Int. J. Environ. Res., 9(1):35-42,Winter 2015
ISSN: 1735-6865

The Effects of CuO and ZnO Nanoparticles on Survival, Reproduction, Absorption, Overweight, and Accumulation in *Eisenia fetida* Earthworm Tissues in Two Substrates

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Received 4 Dec. 2013; Revised 11 July. 2014; Accepted 16 Oct. 2014

ABSTRACT: This study aimed to investigate the effects of tow nanoparticles (NPs) on the absorption, accumulation, reproduction of the *Eisenia fetida*. A 4×2×2 factorial experiment in a randomized complete block design with three replications was conducted. The factors included in the experiment are the content of NPs at four levels (0, 0.4, 0.8, and 1.2gr/kg weight of substrate), the type of NPs at two levels (CuO and ZnO) and the type of substrates at two levels (cow manure and spent mushroom compost (SMC)). After preparing the substrates, adult worms were added to each pot that has 6kg of each type of substrates. The aqua solution of NPs was added to the cultures. The NPs effects on the overweight, mortality, accumulation and reproduction were measured at the end of seven- and fourteen-day two periods after exposure to NPs. The earthworm tissues were enhanced with increasing the NPs concentration. The increases were more in cow manure than SMC, and the accumulation of ZnO were more than CuO. The number of the earthworm egg decreased in both 7th&4th days through increasing the NPs concentration. The ZnO were more effective in reducing the reproduction than the CuO in the seventh day after the substrates treatment. Overweight decreased in both seventh and fourteenth days through increasing the NPs concentration. Nano CuO was more effective in reducing the overweight than nano ZnO, The decrease of the worm tissues in the SMC substrate was more than the cow manure substrate in the fourteenth day after the treatment

Key words: Compressive strength earthworms, Cow manure, Eisenia foetida, Nanoparticles, SMC

INTRODUCTION

The production and use of nanoparticles (NPs) are rapidly growing; therefore, the NPs may be released into the environment intentionally or unintentionally. Anyhow, this causes undesirable effects on the environment. Recent studies have indicated that NPs can induce toxicity effects on the aquatics and rodents, but many of their effects are still unknown (UNESCO, 2006). Therefore, further studies are needed to investigate the toxicity effects of NPs on human and environment (Roco, 2005). In this context, copper oxide (CuO) and zinc oxide (ZnO) NPs are more remarkable due to their wide applications. Copper oxide (CuO) NPs are one of the most important nano metals which are commercially produced and are used in various industries. For example, CuO NPs have been used as anode materials of the lithium ion batteries due to their good electrochemical properties, high rate of effectiveness, low cost, and recyclability (Wang *et al.*, 2009). Also, it can be used as an additive in oil, plastic/ polymer, metallic coating, and ink (Bouvy *et al.*, 2007; Linand Xing, 2007). Recently, ZnO NPs have attracted more attention due to their vast applications such as in UV light emission, piezoelectric properties, photocatalitic activity, antibacterial and deodorizing properties. Also, nano ZnO can be used in sensors, solar cells, laser diodes, and transparent electrodes. Heat and radiation resistance are two factors which expand the ZnO NPs applications under the intense conditions such as those with high frequency and temperature (Bouvy *et al.*, 2007; Lin and Xing, 2007).

Compost is used as a substrate for the production of mushrooms. Because fattening of compost decrease after harvesting the mushrooms therefore, called spent mushroom compost (SMC). The SMC has a high capacity for holding water and nutrients. Usually, the compost is discarded as waste but, it can be used for different applications (Uzun, 2004). Earthworms are

