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Investigating the Toxicity of Cu, CuO and ZnO Nanoparticles on Earthworms in Urban Soils

Mwaanga P[1]*, Mbulwe S[1], Shumbula P[2] and NyirendaJ[3]

[1]Copperbelt University, Department of Environmental Engineering, Kitwe, Zambia
[2]Mintek Nanotechnology Innovation Centre, Mintek, South Africa
[3]University of Zambia, Department of Chemistry, Lusaka, Zambia

Abstract

Even though some studies have shown that copper (Cu), copper oxide (CuO) and zinc oxide (ZnO) nanoparticles (NPs) are toxic to a number of organisms, fewer studies have investigated toxicity of these NPs in actual soils. This study investigated the effect of Cu, CuO and ZnO NPs in both urban and artificial soils. The aim was to examine the differences on these NPs' ability to cause oxidative stress to organisms in urban and artificial soils. Earthworms, Eisenia fetida, (2 months old) were exposed to varying concentrations of NPs and bulk materials (used for comparison purposes) of Cu, CuO and ZnO ranging from 100-500 mg/kg, 100-4000 mg/kg and 100-4000 mg/kg, respectively, for 14 days, for each soil type. Superoxide dismutase (SOD), reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) were measured at the end of exposure period. The accumulation of Cu and Zn in the earthworm tissues was measured using AAS. In urban soils, SOD, GSH and H2O2 all showed an initial increase as the concentration of NPs increased and but decreased at higher concentrations. Similar results for bulk materials were observed, though with less intensity. Cu and Zn accumulation in earthworm tissues increased as a function of NPs/bulk materials concentrations in both soils. Interestingly however, SOD, GSH and H_2O_2 from artificial soils for both NPs and bulk materials did not show significant differences from the controls, except at higher NPs/bulk materials concentrations.

Introduction

The rapid increase in the human population has put immense pressure on consumer products [1]. Thus, most manufacturers have turned to nanotechnology, to meet the demand for their products while improving on the quality at the same time. Nanoparticles (NPs) have unique properties that confer superior quality in consumer and industrial products [2,3]. This is because NPs have unique physicochemical properties that distinguish them from their bulk counterparts with the same composition [4,5]. These properties include optical, mechanical, chemical and biological enhanced characteristics [5-7], which have led to their wide application and as a result the number of NPs is increasing and it is expected that these nanomaterials will be more complex and will have unique chemistries [8]. Such nanomaterials include metal and metal oxide based NPs such as Cu, CuO and ZnO among others.

Metal oxide based NPs such as ZnO are used in products such as toothpaste, sunscreens, coatings and paints; while CuO NPs are used in gas sensors, photovoltaic cells, in catalyst applications and in heat transfer Nano fluids [4,9]. Cu NPs are used as anti-microbial, anti-fungal, copper diet supplements, in optical and electrical appliances [10]. The use of NPs in a wide range of commercial products and industrial applications is expected to increase exponentially during the next decade [11]. This will inevitably lead to an increase in their release into the environment with concomitant adverse effects to organisms [12,13]. Once in the environment, these NPs can undergo transformations, such as dissolution, agglomeration, sedimentation, or change of surface moieties, which greatly affect the pathway and extent of environmental release [14]. NPs have been shown to be more toxic to organisms, both micro and macro, as compared to their bulk counterparts [15-19]. This toxicity is attributed to the high reactivity of nanoparticles, which increases with decrease in size [8,20].

Being much smaller than cells and cellular organelles, NPs can easily penetrate biological structures and bring about their toxic effects [21]. However, it is still not clear whether the toxic effects observed in organisms are as a result of the dissolved metal ions or the NPs themselves [22]. One of the toxic effects that has been shown to be caused by NPs on organisms is oxidative stress [22,23], and can be used to explain toxicity associated with particle exposure [11]. Oxidative stress is a condition in which reactive oxygen species (ROS) or free radicals, generated extra-or intra-cellularly, exert toxic effects to the cells of the organism [24]. Organisms have effective mechanisms to prevent damage resulting from ROS, which mainly include endogenous antioxidant enzymes and non-enzymatic antioxidant such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione (GSH) [25]. If these mechanisms fail to eliminate the ROS, then an organism undergoes oxidative stress. SOD is the first line of defence which catalyses the dismutation of superoxide radical to H_2O_2 [24,26,27]. GSH is equally an important non-enzymatic antioxidant, which protects an organism's tissues against damage by neutralising ROS and free radicals [24]. These mechanisms are found in most organisms including earthworms.

The feeding mechanisms of earthworms easily expose them to soil pollutants [28]. Particularly, earthworms swallow soil or residues and plant litter on the soil surface, which is mixed by strong muscles and moved through the digestive tract [29]. Therefore, earthworms are exposed to any pollutant, including NPs present in soil or in litter.

Earthworms have been selected for this study, because these

*Corresponding author: Phenny Mwaanga, Copperbelt University, Department of Environmental Engineering, Kitwe, Zambia, Tel: +260969156065; E-mail: phennym@yahoo.co.uk

organisms are important in the maintenance of the ecological function of the soil [30] and recycling of nutrients [28]. *Eisenia fetida,* an earthworm species, is a critical ecotoxicological sentinel species because it is constantly exposed to pollutants in the soil [18,28,31] and therefore can be used in ecotoxicological studies. Interestingly, however, most studies done on earthworm species have used surrogate materials or artificial soils [32-36]. In this study, we used urban and artificial soils to investigate the ability of NPs to cause oxidative stress in *E. fetida*. To the best of our knowledge, we have not come across any research that has compared urban and artificial soils' influence on Cu, CuO and ZnO NPs' ability to cause oxidative stress to *E. fetida*. Therefore, there is need to effectively study the effects of these NPs on earthworms in the urban environment, given that natural environments are being urbanised.

