

## Effects of Nickel, Zinc, and Lead-Contaminated Soil on Burrowing Rate and Coelomocytes of the Earthworm, *Allolobophora chlorotica*\*

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We have shown previously that stubby worms *Allolobophora chlorotica* are sensitive to environmental stress, including metal-polluted soil. In order to discern the mechanisms of this sensitivity, adult (clitellate) *Al. chlorotica* were exposed in the laboratory to soil samples soaked with water (control) or Ni (1 and 2 mg/kg), Zn (1.25 and 2.5 g/kg) or Pb (5 and 10 g/kg) chlorides. Worms avoided contact with metal contaminants by prolonging burrowing time in metal-soaked samples, especially in the case of lead. Higher concentrations of the investigated metals were lethal for worms. During a 3 week exposure to lower metal concentrations, nickel and lead readily accumulated in the bodies of worms while zinc was efficiently regulated. However, body weights and numbers of non-invasively retrieved free coelomocytes (consisting of amoebocytes and riboflavin-loaded eleocytes) were significantly lower only in zinc-exposed worms. We assume that zinc regulation in worm bodies is more energy-demanding than nickel or lead bioaccumulation, thus this might be responsible for inhibition of the body gain and diminution of immunocompetent cells in zinc-exposed earthworms. Alternatively, missing free coelomocytes may actually be involved in Zn trafficking and removal through nephridia and/or in the formation of multicellular brown bodies, since metal can unbalance host/bacteria relationships.

Key words: Earthworm burrowing, coelomocytes, eleocytes, autofluorescence, riboflavin.

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Several experimental approaches have been developed on the use of earthworms as bioindicators of environmental quality (e.g. SPURGEON *et al.* 2003; STÜRZENBAUM *et al.* 2009). These include observations of earthworm behaviour (e.g. YEARDLEY *et al.* 1996; STEPHENSON *et al.* 1998; CAPOWIEZ *et al.* 2003; LANGDON *et al.* 2001, 2005; LOUREIRO *et al.* 2005; LUKKARI *et al.* 2005; OWOJORI & REINECKE 2009) and tests of their immunocompetent cells, the coelomocytes, non-invasively retrieved from the coelomic cavity (e.g. PLYTYCZ *et al.* 2007). For the latter type of experiments earthworms can be collected in the field from unpolluted or metalliferous sites (PLYTYCZ *et al.* 2009, 2010a, 2010b), or exposed in the laboratory

to field-collected natural soil samples (HOMA *et al.* 2003; WIECZOREK-OLCHAWA *et al.* 2003; PLYTYCZ *et al.* 2009, 2010a, 2010b; PIOTROWSKA *et al.* 2010). To avoid antagonistic, additive, or synergistic effects of uncontrolled mixture of metals in natural soil, earthworms should be maintained in artificial or natural soil samples experimentally spiked with known concentrations of heavy metals (e.g. KWADRANS *et al.* 2008; DUTKIEWICZ *et al.* 2009), however, metal bioavailability may still be affected by soil properties. Thus, for comparative studies of metal toxicity, exposure to filter papers soaked with metal ions (HOMA *et al.* 2005, 2007, 2010; OLCHAWA *et al.* 2006; PLYTYCZ *et al.* 2011a) has been adapted from OECD (1984).

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The effects of metals on earthworm coelomocytes are known from studies performed on coelomocytes of *Eisenia fetida* (HOMA *et al.* 2005, 2007) and *Dendrobaena veneta* (OLCHAWA *et al.* 2006) as these species are easy to maintain in laboratory conditions. Nevertheless, it is crucial to study also more ecologically relevant species such as *Lumbricus rubellus* (PLYTYCZ *et al.* 2010b), *Aporrectodea caliginosa* (DUTKIEWICZ *et al.* 2009), *Dendrodrilus rubidus* (PLYTYCZ *et al.* 2009, 2010a) and *Allolobophora chlorotica* (HOMA *et al.* 2003, 2007, 2010; KUREK *et al.* 2007; PIOTROWSKA *et al.* 2010). Coelomocytes can be quantitatively retrieved from the coelomic cavity, counted, and analysed by flow cytometry in respect to the percentage of autofluorescent chloragocyte-derived eleocytes (e.g. CHOLEWA *et al.* 2006), accumulating riboflavin in their granular chloragosomes (KOZIOL *et al.* 2006; PLYTYCZ *et al.* 2007). The amount of riboflavin in coelomocyte lysates may be quantified by spectrofluorometry (PLYTYCZ *et al.* 2006). Species with a high content of riboflavin-loaded eleocytes, such as *Al. chlorotica*, *E. andrei*, *D. veneta*, are especially suitable for the detection of toxic factors (PLYTYCZ *et al.* 2011b).

