Avoidance response in three ecologically different earthworm species exposed to heavy metal spiked soils of Cr and Zn: A comparative study

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Abstract --Avoidance response in earthworms is considered as a potential tool for assessing soil toxicity involving minimal experimental effort. In this study three ecologically different earthworms [epigeic- *Eudrilus eugeniea*, aneic- *Lampito mauritii* and endogeic- *Pontoscolex corethrurus*] were used to assess avoidance response upon exposure to Cr and Zn polluted soils. The studied parameters include differences in their lethal mortality and avoidance response to spiked soils of chromium (Cr VI) and zinc(Zn) using a two-section avoidance test procedure. The LC50 values of both metals was conducted according to OECD guidelines and the concentration at which 50% mortality occurs was calculated by Finney et al. method. The LC50 values for Zn was found to be 1067.64ppm (R2=0.8639), 1159.9ppm (R2=0.839) and 1164.3 ppm (R2=0.7561) for *E.eugeniae*, *L. mauritii* and *P. corethrurus* respectively. Likewise, in Cr spiked soils, the 50% mortality was observed at 22.35ppm (R2=0.8393), 16.57ppm (R2=0.9601) and 17.25ppm (R2=0.9601) for *E.eugeniae*, *L. mauritii* and *P. corethrurus* respectively. The two chamber avoidance tests were set up according to Hund-Rinke et al. (2003) and percentage avoidance to both heavy metals were calculated. All three earthworm species avoided Cr and Zn spiked soils at different grades. *E. eugeniea* was found to be the most sensitive species responding to low metal concentrations compared *to L. mauritii*, while *P. corethrurus* avoided Cr only at 75ppm being the least sensitive species. On contrary, all three species exhibited repellence to Zn spiked soils from 400ppm onwards with varying degree of repellence in the order *E.euginiea> L..mauritii > P. corethrurus* respectively. The results indicate that earthworm avoidance behaviour is an ecologically relevant parameter for assessing toxic metal spiked soils. Further the study highlights the choice of species used plays a significant role in the earthworm avoidance test procedure.

Keywords: Earthworms, Avoidance behaviour, Heavy metals (Cr and Zn), Species specificity.

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1. Introduction

Heavy metals from anthropogenic sources are widely distributed in the environment and finally reach the surface soil layers, through different sources, such as pesticides, fertilizers, organic and inorganic amendments, mining waste and sludge residues [1]. As compared to the harmful organic compounds, heavy metals do not decompose and disappear from the soil [2], leading to long term effects on soil organisms and decomposition process. Enhanced use of pesticides containing heavy metal components has indirectly increased the ecological costs associated with the risk posed to non-target soil organisms [3], thereby declining population(s) of many soil organisms which impairs the provision of several ecological services [4]. Several experiments were performed, using different organisms that demonstrate a behavioural response, such as earthworms, collembolans, enchytraeids and isopods [5], [6],[7],[8],[9].

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Earthworms have been used as model organisms to assess the potential ecological risk on soil ecosystems caused by pollutants [10] and hence considered as fundamental organisms which plays an important role in soil functionality [11]. Unfavourable conditions lead to avoidance behaviour which might act as an indicator of stress potential of that particular site [12], as density of soil fauna depends on the soil characteristics. Variation in the habitat has shown to affect the species, consequently the risk assessment of contaminated soils, thereby the degree of exposure to soil contaminants depends on their behaviour. Yeardley et al. [13] showed that earthworms avoided soils containing elevated levels of toxic metals and hazardous waste while, studies of Alvarenga et al. [14] and Langdon et al. [15] reported active avoidance in earthworms to much lower level of metals which can cause direct toxicity, and their results indicate that if earthworms can detect the contaminated soils they may be strongly avoided. Thus, conventional toxicity testing employing only one indicator species may not reveal the extent of risk of soil contaminants to earthworms [16]. So far, no study has been reported showing the comparative sensitivity of avoidance using ecologically different species of earthworms to heavy metals (Cr and Zn). Thereby, this study aims to categorize the sensitivity of avoidance test towards heavy metals (Cr and Zn) employing three ecologically different types of earthworms epigeic-*Eudrilus euginea,* ***aneic****- Lampito mauritii* and endogeic-*Pontoscolex corethrurus* for their metal tolerance in terms of lethal concentration and avoidance behaviour.