Materials and methods

Materials and chemicals

Cu NPs with an average size of 10 nm were supplied by Mintek (Randburg, South Africa). Both CuO and ZnO NPs with an average size of 20 nm and 33 nm respectively, were synthesised at the Copperbelt University. The micro sized (bulk) Cu was prepared from 0.5 mm copper sheet strips of 99.99% purity purchased from Shanghai Metal Corporation [37]. This copper was cut into small pieces and was pulverized to a powder form. The powdered copper was passed through 4.5 μm sieve to obtain the size of the Cu particles used in this study. The analytical grade micro sized (bulk) CuO and ZnO were purchased from BDH Chemicals (England) and were used as purchased. Analytical grade (Analar) HNO_3 used for metal analysis was purchased from Rich Bay (South Africa). Aqueous 5-sulfo-salicylic acid dihydrate (SSA) was purchased from Merck, South Africa. The 1L high-density polyethylene (HDPE) holding containers were purchased from Athol plastics, Kitwe, Zambia. Colorimetric detection kits were purchased from Arbor Assays (Michigan, USA). The kits were; glutathione colorimetric detection kit (K006-H1), superoxide dismutase (SOD) colorimetric activity kit (K028-H1), hydrogen peroxide colorimetric detection kit (K034-H) and bicinchoninic acid (BCA) protein colorimetric detection Kit (K041-H1), used for determination of glutathione (GSH), super oxide dismutase (SOD), hydrogen peroxide (H_2O_2) and protein respectively. A Tecan plate reader (Sunrise-basic Tecan) was used to obtain the optical densities from samples. These optical densities were input in an online four-parameter logistic curve (4PLC) tool found at www.myassays.com to deduce the respective concentrations of the samples. Flame atomic absorption spectrophotometry (F-AAS) was used for metal analysis.

Test organisms and test media

The earthworms, *E. fetida* were purchased from a worm farmer in Mufulira, Copperbelt Province, Zambia. This species is sensitive, easy to culture and has a relatively short generation time in the laboratory of approximately 4-6 weeks. The earthworms were cultured in the Environmental Engineering Laboratory at the Copperbelt University for about 4 months in two types of soils, urban and artificial. This type of earthworms, *E. Fetida*, was selected for this study due to their abundance on the Copperbelt province soils. Urban soil, mainly sandy loam, was obtained from within the Copperbelt University main campus and was free from any pollutants, and its soil organic matter (SOM) was 1.5%. The artificial soil consisted of cow dung (50%), cooked maize meal (15%), *Carica papaya* (10%) and Irish potatoes (*Solanum tuberosum*) (25%) and was equally free from any pollutants and its SOM was >35%. A few priority characteristics and pollutants of the soils used in this study are shown in Table 1. These soils were also screened for commonly used herbicides and none was found. The source of food

Soil Type	pH	SOM (%)	Cu (ppm)	Zn (ppm)	Co (ppm)	Mn (ppm)	CEC (meq/100g)
Urban	6.93	1.5	<0.01	<0.01	0.01	0.04	65
Artificial	6.41	>35	<0.01	<0.01	<0.01	<0.01	-

Table 1: Priority constituents of the test soils.

for the earthworms for the urban soils included decomposed *Carica papaya*, Irish potatoes, cooked maize meal (nshima/pap) and cow dung, added fortnightly, while water for organisms in both urban and artificial soils was added every two days.

Toxicity tests

The toxicity tests for each soil type were conducted in accordance to the OECD guidelines [38,39] some modifications. For Cu NPs and bulk Cu the concentrations used were 0 (control), 100, 200, 300 and 500 mg/kg of soil introduced into 500 ml plastic containers. For both CuO and ZnO NPs and the bulk CuO and ZnO, the concentrations used were 0 (control), 100, 500, 1000, 2000, 2500 and 4000 mg/kg of soil introduced into 1L HDPE containers. For each concentration, three replicates were used. The pH of the soils was 7.5 and the temperature ranged from 25-27°C. Moisture content was maintained at 40% for the 14-day exposure period. Five (5) earthworms of about two (2) months old with fully developed clitellum were introduced into each container, each containing NPs concentrations mentioned above. The number of earthworms used in each container was based on the OECD guidelines [38]. To ensure that organisms did not escape, the containers were covered with parafilms perforated with small holes for air entry.

Determination of biomarkers

All biomarker analyses were conducted within 8 hours of sacrificing the earthworms. For each concentration and for each replicate sample, three earthworms were used for biomarker determination and the other two were used for metal ion determination. For biomarker determination, each of the three earthworms were chopped into three parts (each part for each biomarker) and then one part from each earthworm for the three earthworms were combined to make one sample for a particular biomarker. The earthworm samples where thoroughly homogenized using a hand tissue homogeniser in ice cold 100 mM phosphate buffer solution at pH 7.0. All the samples were centrifuged at 4°C using a Thermo legend micro 21R centrifuge, at varying speeds depending on the assay. The procedures for each specific biomarker are described in sections 2.4.1 to 2.4.4.

Protein

The proteins were assayed according to the BCA colorimetric kit (catalog number K041-H1) from Arbor Assays [40]. In brief, the homogenized tissues were centrifuged at 14000xg for 10 minutes at 4°C. Then 10 μL of each sample and standards were pipetted into duplicate wells of the 96 well plate. 10 μL of water in duplicate wells was used as the zero standard. The BCA color solution (75 μL) was added to each well containing samples and standards using a repeater pipette. The plate was sealed and incubated at 37°C for 2 hours in an oven. The optical density was read at 560 nm using the Tecan plate reader. The obtained optical densities were input in a four-parameter logistic curve (4PLC) fit tool found on www.myassays.com, as described in the kit, for deducing the concentrations of proteins from the optical densities.

