

ASSESSMENTS OF THE IMPACT OF METALS ON JUVENILE EARTHWORMS (*Eisenia fetida*) IN LABORATORY CONDITIONS

Jovana M. Sekulić ^{1*}, Emina M. Mrkalić¹, Mirjana M. Stojanović-Petrović ²,
Filip J. Popović², Tanja B. Trakić²

¹University of Kragujevac, Institute for Information Technologies Kragujevac,
Department of Science, Jovana Cvijića bb, 34000 Kragujevac, Serbia

²University of Kragujevac, Faculty of Science, Institute of Biology and Ecology,
Radoja Domanovića 12, 34000 Kragujevac, Serbia

*Corresponding author; E-mail: jovanas034@gmail.com

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ABSTRACT. The aim of this study was to evaluate the impact of different concentrations of copper and zinc on the mortality and bodyweight of juvenile earthworms in artificial soil. Copper was more toxic with complete mortality at the dose rate of 1000 mg/kg. Bodyweight loss of earthworms was observed immediately, after the first week, except at the lowest concentration. In comparison to control, there were significant differences in the bodyweight loss every week in the concentrations of 333 and 666 mg/kg, and also 167 mg/kg in the fourth, sixth, and eighth week. Zinc has not shown a significant effect on mortality, except in the highest concentration, where survival was less than 25%. Statistically significant effect ($p < 0.05$) on bodyweight was registered only at the highest concentration, every week. The impact of metals on earthworm populations should be more accurately assessed, and data for juvenile earthworms should be considered when proposing a safe concentration of pollutants in the environment, because of the great importance of these animals' presence in the soil.

Keywords: juvenile earthworms, Lumbricidae, metal toxicity, laboratory test, OECD guidelines.

INTRODUCTION

Metals reaching soil may affect their biodiversity and the structural diversity of invertebrate communities in different ways (KOZLOV and ZVEREVA, 2011), thus having significant, and often harmful effects on the ecosystem functioning (HOOPER *et al.*, 2012). Metals accumulate in the litter layer of soil and gradually eliminate, so quite a number of soil organisms are constantly exposed to the toxicity of heavy metals (MUSTONEN *et al.*, 2014). Earthworms have a thin cuticle and can be exposed to heavy metals by direct dermal contact in the soil, or ingesting soil (SCHNUG *et al.*, 2015). Understanding the effects of metal exposure on earthworms is particularly important. First, these animals play an important role in the metabolism, fertility and structure of soil. Earthworms are less capable to achieve their

essential functions in soil after exposure to injurious concentrations of heavy metals (EDWARDS and BOHLEN, 1996). Second, serve as a food source for many animals (SMITH *et al.*, 2010). Their protection may provide a margin of safety for other members of the soil fauna due to the higher sensitivity to the contaminants (VAN GESTEL *et al.*, 2011) and prevent an increase in the metal concentration through the food chains as well (SCHNUG *et al.*, 2015).

Zinc (Zn) and copper (Cu) sources that are becoming increasingly important are municipal refuse, sewage sludge, animal waste, fertilizers, and pesticides (LUKKARI *et al.*, 2005; MOUSAVI KOUHI *et al.*, 2016). These metals are released into the environment through various activities, being among the most prevalent metals in contaminated soils (KIZILKAYA, 2005).

In the last few decades, several studies have been performed on the response of adult earthworms to Zn and Cu. It is confirmed that these metals cause mortality (NEUHAUSER *et al.*, 1985; SPURGEON *et al.*, 1994; SPURGEON and HOPKIN, 1995, 1996a; SPURGEON *et al.*, 2000), induce cocoon production (MA, 1988; SPURGEON and HOPKIN, 1996a; SPURGEON *et al.*, 2000), have a negative impact the sustainability of the cocoon (SPURGEON and HOPKIN, 1996b; REINECKE *et al.*, 2001; SPURGEON *et al.*, 2004a) and earthworm progression (VAN GESTEL *et al.*, 1991; SPURGEON *et al.*, 1994; KHALIL *et al.*, 1996). According to SPURGEON and HOPKIN (1999), the biomass, abundance and species diversity of earthworm communities are decreased by soil contamination with Cu. Furthermore, these metals also lead to lysosomal membrane instability (HØNSI *et al.*, 2003), changes in physiological and genetic levels (SPURGEON *et al.*, 2006), as well as trigger for oxidative stress (SPURGEON *et al.*, 2004a; BERTHELOT *et al.*, 2008).