Coelomocytes of the stubby worm, *Al. chlorotica*, are well-characterised (KUREK & PLYTYCZ 2003; KUREK *et al.* 2007) and sensitive to metals both in animals exposed to filter papers soaked with metal chlorides (HOMA *et al.* 2005, 2007, 2010) and to natural soil samples from the metalliferous areas of Wales polluted mainly with zinc, lead and nickel, at mean concentrations equal to 15.4, 9.1, and 3.6 g/kg, respectively (PIOTROWSKA *et al.* 2010). Therefore the aim of the present work was to compare the effects of 3-week exposure of *Al. chlorotica* to soil samples soaked with high concentrations of zinc, lead, and nickel chlorides on coelomocytes retrieved at the end of the experimental period, with special emphasis on cell counts and riboflavin content. Additionally, earthworm behaviour in contact with contaminated soil was monitored and burrowing rate was recorded. Metal accumulation in worm bodies over a 3-week exposure period was also established.

## Material and Methods

### Earthworms

Adult (sexually mature with a well-developed clitellum) earthworms *Allolobophora chlorotica* were collected by manual digging and hand sorting of soil from the experimental garden of the Institute of Zoology of the Jagiellonian University. All earthworms were maintained in the laboratory at 16–17°C and 12:12 light/darkness regime, and fed

on mixed food (flour, boiled tealeaves, mouse feed and powdered milk) twice per week. Experiments were performed during the summer. Groups of animals (5 individuals per 0.2 kg soil samples per each group) were maintained in plastic boxes covered with perforated lids.

### Soil samples

Air-dried metal-free soil purchased from a commercial supplier (PPUH BIOVITA, Tenczynek) was spiked either with distilled water (control) or with one of the heavy metal chlorides (Sigma) at the nominal final concentrations: Ni (1 and 2 g/kg), Zn (1.25 and 2.5 g/kg), Pb (5 and 10 g/kg). Soils samples were allowed to equilibrate for 24 hours before being used in the experiments.

### Soil burrowing rate

At the start of experiments, earthworms of similar body weights were placed on the top of the control or metal-soaked soil surface and the time necessary for their complete disappearance from the upper surface of the soil (time of burrowing) was measured and expressed in minutes.

### Coelomocyte retrieval

At the end of the experiments, the surviving earthworms were stimulated for 1 minute with a 4.5V electrical current to expel coelomic fluid with coelomocytes through the dorsal pores, according to a procedure described previously (PLYTYCZ *et al.* 2006). Briefly, after weighing, washing and dry-blotting, the earthworms were placed individually in Petri dishes containing 3 ml of extrusion fluid (0.9% saline supplemented with 2 g/l EDTA (Sigma) to prevent cell aggregation). Extruded coelomocytes were counted in a haemocytometer. Cell suspension (2 ml) was lysed in 2% Triton (Sigma) and used for spectrofluorometric detection/measurement of riboflavin content while the remaining parts of samples were fixed in 2% formalin and used for flow cytometric detection of autofluorescent eleocytes.

### Flow cytometric measurements and analysis

Samples of formalin-fixed coelomocytes were analysed with a FACSCalibur flow cytometer (BD Biosciences). 10000 thresholded events per worm sample were collected and analysed on the basis of their forward scatter (FS) (for cell size) and side-ward scatter (SS) (cell complexity) properties. Fluorescence FL1 (for autofluorescence) (emission 530 nm; excitation 488 nm) was recorded. The resulting files were analysed using WinMDI 2.8 software (<http://facs.scripps.edu>) by producing density plots of FL1 autofluorescence.

## Spectrofluorometric measurements and analysis

Measurements were done on a Perkin-Elmer spectrofluorometer LS50B (Beaconsfield, Buckinghamshire, United Kingdom) in 2-ml of 2% Triton coelomocyte lysates in cuvettes with an excitation slit of 5 nm and an emission slit of 5 or 10 nm. Excitation spectra were recorded between 300-510 nm ( $\lambda = 525$  nm), while emission spectra were recorded between 390-700 nm ( $\lambda = 370$  nm). Excitation of a sample at 370 nm resulted in an emission spectrum with a maximum at 525 nm, while monitoring of fluorescence at 525 nm provided the excitation spectra with maxima at 370 nm and 450 nm. Spectrofluorometric analysis of earthworm coelomocyte samples was performed as previously described (CYGAL *et al.* 2007).

