

Heavy metals accumulation from sewage sludge in the Nile tilapia *Oreochromis niloticus* (Trewavas, 1983) during a sludge-earthworm-fish short-term cycling

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Abstract

Municipal sewage sludge from wastewater treatment is an important nutritional source for sustainable agriculture. Here, we report on the assessment of the accumulation of heavy metals in Nile tilapia *Oreochromis niloticus* (Trewavas 1983) fed on earthworms *Eisenia fetida* reared on soil treated with different concentrations of sewage sludge (25% and 100%) during sludge-earthworm-fish short-term cycling. In this short-term cycling the Nile tilapia collected from the White Nile were chosen as final consumers, whereas the earthworms reared on loam soil mixed with different ratios of sludge were used as a feed for the final consumers. Our results indicate that the concentrations of Cd²⁺, Cr²⁺, Pb²⁺ and Zn²⁺ in the sludge treated soil are proportional to the sludge content in the soil. Importantly, the accumulation of these heavy metals was significantly low in the earthworms and the Nile tilapia in comparison with the treated soil and that these concentrations in the Nile tilapia were below the international limits recommended by the US Environmental Protection Agency (2014). Moreover, the growth and overall flesh quality of the fish were improved as indicated by the growth increase up to 146% when fed on earthworm reared in 100% sludge. Additionally, our physico-chemical properties (*i.e.* pH, soil moisture, electric conductivity and organic matters)

evaluation on the soil indicates an improvement of the soil quality when mixed with sewage sludge. These results suggest a sustainable application of sewage sludge in fish culture owing to its high nutritional values, low cost, and low risk of hazardous heavy metals when using primary consumers with heavy metals bioaccumulation capability such as *E. fetida*.

Introduction

Municipal sewage sludge produced from the wastewater treatment plants is an important source of nutrients and soil conditioners, which can be applied for sustainable agricultural purposes. However, such sludge is usually accompanied with high levels of toxic heavy metals that can affect the soil, plants and human. Metal concentrations are directly related to the consumed amounts of such metals by the society and therefore their amounts are relative to the level of consumption (Kirchmann *et al.*, 2017). Therefore, the determination of the concentrations of such metals in the sewage sludge is a prerequisite prior to application (Dolgen *et al.*, 2007). Sewage sludge can be recycled and applied for sustainable agricultural applications, which makes it highly demanded and a subject of great interests, which have encouraged several wastewater treatment plants to redirect these large amounts of sewage sludge and use them for agricultural applications (Ababu *et al.*, 2014; Kirchmann *et al.*, 2017). A previous report conducted on wheat, white head cabbage and tomato under seven crop rotation systems have shown that the use of sewage sludge significantly increased the yield, which was found to be directly related to the content of the sludge in the soil in comparison with that of the control (Mehmet, 2013).

The physio-chemical processes involved in the treatment of the sewage play roles in the concentration of the heavy metals in sludge, as well as assist in accumulating poorly biodegradable trace organic materials and potential pathogenic organisms (NETSP, 2008). For instance, heavy metals such as Zn²⁺, Cd²⁺, Pb²⁺, and Cr²⁺ may be concentrated in high levels in the soil, which can extremely affect the food safety due to the high toxicity of such metals when found in such high concentrations beyond the limits recommended by the US Environmental Protection Agency (EPA, 2014). It's well known that heavy metals accumulations lead to an excessive redundancy of reactive oxygen species and highly oxidant compounds such as methylglyoxal which may lead to lipid peroxidation that cause damage to proteins, DNA and other

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Contributions: NS, SS and AA, designed experiments; AA, conceived and supervised the entire project; NS, performed all experiments, collected and analyzed data, and drafted the initial manuscript; FHMK prepared figures, wrote and finalized the manuscript; SS, performed the atomic absorption analysis of soil elements; and all authors commented on the manuscript and approved the final version before submission.

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biomolecules (Mukti, 2014).