2. Materials and Methods

2.1 Soil preparation and Test organisms

The garden soil was collected from Bangalore university campus with no input of pollution and was sieved with 2mm mesh and mixed with dry cow dung powder (3:1, v/v) moistened to 35- 40 % of the water holding capacity using metal solutions for all the tests, and the pH was measured at the beginning and end of each test. Each metal concentration was tested in five replicates plus dual controls. Metal solutions were prepared to get series of concentrations of Zinc (Zn) (100,200,300,400,500 ppm/kg soil) using $ZnCl_2$ and Chromium, Cr (VI) (25, 50, 75, 100 and 125 ppm/kg soil) using $K_2Cr_20_7$.

To avoid false positive results, and to minimise the interference of soil physicochemical properties, and organic carbon content which may affect the avoidance behaviour, and to mimic the field conditions, single garden soil was used. The garden soil was assayed for its unfavourable and non-toxic characteristics, employing dual controls, where distribution of worms were 50-50 or 60-40 range as detailed in the ISO guidelines. Two validity criteria were assured for the correct performance of the avoidance tests: (a) random distribution of earthworms on both sections of the test chamber when filled with the control soils, (b) no lethality [12].

Earthworms, *Eudrilus eugeniea*, *Lampito mauritii* and *Pontoscolex corethrurus* were collected from a site with no history of the influx of pollutants from selected areas of Bangalore, and cultures were maintained at a constant temperature of 23 ± 2 °C and regime of 16h light, 8h dark cycle. The earthworms were maintained in plastic culture boxes with a mixture of garden soil and cow dung (3:1, v:v) as substrate with pH 6.5–7.0. The individuals used in the tests were mature, clitellate, and with an individual fresh weight between 400-600 mg or 1.0-1.2 g depending on species.

2.2 Lethal concentration

Metal solutions of various concentrations (Zn and Cr) were prepared and spiked to the soil, ten mature clitellate adult earthworms of each species were introduced to all the replicates. Five replicates were made for each concentration and maintained in the laboratory for 14-days and earthworm mortality in each replicate for each metal concentration was recorded to know the toxic effect of metals. Lethal concentrations at which 50% of the worms died were calculated using Finney et al. [17].

2.3 Earthworms avoidance test

The two chamber avoidance tests were set up according to Hund-Rinke et al. [18]. Each plastic container (20 cm L x 15 cm w x 7 cm h) was divided into two equal sections with a plastic card, one-half of the container received 500g (dry wt) of control soil and the other half 500g (dry wt) of spiked soil either with zinc or chromium of different range of concentrations. Each concentration was tested with five replications, so that the behaviour of 50worms were investigated for each concentration [19]. After placing the soils into each container, the divider was removed and ten worms were put on the middle line, container was covered with a perforated lid to facilitate aeration. The containers were incubated at 23 ± 2 °C with a photoperiod of 16h : 8h (light: dark) for 48 hours. After the test period, the divider was put back to containers to separate the control and test soils, and the number of worms in both sections were counted. To evaluate mortality rate, a spatial bias test (dual control, avoidance test) was performed, in which both sections of the test containers were filled with the control soil.

The percentage avoidance to different soil treatments was calculated by counting the mean number of earthworms in each concentration and compared with the mean number of worms in the untreated control soil. The amount of earthworms counted was converted to a percentage of avoidance by the following equation: R (%) = [(C − T) /N] × 100, where R = avoidance; C = number of worms in the control (C0) condition; T = number of worms in each dose in the same soil; N = total number of worms. Thus, positive values account for avoidance of test soil, while neutral or negative responses represent indifference or preference of the test substance.

3 Results

3.1 soil characteristics

The physicochemical characteristics of the soil assessed are given in table (1). Test soils were prepared by spiking the control soil with a solution of $ZnCl_2$ to get the required concentration of Zn (100,200,300,400,500 ppm/kg soil) and solution of $K_2Cr_2O_7$ for chromium concentration (25,50,75,100,125 ppm/kg soil).