Various guidelines for testing xenobiotics on earthworms provide their impact assessment on the survival and reproduction of adult organisms, but juvenile stages are not included. Since the sensitivity of juvenile and adult organisms may be different, using data only for adults can underestimate the impact of chemicals on these important soil organisms (SPURGEON *et al.*, 2004a). Even though exposure of juvenile earthworms to different chemicals can affect the population's demography and dynamics, little attention is still paid to these studies (ŽALTAUSKAITĖ and SODIENĖ, 2014). In this context, we carried out investigations on the toxic effects of Cu and Zn on the juvenile stage of *Eisenia fetida* selecting mortality, body weight, and growth inhibition as endpoints. Earthworm survival results were analyzed using the sublethal sensitivity index (SSI) (CROMMENTUIJN *et al.*, 1995). Therefore, our goal was to get a more comprehensive understanding of the effects of these metals on juvenile earthworm *E. fetida* and to provide more data about the potential ecotoxicological risk of metals on the soil.

MATERIALS AND METHODS

Earthworms

Eisenia fetida juvenile earthworms were taken from laboratory cultures of the agricultural holding (Gornji Milanovac, Serbia). Three- or four-weeks old juveniles of approximately equal body weight and size were used for all tests, cultured in the laboratory in a medium according to the OECD standards (OECD, 1984).

Artificial test soil and chemicals

The test soil was OECD artificial soil (OECD, 1984, 2016). The soil consisted of 70% quartz sand, 20% kaolin clay, 10% sphagnum peat and calcium carbonate to adjust the pH to 6.0 ± 0.5 . The dry components of the artificial soil had been mixed. Solutions of copper(II) nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$) and zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) were mixed with the dry constituents to give the required percentage of water content (40-60%) and metal concentrations in the soil. The concentrations of these metals in the soils (in mg / kg dry weight of soil) were as follows: copper – 83, 167, 333, 666 and 1000; zinc – 260, 347, 432, 867 and 1300. For the control treatment, only distilled water was used.

Experimental design

The earthworms were washed, dried on filter paper, weighed, and thereafter ten earthworms per plastic container ($10.5 \times 9.6 \times 7$ cm) were placed on the soil surface. There were four replicates per concentration, plus the control. Test containers were covered with perforated plastic lids and kept at a temperature of 20 ± 2 °C. The test was carried out under light-dark cycles (16:8). During the eight weeks of the experiment, the earthworms were fed with 5 g of ground cattle dung once a week. Also, the soil moisture was recorded once a week. The mortality and bodyweight of earthworms were monitored weekly. The earthworms were considered dead when they did not respond to a gentle mechanical prodding at the anterior of the body. Before weighing, all the earthworms had been sorted, washed with tap water, and blotted with filter paper. The earthworms were then weighed using an electronic balance and returned to the soil.

The bodyweight of the earthworms exposed to each concentration was reported from the various exposure periods and used to calculate the growth inhibition as follows (SHI *et al.*, 2007):

$$GI_n = \frac{W_0 - W_t}{W_0} \times 100\% \quad (1)$$

where GI_n is the growth inhibition for concentration n , W_0 is the bodyweight on day 0 and W_t is the bodyweight after t days of exposure.

Statistical analysis

LC_{50} (the concentration that is lethal to 50% of individuals), following a 95% confidence interval, was calculated using the CalcuSyn program ver. 2.0 (Biosoft). EC_{10} (the concentration that influences body weight in 10% of test organisms in a period of exposure) was calculated using Microsoft Excel software.

Sublethal sensitivity indices (SSI) were calculated according to CROMMENTUIJN *et al.* (1995) for each metal as follows:

$$SSI = \frac{LC_{50}}{EC_{10}} \quad (2)$$

The Shapiro-Wilk test was used to ensure the normality assumption. Based on that, a one-way ANOVA ($P < 0.05$) or Kruskal-Wallis H test was used for assessing the effects of contaminants on growth. With post hoc, in comparison of means (growth), a Dunnett's test was applied. The data for growth inhibition were subjected to ANOVA using the Student-Newman-Keuls test (S-N-K). Data are presented as mean \pm standard deviation (SD). All

statistical procedures were performed using the SPSS software (BM SPSS Statistics, Version 20, Inc. 1989–2011, USA).

