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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

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ABSTRACT

Heavy metals can influence earthworm behavior and physiology which could drastically impact the soil functioning. To test this hypothesis, under laboratory conditions, the cast production and burrow systems(burrow length and patterns), macropores formed and surface migration of three ecologically different earthworm species (the epigeic *Eudrilus eugeniea*, aneic *Lampito maurutii* and the endogeic *Pontoscolex corethrururs*) in natural spiked soil with different concentrations of heavy metals (chromium Cr(VI) and zinc (Zn)) were performed. The amount of cast produced in all the three species varied with increasing concentrations of heavy metals which is inversely proportional to incidence of surface migration observed. Macropores formed increased significantly (P <0.05) in Zn-spiked soils and reduced significantly (P <0.05) upon chromium exposure. The burrow patterns of three earthworm species varied: *E.eugeniea* being an epigeic exhibited only horizontal movements which showed statistically significant (P<0.05) change in its burrow length at the lower concentration of 10 ppm Cr (VI) and 300ppm of Zn, whereas *L. maurutii* made more continuous, less branched and more vertical burrows and exhibited statistically significant (P <0.05) change at 10ppm of Cr. However upon Zn exposure significant (P <0.05) change was evident in 100ppm of Zn onwards. Similar pattern was also evident in *P. corethrurus.* Some conspicuous changes in *L. maurutii* and *P. corethrurus* burrow systems upon exposure to chromium were they made a smaller and narrower burrow system, at concentration above 20ppm of Cr and 400ppm of Zn. As a consequence, the continuity of the burrow systems made by both species was altered following heavy metal exposure which could be considered as a potential tool for evaluation of heavy metal toxicity.

Keywords: Earthworms, burrowing, cast production, surface migration, macropores, heavy metal toxicity.

1. Introduction

Behavioural changes in earthworms are of fundamental importance for soils, as they can result in adverse effects on soil functions (Capowiez et al., 2006). The most widely used behavioural test for earthworms is the standardized 48 hours avoidance test, and proved to be sensitive in many studies (Yeardley et al., 1996; Natal Ad Luz et al., 2004; Hund-Rinke et al., 2005; Pereira et al., 2010). On the other hand, the non-avoidance of few toxicants (diazinon; chlorpyrifos; imidacloprid; ivermectin) or even significant attraction has been observed (Hodge et al., 2000; Capowiez and Bérard, 2006; Torkhani et al., 2011). Thus, the avoidance test is rather considered to be a measure of repellence than of toxicity (Capowiez et al., 2006). Since the beneficial role of earthworms is highly dependent on their burrowing activity, examining whether and how toxicants impede the earthworms burrowing behaviour has high ecological relevance.

Eisenia fetida and *Eisenia Andrei* are the standard test organisms in few existing standard tests focusing mainly on mortality, reproduction and avoidance behaviour (OECD 1984, ISO 2008). However, studies of Edwards, Spurgeon and weeks, revealed tha *E. fetida* and *E. andrei* are epigeic species ecologically not relevant test organisms since these earthworms are absent from most agricultural soil and often claimed to be less sensitive to environmental toxicants than other earthworm species (Edwards and Coulson 1992; Spurgeon and Weeks 1998). Ditterbner etal. (2010), emphasized on the relevance of a toxicity test based on the possibility to link the response to indispensable soil functions considering some behavioural endpoints as they are important and efforts should be made to develop, optimise and increase their use in earthworm toxicity testing. In the case of earthworms, since their beneficial roles in soils depend highly on their activities like creation of burrows and burial of organic matter, it is crucial to study the sublethal effects of heavy metals that could affect earthworm activity. Studying earthworm behaviour is, however, a complex task since these animals are hidden in the soil but studying the outcomes of this activity (such as burrows) is possible.

The 2D terrarium (Evans, 1947) is an apparatus that enables studies of earthworm behavior (Schrader, 1993) and has been used in several ecotoxicological studies (Hodge et al., 2000; Eijsackers et al., 2001; Capowiez et al., 2003a). Several aspects of the earthworm behavior like burrow length; sinuosity and no of burrows were affected by the heavy metal concentrations. In this preview, we attempted to evaluate the alterations induced by heavy metal (Cr (VI) and Zn) after 48 hours of exposure in terms of surface migration, burrow length, macro pores formed, burrow patterns and cast production in three ecologically different earthworm species.