3.2 Lethal concentration

The 14-day LC_{50} was conducted according to OECD guidelines and 50% mortality in Zn spiked soils were observed at 1067.64 ppm (R^2=0.8639), 1159.9 ppm (R^2=0.839) and 1164.3 ppm (R^2=0.7561) for *E.eugeniae*, *L. mauritii* and *P. corethrurus* respectively. Likewise, in chromium spiked soils, the 50% mortality was observed at 22.35 ppm (R^2=0.8393), 16.57 ppm (R^2=0.9601) and 17.25 ppm (R^2=0.9601) for *E.eugeniae*, *L. mauritii* and *P. corethrurus* respectively(Fig 1).

3.3 Avoidance behaviour

The test was performed in a two chamber system as suggested by Hund-Rinke et al. (2003). Soils spiked with the selected concentrations were not acutely lethal to all the species tested. The distribution of the worms found in the double control was within the range 40%–60% or 50% - 50% after 48h. In Cr spiked soils, 58% of *E.eugeniea* avoided 10ppm of Cr and 100% avoidance was seen at further concentrations. On the contrary, significant ($P<0.05$) avoidance by *L. mauritii* 56%, 86% and 94% in *P. corethrurus* significant ($P<0.05$) avoidance of 50%, 80% and 92% was evident in 30, 40 and 50ppm of Cr respectively.

Table 1: Physico-chemical characteristics of garden soil										
	pH	E.C	O.C (%)	Av P_2O_5 kg/ac	AvK_2O_5 kg/ac	Zn (ppm)	Fe (ppm)	Cu (ppm)	Mn (ppm)	T.Cr (ppm)
Soil	6.9±0.1	0.07±0.01	0.19±0.02	20.0±1.2	214±15.8	1.29±0.3	9.2±0.8	1.6±0.2	35.9±0.5	BDL
Values are Mean ± SE of 3 values (n=3). BDL-below detectable limits										