RESULTS

Both tested metals (Cu and Zn) evaluated with the artificial soil test evinced dissimilar levels of toxicity to *E. fetida* juveniles. Only in the control treatment did all the earthworms survived up till the end of the experiment.

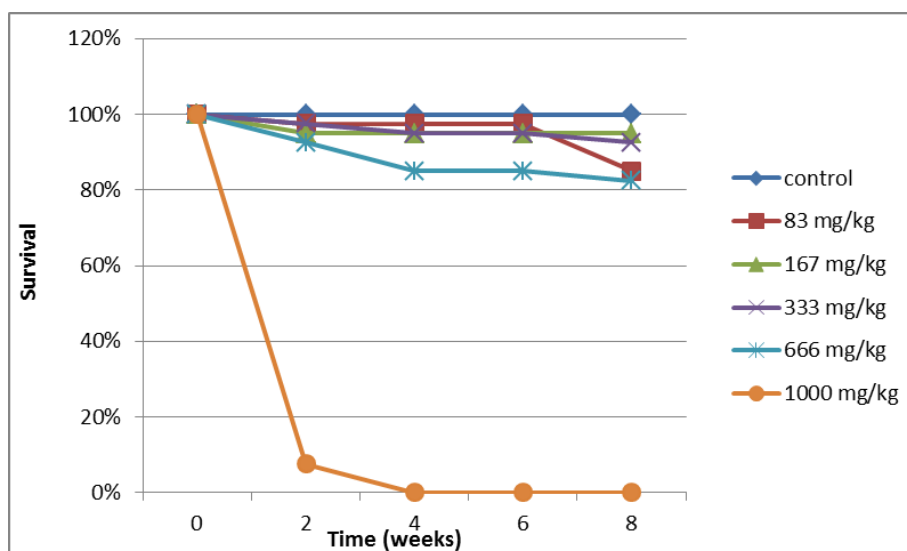


Figure 1. Survival of juvenile earthworms *Eisenia fetida* in OECD artificial soil exposed to copper.

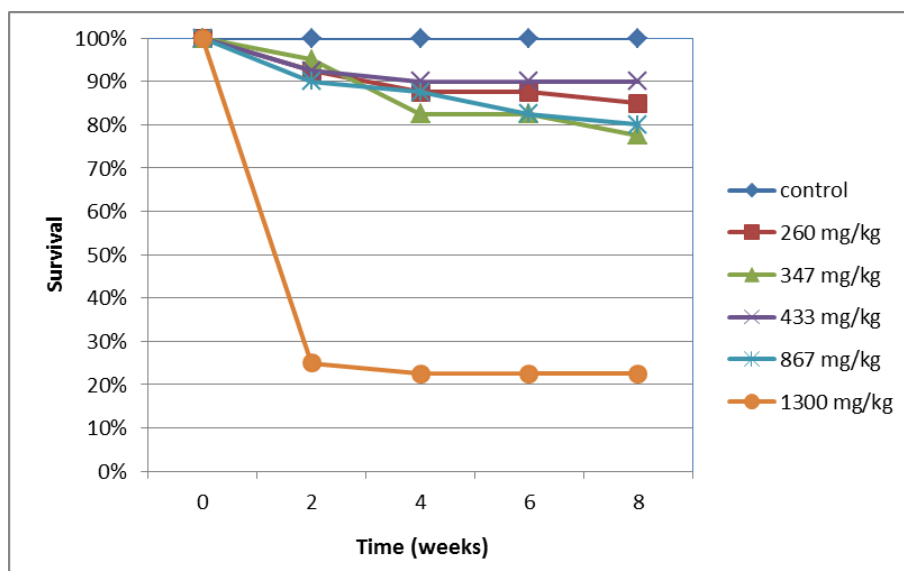


Figure 2. Survival of juvenile earthworms *Eisenia fetida* in OECD artificial soil exposed to zinc.

The first week of the experiment was fatal to most of the earthworms in the test with the metals. Thus, in the second week at the highest Cu-concentration of 1000 mg/kg, only one worm remained alive in each box, while they all died the following week (Fig. 1). After the 7-day exposure, no mortality was recorded at any of the concentrations, except at the highest concentrations of Cu and Zn. Copper was the most toxic with complete mortality at 1000

mg/kg (Fig. 1), and the 28-day LC₅₀ value was 349.74 mg/kg. Zinc has not shown a great reduction in the number of earthworms, except in the highest concentration, where survival is less than 25% (Fig. 2). When there was a high, but not complete mortality (for example, at 1300 mg/kg of Zn), the other earthworms survived until the end of the experiment (Fig. 2). The LC₅₀ value for Zn after 28 days is 1240 mg/kg, which approximates the highest concentration. During the period between the fourth and sixth week for both metals and in all concentrations, except at the concentration of 867 mg/kg for Zn, there are no deaths of earthworms and, after that, there comes a decrease in the number of individuals (Figs. 1 and 2).