Superoxide Dismutase

The SOD was assayed according to the super oxide dismutase colorimetric kit, (catalog number K028-H1), from Arbor Assays [41]. In brief, the homogenized tissues were divided into two portions, one

for protein determination as described in section 2.4.1 and the other was centrifuged at 1,500xg for 10 minutes at 4°C. From the supernatant, 10 μL of each sample and 10 μL of each standard were pipetted into duplicate wells of the 96 well plate. From the supplied assay buffer, 10 μL was pipetted into duplicate wells as the Zero standard. Then 50 μL of the substrate preparation, described in the assay kit, was added to each well containing samples and standards using a repeater pipette. Finally, 25 μL Xanthine oxidase preparation was added to each well containing samples and standards using a repeater pipette. Then the plate was sealed and incubated at room temperature for 20 minutes. The optical density was read at 450 nm using the Tecan plate reader. The concentrations were obtained using 4PLC fit as described in the kit. The amount of SOD was normalized with protein content.

Hydrogen peroxide

The H_2O_2 was assayed as described in the kit (catalog number K034-H1), from Arbor Assays [42]. In brief, the homogenised tissues were divided into two portions, one for protein determination as described in section 2.4.1 and the other was centrifuged at 1,500xg for 10 minutes at 4°C. From the supernatant, 50 μL of each sample and 50 μL of each standard were pipetted into duplicate wells of the 96 well plate. A 50 μL of assay buffer was pipetted into duplicate well as the zero standard. The 25 μL of colorimetric substrate was added to each well containing samples and standards using a repeater pipette. The reaction was initiated by adding 25 μL of the Horseradish peroxidase (HRP) preparation to each well using a repeater pipette. The plate was sealed and incubated at room temperature for 15 minutes. The optical density was read at 560 nm using the Tecan plate reader. The concentrations were obtained using 4PLC fit as described in the kit. The amount of H_2O_2 was normalized with protein content.

Glutathione

The GSH was assayed as described in the kit (catalog number K006-H1), from Arbor Assays [43]. In brief, the homogenized tissues where centrifuged at 14000xg for 10 minutes at 4°C. Then an aliquot of the supernatant was removed for protein determination as described in section 2.4.1. The remaining supernatant was deproteinized using 5-sulfo salicylic acid. From this protein free extract, 50 μL of each sample and 50 μL of each standard was pipetted into the duplicate wells of the 96 well plate. Then 50 μL of the sample diluent was pipetted into duplicate wells as the Zero standard. Thereafter, 25 μL of the colorimetric detection reagent was added to each well containing samples and standards using a repeater pipette. Finally, a 25 μL of the reaction mixture was added to each of the wells containing the samples and standards using a repeater pipette. The sides of the plate were gently tapped to ensure adequate mixing of the reagents. The plate was sealed and incubated at room temperature for 20 minutes. The optical density was read at 450 nm using the Tecan plate reader. The concentrations were obtained using HPLC fit as described in the kit. The amount of GSH was normalized with protein content.

Metal ion determination

The metal accumulation in earthworms was assayed using the atomic absorption spectrophotometer (AAS, Analyst 200, Perkin-Elmer model). For each treatment, the earthworms were depurated for 24 hrs prior to digestion for analysis. The sample preparation was carried out as described by Dai et al. [44] with minor modifications. The modification was that the wet weights of earthworm samples were used and the dilution of final samples was made up to 25 ml.

Statistics

Data presented here correspond to the arithmetical mean of three replicates (n=3). Statistical significances of differences between the control and treatments were determined by use of one-way analysis of variance (ANOVA), this was followed by post-hoc Dunnet's test at 95% confidence level ($p=0.05$). All graphs and statistical analyses were performed using the GraphPad® Prism Software.

Results and Discussion

Accumulation of copper and zinc in earthworms after 14-days exposure

Cu and Zn are essential elements in organisms and most species can regulate essential elements to a certain extent [45]. Therefore, it is not unusual to detect traces of Cu and Zn in organisms. In this study, after 14-days exposure, the concentration of Cu and Zn was measured in the earthworms using F-AAS. The measurements were carried out on earthworm tissues both in urban and artificial soils. The results in both soils showed a correlation between metal ions accumulation in earthworm tissues and the concentration of NPs in soils (Figure 1). Similar trends for the bulk Cu, CuO and ZnO were also observed, albeit with smaller magnitudes. To ensure that only metal ions in earthworm tissues were considered, the worms were depurated for 24 hrs prior to metal concentration determination. The concentration of Cu and Zn metals in earthworms were normalized with protein content. For both metals and in both NPs and bulk materials, there were significant differences ($p<0.05$) in the metal accumulation between treatments and controls. The increase in the metal ion concentration in the earthworm tissues as a function of NPs concentration in soils in this study was an indicator of NPs internalization by the earthworms. The NPs internalization could have been through diffusion and other modes such as ion-gated channels and transporter proteins, which permit NPs to cross the plasma membrane as suggested by Chang et al. [23]. However, as observed elsewhere [46], it is likely that some of this metal may have been absorbed as metal ions due to NPs dissolution within the soils. For bulk materials, the uptake could have been mainly due to dissolution consistent with the observation [47]. Generally, the uptake of metal ions in earthworms corroborated the results of the biomarkers as shown in sections 3.2-3.4. Furthermore, despite mortality not observed in any of the treatments, the results revealed that there was loss of weight of earthworms that was dose related. Contrary to expectation, there was no significant difference ($p>0.05$) in the accumulations of metal ions between urban (1.5% SOM) and artificial (>35% SOM) as shown in Figure 2. These results may partly be attributed to feeding mechanisms of earthworms. Furthermore, these results when taken together with effect results in sections 3.2, 3.3 and 3.4, suggest that organic matter complexation with NPs can modulate the toxic effects [22]. However, these uptake results suggest that further studies are still required to delineate the particle internalization from dissolved metal ion absorption.

