

Original Article

The impact of cow dung augmentation on soil restoration and bio-accumulation of metals (Lead and Cadmium) in *Pheretima posthuma* (Annelida: Clitellata)

O impacto do aumento de esterco de vaca na restauração do solo e bioacumulação de metais (chumbo e cádmio) em *Pheretima posthuma* (Annelida: Clitellata)

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Abstract

To investigate the role of cow dung in soil reclamation and bio assimilation along with bio accumulation of heavy metals in earthworm (*P. posthuma*) (N=900) earthworms were used and treatment groups of CD-soil mixture of different proportion of cow dung were designed. Nonlethal doses of lead acetate and cadmium chloride were added in treatment groups. Mature *P. posthuma* were released in each experimental pot maintaining the favorable conditions. The pH, carbon, nitrogen, phosphorus, exchangeable cations, and heavy metal level of each mixture was evaluated. The results indicated that bio-assimilation of Pb and Cd by *P. posthuma* were significantly ($P < 0.01$) higher in different soil-CD treatments compared to control. Highest bio-assimilation of both metals was observed in T¹ of both groups (Pb = 563.8 mg/kg and Cd = 42.95 mg/kg). The contents of both metals were significantly ($P < 0.05$) lowered in casting. The nutrient concentration in the final castings of all soil-CD treatments were also equally transformed from less or insoluble to more soluble and available for plants, except for carbon level which increased with CD proportion. It is concluded that cow dung as organic matter has a positive effect on soil reclamation and bio-assimilation of metals by *P. posthuma*.

Keywords: cow dung, bio-assimilation, heavy metals, soil reclamation, *Pheretima posthuma*.

RESUMO

Para investigar o papel do esterco de vaca na recuperação do solo e bioassimilação, juntamente com a bioacumulação de metais pesados em minhocas (*P. posthuma*) (N = 900), minhocas foram usadas e grupos de tratamento de mistura CD-solo de diferentes proporções de esterco de vaca foram projetados. Doses não letais de acetato de chumbo e cloreto de cádmio foram adicionadas aos grupos de tratamento. *P. posthuma* maduros foram liberados em cada vaso experimental, mantendo as condições favoráveis. Foram avaliados o pH, carbono, nitrogênio, fósforo, cátions trocáveis e nível de metais pesados de cada mistura. Os resultados indicaram que a bioassimilação de Pb e Cd por *P. posthuma* foi significativamente ($P < 0,01$) maior em diferentes tratamentos de solo-CD em relação ao controle. A maior bioassimilação de ambos os metais foi observada em T¹ de ambos os grupos (Pb = 563,8 mg / kg e Cd = 42,95 mg / kg). O conteúdo de ambos os metais foi significativamente ($P < 0,05$) reduzido na fundição. A concentração de nutrientes nas fundições finais de todos os tratamentos de solo-CD também foi igualmente transformada de menos ou insolúvel para mais solúvel e disponível para as plantas, exceto o nível de carbono que aumenta com a proporção de CD. Conclui-se que o esterco de vaca como matéria orgânica tem um efeito positivo na recuperação do solo e na bioassimilação de metais por *P. posthuma*.

Palavras-chave: esterco de vaca, bioassimilação, metais pesados, recuperação de solo, *Pheretima posthuma*.

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1. Introduction

Cow dung (CD) is undigested fecal matter of buffaloes and cows. With the increasing demand of cow husbandry, cow dung is also a major challenge to clean the environment. Few decades back, CD was utilized as mosquito repellent, burning fuel and as natural fertilizer in India (Gupta et al., 2016). Besides, CD is being used as food material in earthworm culturing and vermicomposting. Vermicomposting is the process by which epigeic earthworm species are used for conversion of organic wastes into vermicompost (Suthar et al., 2017). CD is used as feed for earthworm because it is easily and readily available during composting process. In the same way, CD was mixed in sewage sludge as source of organic food for vermicomposting (Gupta et al., 2016).