The results of the growth of the juvenile earthworm exposed to Cu and Zn for eight weeks are given in Tab. 1. It was noticed that the earthworms exposed to Zn had slightly gained in bodyweight per treatment during the first week, as compared to their initial bodyweight in the same treatment, except at the highest concentration. In the case of Cu, a decrease in body weight was observed after the first week, except at the lowest concentration.

Table 1. Growth of the juvenile earthworm *Eisenia fetida* exposed to copper and zinc during eight weeks.

Treatment	Mean bodyweight per earthworm (mg)				
	0 week	2 weeks	4 weeks	6 weeks	8 weeks
Zinc (mg/kg)					
Control	335±24	335±43	350±24	343±24	365±13
260	308±10	303±38	283±70	285±44	275±41
347	323±15	318±53	310±83	313±62	313±85
433	308±13	300±32	303±33	308±38	303±61
867	333±15	305±59	300±47	268±49	278±37
1300	280±60	98±36*	83±36*	80±23*	98±25*
Copper (mg/kg)					
Control	273±10	285±37	290±26	273±22	273±22
83	268±48	268±28	227±51	223±13	230±28
167	303±30	258±17	253±5*	240±28*	225±13*
333	293±25	245±24*	243±13*	233±17*	250±54*
666	235±62	223±41*	225±13*	218±22*	193±43*
1000	278±72	20±18	/	/	/

*Significant differences ($p < 0.05$) between treatment and control were shown during the experiment.

For Zn, the individual bodyweights ranged from 365 mg to 80 mg. The only significance ($p < 0.05$) was found at the highest concentration for every week.

The juvenile earthworms treated with Cu had body weighted from 285 mg to 20 mg for the second-week of assessment (Tab. 1). The growth data for 1000 mg/kg Cu for this week are not shown because only a few worms survived (7.5%). Consequently, such data could not permit reliable statistical analysis. In comparison to control statistical analyses displayed a significant difference ($p < 0.05$) in the bodyweight loss in the second week in the concentrations of 333 and 666 mg/kg. The earthworms treated with Cu had a mean bodyweight between 290 and 225 mg after the four-week assessment and a mean bodyweight between 273 and 218 mg following the six-week assessment (Tab. 1). The effects were significant ($p < 0.05$) for the concentrations of 167, 333, and 666 mg/kg. At the end of the experiment, the mean bodyweight was between 273 and 193 mg, displaying also a statistically significant difference ($p < 0.05$) between these doses and the control (Tab. 1).

The growth inhibition of earthworms after exposure to studied metals is presented in Figs. 3 and 4. After treatment with Zn, the growth inhibition was negative in the control, which means that the earthworms gained bodyweight, as opposite to all used concentrations and weeks, in which case the growth inhibition was positive, meaning that the earthworms lost their bodyweight (Fig. 3). Regardless of these facts, statistically significant differences ($p < 0.05$) were found only at the highest concentration.

During the eight-week experiment, the growth inhibition for all earthworms cultured in the Cu-treated soil (Fig. 4) was positive, except in the control treatment. Statistical analysis showed significance ($p < 0.05$) in the concentrations of 333 and 666 mg/kg every week, and in the case of 167 mg/kg in the fourth, sixth, and eighth weeks (Fig. 4).

