frontiers | Frontiers in Environmental Science

ORIGINAL RESEARCH
published: 14 June 2022
doi: 10.3389/fenvs.2022.888394

Sewage Sludge-Induced Effect on Growth, Enzyme Inhibition, and Genotoxicity can be Ameliorated Using Wheat Straw and Biochar in *Pheretima posthuma* Earthworms

Hira Khalid[1], *Muhammad Kashif Zahoor*[1]*, *Danish Riaz*[2], *Madeeha Arshad*[2], *Rabia Yaqoob*[2] *and Kanwal Ranian*[1]*

[1]*Department of Zoology, Government College University, Faisalabad, Pakistan,* [2]*Division of Science and Technology, University of Education Lahore, Lahore, Pakistan*

OPEN ACCESS

Edited by:
Mazhar Iqbal Zafar,
Quaid-i-Azam University, Pakistan

Reviewed by:
Sajida Rasheed,
University of Kotli Azad Jammu and Kashmir, Pakistan
Sartaj Ahmad Bhat,
Gifu University, Japan

***Correspondence:**
Muhammad Kashif Zahoor
kashif.zahoor@gcuf.edu.pk
Kanwal Ranian
kanwalranian22@gcuf.edu.pk

Specialty section:
This article was submitted to Toxicology, Pollution and the Environment, a section of the journal Frontiers in Environmental Science

Received: *02 March 2022*
Accepted: *25 April 2022*
Published: *14 June 2022*

Citation:
Khalid H, Kashif Zahoor M, Riaz D, Arshad M, Yaqoob R and Ranian K (2022) Sewage Sludge-Induced Effect on Growth, Enzyme Inhibition, and Genotoxicity can be Ameliorated Using Wheat Straw and Biochar in Pheretima posthuma Earthworms. Front. Environ. Sci. 10:888394. doi: 10.3389/fenvs.2022.888394

Sewage sludge, rich in organic matter and nutrients, is widely used as a fertilizer to increase the fertility of soil. The direct application of sewage sludge without any treatment causes soil contamination as well as significantly affects the earthworm population. In the present study, the effect of sewage sludge-amended soil on growth, enzyme activities, and the DNA damage in *Pheretima posthuma* earthworms was studied under wheat straw and biochar treatment of 30-day laboratory incubation. Wheat straw, biochar, and sewage sludge were applied at 0 (control), 5, 10, and 25% w/w along with combined treatment of sewage sludge with biochar and wheat straw, respectively at 25% each. After the incubation period, the percentage change in mean weight and length was measured and esterase and phosphatase enzyme activities were quantitatively determined. RAPD-PCR and the comet assay were performed to assess the genotoxicity. A significant weight loss was observed (26%) at a 25% rate of sewage sludge and biochar (11%). Similarly, a maximum decrease in length was observed with sewage sludge (2.5%) followed by biochar (0.80%) at a 25% application rate. Mean weight and length both decreased by increasing the rate of sewage sludge and biochar. In addition, no change was observed in the weight and length of *P. posthuma* in the treatment consisting of sewage sludge and wheat straw. Moreover, sewage sludge caused inhibition of esterase and phosphatase activities as well as induced DNA damage. The comet parameters showed that wheat straw and biochar ameliorated the toxic effects of sewage sludge. It is, therefore, concluded that sewage sludge has a tangible impact on earthworms which ultimately disrupts ecosystem functions and wheat straw and biochar can thus be utilized to reduce the toxicity of sewage sludge in *Pheretima posthuma* earthworms.

Keywords: biochar, sewage sludge, vermicompost, earthworm, comet assay, esterases and phosphatases, genotoxicity

INTRODUCTION

Earthworms are commonly defined as "ecosystem engineers" and are considered as the largest element of animal biomass. They take part in many ecosystem services such as soil structure formation, pedogenesis and nutrient cycle in soil, and regulation and movement of water where they make it more available for plants (Valckx et al., 2010; Blouin et al., 2013; Li et al., 2018). They aid in soil formation and stabilization by mixing material present in the soil, reconditioning the soil with the help of various microbes, improving soil structure and aeration, increasing soil porosity, facilitating decomposition of organic matter, and helping to develop vermicompost (Ansari and Ismail, 2012; He et al., 2016). There are more than 3,000 species of earthworm in the natural environment which turnover large quantities of organic matter. Detailed scientific knowledge is only known for about five percent. Two tons per hectare of earthworm populations have been recorded in the natural environment which can be potentially increased up to 10 times under controlled laboratory conditions. The principle function of earthworms in vermicomposting is to increase the surface area available for micro and macro-organisms involved in the decomposition and stabilization of organic matter (Murtaza et al., 2016; Murtaza, 2015).

The *Pheretima posthuma* earthworm belongs to the order Oligochaeta and class Clitella which includes all segmented worms with clitella. The structure of the clitella appears as a modification of the epidermal layer (Martin et al., 2007; Ansari and Saywack 2011). Adult *P. posthuma* is generally brownish in color due to the presence of porphyrin pigment and has a 15–30 cm body length. They are usually terrestrial and also found in the moist soil of agricultural areas, near plantations, and soil near ponds and other water bodies (Elmer et al., 2015). Earthworms are usually classified into three categories on the basis of feeding, burrowing, and the ecological niches they inhabit, i.e., aneic, endogeic, and epigeic. *P. posthuma* are endogeic worms with shallow burrowing only up to 30–45 cm deep in the soil. They generally feed on soil organic matter which is decomposed by microbial decomposers present in their guts (Van Groenigen et al., 2014). The gut, in fact, harbors a wide diversity of microorganisms including bacteria, algae, fungi, protozoa, actinomycetes, and even nematodes (Birkas et al., 2010). Earthworm species are tolerable to a range of contaminants such as herbicides, heavy metals, and the organic pollutants emerging from industries, factories, and hospitals *via* wastewater and sewage sludge (Murtaza et al., 2016; Murtaza, 2015). They reduce the concentrations and toxicity of soil contaminants by accumulating them in their body tissues (Dabke 2013; Ibrahim et al., 2016).

Sewage sludge is a byproduct of wastewater treatment plants rich in organic matter, nutrients (e.g., nitrogen, phosphorus, sodium, potassium, magnesium, and sulfur), and heavy metals including copper, zinc, cadmium, and many others (Singh and Agrawal 2008). Sewage sludge has high organic matter (40–70%) and can manipulate the physical, chemical, and biological properties of soil; thus potentially increasing the crop yield. Approximately 50% of all sludge produced in the United Kingdom and human waste-based sludge imported from the Netherlands is used in agriculture. Approximately 25% of all the sludge disposed to agriculture in the United Kingdom is raw sludge derived from small rural works (Charlton et al., 2016; Murtaza et al., 2016; Edwards and Arancon, 2004). In Pakistan, about 6.75 million tons of solid waste is being formed on a daily basis, and 50–70% of this total is either transported to open dumps or landfills. The remaining 30–50% is left as masses in populous regions that cause numerous health-related issues in dwellers (Pak-Epa, 2005; Ali and Hasan, 2001). In Pakistan, wastewater treatment plants (WWTPs) have been installed on different industrial and municipal sites and remain functional throughout the year (Haydar et al., 2015) and generate huge amounts of sewage sludge on a daily basis (Bareen and Tahira, 2011; Afzal et al., 2016). These WWTPs generate sewage sludge in large volume on a daily basis which is then used by some farmers owing to their nutritious value.

Sewage sludge is considered an inexpensive source of plant nutrients to increase soil fertility; however, the presence of heavy metals and persistent organic pollutants (POPs) in sewage sludge poses a serious threat to soil biota earthworms. The number of earthworms in soil is continuously declining due to the application of raw sewage sludge as fertilizer (Kızılkaya and Hepş;en 2004). Many studies have reported negative effects of sewage sludge on the physiology, activity, and diversity of earthworms (e.g., Kızılkaya and Hepş;en 2004; Liu et al., 2005; Stöven and Schnug 2009; Karimi e al. 2020). The addition of wheat straw to soil is of great importance as it increases the production and heat in the soil. The application of wheat straw with sewage sludge in the soil is greatly degraded by earthworms (Wanga et al., 2014). Wheat straw is an efficient culture medium for large scale production of vermicompost for sustainable land restoration practices at low input. Biochar is an organic amendment developed from pyrolysis of biomass waste in the absence of oxygen which generally consists of recalcitrant organic carbon and improves the soil physio-chemical environment (Riaz et al., 2017; Gul et al., 2015). Biochar has been shown to reduce the toxicity of both organic and inorganic pollutants in the soil environment by reducing their bioavailability to soil organisms (Park et al., 2011; Zhang et al., 2013; Zhang et al., 2017). Subsequently, biochar reduces the negative effects of pollutants on earthworms (e.g., Malińska et al., 2016; Sanchez-Hernandez et al., 2019).