Heavy metals like lead (Pb) and cadmium (Cd) are widely distributed in the terrestrial and aquatic environments from various anthropogenic sources (Selvi et al., 2019). These metals are assimilated in body tissue of earthworms by ingesting contaminated soil (Pattnaik and Reddy, 2011). After absorption, heavy metals combine with enzymes and inhibit the proper function of enzymes (Duruibe et al., 2007). This may cause severe physiological and neurological consequences in human (Žaltauskaitė and Sodienė, 2010). Pb pollution is 10% of total pollution caused by heavy metals because it is abundantly utilized in lead-acid batteries, gasoline alkyl addition, building construction, paints, cable coating, petrol refining and others (Tchounwou et al., 2012). Pb also exerts negative effects on both growth and leaf expansion of plants (84). High concentration of Pb can damage liver, lungs, kidney, digestive tract and nervous system of humans by changing the normal metabolic processes (Jaishankar et al., 2014).

Like Pb, Cd is extremely important with respect to its toxicity, high mobility and water solubility (Duruibe et al.,

2007). The major sources of Cd are fertilizers, Ni-Cd batteries, paint, PVC plastics, fungicides, insecticides, and sludge. Cd concentration is very high in the crops and grains grown in the polluted areas and irrigated with polluted water (Hussain et al., 2019). After absorption, metals like Cd are mainly accumulated in the liver, gall bladder, muscles, thyroid, lungs, kidney, pancreas, and other tissues (Tchounwou et al., 2012).

Earthworms act in the soil as aerators, grinders, crushers, chemical degraders, and the biological stimulators (Sharma et al., 2017a). These worms play significant role in soil restoration process to increase fertility and remove soil contaminants (Butt, 2008). *Pheretima posthuma* (Kinberg, 1867) belongs to the group of epigeic earthworms that extensively found along various crop fields in Pakistan (Sial et al., 2017). Concentration of metal accumulation in earthworm body tissues is concerned with the bioavailable level of heavy metal within soil (Liu et al., 2012). Different environmental factors are correlated with bioaccumulation of heavy metals such as temperature, pH, humidity, and organic matter contents (Sial et al., 2017). With all this background, this current study has been designed to investigate the role of cowdung in soil reclamation with earthworm (*P. posthuma*) by bio-assimilation and its effect on bioaccumulation of heavy metals (Pb and Cd).

2. Materials and Methods

2.1. Sample organism, soil and cowdung

Earthworms (as sample organism) were collected at the depth of 30-50 cm, during August-October of 2019, from the Maralah Ravi Link (32°22'25.4"N 74°24'54.9"E) near Motra Canal Bridge Daska Pakistan, using digging method (Figure 1). Soil samples were also obtained from same site

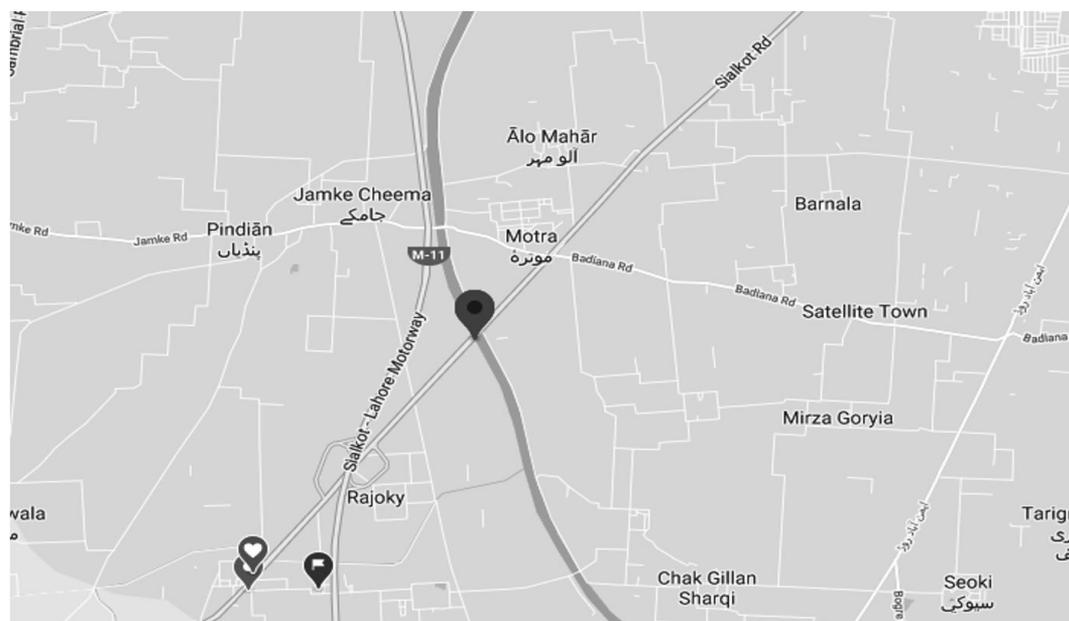


Figure 1. Sampling site near Daska Sialkot.

at same depth, and time. To form soil-CD mixture, cow dung samples were collected from dairy farms in Daska city, Pakistan.