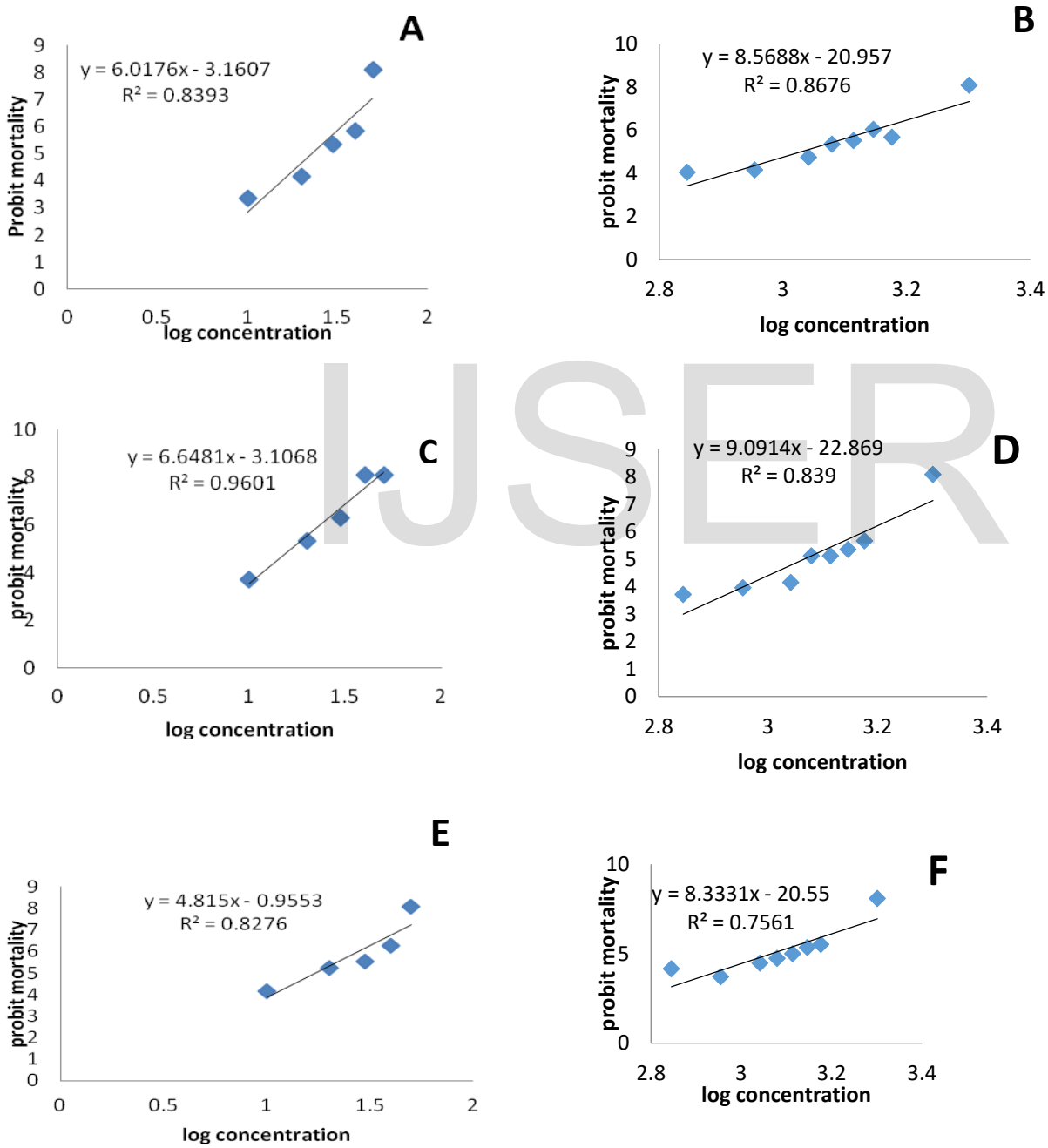

Figure 1: 14-day Probit mortality of three ecologically different earthworm species exposed to different concentrations of Cr^{+6} and Zn^{2+}. 'A' represents probit mortality of *E. eugeniea* exposed to Cr spiked soils; **'B'** represents probit mortality of *E. eugeniea* exposed to Zn spiked soils; **'C'** represents probit mortality of *L. mauritii* exposed to Cr spiked soils; **'D'** represents probit mortality of *L. mauritii* exposed to Zn spiked soils; **'E'** represents probit mortality of *P. corethrurus* exposed to Cr spiked soils; **'F'** represents probit mortality of *P. corethrurus* exposed to Zn spiked soils.

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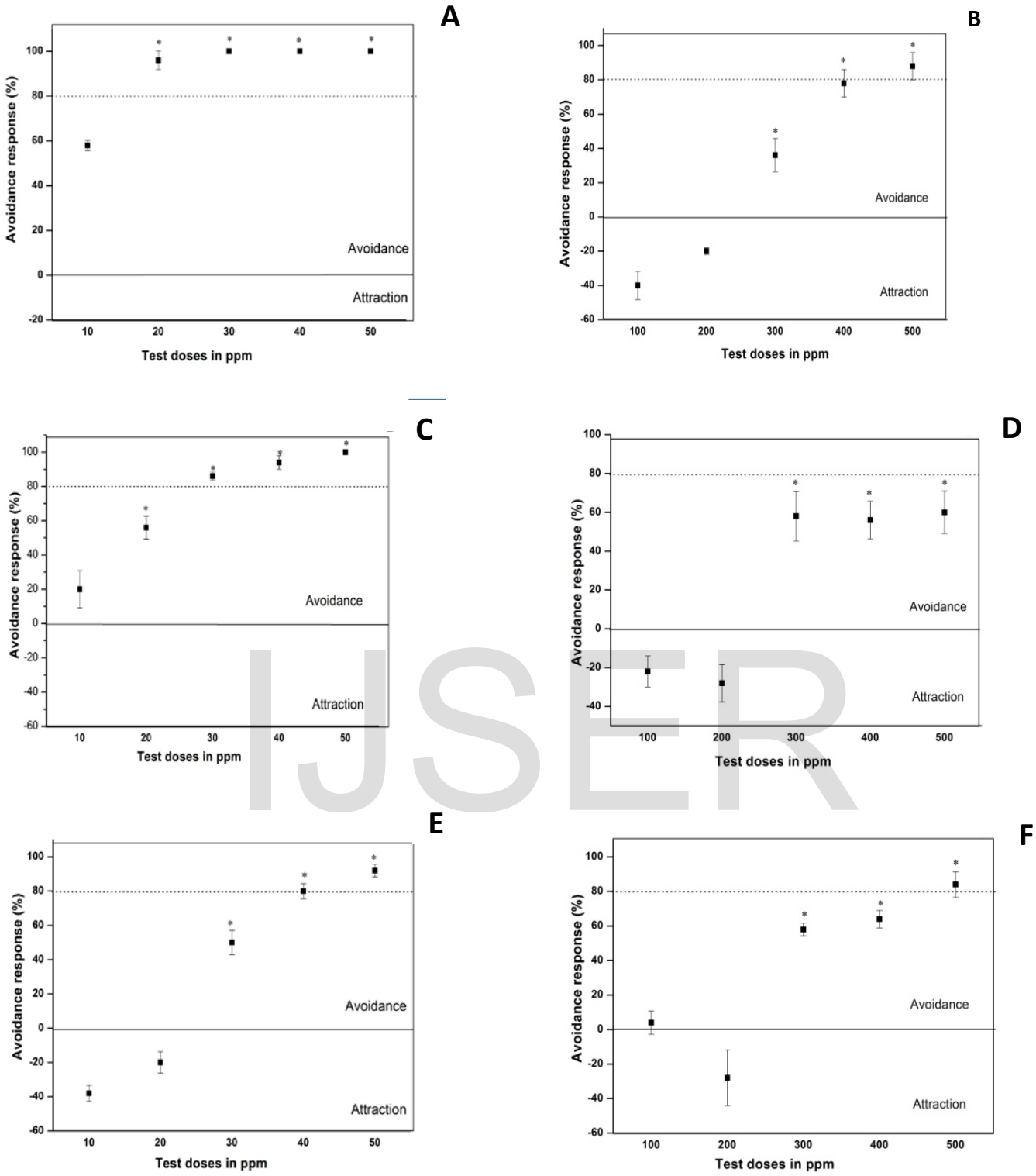