Despite the handful of benefits associated with land application of sewage sludge, the presence of potentially toxic elements in these amendments could be a serious threat to soil, soil fauna, and also, to a certain extent, to food health and quality (Murtaza et al., 2016; Murtaza, 2015). Since, very limited data are available on the toxicity and overall effect of sewage sludge on the growth and enzyme activities in earthworms, the present study was conducted wherein we hypothesized that wheat straw and biochar would decrease the toxic effect of sewage sludge on growth and enzyme activities in *Pheretima posthuma* earthworms. Whereas the addition of wheat straw and biochar would also improve earthworm activities by providing them with food and reducing the negative effects of sewage sludge, and these

effects would largely depend upon the rate of amendment applications. The objectives of this study, therefore, were to investigate the deleterious effects of different concentrations of sewage sludge on the growth, enzyme activities, and genotoxicity in *P. posthuma* earthworms under laboratory conditions.

MATERIAL AND METHODS

Collection of Earthworms and Soil Samples

The samples of *P. posthuma* earthworms with well-defined clitella were collected by hand digging from agricultural field plots in Faisalabad as they are one of the most abundantly found earthworms in the soil of Pakistan. Soil samples were also collected from the area where the earthworms were collected. The soil was dried in air and sieved through a 2.0 mm mesh to remove any vegetation litter and stones. The soil was alkaline calcareous clay loam with pH 8.02, EC 1.34 dS m^{-1}, total organic C 9.21 g $kg^{-1,}$ and total N 0.54 g kg^{-1} (Riaz et al., 2017). Earthworms were kept in plastic buckets containing soil moistened to a 60% water holding capacity (WHC) and mixed with leaf litter (Ali et al., 2019). Simultaneously, the soil was brought to 60% WHC and pre-incubated to recondition the earthworms with optimum microbial activities before the start of the experiment.

Collection of Sewage Sludge, Wheat Straw, and Biochar

Sewage sludge was collected from Water and Sanitation Agency Faisalabad, air-dried, and sieved with 2.0 mm mesh for uniform particle size. It had a pH of 7.06, EC 12.46 dS m^{-1}, total organic C 146 g kg^{-1}, total N 1.19 g kg^{-1} and total P 1.02 g kg^{-1}, and heavy metals including Cd (0.86 mg kg^{-1}), Cr (50 mg kg^{-1}), Ni (56 mg kg^{-1}), and Pb (47 mg kg^{-1}) (Riaz et al., 2020). Wheat straw was collected from an agriculture field in Faisalabad and was crushed in a sieve from a 2.0 mm mesh. It had a total organic C of 427 g kg^{-1}, a total N of 6.6 g kg^{-1}, and a C/N ratio of 65 (Riaz et al., 2017). Biochar was collected from the Department of Environmental Sciences and Engineering, Government College University Faisalabad. Biochar was developed from corncob biomass at 400°C and contained 733 and 10.3 g kg^{-1} of total organic C and total N contents, respectively (Riaz et al., 2017).

Laboratory Incubation Experiment

A microcosm experimental setup was developed in 1 L glass Mason jars filled with 700 g of pre-incubated moist soils mixed with treatments in a completely randomized experimental design. Treatments consisted of control (soil only), wheat straw, and biochar at 5, 10, and 25% w/w basis rates where wheat straw plus biochar received 25% each of wheat straw and biochar. Each treatment was replicated three times. Earthworms were pre-starved by being placed on petri plates on a moistened paper towel overnight to ensure their guts were free of any food before introducing them into the microcosms. After starvation, the body weight and length of each group of earthworms (consisting of three biological replicates) were measured before introducing them into the treatment jars. During the 30 day incubation period, the jars were partially closed with their lids, and moisture contents were maintained on a daily basis by adding distilled water. After 30 days of exposure, at the end of the incubation period, the soil was removed from incubation jars to remove earthworms which were gently washed in tap water, partially dried with paper towels, and their body weights (g) and lengths (cm) were measured. Changes in body weight and length were measured with the following formulae (Givaudan et al., 2014).

$$\text{Change in weight } (\%) = \left(\frac{\text{Final weight} - \text{Initial weight}}{\text{Final weight}}\right) \times 100 \quad (1)$$

$$\text{Change in length } (\%) = \left(\frac{\text{Final length} - \text{Initial length}}{\text{Final length}}\right) \times 100 \quad (2)$$

The worms were preserved in 70% alcohol for further biochemical and molecular assays.

Quantitative Analysis of Esterases and Phosphatases in *Pheretima posthuma* Earthworms

A body extract of earthworm was prepared by following a modified protocol of Hrzenjak et al. (1992). The adult clitellate earthworms (1.35 g/per worm) were washed with running tap water and kept in 0.65% NaCl solution at room temperature for 2 h to ensure their guts were empty. Worms were minced and homogenized in sodium phosphate buffer, centrifuged at 8,000 rpm, and the supernatant was used for the estimation of esterase and phosphatase activities (Callaham et al., 2002; Younes et al., 2011). The percentage inhibition of the enzyme activity in test extracts was calculated as follows:

$$\text{Enzyme Inhibition } (\%) = \left(\frac{\text{OD of control worm} - \text{OD of treated orm}}{\text{OD of control worm}}\right) \times 100 \quad (3)$$

DNA Extraction

Genomic DNA was extracted from earthworms by the TNE (Tris-HCL-NaCl-EDTA) buffer method (Zulhussnain et al., 2020). Earthworms (prostomium, clitella, gut, and tail) were separately homogenized in 400 µl of TNE buffer (0.45 M NaCl, 3 mM EDTA, and 10 mM Tris-HCl; pH: 8.0). The mixture was vortexed and centrifuged at 10,000 rpm for 10 min before the supernatant was collected. After the addition of 50 µl of 20% SDS and 100 µl of 20 mg/ml of proteinase-K to the supernatants, samples were incubated in a water bath at 54°C for 1.5 h. After incubation, 300 µl of 5 M NaCl was added and DNA was precipitated using 350–400 µl of ice-cold 100% ethanol and resuspended in 50 µl of sterile water (D_3H_2O). The concentration of DNA was quantified by measuring the absorption of samples at 260 nm on a UV Spectrophotometer (Dynamic DB-20, United Kingdom).

Random Amplified Polymerase DNA Polymerase Chain Reaction

DNA variations of earthworms were determined by employing the RAPD-PCR assay (Zulhussnain et al., 2020). Six oligomer primers of Gene Link-A series were used for the amplification of DNA (**Supplementary Table S1**). The PCR products were run on 1.5% agarose gel electrophoresis at 80 voltages for 1 h and the gel was observed under UV light. The size of DNA bands was observed with respect to the ladder.

Comet Assay

A comet assay was performed for the detection of DNA damage by following the methodology of Singh et al. (1988). Different parts of dissected earthworms (head, tail, clitellum, prostomium, and gut) were homogenized in 400 µl of buffer (0.07 M NaCl and 0.24 M EDTA). The mixture was centrifugated at 13,000 rpm for 10 min and the palette was resuspended in 1,000 µl of ice-cold homogenizing buffer for nuclei preparation. The earthworm body mixture (100 µl) was mixed with 200 µl of 1% LMPA and evenly distributed on a slide precoated with NMPA. Slides were dipped in freshly prepared cold lysing solution (100 mM EDTA, 3 M NaCL, 12 mM Tris-HCl, pH 10). Then, 10% dimethyl sulphoxide (DMSO) and 1% X-100 Triton were added to the lysing solution 20 min before use. After lysing, the slides were dipped in alkaline buffer (1 mM EDTA, 400 mM NaOH; pH 12.5) for 20 min for DNA unwinding. Electrophoresis was done in electrophoresis buffer (0.18 EDTA, 400 mM NaOH, pH 13) for 20 min, 25 min on 25 V and 300 mA. Slides were neutralized by washing for 5 min with neutralizing buffer (Tris-Base, pH 7.5) and left overnight on a clean dust-free area. Slides were evenly stained with 20 mg ml^{-1} of 75 µl ethidium bromide (EB), with cover slips. After staining, slides were examined at ×400 magnification with Komet 5.5 Image Analysis Systems (Kinetic Imaging, Liverpool, United Kingdom). A total of 100 cells were selected randomly from each slide and were analyzed for damages in DNA showing a definite tail and comet-like shape whereas the cells with no tail and round shape were considered normal.

The actual number of comet cells was scored with the following formula:

$$\text{Total DNA Damage Score} = (0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4)$$

Where 0–4 is the number of comet classes, and n_0–n_4 is the number of comet scores in each class.

Arbitrary unit damage is classified into five levels (0–4). 0 indicates no damage while four represents maximum damage. The other three levels are intermediate between 0 and 4. Computer image analysis ($Casp^R$ software) was used to measure head length, tail length, comet length, head DNA, tail DNA, and tail movement. Cells with maximum damage showed the highest value for tail DNA and minimum value of head DNA (Attullah et al., 2020; Zulhussnain et al., 2020).