2.2. Experimental design

The experiment was performed in two groups each group divided into further four treatments *i.e.* T¹, T², T³, T⁴ and control group (T^c) without any CD dosage (Suthar and Singh 2009). Fifteen plastic spherical pots (10 cm x 10 cm) were utilized for each group and each pot consist of 250 g soil-CD mixture with different CD (%) proportions such as T¹ (2%), T² (4%), T³ (6%), T⁴ (8%) and T^c (0%) (Kumar et al., 2008). Soil-CD mixture and metals were taken in plastic pots as shown in Table 1. Double distilled water was added, yielding moisture of ~50-70% and was kept constant throughout the experiment by spraying (Suthar and Singh, 2009). Prior to adding earthworm, all the experimental pots with soil-CD mixture were placed in shady area for four weeks. Mature *P. posthuma* worms ($n = 10$, \approx weight 220 g) were released in pots with porous lid and kept in dark (~27-37 °C, 50-70% moisture content and pH ~6.0-7.5) throughout the experiment composting period (Eltejani and Abdel, 2017).

After 28 days metal exposure, the earthworms gut content (Pattnaik and Reddy, 2011) and castings were collected (Hidalgo and Harkess, 2002). Gut cleared earthworms were then placed in beakers and dried in a TK/L 4105 (EHRET) at 60 °C for 48 hours and weighed. Dry earthworms were then digested following the method by Wang et al. (2018). Samples were then diluted, centrifuged and subjected to flame atomic absorption spectrophotometer (FAAS) analysis in High Tech Laboratory, University of agriculture Faisalabad following the method of (Zhang and Reynolds, 2019). Like earthworm tissues, 1 g each of soil, cow dung, soil-CD mixture and castings of different treatments were also analyzed to check metal concentrations through FAAS.

Nutrient content analysis of homogenized substrate and cow dung soil sample was done following the method of Al-Busaidi et al. (2005), Yoon et al. (2018), Horta and Torrent (2007) and Le Roux et al. (2016). Bio-assimilation factors BAF was calculated following Mountouris et al. (2002).

2.3. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the significant difference between cow dung treatments of initial substrates, earthworm tissues and

earthworm casting. Duncan's Multiple Range Test (DMR) was applied to compare the means of different cow dung treatments.

3. Results

At the beginning, metal concentrations in initial substrate were analyzed before adding the Pb and Cd concentration. The concentrations of Pb and Cd in soil are shown in Table 2. These non-lethal metal levels were non-significant and statistically similar for Pb ($F = 0.0681$, $P > 0.05$) and Cd ($F = 0.2901$, $P > 0.05$) in all initial substrates for different cow dung treatments. Following incubation period of four weeks, in tissues of *P. posthuma*, the concentration of Pb against different Pb polluted soil-CD treatments were found highly significant ($F = 9.201$, $P < 0.01$) as shown in column 4 of Table 2. The concentration of Pb, after vermicompost, declined in all treatments. According to DMR analysis, T^c, T¹ and T² showed significantly lower metal level as compared to T³ and T⁴.

The content of Pb in earthworm casting was decreased compared to initial soil-CD mixture which assured that it must be assimilated in earthworm tissues as shown in Table 2.

The ranges of BAFs for Pb and Cd metals were recorded between 0.5235-0.6246 and 0.4864-0.6642 respectively. These higher values of BAFs described the bioavailability of Pb and Cd in all treatments for *P. posthuma*. But it was observed highest in T¹ treatments for both Pb and Cd, 0.6246 and 0.6642 respectively as shown in Figure 2.

The results regarding to nutrient contents in different soil-cow dung treatments are summarized in Table 3.

4. Discussion

In the present study, analysis of Pb and Cd concentrations in initial substrate indicated the presence of these metals in soil and CD samples. Additional doses of Pb and Cd concentrations in all treatments were found non-lethal as observed by no death throughout the experimental period. These tolerable values for earthworms were comparable to Pb < 2500 and Cd < 100 mg/kg (Kumar et al., 2008; Zhang and Reynolds, 2019). In regarding to heavy metal toxicity, literature proved the earthworms have a capability to tolerate bioavailable metals because of physiological familiarization and genetic variability (Nirola et al., 2016).