Superoxide Dismutase

SOD has been described as the key enzyme in the natural defence against free radicals [48]. It is the most effective and high catalytic efficient intracellular enzymatic antioxidant, which is present everywhere in all aerobic organisms and in all subcellular compartments prone to ROS ($O_2^{\cdot-}$), mediated oxidative stress [24]. It is a limiting enzymatic activity in the disposal of ROS in tissues [49]. According to Matés [50], Cu and Zn atoms constitute the Cu, Zn SOD active site. The Cu^{2+} ions are directly involved in the dismutation of hydrogen peroxide (H_2O_2) while being reduced to Cu^{1+} ions, while the Zn ions are for stabilizing the
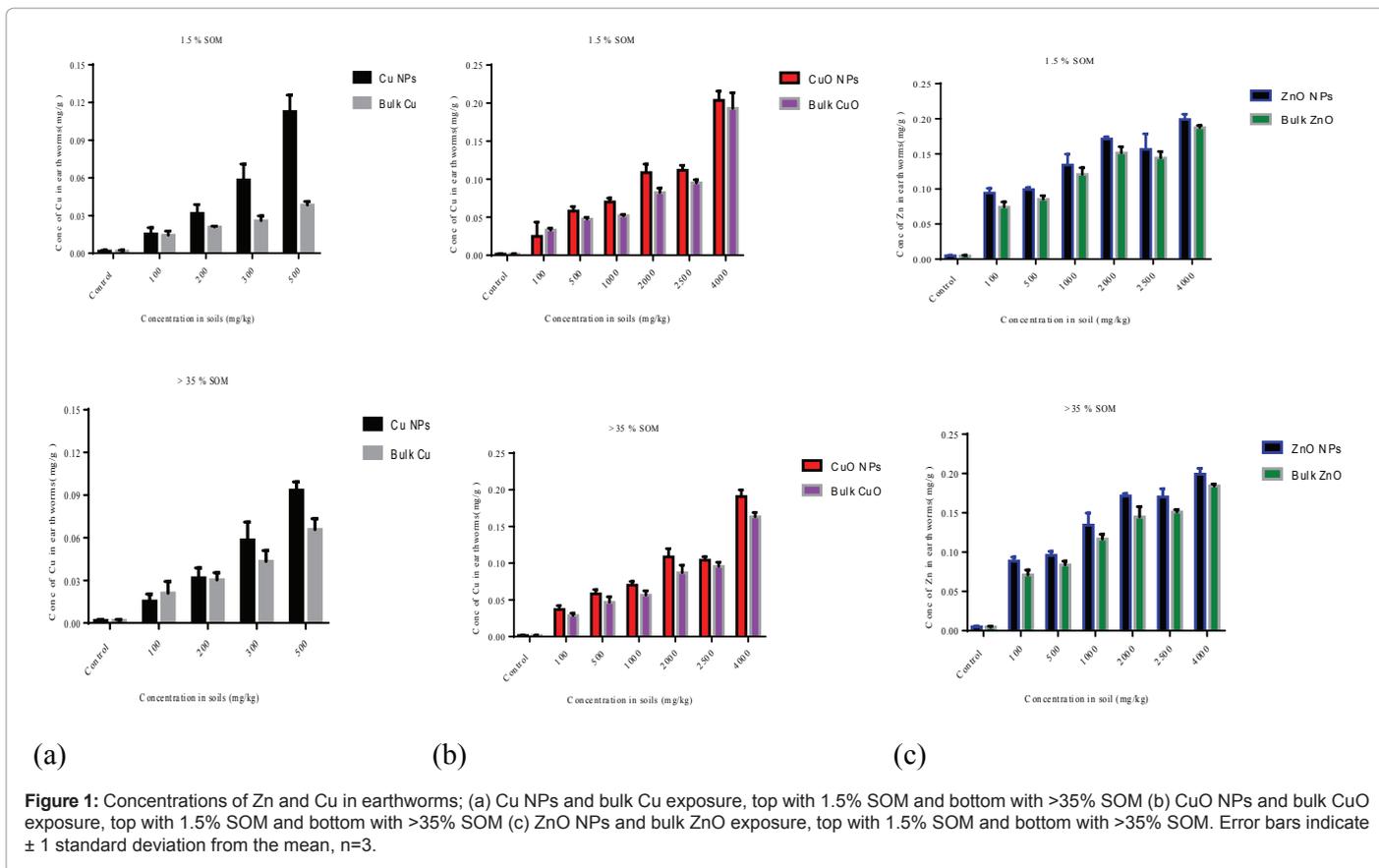
Figure 1: Concentrations of Zn and Cu in earthworms; (a) Cu NPs and bulk Cu exposure, top with 1.5% SOM and bottom with >35% SOM (b) CuO NPs and bulk CuO exposure, top with 1.5% SOM and bottom with >35% SOM (c) ZnO NPs and bulk ZnO exposure, top with 1.5% SOM and bottom with >35% SOM. Error bars indicate ± 1 standard deviation from the mean, n=3.
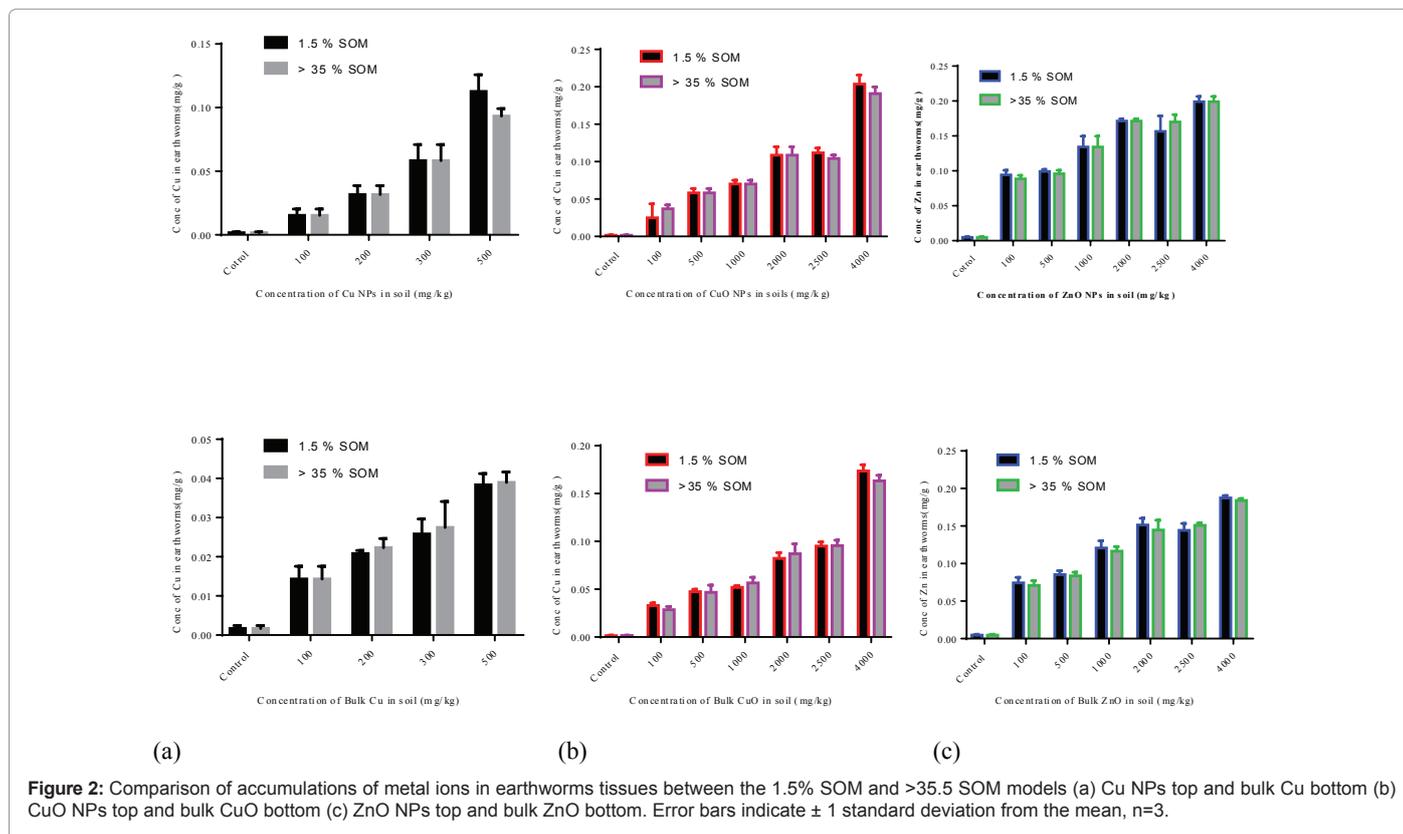