Statistical Analysis

The data were subjected to the analysis of variance (ANOVA) test to find significance of treatment effects on experimental variables using Statistica 13.0 for Windows software. The means were separated using Tukey's HSD post-hoc test at the significance level of 0.05. PCR products were analyzed using gel electrophoresis and the genetic data were analyzed using POPGENE software (Ashraf et al., 2016). For the comet assay, the DNA damage in the earthworm cells was assessed following the assumptions of Dua et al. (2013) and reported by Zulhussnain et al. (2020) and Attullah et al. (2020).

RESULTS

Changes in Weight and Length of *Pheretima posthuma*

The respective change in weight and length of *P. posthuma* after exposing them to sewage sludge, wheat straw, biochar, sewage sludge + wheat straw, and sewage sludge + biochar is shown in **Table 1**. The mean weight was found to decrease significantly with increased concentration of sewage sludge and biochar. The highest weight loss of 26% was observed under sewage sludge at a 25% application rate followed by 11% in 25% biochar-treated soil. Weight loss with the treatment of 5 and 10% of sewage sludge was 2% and 16% respectively, followed by biochar treatment as 1% and 6%, respectively at the same application. However, the decrease in body weight following the sewage sludge + wheat straw treatment was not significant compared to the control and biochar and wheat straw treatments alone. The highest decrease in body weight was observed at a 25% concentration of sewage sludge while the lowest decrease in weight was recorded after the addition of sewage sludge and wheat straw combined (**Table 1**).

Similarly, it was observed that mean length decreased significantly with the increase in rate of sewage sludge application. A maximum decrease in length (3%) was observed at a 25% rate of sewage sludge followed by biochar (1%). At 5 and 10% concentrations of sewage sludge, the decrease in mean length was found to be 1% and 2%, respectively followed by biochar at the same rate. There was a slight change in mean length (0.14%) with the treatment of 25% sewage sludge in combination with the same rate of biochar. No change in length was observed with the combined treatment of sewage sludge + wheat straw (**Table 1**).

Enzymatic Activities in *Pheretima posthuma*

The effects of treatments and application rates on the activities of acetylcholinesterase (AChE), acid phosphate (ACP), and alkaline phosphatase (AKP) in earthworms are presented in **Table 2**. The inhibition in acetylcholinesterase activity was increased significantly with the increase in concentrations of soil amendments. The maximum inhibition in acetylcholinesterase activity was found at 25% sewage sludge (40%) followed by biochar and wheat straw at the same rate. At 5% and 10% sewage sludge, the inhibition in acetylcholinesterase activity was 7 and 28%, respectively. However, biochar amendment at

TABLE 1 | Percentage decrease in mean weight and length after exposure to different concentrations of soil amendments in *Pheretima posthuma*.

Treatments	Rate (%, w/w)	F values; d.f; *p* Value	Percent mean weight decrease after 30 days of exposure	F Value; d.f; *p* Value	Percentage mean length decrease after 30 days of exposure
Control	0	70.669 (df = 2; *p*=<0.05)	0.2 ± 0.05f	0.800 (df = 2; *p*=<0.05)<	0.001 ± 0.05d
WS	5% 10% 25%	70.669 (df = 2; *p*=<0.05)	0.1 ± 0.03f 0.01 ± 0.003f 0.1 ± 0.003f	0.800 (df = 2; *p*=<0.05)	0.02 ± 0.02c 0.01 ± 0.00b 0.01 ± 0.00b
B	5% 10% 25%	1022 (df = 2; *p*=<0.05)	1.3 ± 0.03ef 6.4 ± 0.03d 11.00 ± 0.5c	1594.158 (df = 2; *p*=<0.05)	0.13 ± 0.03a 0.53 ± 0.04a 0.80 ± 0.01c
SS	5% 10% 25%	969.73 (df = 2; *p*=<0.05)	2.3 ± 0.06e 16.07 ± 0.51b 25.75 ± 0.58a	276.117 (df = 2; *p*=<0.05)	0.64 ± 0.01d 1.74 ± 0.05b 2.5 ± 0.00a
SS + WS SS + B	25% + 25% 25% + 25%	1022.323 (df = 2; *p*=<0.05)	0.00 ± 0.005f 0.16 ± 0.03f	0.180 (df = 2; *p*=<0.05)	0.00 ± 0.00d 0.14 ± 0.029c

p < *0.01 = highly significant,* p < *0.001 = highly significant,* p > *0.05 = non-significant,* p < *0.05 = significant. WS, wheat straw; B, biochar; SS, sewage sludge.*

TABLE 2 | Percentage inhibition of different enzyme activities after exposure to different concentrations of soil amendments in *Pheretima posthuma*.

Treatments	Rate (%)	F Value; d.f; *p* Value	Acetylcholinesterase (AChE) activity	F Value; d.f; *p* Value	Acid Phosphatase (ACP) activity	F Value; d.f; *p* Value	Alkaline Phosphatase (AKP) activity
Control	0	0.642 (df = 2; *p*=<0.05)	0.2 ± 0.05F	1.647 (df = 2; *p*=<0.05)	0.0 ± 0.05D	3.250 (df = 2; *p*=<0.05)	0.00 ± 0.05D
WS	5% 10% 25%	0.642 (df = 2; *p*=<0.05)	0.01 ± 0.01b 0.01 ± 0.03b 0.1 ± 0.003a	1.647 (df = 2; *p*=<0.05)	0.006 ± 0.00b 0.01 ± 0.00a 0.003 ± 0.00c	3.250 (df = 2; *p*=<0.05)	0.013 ± 0.01a 0.00 ± 0.00b 0.01 ± 0.00b
B	5% 10% 25%	458 (df = 2; *p*=<0.05)	4.6 ± 0.08c 8.6 ± 0.29b 15.83 ± 1.4a	133.5 (df = 2; *p*=<0.05)	9.0 ± 0.10c 10.8 ± 0.91b 22.70 ± 0.63a	41.98 (df = 2; *p*=<0.05)	8.66 ± 0.21c 16.18 ± 0.83b 20.40 ± 1.33a
SS	5% 10% 25%	209 (df = 2; *p*=<0.05)	6.70 ± 0.32c 27.7 ± 0.6b 39.83 ± 1.8a	267.5 (df = 2; *p*=<0.05)	9.36 ± 0.42c 33.07 ± 1.5b 47.60 ± 1.2a	37.85 (df = 2; *p*=<0.05)	4.70 ± 0.29c 18.03 ± 2.5b 23.05 ± 2.23b
SS + WS SS + B	25% + 25% 25% + 25%	176 (df = 2; *p*=<0.05)	0.00 ± 0.01c 2.96 ± 0.2a	296 (df = 2; *p*=<0.05)	0.003 ± 0.0015c 11.03 ± 0.6a	620 (df = 2; *p*=<0.05)	0.02 ± 0.01c 7.06 ± 0.08a

p < *0.01 = highly significant,* p < *0.001 = highly significant,* p > *0.05 = non-significant,* p < *0.05 = significant. W, wheat straw; B, biochar; SS, sewage sludge.*

5% and 10% rate caused inhibition of 5% and 9%, respectively. A very low inhibition in acetylcholinesterase activity was found with the treatment of sewage sludge and wheat straw combined; while an inhibition of 3% in the acetylcholinesterase activity was observed in the combined addition of sewage sludge and biochar (**Table 2**).

Maximum and significant inhibition in acid phosphatase activity in *P. posthuma* was observed at a 25% application of sewage sludge (50%) followed by biochar (23%) at the same application rate (**Table 2**). At 5% and 10% sewage sludge rates, the decrease in acid phosphatase activity was 9 and 33%, respectively and the inhibition at 5 and 10% biochar rates was 9 and 11%, respectively. The combination of 25% sewage sludge with the same concentration of wheat straw reduced the negative effects of sewage sludge on acid phosphatase activity and resulted in <1% inhibition. Similarly, wheat biochar with sewage sludge also reduced inhibition in acid phosphatase activity (11%; **Table 2**).

The highest percentage inhibition of alkaline-phosphatase activity was found at 25% sewage sludge (23%) followed by biochar (21%) (**Table 2**). At a 10% rate of sewage sludge and biochar separately, the inhibition was found to be 18% and 16%, respectively. However, at a 5% rate, biochar more strongly reduced alkaline-phosphatase activity than that of sewage sludge. In the combined application of sewage sludge and biochar, a 7% inhibition of alkaline-phosphatase activity was found. Whereas, the combination of wheat straw and sewage sludge reduced the negative effects of sewage sludge on alkaline-phosphatase activity and resulted in <1% inhibition (**Table 2**).