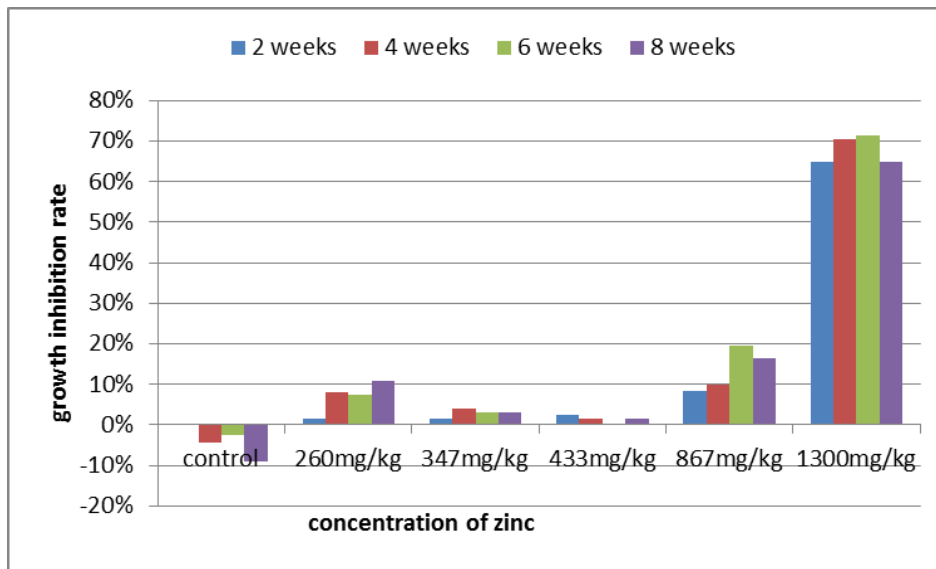


Figure 3. Growth inhibition rates of the juvenile earthworm *Eisenia fetida* after exposure to zinc under testing conditions.

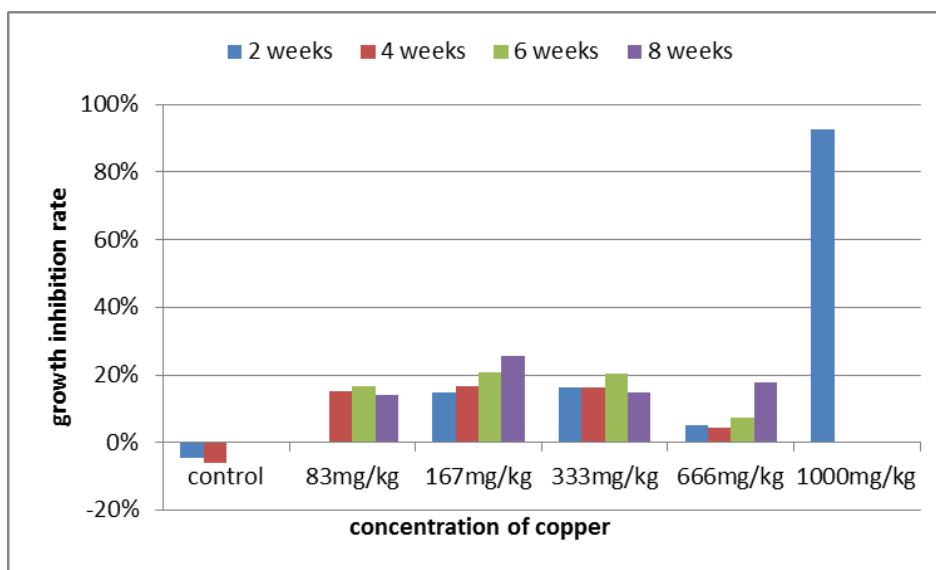


Figure 4. Growth inhibition rates of the juvenile earthworm *Eisenia fetida* after exposure to copper under testing conditions.

To calculate the SSI index, the EC_{10} values were first calculated. The threshold concentrations for sublethal effects for bodyweight fell below the median lethal concentrations

(LC₅₀) for both metals. SSIs calculated for the effects of Zn and Cu on bodyweight change were 34 and 152, respectively.

DISCUSSION

The toxicity of Cu and Zn on juvenile earthworm survival has been investigated in a few studies. SPURGEON and HOPKIN (1996a) reported that juvenile *E. fetida* was more susceptible to metal-contaminated soils than adult earthworms. They also explain that mortality occurs in the first weeks of the treatment, as was the case also in our experiment, and that it is a consequence of the uptake of metals across the body wall, rather than through feeding.

LUKKARI *et al.* (2005) concluded, based on LC₅₀ values which were significantly higher for *E. fetida* than for *Aporrectodea tuberculata*, that *E. fetida* is more tolerant to heavy metals than *A. tuberculata*.

Exposure to copper

The results of our investigation, presented in Table 1 as well as in Figs. 1 and 4, indicate that juveniles were sensitive to Cu. Values of LC₅₀ obtained from our experiments are lower than the values found for adults (ARNOLD *et al.*, 2003; FRAMPTON *et al.*, 2006), thus confirming once more the fact that juvenile worms are more sensitive than adults. Furthermore, ŽALTAUSKAITĖ and SODIENĖ (2014) have found that the mortality rates during exposure to the metals depend on the age of the earthworms. Juvenile earthworms are more susceptible to Cd and Pb than adults, as also reported by VAN GESTEL *et al.* (1991) for *Eisenia andrei* and by SPURGEON *et al.* (2004b) for *Lumbricus rubellus*.