2. Material 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. Metal solutions were prepared to get series of concentrations of Zinc (Zn) (100,200,300,400,500ppm/kg soil) using $ZnCl_2$ and Chromium, Cr (VI) (10, 20, 30, 40 and 50 ppm/kg soil) using $K_2Cr_2O_7$.

Earthworms, *Eudrilus eugeniea, Lampito maurutii* 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 for control and soil was spiked with respective metal solutions of $Zncl_2$ and $K_2Cr_2O_7$ to get required concentrations of Zn and Cr(VI) for treated replicates. Cast production, macro pores formed and surface migration was observed in four replicates of each concentration. Surface casts formed were collected, dried in an oven and weighed; number of macro pores formed was counted in each culture box. Number of worms observed on the surface were counted and expressed in terms of percentage surface migration. The individuals used in the tests were mature, clitellate, and with an individual fresh weight between 400-600 mg and 1.0-1.2 g depending on species.

2.2 Measurements of burrowing behaviour using 2D-terraria

Burrow length and its patterns were studied in three replicates (n = 3) for each metal concentration using Evans (1947) 2D-terraria after 48 hrs of heavy metal exposure. The 2D-terraria consisted of two glass sheets (30 cm to 42 cm) fixed at 3mm apart [18]. The terratia were filled with sieved soil (2 mm) and the soil water content was adjusted to 100 % of the water holding capacity. The earthworms were put individually in the 2D terraria for 48h and were kept in dark climate chambers (at 23±1° C). The burrows were marked after 48h on transparent sheets. Then, a thread was run along the pattern to find the total burrow length and expressed as mean length of three readings for each concentration. The overall patterns were analysed for orientation preferences.

3. Result

3.1 Burrow patterns

The burrow patrrens exhibited by all the three species varied upon exposure to heavy metals Cr and Zn. In control soils *E. eugeniea* exhibited horizontal movements and *L. maurutii* exhibited vertical less branched burrows followed by vertical branched burrows by *P. corethrurus* all over the terratium. On the contrary, in Cr-spiked soils the burrow patterns were limited to the centre of the terratium without much dispersal. However in Zn-treated soils the patterns were much in accordance with the control soils (Figure: 1a).