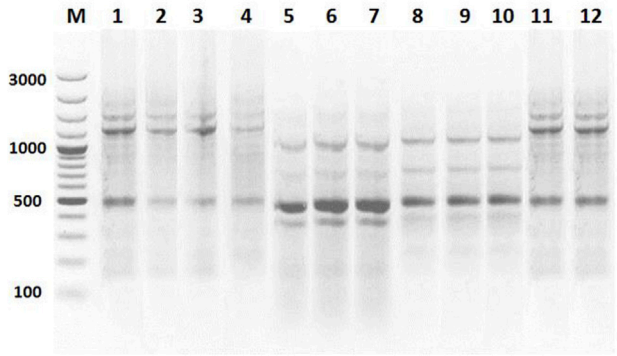

FIGURE 1 | Comparison of RAPD profile for genotoxicity in *Pheretima posthuma* with primer A-05. Lane M = DNA marker (size 100 bp). L_1 = Control; L_2 = Wheat straw (5%); L_3 = Wheat straw (10%); L_4 = Wheat straw (25%), L_5 = Biochar (5%), L_6 = Biochar (10%), L_7 = Biochar (25%), L_8 = Sewage sludge (5%), L_9 = Sewage sludge (10%), L_{10} = Sewage sludge (25%), L_{11} = Sewage sludge + wheat straw, and L_{12} = Sewage sludge + Biochar.

DNA Damage Detection Through Random Amplified Polymerase DNA Polymerase Chain Reaction

DNA damage in *P. posthuma* under various treatments of sewage sludge, biochar, and wheat straw was assessed through RAPD-PCR to evaluate the genotoxic effects. The size of amplified fragments of DNA was analyzed using a 1 kb DNA marker. The lowest size of the band of DNA maker was 100 base pairs (bp) and the band with highest size was of 5,000 bp. A different banding profile was observed in earthworms under treatments than control (**Figure 1**). The maximum number of bands was observed in sewage sludge treatment at all tested concentrations which showed fragmentation in DNA. A similar banding pattern was also observed with biochar. The number of bands was increased with the increase of the concentration of sewage sludge and biochar. No change in banding profile was observed in treatment with wheat straw similar to control. The number of DNA bands also remained similar for control and

TABLE 3 | Comet parameters after exposure to different concentrations of soil amendments in *Pheretima posthuma*.

Comet Parameters	Treatment	F Value; d.f; *p* Value	Rate (%, w/w)			
			Control	5%	10%	25%
Tail length (μm)	WS	(F = 9.50); d.f = 3; *p* (0.005)	3.47 ± 0.28c	4.82 ± 0.20a	3.85 ± 0.10bc	4.39 ± 0.11ab
	B	(F = 448.55); d.f = 3; *p* (0.000)		7.75 ± 0.24b	8.47 ± 0.36b	19.36 ± 0.35a
	SS	(F = 51.51); d.f = 3; *p* (0.000)		8.34 ± 0.34b	10.63 ± 0.20a	9.49 ± 0.72ab
	SS + WS	(F = 52.18); d.f = 3; *p* (0.002)		(SS + WS 25% + 25%) 6.67 ± 0.44a		
	SS + B	(F = 146.3); d.f = 3; *p* (<0.05)		(SS + B 25% + 25%) 10.77 ± 0.23a		
Comet length (μm)	WS	(F = 53.29); d.f = 3; *p* (0.000)	9.29 ± 0.51b	15.74 ± 0.73a	16.30 ± 0.32a	17.73 ± 0.49a
	B	(F = 306.65); d.f = 3; *p* (0.000)		18.82 ± 0.48c	23.37 ± 0.41b	27.49 ± 0.35a
	SS	(F = 181.41); d.f = 3; *p* (0.000)		19.04 ± 0.37c	24.88 ± 0.97b	27.85 ± 0.34a
	SS + WS	(F = 118.16); d.f = 3; *p* (0.000)		(SS + WS 25% + 25%) 18.24 ± 0.64a		
	SS + B	(F = 102.6); d.f = 3; *p* (<0.05)		(SS + B 25% + 25%) 25.21 ± 0.42b		
Tail DNA (%)	WS	(F = 10.46); d.f = 3; *p* (0.004)	1.34 ± 0.08d	2.9 ± 0.17ab	2.91 ± 0.14ab	4.57 ± 0.78a
	B	(F = 43.55); d.f = 3; *p* (<0.000)		11.76 ± 0.29c	28.73 ± 4.14b	44.80 ± 3.66a
	SS	(F = 1596); d.f = 3; *p* (0.000)		46.32 ± 0.88c	76.10 ± 0.88b	80.72 ± 0.67a
	SS + WS	(F = 606.9); d.f = 3; *p* (0.000)		(SS + WS 25% + 25%) 60.86 ± 0.76a		
	SS + B	(F = 128.7); d.f = 3; *p* (<0.05)		(SS + B 25% + 25%) 78.82 ± 0.12b		
Tail movement (μm)	WS	(F = 25.67); d.f = 3; *p* (0.000)	0.13 ± 0.07c	0.4 ± 0.04a	0.27 ± 0.03b	0.42 ± 0.00a
	B	(F = 26.75); d.f = 3; *p* (0.000)		4.89 ± 2.15b	15.10 ± 1.76a	18.66 ± 1.84a
	SS	(F = 5.519); d.f = 3; *p* (0.024)		9.6 ± 0.68ab	9.7 ± 4.2ab	11.63 ± 0.31a
	SS + WS	(F = 166.3); d.f = 3; *p* (0.000)		(SS + WS 25% + 25%) 11.56 ± 0.80a		
	SS + B	(F = 328.72); d.f = 3; *p* (<0.05)		(SS + B 25% + 25%) 12.22 ± 0.18a		
Head length (μm)	WS	(F = 22.12); d.f = 3; *p* (0.000)	8.43 ± 0.37b	10.46 ± 0.95b	14.81 ± 0.59a	16.64 ± 1.09a
	B	(F = 7.285); d.f = 3; *p* (0.011)		13.74 ± 7.0c	18.60 ± 0.30b	30.76 ± 0.79a
	SS	(F = 31.71); d.f = 3; *p* (0.000)		18.47 ± 0.93a	20.11 ± 0.94a	22.70 ± 1.73a
	SS + WS	(F = 153.10); d.f = 3; *p* (<0.000)		(SS + WS 25% + 25%) 17.16 ± 0.60a		
	SS + B	(F = 498.18); d.f = 1; *p* (<0.05)		(SS + B 25% + 25%) 19.87 ± 0.12a		
Head DNA (%)	WS	(F = 21.80); d.f = 3; *p* (0.000)	92.35 ± 1.21a	89.77 ± 1.27a	85.12 ± 0.66b	82.46 ± 0.31b
	B	(F = 155.32); d.f = 3; *p* (0.000)		63.44 ± 2.7b	57.07 ± 1.9b	36.74 ± 0.90c
	SS	(F = 752.73); d.f = 3; *p* (0.000)		49.94 ± 1.32b	21.61 ± 1.64c	18.32 ± 0.5c
	SS + WS	(F = 502.1); d.f = 3; *p* (0.000)		(SS + WS 25% + 25%) 38.56 ± 0.47a		
	SS + B	(F = 262.4); d.f = 3; *p* (<0.05)		(SS + B 25% + 25%) 32.24 ± 0.24c		

p < *0.01 = highly significant,* p < *0.001 = highly significant,* p > *0.05 = non-significant,* p < *0.05 = significant. WS, wheat straw; B, biochar; SS, sewage sludge.*