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used as biological indicators of the environmental metal pollution. Many investigations have been carried out on this terricolous organism. The *Eisenia fetida* species is resistant to the changes of environmental conditions and has a high alimentation and reproduction rate (Coleman *et al.*, 2010). Edwards studies showed that maximum reproduction rate of *Eisenia fetida* in animal wastes is 3.8 cocoons per adult worm per week (Edwards, 1995). In this regard, Reinecke et al. reported 0.35 cocoons per adult worm per week (Reinecke *et al.*, 1992). *Eisenia fetida* produces 900 egg capsules per worm per year (Hashemi Majd, 2003). Each worm of this species produces an egg capsule every seven to ten days and there are two to twenty eggs in each capsule (Smith, 1998). The compost temperature and humidity are the most important environmental factors affecting the growth and development of this earthworm (Edwards, 1995). The proper humidity is 70 to 80% (Hashemi Majd, 2003). Villem et al. studied the toxicities of CuO, ZnO and titanium dioxide (TiO2) NPs on alga *Pseudokirchneriella subcapitata* and showed that NPs affected the growth of the alga and NPs that induced maximum toxicities on the algae are in the sequence above (Aruojaa *et al.*, 2009). The investigations carried out by Mindy et al. on the effects of perchlorate in *Eisenia fetida* showed that the survival and the cocoon production decrease with increasing the perchlorate concentration. The survival and hatchability of perchlorate-exposed earthworms are less than the unexposed ones (Landrum *et al.*, 2006).

The study of the toxicity effects of carbon double-walled nanotubes (DWNT) on the *Eisenia Veneta* earthworms showed that the reproduction of worms are affected by the high concentration of DWNT (above 37 mg DWNT/kg food) ;however, the hatchability, survival, and mortality are not influenced (Scott-Fordsmand *et al.*, 2008). Hu *et al.* studied the toxicity effects of ZnO and TiO2 NPs on *Eisenia fetida* and showed that the toxicity and accumulation increase when the NPs concentration goes up. In addition, the toxicity effects of ZnO NPs are more than TiO2 (Hu *et al.*, 2010). Another study investigated the effects of nano aluminum oxide (Al2O3) on *Eisenia fetida* and its results indicated that the size and concentration of Al2O3 can be effective in the accumulation and the reproduction of worms (Coleman *et al.*, 2010). The study of the C60 NPs effects on the *Lumbricus rubellus* earthworms showed that the mortality, growth rate, and reproduction of the juvenile earthworm populations are more affected than the adult worms. Therefore, with increasing the NPs concentration, the growth rate and reproduction of the juvenile populations decrease; however, their mortality increases (Van Der Ploeg *et al.*, 2011). Griffitt *et al.* investigated the effects of the metallic NPs on the aquatic organisms and found that nano copper and nano silver induce toxicity in all the treated organisms. However, TiO2 NPs do not induce any toxicity in any of them (Griffitt *et al.*, 2010). Studies carried out by Tian et al. on the toxicity effects of ZnO NPs on the zebrafish embryos indicated that nano ZnO causes the decrease of the antioxidant enzymes activity of the catalase and super oxidase dismutase and the inhibition of glutathione in the zebra fish embryo (Wen Jing *et al.*, 2010). Atiyeh *et al.* reported that the *Eisenia andrei* earthworm activity enhances the decomposition rate and the stabilization of cow and pig manure. In turn, the improvement of the manure biochemical characteristics is desirable for plant growth (Atiyeh *et al.*, 2000a; 2000b). Hermands *et al.* used bovine manure as the substrate for the *Eisenia fetida* (Ramirez *et al.*, 1999).This study is designed to investigate the effects of CuO and ZnO NPs on the *Eisenia fetida* earthworm in two substrates.