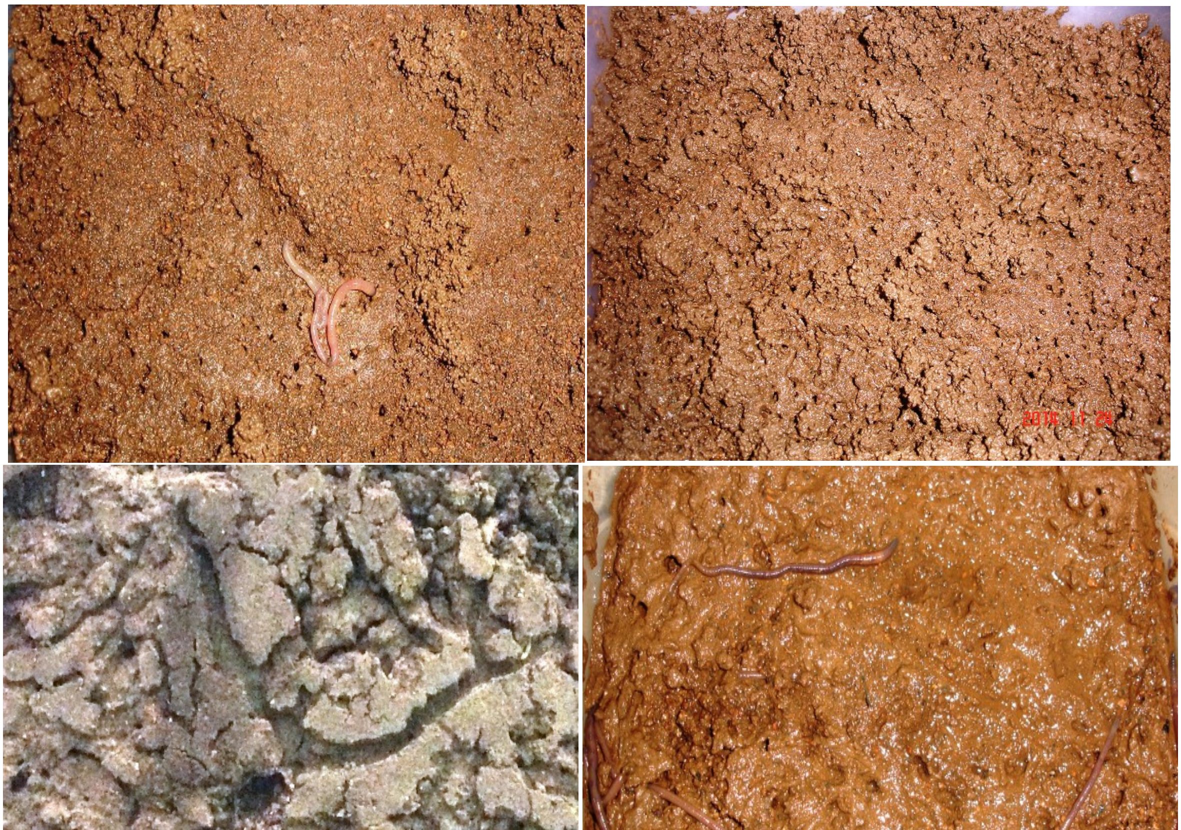

Figure 1: Macropores, burrow patterns, surface cast production and surface migration observed in Zn and Cr(VI) spiked soils.

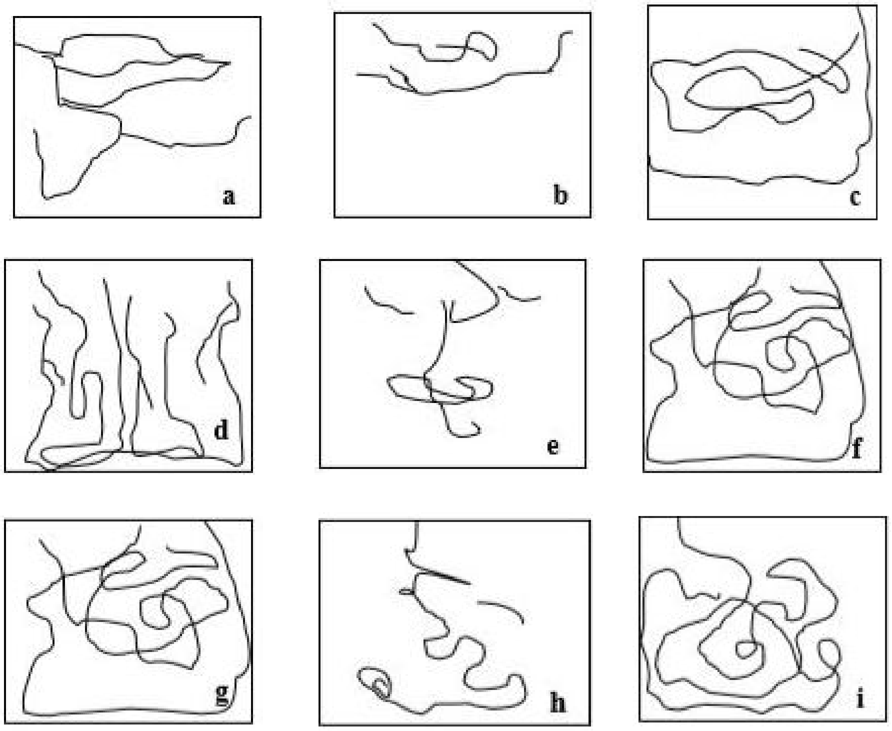

Figure 1a: Schematic representation of heavy metals induced alterations in burrow patterns of three ecologically different earthworm species, epigeic *E.eugeniea*(**a**-control; **b**-Cr(VI) exposed; **c**-Zn exposed), aneic *L.maurutii*(**d**-control; **e**-Cr(VI) exposed; **f**-Zn exposed) and endogeic *P. corethrurus*(**g**-control; **h**-Cr(VI) exposed; **i**-Zn exposed).(not to scale)

3.2 Surface migration

Surface migration observed in *E. eugeniea* and *L. maurutii* varied significantly ($p < 0.05$) from control group upon exposure to chromium at all tested concentrations. On the contrary, in *P.corethrurus*, significant change was observed fron 30 ppm of Cr(VI) exposure onwards. Whereas upon Zn exposure, in *E. eugeniea* significant ($p < 0.05$) difference was observed in 300 ppm but *L. maurutii* and *P.corethrurus* exhibited significant ($p < 0.05$) change in 400 ppm onwards (Figure: 2).