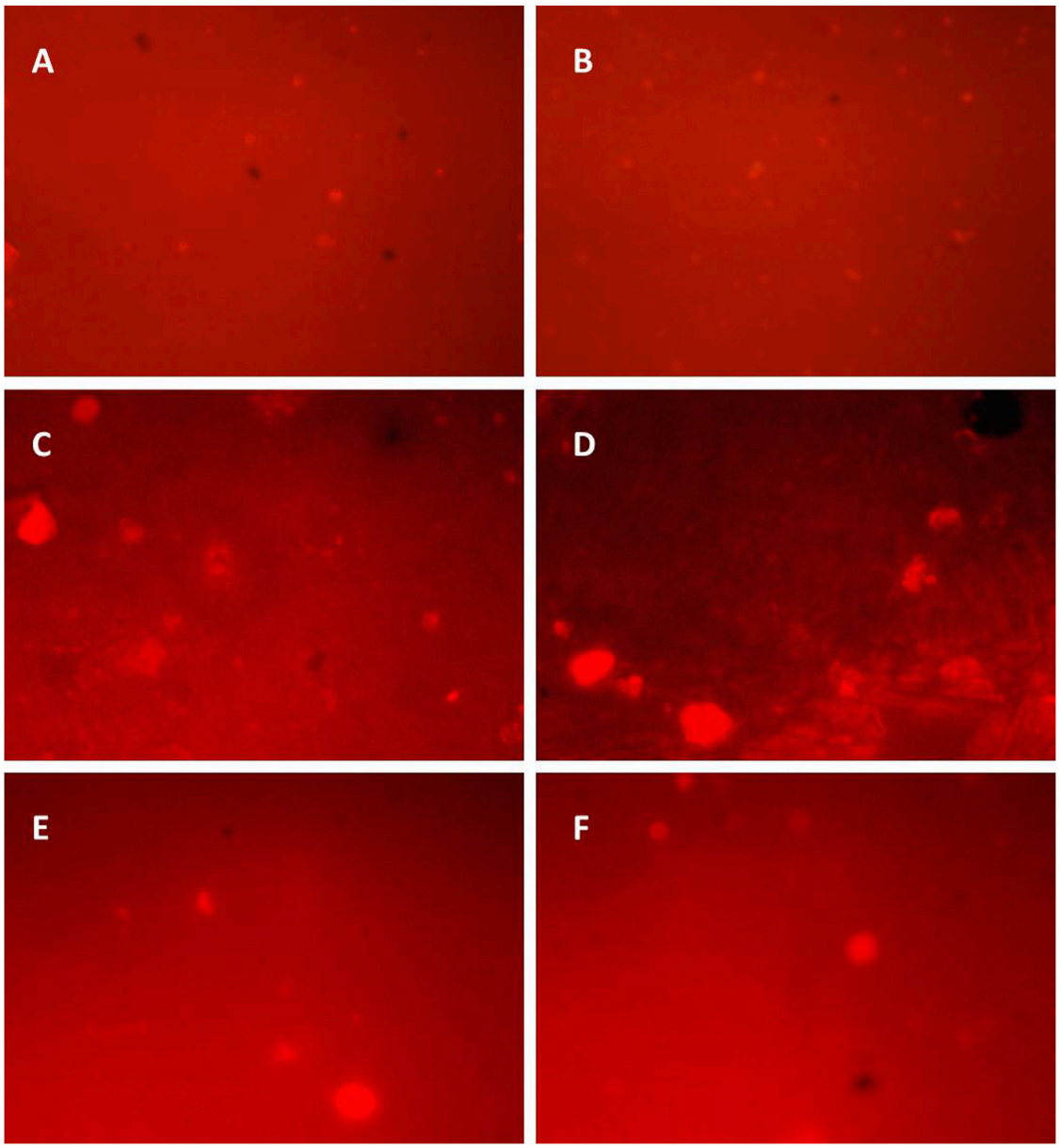

FIGURE 2 | Comet assay profile for genotoxicity in *Pheretima posthuma.* **(A)** = Control; **(B)** = Wheat straw; **(C)** = Biochar; **(D)** = Sewage sludge; **(E)** = Sewage sludge 25% + Biochar 25%; **(F)** = Sewage sludge 25% + Wheat straw 25%.

wheat straw + sewage sludge treatments in *P. posthuma*; meaning thereby, that DNA damage occurred in this combined treatment (**Figure 1**).

Comet Assay

The comet parameters, i.e., head and the tail length of DNA and head and tail movement, were measured to identify the DNA damage. A very minor increase in tail length was observed in earthworms treated with wheat straw at 5, 10, and 25% rates as compared to control. However, under biochar treatment, tail and head lengths increased with an increase in the rate of application. A similar trend was also observed with the treatment of different concentrations of sewage sludge wherein tail DNA (%), head DNA (%), tail length, and head length increased with increasing concentrations. In combined treatments of sewage sludge against wheat straw and biochar, a change in head and tail length was obtained as compared to treatment with wheat straw alone (**Table 3**). The tail length value was found significantly low in treatments with wheat straw at all the concentrations. Cells with maximum damage show the highest value for tail DNA. Hence, the highest value of tail DNA% (46, 76, and 81%, at 5, 10, and 25%, respectively) due to sewage sludge showed high DNA damage which was reduced (18 at 25%) when combined with wheat straw (**Figure 2**).

DISCUSSION

Earthworms assist soil. To regulate water within the soil, their presence influences the presence of nutrients and minerals within soil and the availability of these nutrients for animals and plants. They increase the microbial activity in the soil. They perform vermicomposting of sewage sludge and other pollutants within the soil (Wang et al., 2019). But the application of sewage sludge and heavy metals bioaccumulate in the earthworm's body and cause physiological and biochemical damage (Babic et al., 2016; Kooch and Jalil 2008; Ahmad et al., 2021).

Earthworms cause the fragmentation of sewage sludge, thus converting it into castings and increasing the surface area available for drying and microbial decomposition; improving aeration due to the tunnelling action of worms. In the United Kingdom and mostly in Europe, human waste-based sewage sludge is being used which is high yielding towards crops. In Pakistan, wastewater treatment plants (WWTPs) have been installed on different industrial and municipal sites and remain functional throughout the year to generate huge amounts of sewage sludge on a daily basis (Haydar et al., 2015). Particularly, this sewage sludge potentially causes a negative effect in earthworms. It produces growth defects and induces enzyme inhibition and toxicity potentially at the genetic level. The land application of sewage sludge can damage soil, water, and plants since it contains variable levels of organic and inorganic pollutants, salts, and pathogens (Singh and Agrawal, 2008 and 2010; Latare et al., 2014; Qadir et al., 2015). Despite being considered fertilizer in the farming community, it has been reported that the sewage sludge affects the soil fauna and resulted in low crop yield. The yield reduction could be due to the heavy metal accumulation in the soil which is in line with previous studies reported on maize, pea, and mustard. In fact, the higher contents of metal in sewage sludge like Cd and Pb can lead to a toxic effect on the soil fauna (Liu et al., 2005). Singh and Agrawal (2010) reported that the application of sewage sludge increased the straw yield of rice but at higher rates negatively affected the yield due to higher accumulation of metals in soil fauna presumably earthworms and ultimately resulted in a decreased yield.

The *P. posthuma* earthworm, being abundantly found across Pakistan, plays an important role in our ecosystem, food chain, and soil. Thus, the present study was performed to assess the toxic effects of sewage sludge on *P. posthuma* earthworms and how biochar and wheat straw can ameliorate the toxicity in terms of growth and weight of worms before and after treatment. In addition, the damage at the biochemical and molecular levels in earthworms was also studied (Khan et al., 2017; Wang et al., 2019). Application of sewage sludge alone is toxic for normal growth and reproduction of earthworms as compared to combining treatment with wheat straw and biochar. Growth parameters were suppressed with an increase in the rate of sewage sludge. A decrease in mean weight was observed and a similar rate-dependent decrease in length was also observed. The very low mean decrease in length was observed in lower concentrations of sewage sludge. For a 5% concentration of sewage sludge, the decrease in length was only 0.65%, but when increasing the concentration, a decrease in length was also seen (Dominguez et al., 2012). These results are supported by the study of Zaltauskaite and Sodiene (2010) who reported a smaller decrease in weight at lower concentrations of sewage sludge. However, a higher decrease in weight of earthworms at higher sewage sludge rates have been shown earlier (Yang et al., 2014). The reduction in the length of earthworms after exposure to sewage sludge has been attributed to the poor development of earthworms (Lemtiri et al., 2015; Babic et al., 2016).

The addition of wheat straw with sewage sludge is of great importance for bioremediation of sludge, soil fertility, earthworm growth, functioning to facilitate organic matter turnover, and nutrient availability for all soil life. Wheat straw also facilitated fast and efficient bioremediation of sewage sludge-amended soil by earthworms without genetically and physiologically affecting the earthworms (Yang et al., 2014; Elyamine and AU 2020). In the present study, we found that little or no changes in length and weight of earthworms were observed in the mixed treatment consisting of 25% sewage sludge and wheat straw (Sizmur et al., 2017). The normal growth rates and development of earthworms in wheat straw and sewage sludge-mixed treatments showed a positive correlation between sewage sludge and wheat straw. The results of the present study are in accordance to the findings of Yang et al. (2014) who reported similar positive effects of wheat straw on earthworm growth and functions in sewage sludge applied to soil to enhance active remediation of sewage sludge without affecting the population of earthworms. Wheat straw is a natural organic fertilizer and its combined application with sewage sludge enhances the microbial activity of soil, hence decontamination of sewage sludge containing heavy metals also increases without affecting the population of *Pheretima posthuma* in soils.

The current results showed that the excessive use of sewage sludge as a fertilizer resulted in the accumulation of heavy metals in soils and earthworm bodies and a decrease in percentage weight and length was also reported if worms were treated solely with biochar, and the weight and length decreased with increasing biochar rate. A maximum decrease in weight was observed at a 25% biochar rate followed by 10%. However, when earthworms were treated with sewage sludge and biochar combined, the negative effects of sewage sludge were significantly reduced as was evident from the minor decrease in weight and length. The addition of biochar along with sewage sludge could rescue the negative effect on growth (Zhang et al., 2017; Sanchez-Hernandez et al., 2019). Biochar alone has been shown to have little effect on earthworms which was reflected in recent studies in the low reduction in length and weight in some of the earthworms after biochar application; however, combined application of sewage sludge and biochar moderated their toxic effects as shown by minor changes in length and weight of earthworms. So, the disposal of sewage sludge with biochar is the best and safe way to reuse sludge in an environmentally friendly way (Li et al., 2011).