Table 1. Soil-CD mixture proportions contaminated with Pb and Cd.

Treatments	%age of S+CD	Soil (g)	CD (g)	Soil-CD Mixture (g)	Group I Pb (mg)	Group II Cd (mg)
T ^c	S (100%) + CD (0%)	250	0	250	450	20
T ¹	S (98%) + CD (2%)	245	5	250	450	20
T ²	S (96%) + CD (4%)	240	10	250	450	20
T ³	S (94%) + CD (6%)	235	15	250	450	20
T ⁴	S (92%) + CD (8%)	230	20	250	450	20

Note: T^c (Control treatment); T¹ (Treatment 1); T² (Treatment 2); T³ (Treatment 3); T⁴ (Treatment 4); S (soil); CD (Cow dung); Pb (Lead); and Cd (Cadmium).

Table 2. Comparison of Pb and Cd concentrations in initial substrate, *P. posthuma* casting and tissue.

Treatments ^r	Pb in I.S	Pb in E.C	Pb in E.T	CD in I.S	CD in E.C	CD in E.T
T ^c	904.55 ± 3.41 ^a	378.5 ± 9.98 ^a	481.0 ± 19.81 ^b	63.44 ± 2.2 ^a	25.21 ± 1.5 ^b	30.86 ± 1.16 ^b
T ¹	902.06 ± 4.38 ^a	331.4 ± 10.34 ^b	563.4 ± 11.21 ^a	64.66 ± 1.02 ^a	27.38 ± 1.11 ^b	42.95 ± 1.72 ^a
T ²	903.64 ± 2.85 ^a	369.5 ± 12.72 ^a	501.8 ± 13.92 ^b	64.25 ± 0.37 ^a	26.97 ± 2.2 ^b	34.19 ± 1.85 ^b
T ³	903.20 ± 3.01 ^a	388.6 ± 19.81 ^a	484.8 ± 19.78 ^b	63.96 ± 1.76 ^a	31.43 ± 1.4 ^a	33.63 ± 1.41 ^b
T ⁴	902.72 ± 2.99 ^a	392.4 ± 12.11 ^a	472.6 ± 18.78 ^b	64.74 ± 1.01 ^a	30.15 ± 1.19 ^a	32.14 ± 1.57 ^b
ANOVA						
F value	0.068	5.052	9.201	0.29	9.213	4.228
P value	0.99	0.011	0.002	0.877	0.002	0.029
Significance	N.S	**	**	N.S	**	*

^rRefer to the Table 1 for description. All values represent mean (mg/kg) ± SD of triplicates. I.S (Initial substrate); E.C (Earthworm casting); and E.T (Earthworm tissue). Superscript a and b letters indicate comparison of mean values by Duncan's Multiple Range Test. If P > 0.05 = Non-significant (N.S), P < 0.05 = Significant (*) and P < 0.01= highly significant (**). Superscripts c, 1, 2, 3, and 4 showed the control and number of treatments respectively.

Table 3. Nutrients analysis in initial substrate and final castings after earthworm processing.