## Metal content in whole earthworm bodies

Earthworms were left on wet filter papers in Petri dishes for 48 h to depurate. Metal accumulation was measured in whole worm bodies using an atomic absorption spectrophotometer (Aanalyst 800, Perkin-Elmer) as described in detail in previous papers for Cd, Cu, and Pb exposure (HOMA *et al.* 2003) or Ni-exposure (BEDNARSKA & LASKOWSKI 2008).

## Statistical analysis

Results were expressed as means  $\pm$  standard errors. Differences between means were determined by a Mann-Whitney test with significance at  $P < 0.05$ , using Microsoft Excel v. 97.

## Results

## Worm burrowing rate and survival (Fig. 1)

The behaviour of *Al. chlorotica* differed depending on whether worms were put on the surface of water-soaked uncontaminated soil or on metal chloride-soaked soil. In containers with the control soil, earthworms started burrowing directly after introduction, whereas burrowing was delayed in containers with metal-soaked soils. In higher metal concentrations worms exhibited side-track "searching" behavior. This was especially drastic in the case of higher metal concentrations when worms tried to avoid contact with soil and tried to escape from containers (Fig. 1a), especially from those containing soil soaked with lead at 10 g/kg, when time of burrowing was longer than 30 minutes.

Figure 1b shows the mean times of burrowing in the control and metal-polluted soil samples. Burrowing in polluted soil was always prolonged in comparison with the control group, especially in contact with higher metal chloride concentration. In the case of lead the differences versus control were statistically significant.

All worms in containers with higher metal concentrations (2, 2.5, and 10 g/kg of Ni, Zn, and Pb, respectively) died during the first or second week of exposure. Due to the high mortality after exposure to high metal concentrations in soil, data on metal accumulation, body weights and coelomocyte-connected parameters after 3-week exposure concern only *Al. chlorotica* maintained in soil with

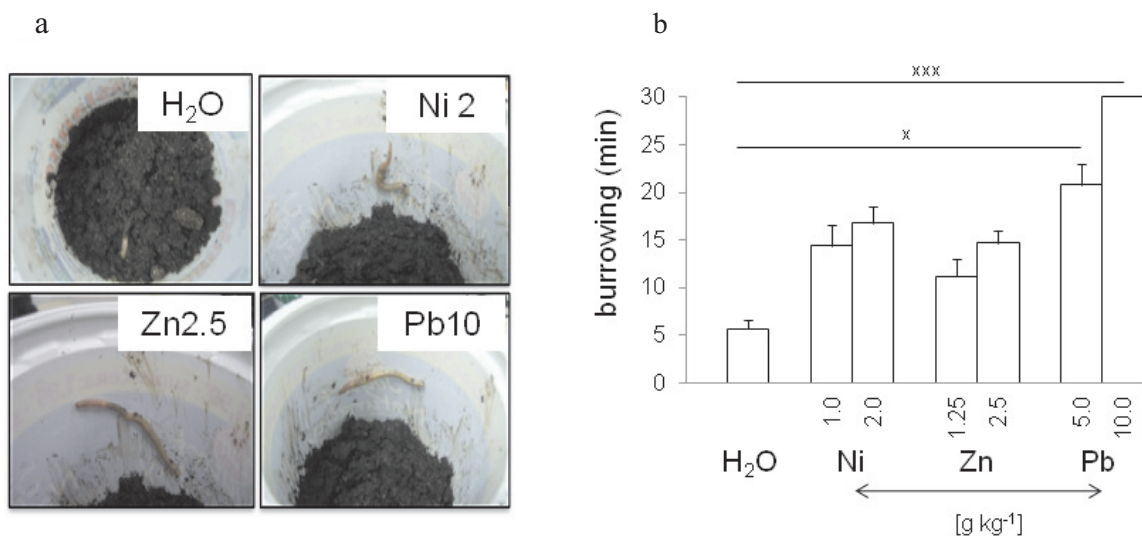


Fig. 1. Avoidance behaviour and burrowing rate of *Allolobophora chlorotica* in contact with soil samples soaked with water (H<sub>2</sub>O – controls) or metal chlorides: Ni (1 and 2 g kg<sup>-1</sup>), Zn (1.25 and 2.50 g kg<sup>-1</sup>) and Pb (5 and 10 g kg<sup>-1</sup>); a) typical examples of burrowing in the control soil and avoidance behaviour in soil samples soaked with Ni, Zn, Pb at 2.0, 2.5, and 10.0 g kg<sup>-1</sup>, respectively; b) time of burrowing [minutes] measured from the contact with particular soil sample until complete worm disappearance from the soil surface. Means  $\pm$  SE, 5 worms per group. Asterisks at means statistically significantly different from those of the water-soaked group ( $P < 0.05$  according to Mann-Whitney's test).

lower metal content, i.e. 1, 1.25, and 5 g/kg of Ni, Zn, and Pb, respectively (Figs 2 & 3).