Sewage sludge, wheat straw, and biochar were also tested for their effects on the enzyme activity of *P. posthuma* for acetylcholinesterases, acid phosphatases, and alkaline

phosphatases; as enzymes are important indicators of toxicant accumulation at the molecular level in worms. The acid phosphatases group of enzymes is related to the class of enzyme hydrolases and the enzyme in acidic conditions catalyzes the hydrolysis of monoester (orthophosphate) (Tewari et al., 2016). Alkaline phosphatase is an enzyme that phosphorylates molecules under alkaline conditions which are present throughout the body of earthworms but occur most excessively within the gizzard/crop and intestine of earthworms. Acetylcholinesterase is a serine group that belongs to the class of hydrolases and is reported to target sites of many toxicants. Alkaline and acidic phosphatases are involved in the detoxification of many toxicants containing heavy metals. The addition of contaminants to soil-inhabiting earthworms directly affects the enzyme activity of earthworms (Wang et al., 2016). Our study showed that the sewage sludge induces changes in the activity of crucial enzymes of earthworms responsible for the metabolism of the energy, neurotransmission, and oxidation systems. The dose-dependent response was observed by increasing the concentration of sewage sludge enzyme inhibition (Sanchez-Hernandez et al., 2020). In the case of sewage sludge, acid phosphatase activity was affected by sewage sludge followed by biochar and at the same concentrations of these amendments also caused inhibition of acetylcholinesterase activity within earthworms. However, alkaline phosphates seemed less influenced by the same concentrations of sewage sludge and biochar. The results further demonstrated that the inhibition in enzyme activities increased with increasing rates of sewage sludge and biochar. However, when wheat straw and biochar were applied in combination with sewage, inhibition in enzymatic activities was significantly reduced suggesting that both wheat straw and biochar salvaged the negative effects of sewage sludge which caused physiological abnormalities in earthworms (Song et al., 2009; Yasmin 2010).

PCR-based molecular approaches are considered to provide a reliable estimation of DNA damage in earthworms exposed to contaminants, as earthworms are bioindicators of toxicant contamination. This technique is relatively inexpensive and technically simple yet provides robust estimates of DNA damages. The RAPD DNA primer marker is commonly used to assess the damage caused by sewage sludge and other contaminants present in soil (Sharma et al., 2011). We found that the maximum number of bands (fragmented DNA; damaged) were observed at a 25% sewage sludge application rate followed by biochar treatment at the same rate. Fewer bands were observed in treatments with sewage sludge applied in combination with biochar and wheat straw. The sewage sludge caused DNA damage in *P. posthuma* which can be diminished by using wheat straw and biochar. It has been reported that sewage sludge applied to soils without any pre-treatment causes lower development and reproduction and genotoxicity to earthworms which can be reduced by applying other organic amendments such as wheat straw and biochar along with sewage sludge (Yadhav and Mullah 2017; Fouche et al., 2022). However, there can be unintended negative effects of the application of biochar alone in soil on DNA and enzymatic activities in earthworms as were evident from our study and also suggested by Kim et al. (2014). The application of biochar (black carbon) alone on soil influences earthworm behavior, growth, and reproduction and also causes damage at the molecular level. The number of castings by earthworms is also declined in soils with biochar which indicate a change in the feeding habitat of earthworms under the influence of biochar (Weyears et al., 2011).

The comet assay is considered as a very reliable technique used for detecting damage and repairing DNA to assess the genotoxicity caused by the application of chemicals and pesticides (Attullah et al., 2020; Zulhussnain et al., 2020). Hence, in the present study, a comet assay was performed to assess the genotoxicity in *P. posthuma* caused by the application of sewage sludge. It was observed that DNA damage increased as observed with DNA tail length and tail movement increases with the increasing concentration of sewage sludge (25%) followed by biochar (25%) as compared to control which showed damage at the DNA level of *P. posthuma*. There was a much smaller increase in DNA head length and movement in the case of control and wheat straw-treated *P. posthuma* at all the concentrations. The sewage sludge when combined with wheat straw and biochar resulted in a change in DNA head and tail length compared to treatment with wheat straw and biochar alone. The high value of tail DNA in the comet assay showed maximum damage; thus, the high value of tail DNA% due to sewage sludge revealed high DNA damage which was reduced with treatment of wheat straw (Button et al., 2010; Fouche et al., 2022).

CONCLUSION

The present study revealed the negative effects of sewage sludge on growth, enzyme inhibitory activity, and genotoxicity in *P. posthuma* earthworms. It was found that sewage sludge showed more toxic effects individually, which caused a decrease in length and body weight as well as inhibition of enzyme activity and induced DNA damage. Being a good fertilizer for crops, sewage sludge should be used only after the addition of proper soil amendments like wheat straw and biochar, otherwise it causes severe damage to growth, development, and functioning of earthworms. Wheat straw can rescue the negative effects produced in *P. posthuma* earthworms and helps increase the production of vermicompost. Based on the current work, it is suggested that the use of wheat straw and biochar can rescue the negative effects of sewage sludge in *P. posthuma* earthworms. The research work needs to be extended to examine sewage sludge for the presence of heavy metals. The study can also be further extended for other industrial wastes: effluents normally used for irrigation purpose and herbicides frequently used in agriculture. We also emphasize that these effects need to be tested on other species of earthworms under field conditions for better risk assessment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MKZ designed and supervised the work. HK and KR performed the work. DR helped during sampling and experimentation. MA and RY provided help during the biochemical assay and data analyses. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

The facilities provided by Department of Zoology and Environmental Sciences and Engineering, GCUF are highly acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.888394/full#supplementary-material

REFERENCES

Afzal, M. Z., Ikram, M., and Israr, S. (2016). Study of the Pollutants in Wastewater from Edible Oil/ghee Industries and Their Impacts on Plant Life, Islamabad, Pakistan. *Environ. Sci.* 12, 112–117.

Ahmad, A., Aslam, Z., Bellitürk, K., Iqbal, N., Idrees, M., Nawaz, M., et al. (2021). Earth Worms and Vermicomposting: A Review on the Story of Black Gold. *J. Innovative Sci.* 7 (1), 167–173. doi:10.17582/journal.jis/2021/7.1.167.173

Ali, K., Wang, X., Riaz, M., Islam, B., Khan, Z. H., Shah, F., et al. (2019). Biochar: an Eco-Friendly Approach to Improve Wheat Yield and Associated Soil Properties on Sustainable Basis. *Pak. J. Bot.* 51 (4), 1255–1261. doi:10.30848/pjb2019-4(7)

Ali, M., and Hasan, A. (2001). *Integrating Recycling and Disposal System for Solid Waste Management in Karachi*. Karachi, Pakistan: Report Urban Resource Center.

Ansari, A. A., and Saywack, P. (2011). Identification and Classification of Earthworm Species in Guyana. *Int. J. Zoological Res.* 7, 93–99. doi:10.3923/ijp.2011.212.216

Ansari, A., and Ismail, S. (2012). Earthworms and Vermiculture Biotechnology Management of Organic Waste. *Soil Biol. Biochem.* 41 (5), 905–909. doi:10.5772/30957

Ashraf, H. M., Zahoor, M. K., Nasir, S., Majeed, H. N., and Zahoor, S. (2016). Genetic Analysis of Aedes aegypti Using Random Amplified Polymorphic DNA (RAPD) Markers from Dengue Outbreaks in Pakistan. *J. Arthropod Borne Dis.* 10 (4), 546–559.

Attaullah, Zahoor, M. K., Zahoor, M. A., Mubarik, M. S., Rizvi, H., Majeed, H. N., et al. (2020). Insecticidal, Biological and Biochemical Response of *Musca domestica* (Diptera: Muscidae) to Some Indigenous Weed Plant Extracts. *Saudi J. Biol. Sci.* 27 (1), 106–116. doi:10.1016/j.sjbs.2019.05.009

Babić, S., Barišić, J., Malev, O., Klobučar, G., Popović, N. T., Strunjak-Perović, I., et al. (2016). Sewage Sludge Toxicity Assessment Using Earthworm Eisenia fetida: Can Biochemical and Histopathological Analysis Provide Fast and Accurate Insight? *Environ. Sci. Pollut. Res. Int.* 23 (12), 12150–12163. doi:10.1007/s11356-016-6097-3

Bareen, F.-e., and Tahira, S. A. (2011). Metal Accumulation Potential of Wild Plants in Tannery Effluent Contaminated Soil of Kasur, Pakistan: Field Trials for Toxic Metal Cleanup Using Suaeda Fruticosa. *J. Hazard. Mater.* 186, 443–450. doi:10.1016/j.jhazmat.2010.11.022

Birkas, M., Bottlik, L., Stingli, A., Gyuricza, C., and Jolánkai, M. (2010). Effect of Soil Physical State on the Earthworms in Hungary. *Appl. Environ. Soil Sci.* 20 (8). doi:10.1155/2010/830853

Blouin, M., Hodson, M. E., Delgado, E. A., Baker, G., Brussaard, L., Butt, K. R., et al. (2013). A Review of Earthworm Impact on Soil Function and Ecosystem Services. *Eur. J. Soil Sci.* 64 (2), 161–182. doi:10.1111/ejss.12025

Button, M., Jenkin, G. R. T., Bowman, K. J., Harrington, C. F., Brewer, T. S., Jones, G. D. D., et al. (2010). DNA Damage in Earthworms from Highly Contaminated Soils: Assessing Resistance to Arsenic Toxicity by Use of the Comet Assay. *Mutat. Research/Genetic Toxicol. Environ. Mutagen.* 696 (2), 95–100. doi:10.1016/j.mrgentox.2009.12.009