Figure 2: Comparison of accumulations of metal ions in earthworms tissues between the 1.5% SOM and >35.5 SOM models (a) Cu NPs top and bulk Cu bottom (b) CuO NPs top and bulk CuO bottom (c) ZnO NPs top and bulk ZnO bottom. Error bars indicate ± 1 standard deviation from the mean, n=3.

SOD [51]. Thus, the SOD destroys the free radical superoxide ($O_2^{\bullet-}$) by converting it to hydrogen peroxide that is in turn reduced to O_2 and H_2O by catalase (CAT) or glutathione peroxidase (GPX) reactions [48,50]. Therefore, the induction or inhibition of SOD during the exposure of organisms to NPs is one of the critical biomarkers of oxidative stress. In this study, the exposure of earthworms to Cu, CuO and ZnO NPs and bulk Cu, CuO and ZnO was carried out in two different soils, the urban and artificial. Given that the majority of studies on NPs toxicity on organisms have been carried out on artificial or surrogate media, it was necessary to understand whether NPs effects on organisms in urban soil would be significantly different from that of the artificial soils. The bulk Cu, CuO and ZnO, in this study was used for comparison purposes. The results of SOD for the exposures of earthworms to both NPs and bulk materials of Cu, CuO and ZnO for both soil types were shown in Figure 3.

In these results, the earthworms exposed to Cu, CuO and ZnO NPs in urban soils (1.5% SOM) exhibited a similar trend, thus initial increase in the SOD activity as the NPs concentration increased. However, as NPs concentration was further increased, a peak was reached and then a decrease in SOD activity was observed. The observed trend is attributed to the fact that initially, the SOD enzyme was up regulated, in response to the increase in super oxide due to increase in NPs' concentration. However, as the NPs' concentrations reached 200 mg/kg for Cu NPs and 1000 mg/kg for both CuO and ZnO NPs (Figure 3), SOD's capacity to detoxify the $O_2^{\bullet-}$ was overwhelmed [24,52], leading to decreased SOD activity and ultimately this would normally result into decrease in H_2O_2. However, during the increase in the expression of SOD, the production of H_2O_2 increases [53,54]. Since, according to Matés [50], H_2O_2 can inactivate Cu, Zn-SOD at certain concentrations, the observed reduction in SOD activity, as the concentration NPs was increasing, in this study, could have been partly due to H_2O_2. Furthermore, Cu, Zn SOD is known to have peroxidase activity and therefore increased expression of this enzyme leads to increased lipid peroxidation and hypersentivity to oxidative stress [54]. Thus, the

complexity and interrelatedness of oxidative stress processes requires caution, when interpreting oxidative stress results.