#### Metal accumulation in whole earthworm bodies (Fig. 2)

At the end of exposure to metal-soaked or control soil samples, metal concentrations in *Al. chlorotica* bodies were significantly higher in worms kept in Ni and Pb-soaked soils than in the control group. In contrast, Zn concentrations were similar in the whole bodies of the control worms and those exposed to Zn-soaked soil (Fig. 2).

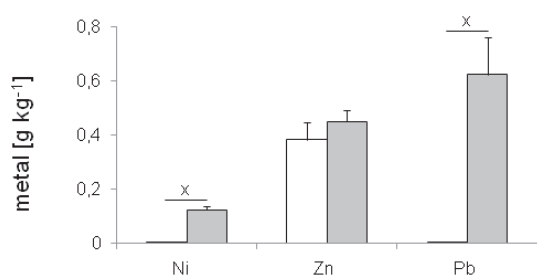


Fig. 2. Metal concentrations ( $\text{g kg}^{-1}$  dry weight) in whole bodies of *Allolobophora chlorotica* maintained for 3 weeks in soil samples soaked with water only (empty bars) or metal chlorides at 1.0, 1.25, and 5  $\text{g kg}^{-1}$  dry soil weight for Ni, Zn, and Pb, respectively (solid bars). Means + SE, 5 worms per group. Asterisks at means statistically significantly different from those of the water-soaked group ( $P < 0.05$  according to Mann-Whitney's test).

Earthworm body weights and coelomocytes (Fig. 3)

At the end of the experiments, body weights of *Al. chlorotica* kept in the Ni- and Pb-chloride soaked soils were similar to their control counterparts. In contrast, body weights of worms exposed to Zn-chloride were significantly lower (Fig. 3a). The number of coelomocytes extruded by electric shock was significantly reduced in Zn-exposed animals but was only slightly decreased in Ni- or Pb-exposed worms (Fig. 3b). However, when coelomocyte numbers were adjusted per fresh body weight (i.e. expressed as CN/BW) the mean values did not differ significantly between the four treatment groups (Fig. 3c).

Flow cytometric analysis of coelomocyte samples confirmed the presence of both agranular amoebocytes and granular autofluorescent eleocytes. The latter exhibited strong FL-1 autofluorescence, similar to that described previously (HOMA *et al.* 2010). The percentages of autofluorescent eleocytes (E %) were similar in the four treatment groups (Fig. 3d). The total number of eleocytes (EN) was lowest in Zn-exposed worms (Fig. 3e); the Zn-induced effect became statistically insignificant in the EN/BW groups (Fig. 3f).

Spectrofluorometric analysis of coelomocyte lysates revealed riboflavin-specific emission and excitation spectra, as described previously (HOMA *et al.* 2010). The amount of riboflavin expressed in arbitrary units (RF [AU]) was significantly lower