MATERIALS & METHODS

This study was conducted in the laboratory of Tarbiat Modares University. A 4×2×2 factorial experiment in a completely randomized block design with three replications was conducted. The factors included in the experiment are the contents of NPs at four levels (0, 0.4, 0.8 and 1.2 gr/kg weight of substrate), the type of NPs at two levels (CuO and ZnO), and the type of substrates at two levels (cow manure and spent mushroom compost (SMC)). The *Eisenia fetida* earthworms, completely rotted cow manure, and the SMC were obtained from this university. Table 1 shows some properties of the cow manure and SMC that were used in this study. The SMC contained straw, poultry manure, molasses, and urea. CuO NPs were obtained from Neutrino Company and ZnO NPs were purchased from the Institute of Petroleum Industry. Table 2 shows the properties of these NPs. Before starting the experiment, we needed to prepare the substrates. For this reason, the completely rotted and screened (through a 3mm mesh sieve) cow manure was poured into 17kg pots and then completely submerged to remove the leachate from the bottom of the pots. This operation was performed for one week. The SMC compost was placed on the floor of the rest room so that it was aerated and watered for 10 days to remove ammonia and salt in order to prevent the mortality of worms. Then, the SMC was poured into the pots. Totally, 48 pots (24 pots of each substrate) containing about 6 kg of the substrates were prepared. Fine mesh was created at the bottom of all the pots for running the excess water out. Also, we placed cloth screen to prevent the earthworms from crawling out. Then, 30 of the adult worms with an approximate weight of 500 to 800 mg which their Klitelium ring had been formed were added to each pot. The earthworms were kept at the 20 to 30°C temperature and the humidity of 60-80% for one week to get used to the new conditions. In this period, the humidity of the substrates was controlled through spraying water over them. After one week, 0.0, 0.4, 0.8, and 1.2 gr/kg weight of the substrate of CuO and ZnO NPs were solved in 40 cc of water and then added to each substrates. The worms were kept in this condition and at 24±2°C temperature and the humidity of 70% for one week. Then, the substrates were screened through a 6mm mesh sieve and the number of the worms was counted. The earthworms were separated from the substrates and were placed in the mesh basket. Then, they were washed with water to remove the adhering substrates from their bodies and the weight of worms was measured using a digital balance. After screening substrates through a 6mm mesh sieve two times, they were screened through a 3mm mesh sieve as well and the number of cocoons was counted. To measure the content of the accumulated NPs in the earthworm tissues, 10 worms completely random were completely randomly selected from each pot and were placed into petri dishes with wet paper filters for 24 h. After that the stomach contents were emptied into a petri dish. The weight of worms was measured while they were kept at the temperature of -20 °C. The accumulated NPs in the worm tissues were measured in the veterinary faculty of Tehran University. So, the amount of 0.5 gr of the sample was solved in a 10 ml of nitric acid and were put into a 25 mL volumetric flask and then diluted. After that, Cu and Zn elements were measured using ICP-OE (JOBIN ULTIMA2). These steps were repeated to re-measure the traits of the earthworm in the second week after the exposure to NPs. The data were statistically analyzed using the SAS software (GLM procedure). Also comparisons between the mean values were carried out through the Duncan´s test (α=%5).

RESULTS & DISCUSSION

The results obtained from the analysis of covariance showed that there was no significant relationship between the contents of the accumulated NPs in the tissues of earthworms and the weight of worms after seven days. Similar results were obtained after fourteen days. The results also indicated that there was a significant difference among singlet, doublet and triplet reciprocal effects ($P<0.01$) (Table 3). The comparison between the mean values showed that the absorption and accumulation in the earthworm tissues was enhanced when the NPs concentration went up after seven and fourteen days (Figs. 1 and 2). The accumulation of NPs in the worm tissues in cow manure was more than the SMC that may be due to favorable nutrition for worms in this substrate. In addition, the accumulation of nano ZnO was more than nano CuO in both of the substrates. The results of the variance analysis of the produced egg capsules by 30 worms per week indicated that the effects of content and type of NPs were significant at the 1% and 5% levels of confidence, respectively (Table 4). The results of the comparison between the mean values showed that the number of the earthworm egg capsules decreased when the NPs concentration rose in the seventh day. In addition, ZnO NPs were more effective in reducing the reproduction than CuO (Figs. 3 and 4). The results of the variance analysis of the produced egg capsules by 20 worms in fourteen days after the substrates treatment indicated that the content of NPs in earthworms were significantly different at $P<0.01$. However, there were not significantly different in other traits. The comparison between the mean values showed that the number of the earthworm egg capsules decreased with increasing the NPs concentration in the fourteenth day (Fig. 5).