Interestingly, the concentrations of NPs that have caused significant changes (increase/decrease) in the SOD activities in the urban soils, in this study, are several orders of magnitude greater than the current concentrations of NPs predicted to be in the environment [47]. Thus, current environmental NPs concentrations do not pose a serious threat to soil organisms. However, current and future investigation on the safety of NPs in the actual environmental compartments needs to emphasize on chronic and delayed toxic effects as suggested by Huang et al. [47].

The results of the exposure of earthworms to Cu, CuO and ZnO NPs in artificial soils (>35% SOM) showed less dramatic changes in SOD activities. The observed results confirm the conclusion by Wang et al. [55] that adverse effects of NPs in an environmental system depends on the amount and nature of organic matter. Furthermore, the results of bulk Cu, CuO and ZnO in both soils showed a much less effect in comparison to the effect exhibited by organisms exposed to NPs. This is consistent to expectation, given that NPs can easily enter into the organisms due to their small sizes [21], whereas bulk materials effect could be due to dissolved ions. The high amount of organic matter in the artificial soil as reported in this study could be the reason for the reduced activity of SOD. Thus as observed elsewhere [22], particles and organic matter can undergo complexation reactions leading to formation of chemical-complex species that are non-bioavailable, resulting in reduced toxicity.

Glutathione (GSH)

As noted by researchers elsewhere [24], GSH plays an important role as an antioxidant, preserving protein and enzyme integrity, and serves as a co-factor for the GST enzyme. Therefore, an increase or depletion of GSH in an organism after exposure to a xenobiotic can be used as a biochemical indicator of oxidative stress. The production
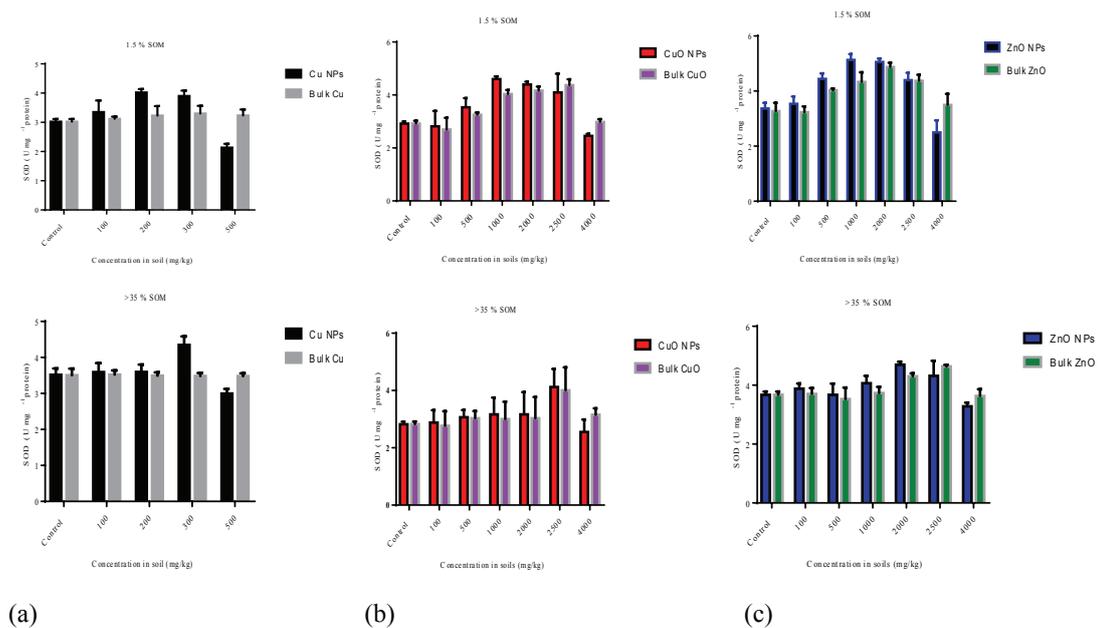

Figure 3: SOD activity: (a) Cu NPs and bulk Cu exposure, top with 1.5% SOM and bottom with >35% SOM (b) CuO NPs and bulk CuO exposure, top with 1.5% SOM and bottom with >35% SOM (c) ZnO NPs and bulk ZnO exposure, top with 1.5% SOM and bottom with >35% SOM. Error bars indicate ± 1 standard deviation from the mean, n=3.

of ROS in oxidative stress can lead to the damage of macromolecules such as DNA, lipids and proteins [56] and eventually to the death of an organism. Other researchers elsewhere Mwaanga et al. [13] used oxidized glutathione (GSSG) as an index of oxidative stress. However, as stated by Gill and Tuteja [24] both GSSG and GSH can be used as indicators of oxidative stress. In this study, as explained in the method section, the earthworms were exposed to Cu, CuO and ZnO NPs in both artificial and urban soils. The resulting changes in GSH were shown in Figure 4. In the urban soils, the results for all the three NPs indicated an initial increase of GSH, but as NPs concentration increased, there was a decrease in the amounts of GSH. This decrease in GSH concentration corresponded with a decrease in H_2O_2 at similar NPs concentrations in soils as shown in Figure 5. The observed trend can be explained as follows: GSH biosynthesis is stimulated under mild oxidative stress owing to increased expression of glutamate cysteine ligase, a key enzyme for its biosynthesis [57] and hence the observed initial increase of GSH. However, as the concentrations of NPs increased, oxidative stress became intensive. Thus, more $O_2^{\bullet-}$ ions were generated and this in turn increased the amount of H_2O_2, which required more molecules of GSH for its conversion to water and oxygen in the presence of glutathione peroxidase. In the process of converting H_2O_2 to oxygen and water, GSH is converted to the oxidized form (GSSG). The reduction of GSH in the anti-oxidative processes is known to be carried out by several other processes [57]. Thus, in this study several processes could have caused the depletion of GSH.

