of the total variance, showing that the percentage of earthworms in the field-collected soil increases with an increase in OM content (Table 2). This result is in agreement with those of Chelinho et al. (2011) and Natal-Da-Luz et al. (2008), who found that avoidance response is related to OM. This behavior is because *Eisenia fetida* generally inhabits soils rich in OM, as this is its principal food source (Jänsch et al., 2005).

**3.3 Effect of adjusting the OM and EC of the artificial substrate on avoidance response in uncontaminated soils (ISOOM and ISOOM-EC)**

When the organic matter content of the artificial substrate was adjusted to match that of the compared uncontaminated soil (ISOOM), the avoidance response was dependent on EC and pH (Table 2). However, pH was not significant by itself (R2=0.03; p=0.37), possibly due to the small range of pH values of the studied soils. This is in contrast to the study by Chelinho et al. (2011), which found an effect of pH on avoidance behavior, possibly due to the wider range of pH values in that study (4.2-7.7). The ISOOM data were used to determine values for EC50 and EC80 which were 0.6 (0.5-0.7) and 1.1 (0.8-1.3) dS m-1, respectively (95% confidence intervals in brackets) (Fig. 1). These results differ slightly from those of Owojori and Reinecke (2009), who obtained an EC50 of 1.0 dS m-1 using natural soils enriched with NaCl.

No significant relationships were indicated by regression analysis for avoidance in the ISOOM-EC bioassay. This suggests that the avoidance test with *Eisenia fetida* is mainly sensitive to OM and EC, for this particular set of soils. These variables may be confounding factors when assessing field-collected metal-contaminated soils using avoidance tests. However, by matching the values of OM and EC in the field-collected soils and the artificial substrate, it is possible to suppress the effect of these physico-chemical properties on avoidance response.{Ginocchio, 2006 #1}

**3.4 Effect of total Cu concentration on avoidance response**

Simple and multiple regression analyses were carried out on all the collected soils (n=52) in order to assess the effect of the physico-chemical properties and the metal content on avoidance response. Simple regression suggests that total Cu explained a low percentage of the variance of the avoidance response in the ISOC bioassay (Table 3, Supplementary Figure 2). The best regression model suggests that in ISOC the avoidance response was dependent on OM, EC and total Cu.

In ISOOM, OM was not a significant driver of avoidance behaviour, while EC and total Cu remained in the model. Again, total Cu explained a low percentage of the variance of the avoidance response (Table 3, Supplementary Figure 2). Finally, in ISOOM-EC the avoidance response was dependent only on total Cu content (Table 3, Figure 2) and total Cu explained a greater percentage of the variance of the avoidance response than in ISOC or ISOOM. The effect of other metals was not significant. Therefore, by adjusting OM and EC in the artificial substrate, it was possible to suppress the effect of the physico-chemical properties on avoidance response.

**3.5 Total concentrations of Cu in soil that drive avoidance**

ECx values for total Cu that cause avoidance without other confounding variables were obtained from ISOOM-EC. An EC80 of 433 mg Cu kg-1 was obtained with a 95% confidence interval of 339-528 mg Cu kg-1 (Figure 2). Therefore, soils with a total Cu content above 339 mg Cu kg-1 may represent a limiting habitat for earthworms.

The values obtained are relatively similar to those of Holmstrup and Hornum (2012), in a study in the region of Hygum (Jutland, Denmark) where copper is found as the single contaminant in a gradient. These researchers reported that biomass and population density of earthworms decreased at total Cu concentrations above 300 mg Cu kg-1. It is important to state that in both the Danish study and the study reported here, the metals have been aged in the studied soils for several decades.

The results obtained in the present study are also relatively similar to those of Lukkari and Haimi (2005), who used avoidance tests with metal-spiked soils, finding significant avoidance at concentrations of 300 mg Cu kg-1for *Lumbricus rubellus*. Our own results are more environmentally relevant, since field-collected soils were used (Nahmani et al., 2007a; Nahmani et al., 2007b). In summary, the modifications to the artificial substrate in the avoidance tests allowed detection of toxicity thresholds in the field-collected soils.

**4. Conclusion**

The earthworm avoidance test is affected by the difference in OM and EC of field-collected soils and the artificial control substrate recommended for use in the standardized avoidance test protocols. Adjusting the physico-chemical properties of the artificial control substrate suppresses the effect of these factors on avoidance response. The improved method can be used to determine toxicity thresholds of total Cu in the soil.

**5. Research needs**

Given the narrow range of pH values in the present study, future research is needed into the benefit of adjusting the pH of the artificial substrate. Additionally, performing the procedure described in this study with endogeic and anecic earthworms would produce results with higher ecological relevance.

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