Bio-accumulation and bio-transformation of heavy metals and many other inorganic and organic chemical contaminants are important factors that can be utilized to cleanup sewage sludge from these contaminants and help in re-developing the agricultural land. Many studies demonstrated that earthworms have a capability of cleanup and removal of heavy metals and other chemical pollutants from contaminated soils due to its ability in bio-accumulation and bio-transformation for many chemicals (Shahmansouri *et al.*, 2005). It was previously shown that *Eisenia fetida* has especial capability in the vermicomposting of heavy metals such as Cr²⁺, Cd²⁺, Pb²⁺, Cu²⁺, and Zn²⁺ and also has been shown to have bio-

accumulation capability a factor that can efficiently help *E. fetida* to remove heavy metals from contaminated materials (Shahmansouri *et al.*, 2005; Fei *et al.*, 2012; Seema and Vineeta, 2014). On the other hand, the fish tilapia has been known for its capability to tolerate pollution and accumulation of heavy metals (Maximilian *et al.*, 2015). In support of this two preponderant fishes *Synodontis claris* and *Tilapia nilotica* were examined for their ability to tolerate heavy metals accumulations in the Anambra River. As a result, *T. nilotica* was found to be more tolerant and accumulated less-heavy metals in their tissues (Maximilian *et al.*, 2015).

Generally, it is widely accepted that sewage sludge generated from the municipal wastewater treatment plants can be beneficial to sustainable agriculture as rich and cheap sources for plants and fish nutrition. Hence, it can be potential alternative source to that of the traditional feed source, recyclable, and environment-friendly, which can be applied to sustainable fish culture. Nonetheless, such usages need concrete measures and precautions to ensure the safety of sludge usages. Here, we aimed at evaluating the potential hazards of Cd²⁺, Cr²⁺, Pb²⁺, and Zn²⁺ accumulations in animals reared in sewage soil and their consumption based on the recommended safe limits by internationally recognized organization such as US Environmental Protection Agency (EPA) (2014).

Materials and Methods

Sludge, Earthworms and Nile tilapia

Sludge was collected from Soba Wastewater Stabilization Treatment Plant (SWSTP) in Central Khartoum, Sudan. Nile tilapia *Oreochromis niloticus* (Trewavas, 1983) were collected from Al Shagara location in the White Nile and were taxonomically authenticated by Dr. Asma Abdelrahman Ahmed at the Department of Biology and Biotechnology, Faculty of Science & Technology, Al Neelain University. The worm *E. fetida* were collected from Tuti Island, Khartoum, Sudan.

Physicochemical analysis of sludge-treated soil

The physicochemical properties of soil samples treated with different concentrations of sewage sludge were evaluated according to standard protocols. Moisture, pH, electric conductivity (EC), and organic matter of different soils were measured. For moisture measurement, 5 g each of soil control containing 50% clay and 50% sand, and

soil treated with 25% sludge and 100% sewage sludge were sampled from each basin in metal dishes and heated in an oven at 105°C for 18 h. Samples were then dried in an oven at 60°C for 1 h and same steps were repeated at least three times. Moisture was then measured by calculating the difference between wet and dried weights. To evaluate the pH level and EC of the soil, 5 g of each sample taken from the basins was dissolved in 30 mL of distilled water and centrifuged for 10 min with 4000 rpm at 25°C and then the pH and EC were measured in the supernatant using pH meter and EC meter, respectively. For organic matter assessment, samples were put into small crucibles and heated in an oven at 800°C for 3 h and then the resulting ashes were weighed and the difference in weights represents the organic matter.

Short-term cycling experimental design

A number of 150 to 175 earthworms were put in a fiberglass trough of 30 × 30 × 40 cm dimensions that was pre-filled with soil up to 30 cm depth as follows: i) the control trough was filled with loam soil that is composed of 50% clay soil and 50% sandy soil, and ii) the other troughs were divided into two groups; the first group contains 25% sewage sludge and 75% loam soil, while the second group contains 100% sewage sludge with additional loam soil. These troughs were kept moistened by spraying distilled water in a daily basis to provide suitable environment for earthworms. The earthworms were reared for up to month before harvested. The earthworms were then placed in metal Petri dishes, washed, weighed and dried at 90°C for 18 h. The dried earthworms were ground to a fine powder, of which 50 g was mixed with sorghum (*Sorghum bicolor*), wheat (*Triticum sativum*) and parsley (*Petroselinum crispum*) in a ratio of 1:1:1:0.2, respectively, and mixed with 100 mL distilled water and pelted to small balls and used as feed formulation. For the final consumers, 75 tilapia fishes in a weight range of 20-40 g were divided into three groups with 25 fishes in each group; i) control group which was fed with earthworms reared in loam soil control, ii) 25% group which was fed with earthworms reared in 25% sewage sludge plus 75% loam soil, and iii) 100% group which was fed with earthworms reared in 100% sewage sludge. The fishes were collected after 8 weeks before dissected, from which muscle tissues were removed at the lateral and caudal regions. These samples were immediately wrapped in aluminum foil, weighed and dried in a dry oven at 90°C.