In the artificial soils, with >35% SOM, NPs effects on the increase or depletion of GSH appeared to have been minimized by organic matter content. As already discussed in the previous sections (3.1 and 3.2), these results could be attributed to NPs-SOM complex formation that ultimately modulate NPs toxicity. The results of bulk Cu, CuO and ZnO on GSH in urban soil mirrored those of the NPs, though with much reduced intensity. This reduction in the intensity of the effects could be attributed to differences in the ability of NPs to easily enter organisms due to their small sizes as stated in section 3.2 above. Interestingly,

however, there was generally no significance difference between the results of NPs and bulk materials of Cu, CuO and ZnO in artificial soils, on their effect in the levels of GSH. The reduced effect of micro sized particles on GSH could also be attributed to reduced release of metals by bulk materials as observed elsewhere. Again, these results call for more studies to understand whether the effects observed was to due to NPs or metal ions.

Hydrogen peroxide

The production of H_2O_2 in organisms exposed to xenobiotics can lead to the production of highly reactive and toxic hydroxyl radicals ($\cdot$OH), through Fenton reaction. The production of OH- radicals can lead to lipid peroxidation, which ultimately can create a cyclic destructive pathway, because some of the breakdown products are strongly electrophilic in nature and can further increase oxidative stress, with eventual death to organisms. The conversion of $O_2^{\bullet-}$ into H_2O_2 requires the enzyme Cu, Zn, SOD as already described in section 3.2 above. According to Kowald et al. [54], SOD has two forms, the oxidized (Cu(I)Zn SOD) and the reduced form (Cu(II)Zn SOD). Depending on the ratio of the reduced to the oxidized forms, increase in the total SOD expression may result in increased, decreased or constant amount of H_2O_2. As already described in section 3.2 above, increase in SOD expression can increase lipid peroxidation and hypersensitivity to oxidative stress in organisms. Interestingly, the concentrations of NPs at which such events can occur depend on the environmental medium. For instance, in urban and artificial soils of this study, the same NPs concentrations elicited different responses that were significantly different in terms of increase and depletion of H_2O_2 (Figure 5). The trend of results observed for H_2O_2 is similar to the results observed for SOD and GSH, for both artificial and urban soils. In the urban soils, the results can be rationalized as follows: The initial increase in the H_2O_2 at low NPs concentrations was because of steady increase in SOD expression, which was required to convert the super oxide ion to H_2O_2. According to some reports Gardener et al. and Kowald et al. [53,54],
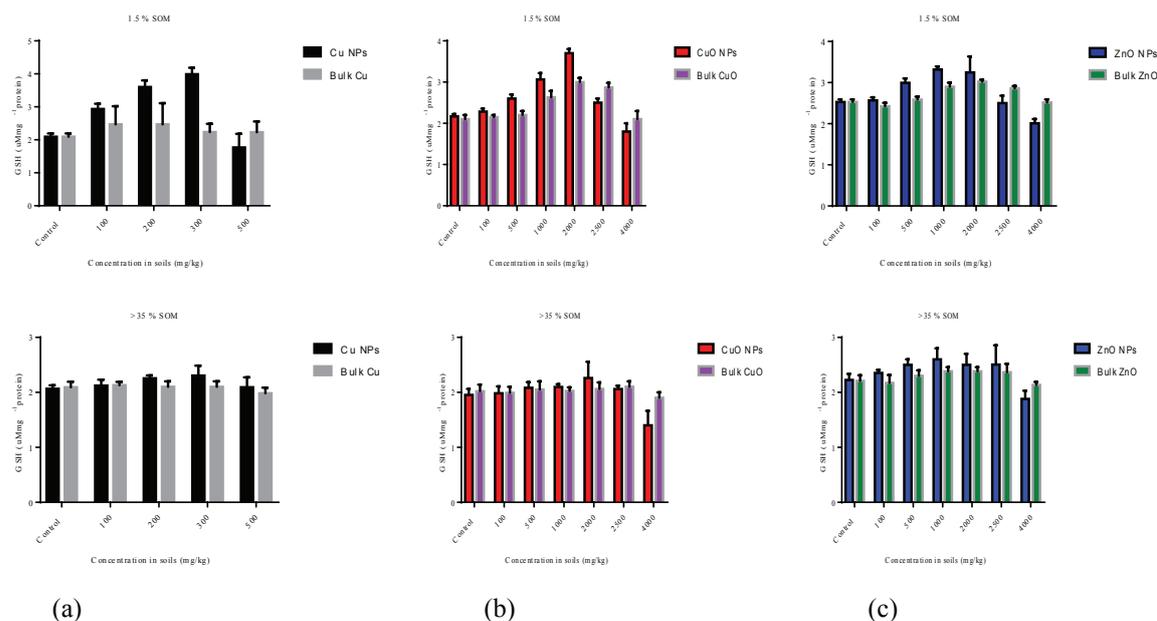