Callaham, M. A., Stewart, A. J., Alarcón, C., and McMillen, S. J. (2002). Effects of Earthworm (Eisenia Fetida) and Wheat (Triticum Aestivum) Straw Additions on Selected Properties of Petroleum-Contaminated Soils. *Environ. Toxicol. Chem.* 21 (8), 1658–1663. doi:10.1897/1551-5028(2002)021<1658:eoeefa>2.0.co;2

Charlton, A., Sakrabani, R., Tyrrel, S., Rivas Casado, M., McGrath, S. P., Crooks, B., et al. (2016). Long-term Impact of Sewage Sludge Application on Soil Microbial Biomass: An Evaluation Using Meta-Analysis. *Environ. Pollut.* 219, 1021–1035. doi:10.1016/j.envpol.2016.07.050

Dabke, S. V. (2013). Vermi-remediation of Heavy Metal-Contaminated Soil. *J. Health Pollut.* 3 (4), 4–10. doi:10.5696/2156-9614-3.4.4

Domínguez-Crespo, M. A., Sánchez-Hernández, Z. E., Torres-Huerta, A. M., Negrete-Rodríguez, M., de la Luz, X., Conde-Barajas, E., et al. (2012). Effect of the Heavy Metals Cu, Ni, Cd and Zn on the Growth and Reproduction of Epigeic Earthworms (E. Fetida) during the Vermistabilization of Municipal Sewage Sludge. *Water, Air Soil Pollut.* 223 (2), 915–931. doi:10.1007/s11270-011-0913-7

Dua, V. K., Kumar, A., Pandey, A. C., and Kumar, S. (2013). Insecticidal and Genotoxic Activity of Psoralea Corylifolia Linn. (Fabaceae) against Culex quinquefasciatus Say, 1823. *Parasites Vectors* 6 (1), 30. doi:10.1186/1756-3305-6-30

Edwards, C. A., and Arancon, N. Q. (2004). The Use of Earthworms in the Breakdown of Organic Wastes to Produce Vermicomposts and Animal Feed Protein. *Earthworm Ecol.* 2, 345–380.

Elmer, W. H., Lattao, C. V., and Pignatello, J. J. (2015). Active Removal of Biochar by Earthworms (Lumbricus Terrestris). *Pedobiologia* 58 (1), 1–6. doi:10.1016/j.pedobi.2014.11.001

Elyamine, A. M., and Hu, C. (2020). Earthworms and Rice Straw Enhanced Soil Bacterial Diversity and Promoted the Degradation of Phenanthrene. *Environ. Sci. Eur.* 32 (1), 1–12. doi:10.1186/s12302-020-00400-y

Fouché, T., Claassens, S., and Maboeta, M. S. (2022). Ecotoxicological Effects of Aflatoxins on Earthworms under Different Temperature and Moisture Conditions. *Toxins* 14 (2), 75. doi:10.3390/toxins14020075

Givaudan, N., Wiegand, C., Le Bot, B., Renault, D., Pallois, F., Llopis, S., et al. (2014). Acclimation of Earthworms to Chemicals in Anthropogenic Landscapes, Physiological Mechanisms and Soil Ecological Implications. *Soil Biol. Biochem.* 73, 49–58. doi:10.1016/j.soilbio.2014.01.032

Gul, S., Whalen, J. K., Thomas, B. W., Sachdeva, V., and Deng, H. (2015). Physico-chemical Properties and Microbial Responses in Biochar-Amended Soils: Mechanisms and Future Directions. *Agric. Ecosyst. Environ.* 206, 46–59. doi:10.1016/j.agee.2015.03.015

Haydar, S., Hussain, G., Nadeem, O., Aziz, J. A., Bari, A. J., and Asif, M. (2015). Water Conservation Initiatives and Performance Evaluation of Wastewater Treatment Facility in a Local Beverage Industry in Lahore. *Pak. J. Engg. Appl. Sci.* 16, 100–109.

He, X., Zhang, Y., Shen, M., Zeng, G., Zhou, M., and Li, M. (2016). Effect of Vermicomposting on Concentration and Speciation of Heavy Metals in Sewage Sludge with Additive Materials. *Bioresour. Technol.* 218, 867–873. doi:10.1016/j.biortech.2016.07.045

Hrzenjak, T., Hrzenjak, M., Kasuba, V., Efenberger-Marinculić, P., and Levanat, S. (1992). A New Source of Biologically Active Compounds-Eearthworm Tissue (Eisenia Foetida, Lumbricus Rubelus). *Comp. Biochem. Physiol. Comp. Physiol.* 102, 441–447. doi:10.1016/0300-9629(92)90191-r

Ibrahim, M. H., Quaik, S., and Ismail, S. A. (2016). "General Introduction to Earthworms, Their Classifications, and Biology," in *Prospects of Organic Waste Management and the Significance of Earthworms*(Springer, Cham) 22 (69–103).

Karimi, F., Rahimi, G., and Kolahchi, Z. (2020). Interaction Effects of Salinity, Sewage Sludge, and Earthworms on the Fractionations of Zn and Cu, and the Metals Uptake by the Earthworms in a Zn- and Cu-Contaminated Calcareous Soil. *Environ. Sci. Pollut. Res.* 27, 10565–10580. doi:10.1007/s11356-020-07719-2

Khan, M. U., Ahmed, M., and Nazim, K. (2017). The Population Behavior of Earth Worm (*Pheritema Posthuma Kinberg*) under the Influence of Industrial Waste. *Fuuast J. Biol.* 7 (1), 1–8.

Kim, W. I., Kunhikrishnan, A., Go, W. R., Jeong, S. H., Kim, G. J., Lee, S., et al. (2014). Influence of Various Biochars on the Survival, Growth, and Oxidative DNA Damage in the Earthworm *Eisenia Fetida*. *Korean J. Environ. Agric.* 33, 231–238. doi:10.5338/kjea.2014.33.4.231

Kızılkaya, R., and Hepşen, S. (2004). Effect of Biosolid Amendment on Enzyme Activities in Earthworm (*Lumbricus Terrestris*) Casts. *J. Plant Nutr. Soil Sci.* 167, 202–208. doi:10.1002/jpln.200321263

Kooch, Y., and Jalilvand, H. (2008). Earthworms as Ecosystem Engineers and the Most Important Detritivors in Forest Soils. *Pak. J. Biol. Sci.* 11 (6), 819–825. doi:10.3923/pjbs.2008.819.825

Latare, A. M., Kumar, O., Singh, S. K., and Gupta, A. (2014). Direct and Residual Effect of Sewage Sludge on Yield, Heavy Metals Content and Soil Fertility under Rice-Wheat System. *Ecol. Eng.* 69, 17–24. doi:10.1016/j.ecoleng.2014.03.066

Lemtiri, A., Liénarda, A., Alabib, T., Brostauxc, Y., Cluzeaud, D., Francisb, F., et al. (2015). Earthworms Eisenia fetida Affect the Uptake of Heavy Metals by Plants Vicia faba and Zea mays in Metal-Contaminated Soils. *Appl. Soil Ecol.* 11 (6), 819–825. doi:10.1016/j.apsoil.2015.11.021

Li, D., Hockaday, W. C., Masiello, C. A., and Alvarez, P. J. J. (2011). Earthworm Avoidance of Biochar Can Be Mitigated by Wetting. *Soil Biol. Biochem.* 43 (8), 1732–1737. doi:10.1016/j.soilbio.2011.04.019

Li, Y., Luo, J., Yu, J., Xia, L., Zhou, C., Cai, L., et al. (2018). Improvement of the Phytoremediation Efficiency of *Neyraudia Reynaudiana* for Lead-Zinc Mine-Contaminated Soil under the Interactive Effect of Earthworms and EDTA. *Sci. Rep.* 8 (1), 6417. doi:10.1038/s41598-018-24715-2

Liu, X., Hu, C., and Zhang, S. (2005). Effects of Earthworm Activity on Fertility and Heavy Metal Bioavailability in Sewage Sludge. *Environ. Int.* 31, 874–879. doi:10.1016/j.envint.2005.05.033

Malińska, K., Zabochnicka-Świątek, M., Cáceres, R., and Marfà, O. (2016). The Effect of Precomposted Sewage Sludge Mixture Amended with Biochar on the Growth and Reproduction of *Eisenia fetida* during Laboratory Vermicomposting. *Ecol. Eng.* 90, 35–41. doi:10.1016/j.ecoleng.2016.01.042

Martin, P., Martinez-Ansemil, E., Pinder, A., Timm, T., and Wetzel, M. J. (2007). Global Diversity of Oligochaetous Clitellates ("Oligochaeta"; Clitellata) in Freshwater. *Hydrobiologia* 595, 117–127. doi:10.1007/s10750-007-9009-1

Murtaza, B., Murtaza, G., Sabir, M., Owens, G., Abbas, G., Imran, M., et al. (2016). Amelioration of Saline-Sodic Soil with Gypsum Can Increase Yield and Nitrogen Use Efficiency in Rice-Wheat Cropping System. *Arch. Agron. Soil Sci.* 63, 1267–1280. doi:10.1080/03650340.2016.1276285

Murtaza, G. (2015). "Evaluation and Management of Sludge and Compost from Different Sources for Sustainable Agriculture," in Proceedings of the First Annual Technical Progress Report for Higher Education Commission (HEC) funded project (April 2014 to March 2017). (Inst. Soil Environ. Sci., Univ. Agric., Faisalabad, Pakistan)

Pak-Epa (2005). *Guidelines for Soil Waste Management*. Islamabad, Pakistan: Pakistan Environmental Protection Agency Pakistan.