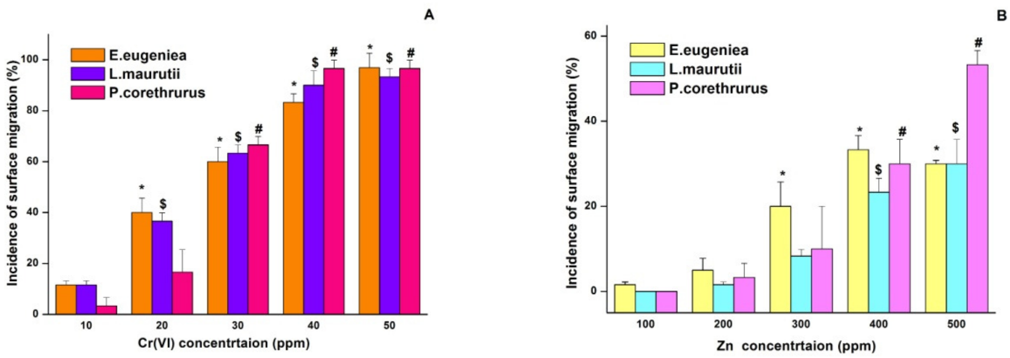

Figure2: Heavy metals induced alterations in surface migration in three ecologically different earthworm species, *E.eugeniea*, *L.maurutii* , *P.corethrurus*. Each bar represents mean± Std error (n=3). *- indicates statistically significant (P<0.05) change in *E.eugeniea* ; **$**- indicates statistically significant (P<0.05) change in *L. maurutii* ; #- indicates statistically significant (P<0.05) change in *P. corethrurus* upon exposure to heavy metals when compared to control, using one- way ANOVA, SPSS(version 20.0) post hoc Dunnett's test.

3.3 Macro pores

Macropores formed by three ecologically different species showed significant ($p < 0.05$) decrease upon exposure to Cr(VI) in the order *P. corethrurus* > *L. maurutii*> *E.eugeniea*. on the contrary, in Zn-spiked soils formation of macropores increased in the order *E.eugeniea* > *L. maurutii*> *P. corethrurus* (Figure: 3).

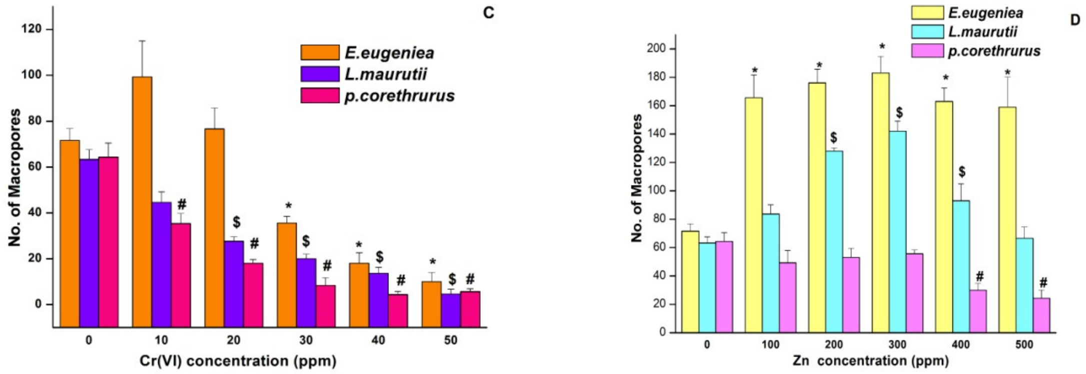

Figure 3: Heavy metals induced alterations in macropores formed in three ecologically different earthworm species, *E.eugeniea*, *L.maurutii* , *P.corethrurus.* Each bar represents mean± Std error (n=3). *- indicates statistically significant (P<0.05) change in *E.eugeniea* ; **$**- indicates statistically significant (P<0.05) change in *L. maurutii* ; **#**- indicates statistically significant (P<0.05) change in *P. corethrurus* upon exposure to heavy metals when compared to control, using one- way ANOVA, SPSS(version 20.0) post hoc Dunnett's test.