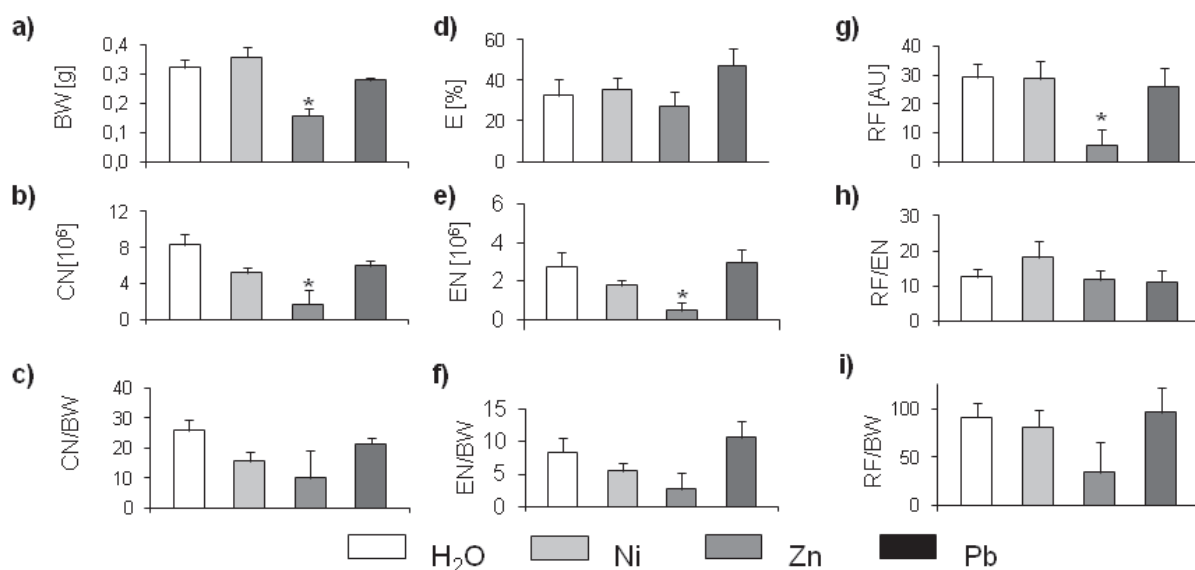


Fig. 3. Effects of soil samples soaked with water ( $\text{H}_2\text{O}$  – control group), Ni ( $1 \text{ g kg}^{-1}$ ), Zn ( $1.25 \text{ g kg}^{-1}$ ), and Pb ( $5 \text{ g kg}^{-1}$ ) on body weights (a) and coelomocytes (b-i) of *Allolobophora chlorotica* during 3-week exposure. a) fresh body weights (BW); b) total number of coelomocytes (CN); c) coelomocytes per fresh body weight (CN/BW); d) percentages of eleocytes (E); e) total numbers of eleocytes (EN); f) eleocytes per body weight (EN/BW); g) riboflavin (RF) content in coelomocyte lysates in arbitrary units [AU]; h) riboflavin recalculated per eleocyte numbers (RF/EN); i) riboflavin per body weight (RF/BW); X+SE, n= samples from 4-5 worms per group. Asterisks at means statistically significantly different from those of the water-soaked group ( $P < 0.05$  according to Mann-Whitney's test).



in samples from Zn-exposed worms than in the control and Ni- or Pb-exposed groups of animals (Fig. 3g). However, when riboflavin content was adjusted to numbers of autofluorescent eleocytes (RF/EN) or to fresh body weights (RF/BW) the mean values did not differ significantly between groups (Fig. 3h, i).

## Discussion

Earthworms can avoid soils containing elevated amounts of heavy metals and hazardous waste (YEARDLEY *et al.* 1996) as they have chemoreceptors in the prostomium and the sensory tubercle on the body (LAVERACK 1961; STEPHENSON *et al.* 1998). In most behavioural studies, earthworms are given a choice between adjacent soils, contaminant-free soil and a contaminant-bearing soil. Avoidance behaviour of earthworms to various chemicals has been reported by many authors (e.g. YEARDLEY *et al.* 1996; LANGDON *et al.* 2001, 2005; GARCIA 2008; EIJSACKERS *et al.* 2005; LOUREIRO *et al.* 2005; GARCIA *et al.* 2008; LUKKARI *et al.* 2005; OWOJORI & REINECKE 2009). In some cases earthworms tolerate certain chemicals, i.e. organophosphate pesticides (HODGE *et al.* 2000) or lead nitrate (REINECKE *et al.* 2002). In several experiments earthworm burrowing rate (i.e. time taken to burrow into soil) was recorded (e.g. EIJSACKERS *et al.* 2005; LANGDON *et al.* 2001) while in others earthworm trajectories within soil were also reconstructed and measured (CAPOWIEZ *et al.* 2003).

*Al. chlorotica* is a soil-dwelling geophagous species which is typically found close to the soil surface when soil conditions are favourable but will burrow deeper to avoid extremes of temperature or dry soil at the surface (GERARD 1967). ELLIS *et al.* (2010) used a vertical arrangement of containers with control soil and soil contaminated with a toxic fungicide – carbendazim. *Al. chlorotica* avoided toxic fungicide-bearing soil samples in all instances except that when worms were put directly on the top of the polluted soil. In this case the majority remained in the toxic soil, perhaps due to impaired mobility caused by the neurotoxic effects of fungicide (ELLIS *et al.* 2010). Observations of *Al. chlorotica* from previous (PIOTROWSKA *et al.* 2010) and present experiments indicate that earthworms can also detect metal soil pollution and change accordingly their behaviour. The results fully confirmed that avoidance behaviour and burrowing rate are fast and simple screening tools to assess soil quality.