Figs. 3, 4, and 5 indicate that CuO and ZnO NPs are effective on the reproductive system and cause the decrease of the reproduction. The results obtained from the variance analysis of the overweight of 30 worms showed that the effects of content and type of NPs were significant at the 1% and 5% levels of confidence, respectively. The comparison between the mean values showed that the overweight of 30 earthworms decreased with increasing the NPs concentration (Fig. 6). In addition, ZnO NPs were more effective in reducing the overweight than CuO (Fig. 7).

The results obtained from the variance analysis of the overweight of 20 worms showed that the effects of content and type of NPs as well as the type of the substrates were significant at the 1% level of confidence. However, the reciprocal effects of the content and type of NPs were significant at the 5% level of confidence (Table 5). The comparison between the mean values indicated that the overweight decreased with increasing the NPs concentration and CuO NPs were more effective in reducing the overweight than ZnO (Fig. 8). Such a decrease in was more in the SMC than cow manure that may be due to the favorable nutrition for worms in the cow manure. Dominguez *et al*. reported that the mean values of the daily weight of earthworms depend on the aggregation of population and the type of nutrients (Dominguez and Edwards, 1997**;** Dominguez *et al*., 2000).

Table 1. Some properties of used cow manure and SMC

Properties	Cow manure	SMC
EC dS/m	12.5	12.37
pH	7.5	7.6
Na^+/ppm	62	392
K^+/ppm	1700	2500
Ca^{++}/ppm	3400	36
Cu^{++}/ppm	31	18
Zn^{++}/ppm	95	*192*
O.C%	15.9	16.61

O.C% refers to the percentage of organic carbon.

Table 2. Some Properties of used CuO and ZnO NPs

Properties	CuO	ZnO
%Purity	%98	%99
Particles mean size	<60 nm	<50 nm
specific surface area (SSA)	>80 m^2/g	>80 m^2/g
Color	Black	White

Table 3. Variance analysis (mean squares) of CuO of ZnO NPs effects and substrates on accumulation of NPs in earthworm tissues

Mean squares			
Accumulation in the fourteenth day (ppm)	Accumulation in the seventh day (ppm)	Degree of freedom	Source of variation
7.41 ns	2.45 ns	2	Replication
754.24 **	701/98 **	3	A factor (NPs)
105.02 **	48.00 **	1	B factor (Substrate)
1668.52 **	1752.08 **	1	C factor (Types of NPs)
214.57 **	196.01 **	3	A×B reciprocal effect
28.85 **	29.04 **	3	A×C reciprocal effect
88.02 **	29.04 **	1	B×C reciprocal effect
29.68 **	23.95 **	3	A×B×C reciprocal effect
5.78	2.13	30	Experimental error
13.25	8.16	-	Coefficient of Variation (%cv)

**,* and ns are significant at the 1% and 5% levels of confidence and no significant, respectively.

Table 4. Variance analysis (mean squares) of CuO of ZnO NPs effects and substrates on numbers of earthworm egg capsules