Heavy metal assessment

To assess the heavy metals on all soils used for the current experiments, a sample of 5.0 g of an air dried and ground soil were sieved and placed in an Erlenmeyer flask and a volume of 20 mL extracting solution containing 0.05 HCl and 0.025 H₂SO₄ was added to each sample and placed in a mechanical shaker for 15 min before filtered through Whatman (No. 42) paper in a 50 mL volumetric flask and the volume was completed to 50 mL with extracting solution (Elmer, 1996). On the other hand, 1.0 g of dried earthworm powder and tilapia muscle samples were weighed and placed in a porcelain crucible and ashed in a muffled furnace at 500 °C for 8 h. Then the ashes were cooled and dissolved in 5 mL of 20% HCl. The mixtures were filtered through an acid washed filter paper into a 50 mL volumetric flask and the volume was completed to 50 mL with deionized water and mixed well. For quantitative measurements of Cd²⁺, Cr²⁺, Pb²⁺ and Zn²⁺ an atomic absorption spectrophotometer (A.A.S. 3110 Model, 210 VGP Buck scientific) was used according to the manufacturer protocol. Metal concentrations expressed as mg/L were measured according to Perkin Elmer Manual (1996).

Results

Changes in physicochemical properties of sewage sludge treated soils

Municipal sewage sludge is a rich source of organic matters that conserves soil intactness from overuse and thus it is recommended for agricultural applications by the European Commission (Hussein *et al.*, 2010; Surajit *et al.*, 2015). In the present study, we evaluated different physicochemical properties of soil treated with different concentrations of sewage sludge collected from a wastewater stabilization treatment plant. As shown in Table 1, the addition of sludge to the soil has changed the physicochemical properties of pH, EC, organic matter and moisture of the treated soil and that the addition of earthworm has also resulted in slight to significant changes in pH and EC. These results indicate that the sewage sludge treatment has no significant effect on the pH property of the soil, whereas the addition of earthworms has slightly changed the pH to more acidic. On the other hand, EC was increased significantly with up to 8-fold in 100% sewage sludge and the addition of earthworms resulted in a significant decrease of the EC, indicating that earthworms have consumed significant amounts of micro- and macro-elements.

Organic matters and moistures were increased up on the addition of sludge indicating that sewage sludge can be a source of essential nutrition such as nitrogen, phosphate and potassium and the moisture preservation is an important enhancer to soil quality (data not shown).

Growth of *Oreochromis niloticus* fed with earthworm reared on different sludge concentrations

Recycling the sludge nutrients from soils to grow animals as a feed source with an aim at producing valuable animal proteins in high amounts through soil-animal-animal cycle is a decent idea in sustainable agriculture (Dolgen *et al.*, 2007; Ababu *et al.*, 2014). Figure 1 shows that the growth of fish fed with earthworms reared on sewage sludge was improved in comparison to the control fish. Interestingly, this improvement was highly related to the increments in the concentrations of sludge in the soil. The growth increases were 134.89%, 146.17%, and 131.21% for sludge concentrations of 25%, 100%, and control, respectively (Figure 1). This result indicates that sludge nutrients can be a potential source of feed for fish growth in soil-earthworm-fish short-cycling system. As shown in Figure 2A-C, the general features of fish grown in up to 100% sewage sludge remained unaltered and further indicate effectiveness of sludge as cheap and rich source of organic nutrients. This agrees with Mondal *et al.*, (2015) who reported similar results on the short-term effects of municipal sewage sludge of the cowpea-wheat cropping system.