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Figure 2 : Avoidance response in three ecologically different earthworm species exposed to different concentrations of Cr^{6+} and Zn^{2+} . **'A'** represents Avoidance response of *E. eugeniea* exposed to Cr spiked soils**; 'B'** represents Avoidance response of *E. eugeniea* exposed to Zn spiked soils; **'C'** represents Avoidance response of *L. mauritii* exposed to Cr spiked soils; **'D'** represents Avoidance response of *L. mauritii* exposed to Zn spiked soils; **'E'** represents Avoidance response of *P. corethrurus* exposed to Cr spiked soils; **'F'** represents Avoidance response of *P. corethrurus* exposed to Zn spiked soils.

It is evident from present study that *E. eugeniea* were most sensitive to chromium compared to *L. mauritii* and *P. corethrurus*. In Zn spiked soils, eventhough avoidance was evident at above 400 ppm of Zn the significant ($P<0.05$) percentage avoidance varied among

species. About 36% of *E.eugeniea* avoided 30 ppm of Zn followed by 78% in 400 ppm and 88% avoidance in 500 ppm of Zn spiked soils. Whereas in *L. maurutii* significant($P<0.05$) avoidance observed at 58%, 56% and 60% in 300, 400 and 500 ppm of Zn was evident. Likewise, in *P. corethrurus* significant ($P<0.05$) avoidance observed at 58%, 64% and 84% in 300, 400 and 500 ppm of Zn respectively. While, attraction towards Zn was evident at lower concentrations, in *E. eudrilus* (40%) and (20%); *L. maurutii* (22%) and (28%); *P. corethrurus* (40%) and (28%) at 100 and 200 ppm of Zn respectively (Fig 2).