3.4 Burrow length

The burrow length measured after 48 hours of exposure decreased significantly ($p < 0.05$) with increasing concentrations of Cr (VI) in all the three species studied. Whereas, in Zn-spiked soils statistically significant ($p < 0.05$) change was observed, with an initial increase in burrow length (100 and 200 ppm) and a decrease in 300, 400 and 500 ppm in all the three species studied (Figure: 4 E & F).

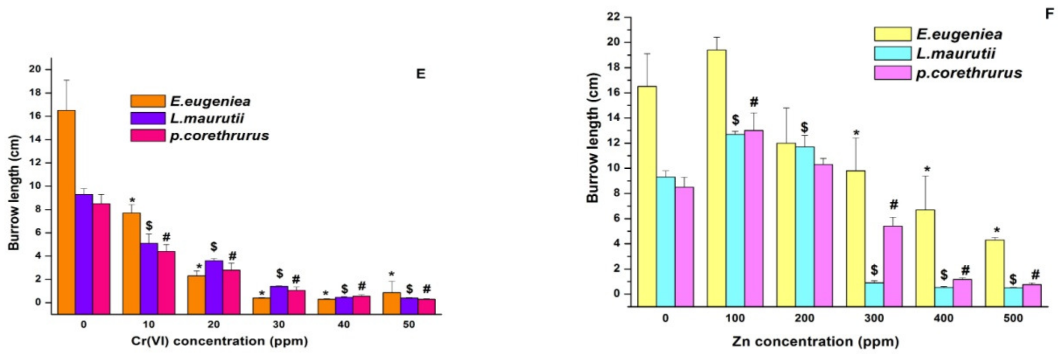

Figure 4: Heavy metals induced alterations in burrow length in three ecologically different earthworm species, *E.eugeniea*, *L.maurutii* , *P.corethrurus.* Each bar represents mean± Std error (n=3). *- indicates statistically significant (P<0.05) change in *E.eugeniea* ; **$**- indicates statistically significant (P<0.05) change in *L. maurutii* ; **#**- indicates statistically significant (P<0.05) change in *P. corethrurus* upon exposure to heavy metals when compared to control, using one- way ANOVA, SPSS(version 20.0) post hoc Dunnett's test.

3.5 Cast production

In *E.eugeniea* cast production observed after 48hours decreased significantly ($p < 0.05$) with increasing concentrations of Cr (VI). Whereas upon Zn exposure only the following concentrations of 300,400 and 500 ppm differed significantly from the respective control groups ($p < 0.05$). Likewise, in *L.maurutii* cast production decreased significantly ($p < 0.05$) with increasing concentrations of Cr(VI), but increased at 20ppm of Cr(VI) whereas, upon Zn exposure only the following concentrations of 100, 200 and 500 ppm differed significantly from the respective control groups ($p < 0.05$). Similarly, in *P.corethrurus* observed cast production upon exposure to heavy metals decreased significantly ($p < 0.05$) with increasing concentrations of Cr(VI) except at 10 ppm, however upon Zn exposure only the following concentrations of 200 to 500 ppm differed significantly from the respective control groups ($p < 0.05$) (Figure: 5(A-F)).