Bioaccumulations of heavy metals in the soil-earthworm-fish short-term cycling system

Four heavy metals; Cd^{2+} , Cr^{2+} , Pb^{2+} and Zn^{2+} were monitored during the short-term cycling of soil-earthworm-fish system as detailed in the Materials and Methods. Figure 3A shows that Cd^{2+} levels in the sludge treated soil are directly influenced by the increase of the sludge concentrations in the soil, where Cd^{2+} concentrations showed the highest levels in 100% sludge treated soil. Intriguingly, these increments in the Cd^{2+} concentrations in sludge treated soil were not obvious in the earthworms and

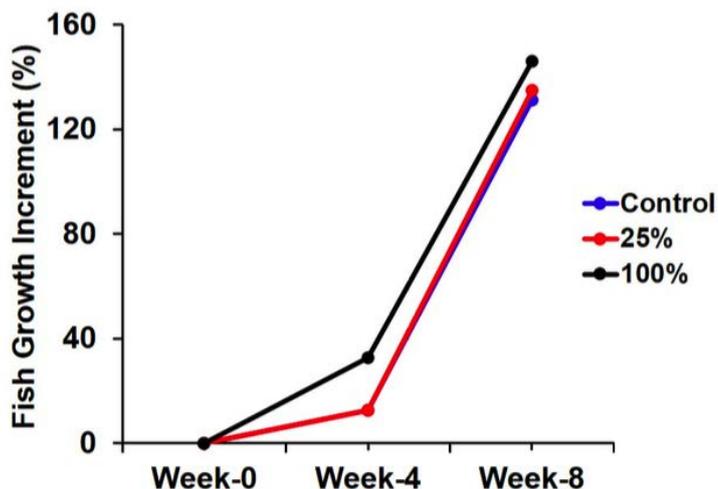


Figure 1. The effect of earthworm reared on different concentrations of sewage sludge treated soil on the growth of Nile tilapia over eight weeks. Percentages were calculated from an average of at least three fishes' weights (g) at week-0, week-4 and week-8. The control fishes are those fed on earthworm reared on control soil (50% clay and 50% sand).



Figure 2. Morphological features of Nile tilapia fed on earthworm reared on soil sludge. The figure shows Nile tilapia fed on earthworm reared on soil treated with various concentrations of sewage sludge. A) Showing the control Nile tilapia that was grown on control soil containing no sewage sludge, B) is a representative of Nile tilapia that were fed on earthworm reared on soil treated with 25% sewage sludge, and C) Nile tilapia fed with earthworm reared on 100% sewage sludge.

Table 1. The physicochemical properties of soil treated with different concentrations of sewage sludge before and after the addition of earthworm consumers.

Sample	pH (before)	pH (after)	EC μ S/cm (before)	EC μ S/cm (after)	Organic matter (%)	Moisture (%)
Control soil*	7.85	6.76	0.558	0.415	18.81	17
25 % sludge	7.77	6.45	1.41	1.502	36	18
100 % sludge	7.78	7.04	4.18	1.90	63	40

*Control soil is composed of clay and sand with 50% each. EC is the electric conductivity in μ S/cm.

the last consumer *O. niloticus*. The later has revealed the lowest concentrations of Cd^{2+} with comparison to the sludge soil and the earthworms. In contrast, Cr^{2+} levels were significantly increased in the soil and earthworm and reached the peak in the 100% sludge (Figure 3B). The Pb^{2+} concentrations were exceptionally high in 100% even higher than that of the pure sludge (Figure 3C). Importantly, its concentrations in our last consumer *O. niloticus* did not increase significantly and kept below the limit of US EPA (2014). This result might be due to the bio-accumulation capability of the earthworm as reported previously (Shahmansouri *et al.*, 2005). On the other hand, Zn^{2+} was the most abundant metal among these heavy metals and found in high concentrations in all samples with approximately 3-folds higher than its counterparts in soil, earthworm and *O. niloticus* (Figure 3D). Regardless of its proportional increase to the increase of sludge contents, Zn^{2+} remained in low concentrations in the final consumers below the limits of US EPA (2014), whereas earthworm, the first consumers, bio-accumulated significant amounts of Zn^{2+} (Figure 3D).