Nickel and lead accumulated significantly in worm bodies during the 3-week exposure whereas zinc accumulation was efficiently regulated. Nev-

ertheless zinc exposure significantly inhibited weight gain. This corresponded with a significant decrease of total numbers of coelomocytes, among them riboflavin-loaded eleocytes, and as a consequence a significant decrease of riboflavin content in coelomocyte lysates.

Inhibition of weight gain in zinc-exposed worms may be putatively explained by the high energetic costs of efficient zinc regulation. Diminution of coelomocyte number in the extruded coelomic fluid may be connected with coelomocyte participation in Zn trafficking and removal through nephridia (STÜRZENBAUM *et al.* 2001; HOMA *et al.* 2005). Alternatively missing free coelomocytes may be involved in the formation of multicellular brown bodies encapsulating intracoelomic microbes (VALEMBOIS 1992, 1994).

We are unaware of studies on the energetic costs of metal regulation or bioaccumulation in earthworm bodies. Until now, the energetic costs of detoxification systems were explored in herbivores feeding on chemically defended host plants. In the case of the grain aphid, *Sitobion avenae*, reared on host plants with differing levels of hydroxamic acid, the energetic costs of detoxification were low (CASTANEDA *et al.* 2009). We assumed that zinc regulation in *Al. chlorotica* bodies was more energy-demanding than nickel or lead bioaccumulation.

In previous experiments, *D. veneta* (KWADRANS *et al.* 2008) and *Aporrectodea caliginosa* (DUTKIEWICZ *et al.* 2009) were maintained for 4 and 8 weeks, respectively, in soil samples soaked with Cd, Cu, Pb, or Ni chlorides. Body weights of *D. veneta* were unaffected by 4-week metal exposure, but eleocyte numbers and riboflavin content were increased in Pb- and/or decreased in Ni-exposed groups of worms. In contrast, after 8-week experiments on *A. caliginosa*, body weight gain was inhibited in all metal-exposed groups while coelomocyte number was significantly increased in Pb-exposed worms. This indicates that the effects of metal soil pollution on the earthworm immune system are species-specific and do not always correspond with the general condition of worms. We may assume that effects of metal exposure on immunity are rather associated with the disrupted balance between the worm immune system and microbial impact from surrounding metal-polluted soil (SALICE & ROESIJADI 2002; WIECZOREK-OLCHAWA *et al.* 2003; OLCHAWA *et al.* 2006).

The earthworm coelom is inhabited by a variety of prokaryotic and eukaryotic organisms, including bacteria, protozoans, fungi, and nematodes, which are effectively controlled by the immune system (FIELD *et al.* 2004). However, metal expo-

sure can unbalance the host-bacteria relationship, as evidenced in *D. veneta* after 3-day exposure to filter paper soaked with water (controls) or metal (Zn, Cu, or Cd) chlorides. Zn was efficiently regulated, while Cu and Cd accumulated in worm bodies. Numbers of coelomocytes were unaffected by Zn exposure but were significantly decreased in Cu and Cd-exposed worms. In the same animals, the contents of bacteria in coelomic cavities increased in Zn and Cu-exposed worms, but decreased in Cd-exposed animals. Moreover, when coelom-derived bacteria were *in vitro* incubated in the presence of metal chlorides, bacterial growth was supported by zinc ions but was significantly or almost completely inhibited by Cu and Cd ions, respectively (OLCHAWA *et al.* 2006). For the experimental conditions used in present experiments on *Al. chlorotica*, zinc contamination acted in favour of microbes inhabiting the soil and the earthworm coelomic cavity, leading to impaired body mass gain and a decrease in free coelomocyte numbers. In contrast, Ni and Pb soil pollution may reduce the bacterial content of soil and worm bodies, therefore the metal burden did not impair worm body weights and their immunocompetent cells.

In conclusion, a soil avoidance test and burrowing rate of *Al. chlorotica* can be used as a quick preliminary method to determine the potential contamination of soil. Metals may be either regulated (Zn) or accumulated (Ni, Pb) in worm bodies, with or without deleterious effects on body weights and immunocompetent cells, putatively due to their differential impact on soil- and coelom-inhabiting microbes.

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