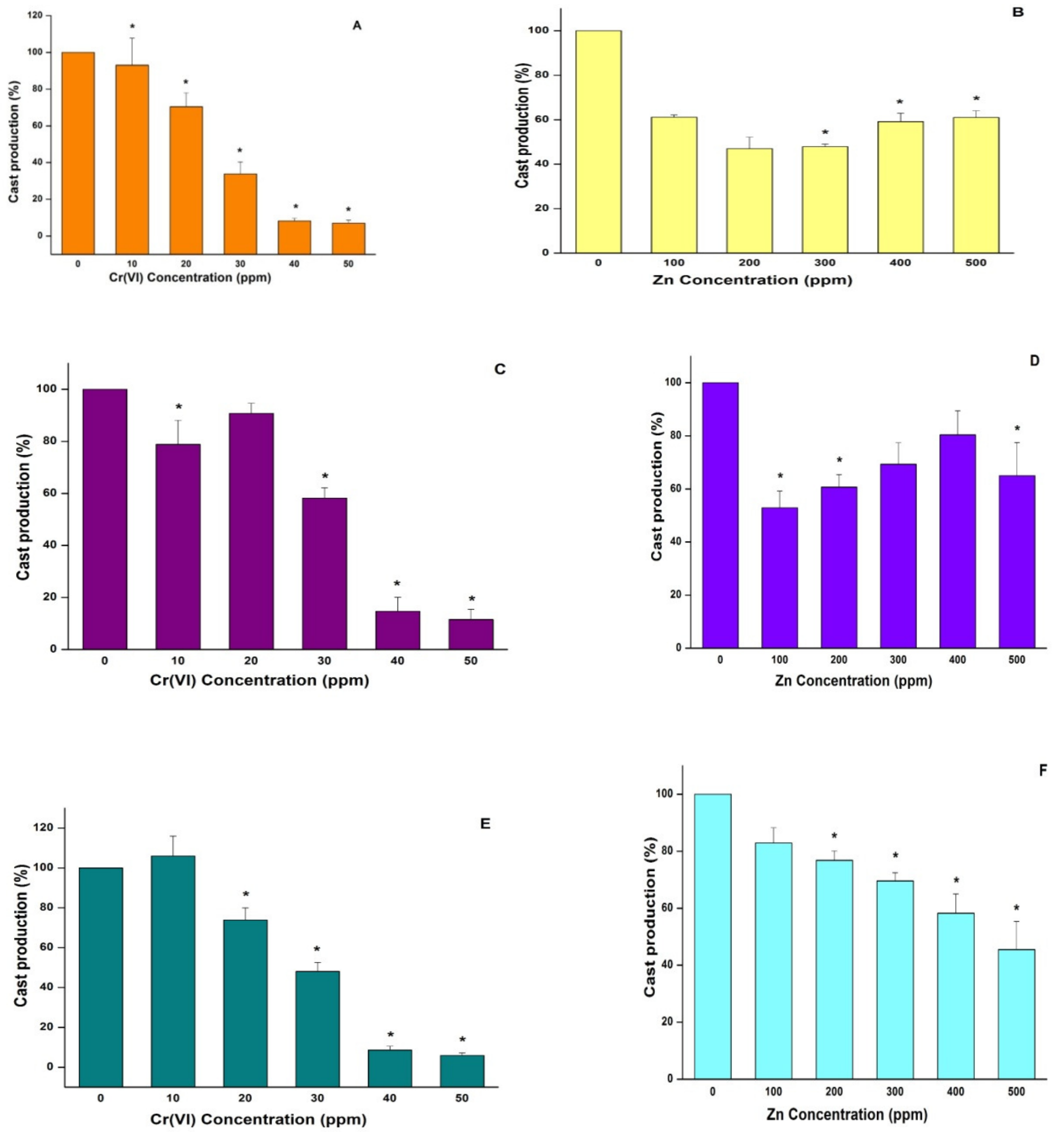

Figure 5: Percentage cast production in *E.eugeniea* ((**A**: Cr(VI); **B**: Zn) , *L.maurutii* ((**C**: Cr(VI); **D**: Zn) , *P.corethrurus* ((**E**: Cr(VI); **F**: Zn) upon exposure to havy metals when compared to control, using one- way ANOVA, SPSS(version 20.0) post hoc Dunnett's test. Each bar represents mean± Std error of 3 replicates. *- indicates statistically significant (P<0.05) difference compared to control.