Park, J. H., Choppala, G. K., Bolan, N. S., Chung, J. W., and Chuasavathi, T. (2011). Biochar Reduces the Bioavailability and Phytotoxicity of Heavy Metals. *Plant Soil* 348, 439–451. doi:10.1007/s11104-011-0948-y

Qadir, M., Mateo-Sagasta, J., Jiménez, B., Siebe, C., Siemens, J., and Hanjra, M. A. (2015). "Environmental Risks and Cost-Effective Risk Management in Wastewater Use Systems," in *Wastewater* (Springer, Netherlands), 55–72. doi:10.1007/978-94-017-9545-6_4

Riaz, M., Roohi, M., Arif, M. S., Hussain, Q., Yasmeen, T., Shahzad, T., et al. (2017). Corncob-derived Biochar Decelerates Mineralization of Native and Added Organic Matter (AOM) in Organic Matter Depleted Alkaline Soil. *Geoderma* 294, 19–28. doi:10.1016/j.geoderma.2017.02.002

Riaz, U., Murtaza, G., SaifullahFarooq, M., Farooq, M., Aziz, H., Qadir, A. A., et al. (2020). Chemical Fractionation and Risk Assessment of Trace Elements in Sewage Sludge Generated from Various States of Pakistan. *Environ. Sci. Pollut. Res.* 27, 39742–39752. In press. doi:10.1007/s11356-020-07795-4

Sanchez-Hernandez, J. C., Ro, K. S., and Díaz, F. J. (2019). Biochar and Earthworms Working in Tandem: Research Opportunities for Soil Bioremediation. *Sci. Total Environ.* 688, 574–583. doi:10.1016/j.scitotenv.2019.06.212

Sanchez-Hernandez, J. C., Sáez, J. A., Vico, A., Moreno, J., and Moral, R. (2020). Evaluating Earthworms' Potential for Remediating Soils Contaminated with Olive Mill Waste Sediments. *Appl. Sci.* 10 (7), 2624. doi:10.3390/app10072624

Sharma, A., Sonah, H., Deshmukh, R. K., Gupta, N. K., Singh, N. K., and Sharma, T. R. (2011). Analysis of Genetic Diversity in Earthworms Using DNA Markers. *Zoological Sci.* 28 (1), 25–31. doi:10.2108/zsj.28.25

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells. *Exp. Cell. Res.* 175, 184–191. doi:10.1016/0014-4827(88)90265-0

Singh, R. P., and Agrawal, M. (2010). Effect of Different Sewage Sludge Applications on Growth and Yield of Vigna Radiata L. Field Crop: Metal Uptake by Plant. *Ecol. Eng.* 36, 969–972. doi:10.1016/j.ecoleng.2010.03.008

Singh, R. P., and Agrawal, M. (2008). Potential Benefits and Risks of Land Application of Sewage Sludge. *Waste Manag.* 28, 347–358. doi:10.1016/j.wasman.2006.12.010

Sizmur, T., Martin, E., Wagner, K., Parmentier, E., Watts, C., and Whitmore, A. P. (2017). Milled Cereal Straw Accelerates Earthworm (Lumbricus Terrestris) Growth More Than Selected Organic Amendments. *Appl. Soil Ecol.* 113, 166–177. doi:10.1016/j.apsoil.2016.12.006

Song, Y., Zhu, L. S., Wang, J., Wang, J. H., Liu, W., and Xie, H. (2009). DNA Damage and Effects on Antioxidative Enzymes in Earthworm (Eisenia Foetida) Induced by Atrazine. *Soil Biol. Biochem.* 41 (5), 905–909. doi:10.1016/j.soilbio.2008.09.009

Stövenm, K., and Schnug, E. (2009). Long Term Effects of Heavy Metal Enriched Sewage Sludge Disposal in Agriculture on Soil Biota. *Agric. For. Res.* 2 (59), 131–138.

Tiwari, R. K., Singh, S., Pandey, R. S., and Sharma, B. (2016). Enzymes of Earthworm as Indicators of Pesticide Pollution in Soil. *Aer* 4 (4), 113–124. doi:10.4236/aer.2016.44011

Valckx, J., Govers, G., Hermy, M., and Muys, B. (2010). Manipulated Earthworm Populations Decrease Runoff and Erosion Rates in Arable Land with Distinct Soil Tillage Treatments. *Soil Biol. Biochem.* 41 (5), 905–909. doi:10.1007/978-3-642-14636-7_2

Van Groenigen, J. W., Lubbers, I. M., Vos, H. M. J., Brown, G. G., De Deyn, G. B., and Van Groenigen, K. J. (2014). Earthworms Increase Plant Production: a Meta-Analysis. *Sci. Rep.* 4, 6365. doi:10.1038/srep06365

Wang, F., Wang, H., Li, S., and Diao, X. (2019). Effects of Earthworms and Effective Microorganisms on the Composting of Sewage Sludge with Cassava Dregs in the Tropics. *J. Air Waste Manag. Assoc.* 69 (6), 710–716. doi:10.1080/10962247.2018.1552215

Wang, Z., Cui, Z., Liu, L., Ma, Q., and Xu, X. (2016). Toxicological and Biochemical Responses of the Earthworm *Eisenia fetida* Exposed to Contaminated Soil: Effects of Arsenic Species. *Chemosphere* 154, 161–170. doi:10.1016/j.chemosphere.2016.03.070

Wanga, L., Løvåsb, T., and Houshfarb, E. (2014). Effect of Sewage Sludge Addition on Potassium Release and Ash Transformation during Wheat Straw Combustion. *Chem. Eng.* 37, 7–12. doi:10.3303/CET1437002

Weyers, S. L., and Spokas, K. A. (2011). Impact of Biochar on Earthworm Populations: A Review. *Appl. Environ. Soil. Sci.* doi:10.1155/2011/541592

Yadav, S., and Mullah, M. (2017). A Review on Molecular Markers as Tools to Study Earthworm Diversity. *Int. J. Pure Appl. Zoology* 5 (1), 62–69.

Yang, J., Lv, B., Zhang, J., and Xing, M. (2014). Insight into the Roles of Earthworm in Vermicomposting of Sewage Sludge by Determining the Water-Extracts through Chemical and Spectroscopic Methods. *Bioresour. Technol.* 154, 94–100. doi:10.1016/j.biortech.2013.12.023

Yasmin, S., and D'Souza, D. (2010). Effects of Pesticides on the Growth and Reproduction of Earthworm: A Review. *Appl. Environ. Soil. Sci.* doi:10.1155/2010/678360

Younes, M., Othman, S. E., Elkersh, M. A., Youssef, N. S., and Omar, G. A. (2011). Effect of Seven Plant Oils on Some Biochemical Parameters in Khapra Beetle

Trogoderma Granarium Everts (Coleoptera: Dermestidae). *Egypt. J. Exp. Biol.* 7 (1), 53–61.

Zaltauskaitė, J., and Sodienė, I. (2010). Effects of Total Cadmium and Lead Concentrations in Soil on the Growth, Reproduction and Survival of Earthworm Eisenia fetida. *Ekologija* 56 (1-2), 10–16. doi:10.2478/v10055-010-0002-z

Zhang, R. H., Li, Z. G., Liu, X. D., Wang, B., Zhou, G. L., Huang, X. X., et al. (2017). Immobilization and Bioavailability of Heavy Metals in Greenhouse Soils Amended with Rice Straw-Derived Biochar. *Ecol. Eng.* 98, 183–188. doi:10.1016/j.ecoleng.2016.10.057

Zhang, X., Wang, H., He, L., Lu, K., Sarmah, A., Li, J., et al. (2013). Using Biochar for Remediation of Soils Contaminated with Heavy Metals and Organic Pollutants. *Environ. Sci. Pollut. Res.* 20, 8472–8483. doi:10.1007/s11356-013-1659-0

Zulhussnain, M., Zahoor, M. K., Rizvi, H., Zahoor, M. A., Rasul, A., Ahmad, A., et al. (2020). Insecticidal and Genotoxic Effects of Some Indigenous Plant Extracts in Culex quinquefasciatus Say Mosquitoes. *Sci. Rep.* 10 (1), 6826. doi:10.1038/s41598-020-63815-w

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.