Overall, the results presented here indicate that Cd^{2+} has the lowest concentrations among the heavy metals monitored and that the levels of Cr^{2+} and Pb^{2+} were moderate in all samples, while Zn^{2+} had the highest level.

Discussion

Despite the fact that sewage sludge is an important nutritional source that is often improve the soil quality in terms of organic matters and moisture as indicated in our physicochemical properties results, the present study demonstrates that the heavy metal concentrations in soil treated with sewage sludge directly associated with the concentrations of the sludge. This is in agreement with a previous a study, which reported that heavy metal levels in the soil are highly related to the sludge concentrations (Abubaker and Ram, 2014). In contrast, another study conducted on the cauliflower has shown that the heavy metal levels have increased in soil treated with brewery sludge resulting in high concentrations of heavy metals in the plant with comparison to the plants that were grown in an

untreated soil (Khanal *et al.*, 2014). The heavy metals investigated in this study, i.e., Cd^{2+} , Pb^{2+} , Cr^{2+} , and Zn^{2+} , have exhibited differences in their levels in the soil, earthworms and fish, with Pb^{2+} having the lowest levels. The Pb^{2+} , Cr^{2+} and Zn^{2+} metals have shown higher levels in earthworms, especially the level of Pb^{2+} , which was even higher than that of the sludge treated soil, the original source. This is intriguing and can be attributed to the bio-accumulation capability of the earthworm *E. fetida* to the heavy metal Pb^{2+} . Similar results have been demonstrated in previous reports showing that *E. fetida* can be used to clean up contaminated lands, which helps in the re-development of lands owing to its bio-accumulation capability (Seema and Vineeta, 2014). Moreover, previous studies have also demonstrated that heavy metals such as Cu^{2+} , Ni^{2+} , Cd^{2+} , Pb^{2+} , and Zn^{2+} in soil can be reduced by vermicomposting using earthworm *E. fetida* as indicated by the bio-accumulation factor (Shahmansouri *et al.*, 2005; Fei *et al.*, 2012; Ademola *et al.*, 2013). This was also confirmed by other researchers who studied the effects of earthworms on the sludge treated soil quality as