4 Discussion

Anthropogenic activities that lead to increase metal concentrations of toxic metals in surface soils which tend to decrease earthworm density [20], and its extinction caused by metal overload has been correlated to soil compaction and disproportionate litter build up which are detrimental to soil quality [21]. Behaviour is a final integrated result of sensory, hormonal, neurological and metabolic processes which involves interaction with biotic and abiotic components of the environment. Capowiez [22] suggests that behaviour seems to be a promising biomarker in earthworm studies, as it can give different end points that could perhaps be linked, to soil functioning. Avoidance response tests reflect these behavioural properties of the earthworms, which works on the principle of preference or avoidance of substrates after a specific exposure period. Earthworms are the major agents of the soil fauna biomass, providing soil aeration and drainage [23], and act as primary decomposers of organic matter whose functions may be suppressed due to pollutants. Earthworms can sense the chemicals in their environment through highly prejudiced sensory mechanisms in navigating and manipulating their environments with high number of chemoreceptors, concentrated in the prostomium and anterior segments coupled with epidermal tubercles and nerve endings in and around each body segments [24]. The sensory cells in the epithelium of mouth region enables the earthworms to avoid adverse habitats [25]. However, earthworm avoidance to various inorganic and organic contaminants is well reported [14], [20], [13]. The degree of avoidance varies with different stressors or different earthworm species, as concentrations that do not induce an avoidance response in earthworms after 48h of exposure could affect the reproduction of these organisms upon longer exposures [26]. In this study epigeic *E.eugeniea* found to be more sensitive to both metals in soil, while less sensitivity observed in aneics *L. mauritii* and endogeic *P.corethrurus*. The variability observed in the results is due to the use of ecologically different species which may turn some species more susceptible to toxicants in the soil than others, as it influences the energy budget of animals by dispensing energy on detecting and escaping from hazard and also decreasing their feeding activity in contaminated areas. In our earlier studies, *Eudrilus eugeniae* was used for its sensitivity to sub lethal doses of Cr and Zn in terms of alterations occurred in oxidative stress indices and reproductive parameters in the testis and results inferred that the excessive free radical production and inadequate antioxidant defenses have led to morphological alterations in sperms which sequentially reduced the reproductive rate [27]. A significant decline in hatchability rate and reproductive function was evident upon exposure to Cr spiked soils contrarily, increased hatchability was observed in Zn spiked soils [28]. Likewise studies of Dominguez et al. [29] reported increased variability in their results by using two test species, *E. fetida* and *E. Andrei*, and concluded that despite their ecological and physiological similarities, their responses to stress can be different. This could be due to the fact that epigeics encounter the pollutants at first instance whereas aneics are exposed once it is infiltrated into the burrows as, they are known to oscillate daily between the surface (where they feed and cast) and deeper zones of their burrows where they escape from excessive dryness and heat [30]. Studies of Renoux et al. [31] observed earthworms on the soil surface instead of burrowing in trinitrotoulene contaminated soil, interpreting it as a behavioural response. Similarly, in our previous study surface migration was observed at higher concentrations of Cr and Zn tested [32]. Several studies indicated that the avoidance test had a comparable or higher sensitivity than the reproduction test [33] hence, can be considered as short-term sub lethal indirect predictor of detrimental effects on ecosystem functioning [34].

5 Conclusion

Endpoints in standardised earthworm test reflect direct lethal and sub lethal effect(s) of chemicals, whereas behavioural tests focus on indirect effects. The decline in population size due to mortality or altered reproduction is an ecological outcome of exposure to chemicals in soils. However, behavioural changes such as substrate avoidance can also be considered as ecologically relevant, as emigration of earthworms leads to soil degradation as they avoid soils with undesirable chemistry [35]. From our study, we can conclude that avoidance response tests were sensitive tools in detecting the effects of sub lethal concentration of heavy metals, Cr and Zn using earthworms, and a responsive parameter compared to mortality as an end point. Even though avoidance test is widely used in ecotoxicological assessments, our study highlights the use of epigeics like *Eudrilus eugeniea* is recommended, as they are more sensitive even to the minimal alteration in its environment compared to aneics- *Lampito mauritii* or endogeic – *Pontoscolex corethrurus.*

6. References

1. Capri, Ettore, and Marco Trevisan, eds. *I metalli pesanti di origine agricola nei suoli e nelle acque sotterranee*. No. 2479. Pitagora, 2002.