According to our results, decrease in weight was noticed immediately, after the first week. HAQUE and EBING (1983) considered earthworm body weight as an important criterium for determination of sublethal effects. MOSLEH *et al.* (2003) assumed that weight loss may indicate reduced food consumption, by which earthworms regulate taking of heavy metals and thus lead to growth inhibition. This strategy is usually used to prevent the poisoning of the organism with heavy metals and pesticides (RIBEIRO *et al.*, 2001).

One of the possible explanations is that pollutants may modify the growth of earthworms either directly, by influencing physiology, or indirectly by driving up to changes in the energy budget as individual attempts to prevent metal accumulation in sensitive tissues (SPURGEON and HOPKIN, 1996a). The mechanisms of metal elimination have metabolic costs which are peculiarly meaningful for juvenile earthworms, due to the reduced amount of energy available for growth and development (BOOTH and O'HALLORAN, 2001). Additionally, this sensitivity is not only found in heavy metals, but for pesticides also. ZHOU *et al.* (2008) examined the pesticide cypermethrin and found lower values of LC₅₀ for juveniles than for adults of *E. fetida*. They also noticed that the juvenile worms were more susceptible to sublethal effects in contrast to adult worms. JORDAAN *et al.* (2012) investigated the effects of the pesticide azinphos-methyl on juvenile earthworm *E. andrei*. The value of LC₅₀ of this pesticide was much lower than the value for adult animals previously reported. MATSUMURA (1975) even opined that the mechanisms of detoxification in juvenile animals might not be developed as in adults.

Exposure to zinc

According to obtained results, the LC₅₀ values for Zn are close to the highest concentration used in the experiment. SPURGEON *et al.* (1994) found LC₅₀ values of 1010 µg/g for two weeks and 745 µg/g for eight weeks days for adult *E. fetida*, which is slightly

less than we obtained. In the study by NEUHAUSER *et al.* (1985), a 14-day LC₅₀ value of 662 µg/g in artificial soil was designated. Available data do not indicate an increased sensitivity in juvenile in relation to adult earthworms. However, previous studies have paid little attention to the sublethal effects of Zn such as bodyweight. When we compare our results regarding body weight with the data obtained by SPURGEON and HOPKIN (1996b), juvenile earthworms are prone to lose more weight than adults.

The SSI was created as a parameter that reflects the extent to which lethal concentrations are affected by some endpoints (CROMMENTUIJN *et al.*, 1995). The differences in the SSI in our results demonstrate variations in earthworm responses to pollutant stress. In contrary to Zn, the sublethal effect for Cu occurs at a concentration that is far below the lethal one. For example, comparing reproductive SSIs for earthworms and other soil invertebrates the values for earthworms are relatively high. A high SSI can be explained by the fact that the already at low concentrations earthworms start to save energy for the benefit of survival (SPURGEON *et al.*, 2000, CROMMENTUIJN *et al.*, 1995). It means that reproduction is affected at pollutant concentrations far below those affecting survival (SPURGEON *et al.*, 2000).

CONCLUSION

It can be concluded that juvenile earthworms have different sensitivity to Zn and Cu. When compared LC₅₀ and SSI values between Cu and Zn, Cu was more toxic than Zn. Also, growth inhibition appeared to be more severely affected by Cu than by Zn. In case of Cu, a decrease in bodyweight was observed immediately, except in the lowest concentration, while the effect of Zn was registered only at the highest concentration every week. Our results are in agreement with previous findings (REINECKE *et al.*, 1997). If we compare the juvenile *E. fetida* sensitivity found in the present study with the results of previous (SPURGEON *et al.*, 2004a; ŽALTAUSKAITĖ and SODIENĖ, 2014) works, there can be noticed a greater sensitivity to the metals for a juvenile than adult earthworms.

Our endpoints bring us one step closer to understanding the possible negative effects on the population and community levels in the field. On the other hand, the impact of heavy metals on earthworm populations should be more accurately assessed, and data for juvenile earthworms should be considered when proposing a safe concentration of pollutants in the environment.

Acknowledgments

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