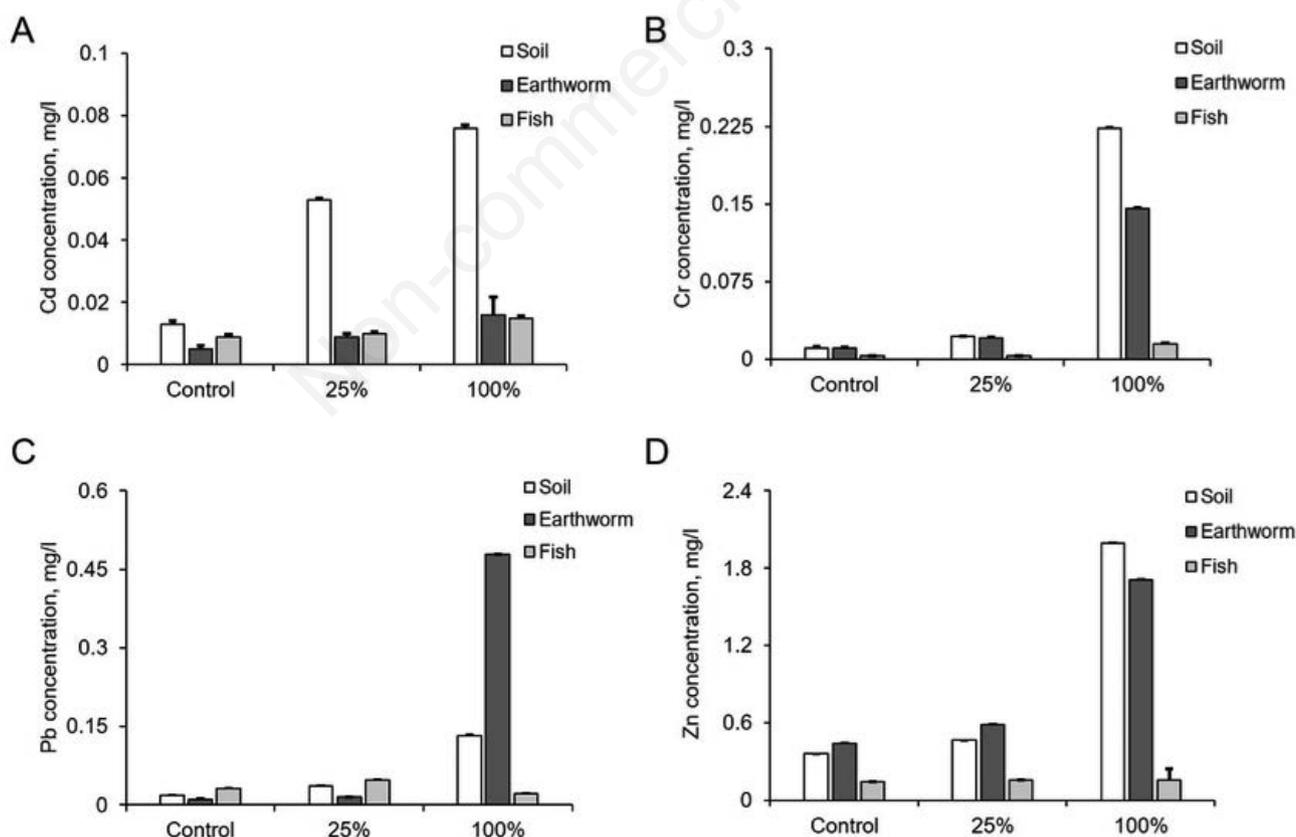


Figure 3. Heavy metal accumulations during short-term cycling of Sludge-Earthworm-Nile tilapia indicating the accumulations of A) Cd^{2+} , B) Cr^{2+} , C) Pb^{2+} , and D) Zn^{2+} in sludge-treated soil, earthworm reared on that soil and Nile tilapia fed on the earthworm.

a fertilizer, indicating that earthworms can help in the reduction of dangerous heavy metals in sludge treated soil (Suther, 2010).

The direct heavy metals mobility from the first consumer *E. fetida* to the final consumer *O. niloticus* appears to be limited as shown in the results, where all of the heavy metals have shown to have low levels in these consumers with comparison to the sludge treated soil. This might not be directly associated with the ability of these consumers to selectively filtrate heavy metals, but there is a possibility that our short-term food cycling system could be the reasons behind these low levels of heavy metals. Our results are consistent with the study reported by Abdel-Mohsien and Mahmoud (2015), who showed similar results in the flesh of *O. niloticus* fished from a water resource that was found to be highly contaminated with heavy metals such as Cd^{2+} , Pb^{2+} and Zn^{2+} . On the other hand, this result contradicts an earlier study, which reported high levels of sewage sludge heavy metals such as Pb^{2+} and Cd^{2+} with up to 10-fold and 2-fold, respectively, in the cattle's liver and kidney (Baxter, 1982; Keinholz *et al.*, 1976). This was attributed to the fact that liver and kidney are naturally more efficient in depositing toxic substances than other tissues such as those of the muscle. Generally, the low levels of metals in the flesh of tilapia might possibly be due to the capability of Nile tilapia to release considerable amounts of heavy metals, in addition to the use of the bio-accumulators' earthworms in this case.

Conclusions

In the present study, short-cycling term consisting of sludge soil, earthworm and fish has been evaluated. The heavy metals accumulations, i.e., Cd^{2+} , Cr^{2+} , Pb^{2+} , and Zn^{2+} of the sludge were assessed in the soils treated with sludge, the earthworm fed on that sludge and flesh of the fishes which were fed on the earthworms. We demonstrated that sludge could be a good source of food for fish production and that using earthworms can limit the risk of heavy metal accumulations in the fish flesh, which is crucially significant when considering such cycling for fish culture with sludge as a primary nutritional source. Regardless of the significant increase in the levels of Pb^{2+} and Zn^{2+} in the soils and earthworms, the overall concentrations of these heavy metals were below the risk limits recommended by FAO/WHO and US

Environmental Protection Agencies. This might be due to bioaccumulation and transformation capability in the fishes which may limit the transformation between earthworm and fishes and accumulation of heavy metals in the flesh. Therefore, the recycling of sludge as fish feed and forages for other animals could be considered as a new nutritional source and a promising alternative management of sludge toward effective sustainable agriculture.

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