Figure 4: Concentration of GSH: (a) Cu NPs and bulk Cu exposure, top with 1.5% SOM and bottom with >35% SOM, (b) CuO NPs and bulk CuO exposure, top with 1.5% SOM and bottom with >35% SOM, (c) ZnO NPs and bulk ZnO exposure, top with 1.5% SOM and bottom with >35% SOM. Error bars indicate ± 1 standard deviation from the mean, n=3.
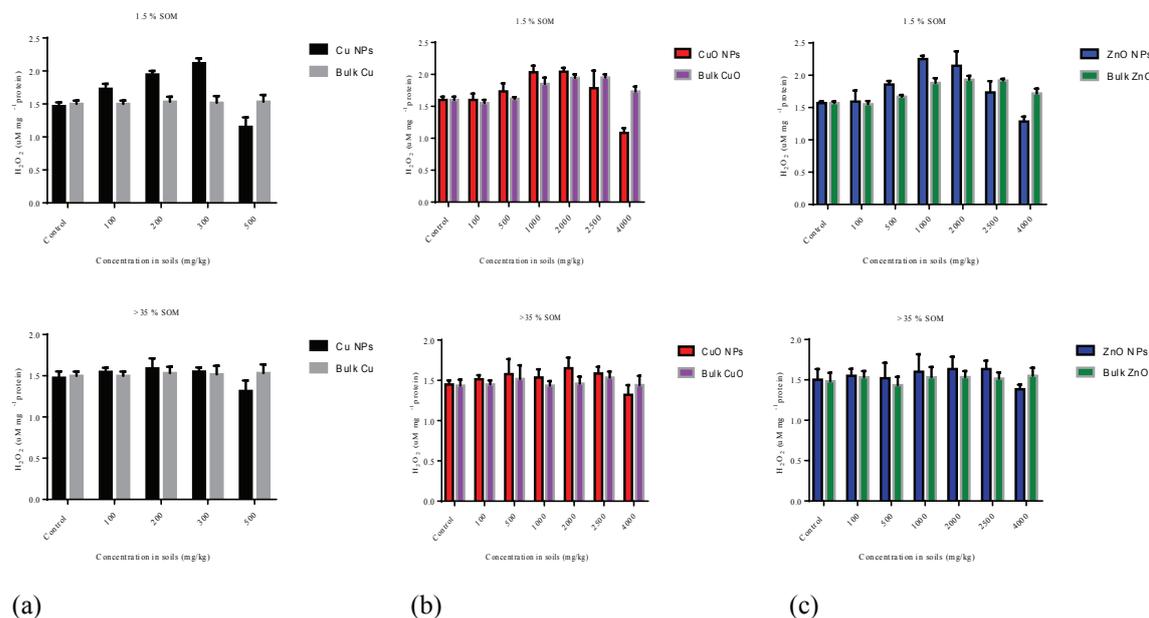
Figure 5: Concentration of H_2O_2: (a) Cu NPs and bulk Cu exposure, top with 1.5% SOM and bottom with >35% SOM, (b) CuO NPs and bulk CuO exposure, top with 1.5% SOM and bottom with >35% SOM, (c) ZnO NPs exposure, top with 1.5% SOM and bottom with >35% SOM. Error bars indicate ± 1 standard deviation from the mean, n=3.

this is likely to happen under steady state condition, where the ratio of the reduced to oxidized forms is approximately unit. Thus, under these conditions, as SOD increased, H_2O_2 can also increase. The results of this study seemed to mirror the description by Gardener et al. and Kowald et al. [53,54], as reflected in both Figures 4 and 5. However, as NPs concentration increased, this increase led to intensive oxidative stress [52], which eventually inhibited the enzyme SOD, which converts $O_2^{\cdot-}$ to H_2O_2 and hence leading to a decrease in H_2O_2. Nevertheless, according to Lushchak and Kowald et al. [52,54], several other processes can lead to decrease in H_2O_2, in addition to others that convert H_2O_2 to water and oxygen.

Similarly, as was the case for SOD and GSH, the effects of NPs to organisms in artificial soil were minimal in comparison to the effects of these same NPs in urban soil. The same reason as outlined in section 3.2 above can be attributed to the observed minimal effects in the artificial soil. For the bulk materials, the effects in urban soils mirrored those of NPs, albeit with less intensity. However, in the artificial soils, there was generally no significant difference between the effects of NPs and that of bulk materials. The same reasons outlined in section 3.3 above can be attributed to the observed minimal effects of the bulk materials in the artificial soil.

The results of this study are interesting and significant for several reasons. Few studies in literature have documented the biochemical effects of Cu, CuO and ZnO NPs on earthworms in urban soils. The concentrations of NPs found to exhibit adverse effects on earthworms in artificial soils or natural soils tend to be several orders of magnitude lower [32,58,59] than what has been found in this study and other similar studies elsewhere [33,46]. These differences could be due to differences in specific soil characteristics. This implies that extensive characterization of test media is important. However, one of the challenges of carrying out effective evaluation of the impacts of NPs in

soils is difficulty in tracking NPs in soil matrix where similar NPs may be naturally occurring. Thus, for studies in urban soils, the existence of metal ions of the same metals that make up the NPs can further confound the results [60]. Another challenge involves the difficulty to delineating the effects of NPs from that of dissolved ions in urban soils.

Conclusion

This study has shown that the ability of Cu, CuO and ZnO NPs to cause oxidative stress is different in different soil media. Thus, the levels of oxidative stress caused by Cu, CuO and ZnO NPs in urban and artificial soils are different even at the same NPs concentrations. This study has also shown that the soil organic matter had a huge influence on the impacts of NPs in causing oxidative stress to earthworms. The study has further showed that there is a correlation between accumulation of metal ion and the concentration of NPs in soils and that the accumulation was significantly higher (p<0.05) for NPs than for bulk materials. Thus, the results of this study suggest that it is the form in which the NPs (or metal ions) exist that can cause severe oxidative stress. Furthermore, the concentrations observed to have caused oxidative stress in this study were several orders of magnitude higher than the predicted current concentrations of these NPs in the actual environment. Interestingly, the soils used in this study were free from any contaminants, a situation that rarely happens in many urban environments. Therefore, evaluation of the adverse effects of NPs in the urban soils has to focus on the chronic and delayed effects with full soil characterization. Furthermore, additional studies are required to delineate the effects of particles from that of metal ions in soils of different organic matter content.

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