Treatments ^r	pH		C _{organic} (g/kg)		N _{total} (g/kg)		C/N ratio	
	IS	FC	IS	FC	IS	FC	IS	FC
T ^c	7.55 ± 0.09	6.9 ± 0.12	285.43 ± 0.52	235.43 ± 0.72	16.43 ± 0.40	25.87 ± 0.23	14.32 ± 0.20	9.10 ± 0.34
T ¹	7.70 ± 0.11	7.3 ± 0.17	295.85 ± 0.23	245.85 ± 0.61	18.98 ± 0.36	30.98 ± 0.30	12.95 ± 0.34	7.93 ± 0.46
T ²	7.16 ± 0.13	7.5 ± 0.50	310.86 ± 0.45	259.18 ± 0.49	21.34 ± 0.56	37.67 ± 0.49	12.14 ± 0.41	6.88 ± 0.50
T ³	7.55 ± 0.19	7.6 ± 0.15	321.72 ± 0.32	268.57 ± 0.71	25.78 ± 0.69	40.31 ± 0.56	10.41 ± 0.59	6.66 ± 0.41
T ⁴	7.32 ± 0.08	7.2 ± 0.13	330.21 ± 0.61	278.71 ± 0.32	28.61 ± 0.82	32.65 ± 0.182	9.74 ± 0.53	8.53 ± 0.25
Treatments ^r	P _{Bio-a} (g/kg)		K ⁺ _{Exch} (g/kg)		Ca ²⁺ _{Exch} (g/kg)		Mg ²⁺ _{Exch} (g/kg)	
	IS	FC	IS	FC	IS	FC	IS	FC
T ^c	8.45 ± 0.15	10.7 ± 0.12	3.25 ± 0.11	7.28 ± 0.08	18.85 ± 0.15	20.23 ± 0.18	23.1 ± 0.34	30.73 ± 0.29
T ¹	10.20 ± 0.20	15.65 ± 0.15	5.85 ± 0.15	9.25 ± 0.21	26.45 ± 0.25	29.15 ± 0.13	21.45 ± 0.25	34.32 ± 0.33
T ²	15.95 ± 0.12	20.5 ± 0.16	7.71 ± 0.17	12.05 ± 0.27	29.34 ± 0.37	32.35 ± 0.37	27.31 ± 0.27	30.19 ± 0.48
T ³	24.80 ± 0.09	28.65 ± 0.19	9.65 ± 0.21	19.45 ± 0.22	35.25 ± 0.11	40.41 ± 0.29	25.45 ± 0.41	33.51 ± 0.49
T ⁴	29.25 ± 0.14	35.85 ± 0.18	10.45 ± 0.19	17.35 ± 0.46	40.23 ± 0.23	46.15 ± 0.36	28.31 ± 0.63	32.53 ± 0.62

Refer to the Table 1 for description. All values showed mean ± SD of 3n, Where IS (initial substrate) and FC (Final casting).

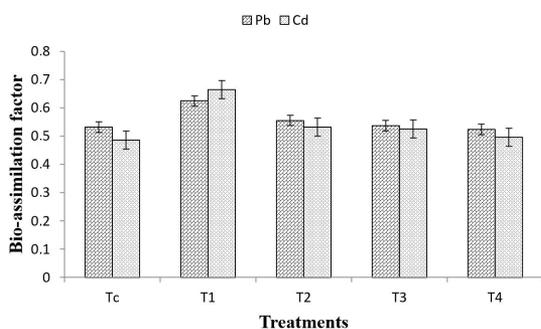


Figure 2. Bio-assimilation factor of Pb and Cd in all treatments.

The findings of this study clearly revealed that cow dung showed significant effect on bio-assimilation of Pb and Cd in the *P. posthuma* tissues during composting or remediation process. Previous studies reported that the effect of organic material enhanced the uptake of the metal concentration due to amendment of poultry, pig and cow manure as organic waste (Li et al., 2010). Likewise, heavy metal bioaccumulations in the tissues of *Pheretima* genera of earthworm were also reported (Muhammad et al., 2010). The use of cow dung as an additional dose to observe its effect on bioaccumulations/ bioassimilations of available heavy metals (Zn, Pb and Cd) in earthworm tissues (*L. violaceus*) was generally low and time dependent (Dada et al., 2016). These variations in

results might be due to different species (Nannoni et al., 2014), metal bioavailable concentrations in substrates (Liu et al., 2017) and metal physiological tolerance or ecological habitat (Demuyne et al., 2014). The positive effect of cow dung on bio-assimilation of heavy metals (Pb-Cd) from environment and aqueous solution was due to its absorbent property with metals ions (Ojedokun and Bello, 2016; Vijver et al., 2003).

It was also observed that *P. posthuma* had more Pb than Cd showing higher availability of Pb in soil-CD mixture compared to Cd. But, Cd accumulation in earthworm (*Eudrilus eugeniae*) tissues was more than that of Pb which was opposite to present study (Pattnaik and Reddy, 2011). This change in bio-assimilation and uptake of heavy metals by earthworm tissues could be due to exposure route or particle size of amendments, dietary habits (Vijver et al., 2005) and behavioral adaptations (Pattnaik and Reddy, 2011; Wang et al., 2018). Furthermore, ingestion of any organic matter containing metals is big source of metal uptake by earthworms (Nannoni et al., 2014). Meanwhile, small sized cysteine rich proteins (i.e. metallothioneins) found in tissues bind metal elements, which were regulated and eliminated by earthworms (He et al., 2016).