4. Discussion

Exposure to sub lethal concentrations of heavy metals modified the burrowing behaviour of earthworms. The differences seen between the patterns appear, to be due to either reduced activity or to altered behavior by modifying several characteristics of the burrow systems, which directly reflect the sub lethal effects of heavy metals Cr and Zn. As a consequence of the different burrowing behaviors the impact on water flow through soil varies among the different ecological earthworm groups (Edwards, 1990). Changes in burrowing behaviour were due to physiological damage in the earthworms and might generally be due to species-specific differences in sensitivity, higher ingestion rates and/or to the relatively low surface-volume ratio. In addition the burrow systems of aneics are less complex than the ones of endogeic species (Bastardie et al., 2003a) indicating that behavioural effects might become more obvious at an earlier stage.

The characteristics of the burrow system made by P.corethrurus were numerous, discontinuous and without preferential orientation as often observed with endogeic species (Lee and Foster, 1991). Earthworm distribution, particularly that of anecic species forming vertical burrows, facilitates larger pores conducting water and contributes to infiltration when water is supplied in large quantities (Chan, 2004). Total burrow length represents a very important behavioral aspect, since it indicates general burrowing activity. Changes in maximal burrow depth should also be of high ecological importance, since reduced depths of earthworm burrow systems under natural conditions might have detrimental effects on gas/water transfer properties of soils and as a consequence might affect the whole ecosystem (Bastardie et al., 2003b; Capowiez et al., 2006). In brief, the toxic effects of heavy metals resulted in shorter burrows or fewer burrows, an increase in the sinuosity of the burrow or a narrowing of the burrow. Due to these changes, the burrow systems made by species appeared to be less continuous. The study could not reveal whether the detected effects were only due to physiological damage caused by the toxicant or whether the effects were influenced by avoidance behaviour.

Macro pores formed by earthworms range between 2 to 11 mm in diameter depending on the ecological group of earthworms, endogeic and epigeic earthworms that live in upper mineral soil mainly from small and tortuous pores ranging between 2 and 5 mm in diameter. In contrast, anecic species form pores larger than 5 mm in diameter, which may reach as deep as 2 m into the soil and thus enhance infiltration into deep soil layers Shuster (2002). In our study decreased macropore formation upon Cr exposure explains changes in earthworm activities like restricted movement at higher concentrations resulting in altered burrowing behaviour.

Decrease in surface cast production by all the species upon exposure at the highest heavy metals concentration was evident which inversely correlates with the percentage of surface migration observed. This could be related to the observed decrease in burrow length and specifically for anecic species, reduced surface cast production could be an indicator of reduced burrowing activity. Studies of Lal et al. (2001) under field conditions observed that the use of treated okra seeds containing imidacloprid (normal application rate) resulted in a significant decrease in surface cast production.

The cast production is an important indication of earthworm activity and some studies have shown reduced cast production of earthworms (Cook et al. 1980; Lal et al. 2001) or reduced ingestion rates of earthworms after pesticide treatment (Gomez-Eyles et al. 2009). Capowiez et al. (2010) have found a significant decrease of cast production after exposure to imidacloprid, at concentrations as low as its application rate. The measurement of changes in cast production is a new and promising behavioural biomarker in ecotoxicology. It is of ecological relevance, since reduced cast production demonstrates a reduced (feeding) activity and in consequence might have indirect impacts on soils (Capowiez et al. 2010). In our study, cast production increased after exposure to the lowest concentration of Cr , but significant ($P<0.05$) decrease was observed for 20ppm onwards, while the exposures to higher concentrations of 300ppm Zn caused significant decreases for all the species.

In general, such biphasic dose responses (low-dose stimulation and high-dose inhibitory effect) are referred to as the phenomenon of hormesis and can often be observed in earthworms after exposure to environmental agents (Spurgeon et al. 2004; Hackenberger et al. 2008; Zhang et al. 2009). Other studies have observed reduced total or surface cast production after imidacloprid exposure for *L. terrestris*. Lavelle et al. (1989) observed four times higher cast production rate in the control groups in *A. caliginosa* compared to *L. terrestris*,and explained possibly be due to higher ingestion/ egestion rates of endogeic species. In this study, the increase in cast production after exposure to the lowest concentration might be explained by higher earthworm activity due to escaping or avoidance behaviour and/or an increased metabolic rate possibly caused by detoxification processes. Hence, developing eco-toxicological tests, involving earthworm behavior viz burrow length and cast production appears to be the most promising design in risk assessment of heavy metal pollution.

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