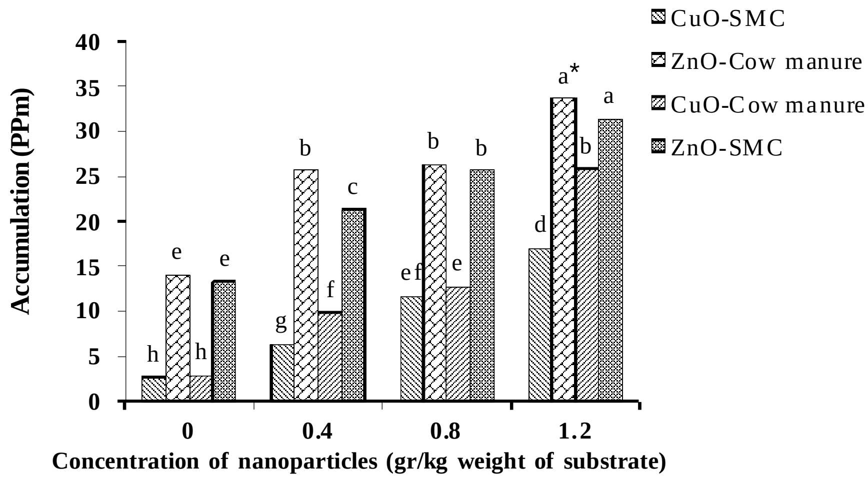
Mean squares			
The numbers of egg capsules (fourteenth day)	The numbers of egg capsules (seventh day)	Degree of freedom	Source of variation
5.81 ns	5.89 ns	2	Replication
840.18 **	1793.72 **	3	A factor (NPs)
1.68 ns	0.08 ns	1	B factor (Substrate)
38.52 ns	52.08 *	1	C factor (Types of NPs)
11.85 ns	6.47 ns	3	A×B reciprocal effect
10.02 ns	4.13 ns	3	A×C reciprocal effect
1.02 ns	12.00 ns	1	B×C reciprocal effect
8.18 ns	9.05 ns	3	A×B×C reciprocal effect
11.99	7.91	30	Experimental error
20.14	12.74	-	Coefficient of Variation (%cv)

**,* and ns are significant at the 1% and 5% levels of confidence and no significant, respectively.

Table 5. Variance analysis (mean squares) of CuO of ZnO NPs effects and substrates on overweight of earthworms

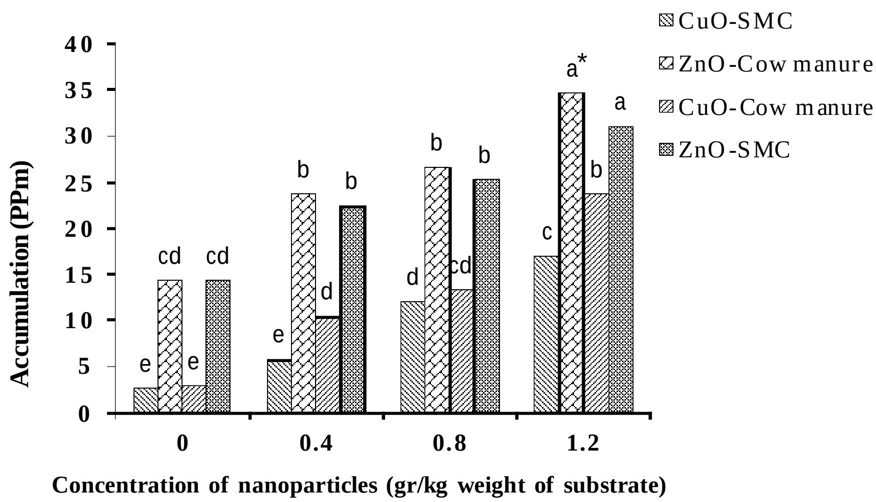
Mean squares			
Overweight (gr) in 14th day	Overweight (gr) in seventh day	Degree of freedom	Source of variation
0.003 ns	0.055 ns	2	Replication
0.075 **	0.249 **	3	A factor (NPs)
0.060 **	0.063 ns	1	B factor (Substrate)
0.065 **	0.308 *	1	C factor (Types of NPs)
0.018 ns	0.146 ns	3	A×B reciprocal effect
0.031 *	0.025 ns	3	A×C reciprocal effect
0.005 ns	0.029 ns	1	B×C reciprocal effect
0.002 ns	0.125 ns	3	A×B×C reciprocal effect
0.007	0.054	30	Experimental error
13.445	20.285	-	Coefficient of Variation (%cv)