Pb and Cd concentrations in earthworm casting or vermicompost were reduced due to bio-assimilation of these metal elements in *P. posthuma* tissues. In a same way, some researcher reported the reduced level of heavy metals (Pb and Cd) in worm casting in relation to the parent mixtures (Kujawska and Wójcik-Oliveira, 2019). As the organic waste mixed in soil passing through elementary tract of earthworms, where the soluble form of heavy metals assimilated and increased in worm's tissues which ultimately decrease the metal concentration in worm's casting (Pattnaik and Reddy, 2011). These results also collaborate with the findings of previous works which indicated the capabilities of earthworms in eliminating bioavailable metals in industrial waste (Azizi et al., 2013).

The values of bio-assimilation factor (BAF) in all soil-CD treatments suggested that both Pb and Cd were greatly assimilated in body tissues of earthworm *P. posthuma* while it was highest in T¹ treatments. These values of BAFs also indicated that the bioavailability of Pb and Cd were higher in *P. posthuma* tissues and lowered in soil-CD (Suthar and Singh, 2009). This reduction in bioavailable metals in soil-CD was due to assimilation in *P. posthuma* during composting process through two physiological adaptations in earthworm's cellular tissues: first, binding of these metals with nuclear proteins; second, increase in production level of special binding proteins, metallothionein, within earthworm chloragogenous tissues (Singh and Kalamdhad, 2016).

As the concentrations of CD increased, in soil-CD treatments, the BAFs values were decreased in all treatments (except T¹) due to formation of organometallic complexes (CD as organic source) which reduce the uptake of heavy metals (Singh and Kalamdhad, 2013). However, the BAFs values examined in this work were decreased compared to previous workers (Wang et al., 2018). This BAFs values difference might be due to varies concentrations of the metals (Hobbelen et al., 2006), exposure period (Pattnaik and Reddy, 2011), habitat variations, food

preferences (Quenea et al., 2009), metabolic physiology and earthworm species specificity (Wu et al., 2020). The high levels of BAFs for Pb and Cd metals were not only helpful in soil reclamation but could also be a big threat due to the entrance of toxic metals in the food chain via earthworms, if inoculated earthworms are not properly managed.

The nutrient level of processed soil contents indicate that earthworm have potential role in soil reclamation and soil structure (Sharma et al., 2017b). Similarly, Literature revealed that earthworm played a significant role in recycling and transformation of nutrients by changing soil biological, chemical and physical properties. In this work, the nutrient available carbon contents, CD as additional organic carbon source, in final substrate was reduced by vermicomposting activity. The findings were parallel with results of the worm composting in sewage sludge (Suthar and Singh, 2009) and green weeds substrate (Singh and Kalamdhad, 2013). This is because, most of the carbohydrates (i.e polysaccharides) were metabolically assimilated in the *P. posthuma* body from substrates and some were loss as CO₂ in atmosphere due to microbial respiration by decomposers (Liu et al., 2012). The data of current study indicated that the metabolic activities in earthworm body enhance the formation mineral nitrogen in soil. As the earthworm intake soil, containing plant's particles, microbial flora and organic-mineral aggregates, the digestion and metabolism of these contents increase the potential level of mineral nitrogen in composted substrate (Araujo et al., 2004). Similarly, the composted soil-CD also showed a reliable increase in level of available plant's nutrients such as phosphorus and exchangeable ions (K¹⁺, Ca²⁺ & Mg²⁺). It was found that the level of total minerals in all treatments was increased during after vermicomposting. These findings agreed with the results of many pervious works (Hait and Tare, 2011). As the nutrients like inorganic nitrogen, phosphorous and exchangeable cations are required in very small amount for metabolic activities and body maintenance in earthworm, so most of the mineral contents released through undigested matter (Aira et al., 2007). The engulfing process of earthworms, occurrence of numerous types of microbial communities and enzymes during substrate composting transformed the insoluble large plants contents into more available and soluble forms, thereby enhancing their level in the composted substrate (Singh and Kalamdhad, 2013).

5. Conclusion

The study of CD effect on soil reclamation and bio-assimilation of heavy metals (i.e. Pb and Cd) showed that it has positive effect in all treatments, as organic material bind dissolve metal ions and enter in earthworm body through digestive route. The addition of cow dung in soil enhance the assimilation of heavy metals (Pb and Cd) in all treatments and highest in CD^a. For soil reclamation, it was concluded that carbon contents were reduced in composted soil due to absorption and consumption in earthworm body while all other available and exchangeable

mineral cations were increased in castings, as these are required in small quantity.

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