2. Brusseau M. L, Transport and fate of toxicants in soils. In: Soil Ecotoxicology, J.Tarradellas G. Bitton and D. Rossel (Eds.) pp. 33–53, Lewis Publishers ISBN 978-1566701341 New York USA (1997)
3. Santos, M. J. G., M. F. L. Ferreira, A. Cachada, A. C. Duarte, and J. P. Sousa. "Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei." *Ecotoxicology* 21, no. 8 (2012): 2113-2122.
4. Frampton, Geoff K., Stephan Jänsch, Janeck J. Scott-Fordsmand, Jörg Römbke, and Paul J. Van den Brink. "Effects of pesticides on soil invertebrates in laboratory studies: a review and analysis using species sensitivity distributions." *Environmental Toxicology and Chemistry: An International Journal* 25, no. 9 (2006): 2480-2489.
5. Da Luz, Tiago Natal, Rui Ribeiro, and José Paulo Sousa. "Avoidance tests with collembola and earthworms as early screening tools for site-specific assessment of polluted soils." *Environmental Toxicology and Chemistry: An International Journal* 23, no. 9 (2004): 2188-2193.
6. de Barros Amorim, Mónica João, Jorg Römbke, Hans-Joachim Schallnaß, and Amadeu Mortágua Velho Maia Soares. "Effect of soil properties and aging on the toxicity of copper for Enchytraeus albidus, Enchytraeus luxuriosus, and Folsomia candida." *Environmental Toxicology and Chemistry*24, no. 8 (2005): 1875-1885.
7. Loureiro, Susana, Amadeu MVM Soares, and Antonio JA Nogueira. "Terrestrial avoidance behaviour tests as screening tool to assess soil contamination." *Environmental pollution*138, no. 1 (2005): 121-131.
8. Lukkari, Tuomas, and Jari Haimi. "Avoidance of Cu-and Zn-contaminated soil by three ecologically different earthworm species." *Ecotoxicology and environmental safety* 62, no. 1 (2005): 35-41.
9. Aldaya, Maite Martínez, Christine Lors, Sandrine Salmon, and Jean-François Ponge. "Avoidance bio-assays may help to test the ecological significance of soil pollution." *Environmental pollution* 140, no. 1 (2006): 173-180.
10. Ribeiro, Sónia, J. P. Sousa, A. J. A. Nogueira, and A. M. V. M. Soares. "Effect of endosulfan and parathion on energy reserves and physiological parameters of the terrestrial isopod Porcellio dilatatus." *Ecotoxicology and Environmental Safety*49, no. 2 (2001): 131-138.
11. Edwards, C. A. "Bohlen., PJ, 1996. Biology and Ecology of Earthworms."
12. Hund-Rinke, Kerstin, and Hendrik Wiechering. "Earthworm avoidance test for soil assessments." *Journal of Soils and Sediments* 1, no. 1 (2001): 15-20.
13. Yeardley, Roger B., Laura C. Gast, and James M. Lazorchak. "The potential of an earthworm avoidance test for evaluation of hazardous waste sites." *Environmental toxicology and chemistry* 15, no. 9 (1996): 1532-1537.
14. Alvarenga, P., P. Palma, A. P. Gonçalves, R. M. Fernandes, A. De Varennes, G. Vallini, E. Duarte, and A. C. Cunha-Queda. "Evaluation of tests to assess the quality of mine-contaminated soils." *Environmental Geochemistry and Health*30, no. 2 (2008): 95-99.
15. Langdon, Caroline J., Mark E. Hodson, Rebecca E. Arnold, and Stuart Black. "Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test." *Environmental Pollution* 138, no. 2 (2005): 368-375.
16. Tomlin, A. D. "Behaviour as a source of earthworm susceptibility to ecotoxicants." *Ecotoxicology of earthworms. Intercept, Andover* (1992): 116-125.
17. Finney, D. J. "Statistical Method in Biological Assay, Griffin & Co." *Ltd. London* (1978).
18. Hund-Rinke, Kerstin, Rudolf Achazi, Jörg Römbke, and Dietmar Warnecke. "Avoidance test withEisenia fetida as indicator for the habitat function of soils: Results of a laboratory comparison test." *Journal of soils and Sediments* 3, no. 1 (2003): 7-12.
19. Hund-Rinke, Kerstin, Monika Lindemann, and Markus Simon. "Experiences with novel approaches in earthworm testing alternatives (7 pp)." *Journal of Soils and Sediments* 5, no. 4 (2005): 233-239.
20. Gongalsky, Konstantin B., Svetlana A. Belorustseva, Daria M. Kuznetsova, Alexander V. Matyukhin, Lyubov A. Pelgunova, Fyodor A. Savin, and Alexander S. Shapovalov. "Spatial avoidance of patches of polluted chernozem soils by soil invertebrates." *Insect Science* 16, no. 1 (2009): 99-105.
21. Eijsackers, H., P. Beneke, M. Maboeta, J. P. E. Louw, and A. J. Reinecke. "The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality." *Ecotoxicology and Environmental Safety* 62, no. 1 (2005): 99-111.
22. Capowiez, Yvan, and Annette Berard. "Assessment of the effects of imidacloprid on the behavior of two earthworm species (Aporrectodea nocturna and Allolobophora icterica) using 2D terraria." *Ecotoxicology and Environmental Safety*64, no. 2 (2006): 198-206.
23. Davies, Nicola A., Mark E. Hodson, and Stuart Black. "Is the OECD acute worm toxicity test environmentally relevant? The effect of mineral form on calculated lead toxicity." *Environmental Pollution* 121, no. 1 (2003): 49-54.
24. Wallwork J A (1983) Earthworm Biology. Studies in Biology No 161 Southhampton England: Camelot Press
25. Stephenson, G. L., A. Kaushik, N. K. Kaushik, K. R. Solomon, T. Steele, and R. P. Scroggins. "Use of an avoidance-response test to assess the toxicity of contaminated soils to earthworms." *Advances in earthworm ecotoxicology* (1998): 67-81.
26. Santos, M. J. G., M. F. L. Ferreira, A. Cachada, A. C. Duarte, and J. P. Sousa. "Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei." *Ecotoxicology* 21, no. 8 (2012): 2113-2122.