**,* and ns are significant at the 1% and 5% levels of confidence and no significant, respectively.



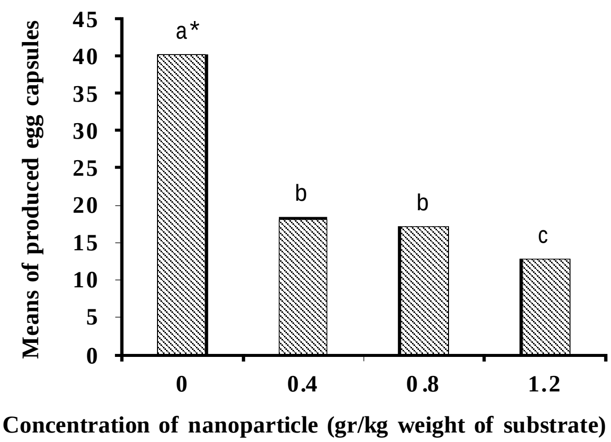
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 1. Comparison between means of accumulation of CuO and ZnO NPs in E.*foetida* in the seventh day after treatment



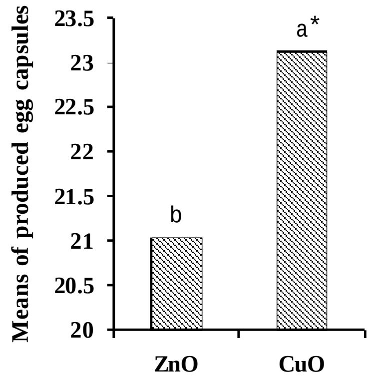
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 2. Comparison between means of accumulation of CuO and ZnO NPs in E.*foetida* in the fourteenth day after treatment



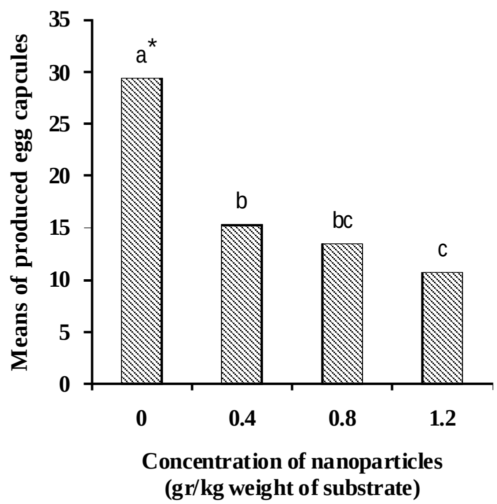
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 3. Comparison between means of produced egg capsules in two substrates (cow manure and SMC) in the seventh day after treatment with NPs



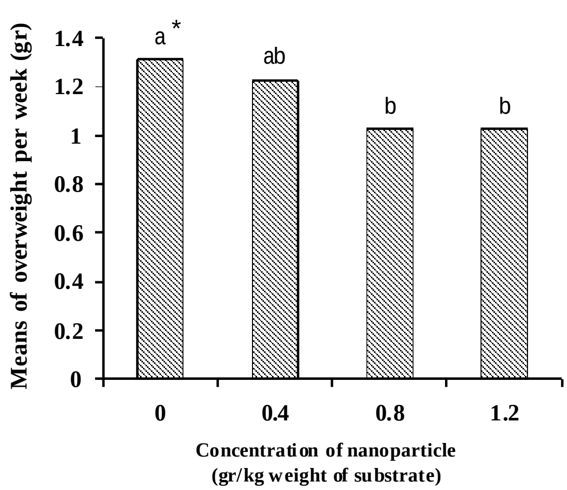
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 4. Comparison between means of produced egg capsules in two substrates (cow manure and SMC) in the seventh day after treatment with CuO and ZnO NPs