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27. Basha, P. Mahaboob, and V. Latha. "Evaluation of sublethal toxicity of zinc and chromium in Eudrilus eugeniae using biochemical and reproductive parameters." *Ecotoxicology* 25, no. 4 (2016): 802-813.
28. Latha, V., and P. Mahaboob Basha. "Extent of Heavy Metal Accumulation in Sewage Irrigated Soils and Their Impact on Distribution of Earthworm Communities: Linking Chromium and Zinc Toxicity on Growth and Reproduction in Selected Earthworm Species." *Current World Environment* 11, no. 1 (2016): 279.
29. Domínguez, Jorge, Alberto Velando, and Alfredo Ferreiro. "Are Eisenia fetida (Savigny, 1826) and Eisenia andrei (Oligochaeta, Lumbricidae) different biological species?." *Pedobiologia* 49, no. 1 (2005): 81-87.
30. Bastardie, François, Yvan Capowiez, and Daniel Cluzeau. "Burrowing behaviour of radio-labelled earthworms revealed by analysis of 3D-trajectories in artificial soil cores: The 7th international symposium on earthworm ecology· Cardiff· Wales· 2002." *Pedobiologia* 47, no. 5-6 (2003): 554-559.
31. Renoux A Y, Sarrazin M, Hawari J, Sunhara G I (2000) Transformation of 2, 4, 6-Trinitrotoluene in soil in the presence of the earthworm Eisenia andrei. Environmental Toxicology and Chemistry 19:1473–1480
32. Latha, V., and Mahaboob P. Basha. "Heavy metals (Cr and Zn) induced alterations in cast production, burrowing behaviour, surface migration and macropore formation in three ecologically different earthworm species: A comparative study." *International Journal of Environmental Sciences* 6, no. 2 (2015): 260.
33. Schaefer, Maike. "Behavioural endpoints in earthworm ecotoxicology." *Journal of Soils and Sediments* 3, no. 2 (2003): 79-84.
34. Smith, Richard, Simon JT Pollard, J. M. Weeks, and C. P. Nathanail. "Assessing significant harm to terrestrial ecosystems from contaminated land." *Soil Use and Management* 21 (2005): 527-540.
35. Shoults-Wilson, W. A., Oksana I. Zhurbich, David H. McNear, Olga V. Tsyusko, Paul M. Bertsch, and Jason M. Unrine. "Evidence for avoidance of Ag nanoparticles by earthworms (Eisenia fetida)." *Ecotoxicology* 20, no. 2 (2011): 385-396.

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