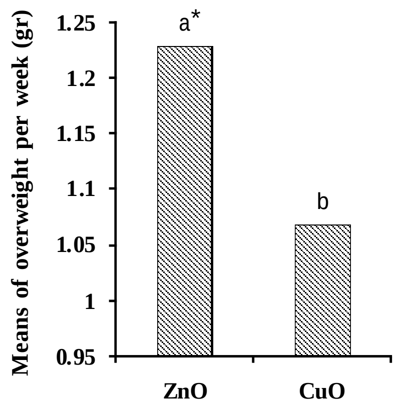
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 5. Comparison between means of produced egg capsules in two substrates (cow manure and SMC) in the fourteenth day after treatment with NPs



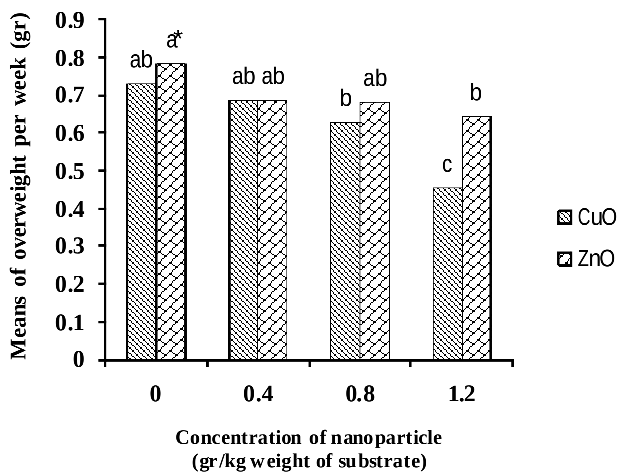
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 6. Comparison between means of overweight of 30 earthworms in two substrates (cow manure and SMC) in the seventh day after treatment with NPs



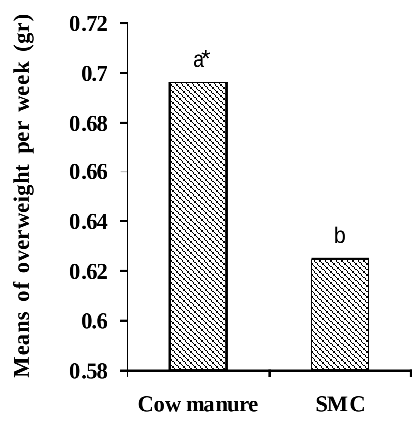
*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 7. Comparison between means of overweight of 30 earthworms in two substrates (cow manure and SMC) in the seventh day after treatment with CuO and ZnO NPs



*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 8. Comparison between means of overweight of 20 earthworms in two substrates (cow manure and SMC) in the fourteenth day after treatment with CuO and ZnO NPs



*According to Duncan´s test (α=%5), means with the same letter are not significantly different.

Fig. 9. Comparison between means of overweight of 20 earthworms in two substrates (cow manure and SMC) in the fourteenth day after treatment with CuO and ZnO NPs

CONCLUSIONS

The environmental effects of CuO and ZnO NPs on the *Eisenia fetida* earthworm in two substrates (cow manure and SMC) was studied and the results showed that there was no significant relationship between the NPs concentration and mortality in the seventh and fourteenth days after the substrates treatment. However, the absorption and accumulation of NPs increased in the worm tissues when the NPs concentration went up. The absorption and accumulation of nano ZnO were more than nano CuO and the accumulation in the worm tissues in the cow manure was more than the SMC. In addition, the number of the earthworm egg capsules decreased when the NPs concentration increased. In addition, ZnO NPs were more effective in reducing the reproduction than CuO in the seventh days after the treatment. The overweight decreased when the NPs concentration increased. Nano CuO was more effective in reducing the overweight than ZnO and this decrease in the worm tissues in the SMC substrate was more than the cow manure in the fourteenth day after the treatment.

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