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Trace Metal Levels and Oxidative Stress Biomarkers in Land Snails, *Achatina fulica*, Exposed to Soils from a Coal Mining Area in Zimbabwe

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ABSTRACT

The aim of the study was to evaluate biological effects of snail exposure to soil collected from a coal mining area in Zimbabwe. Land snails, *Achatina fulica*, collected from a relatively pristine environment and reared under laboratory conditions (for 1 year) were exposed for 45 days to the soils collected from four different sites. Heavy metals were assayed in soils and sail tissues whilst stress enzyme activities were assayed in snail tissues. Levels of cadmium and zinc measured in the soils were found to be 0.92 ± 0.21 mg/kg and 193.50 ± 79.83 mg/kg respectively above the world health organization maximum permissible limits of 0.8 mg/kg and 50 mg/kg. Cadmium and zinc were found to be approximately 4 fold higher in snail tissues exposed to the soil compared to snail tissues exposed to reference soils. The activities of superoxide dismutase, catalase, glutathione peroxidase and DT diaphoresis were significantly increased in snails exposed to soils from the coal mining area compared to the control soil ($p<0.05$). The highest enzyme activities were recorded in snails exposed to soil close to coal processing plant (Site C) and close to coal fired power plant (Site D). Metallothionein concentrations were highest in snail tissue exposed to soil from site D. Our results show that invertebrates exposed to soil at the coal mining area are under oxidative stress. There is need, therefore, to continually assess contaminants in soils at coal mining areas.

Keywords: Soil; Antioxidant enzymes; Metallothioneins; Coal pollutants; *Achatina fulica*

INTRODUCTION

Coal is a key energy resource in Zimbabwe and contributes significantly in the generation of electricity. However, the mining of coal and related activities threaten the capacity of the environment to sustain life. Coal mining and coal fired power plants result in the release of several pollutants such as gaseous oxides of sulphur, nitrogen and carbon into the air, water and land. Dioxins, polyromantic hydrocarbons, particulate matter and heavy metals are also generated by coal mining and coal fired power plants. Environmental contamination by metals is a problem in the world and continues unabated due to extensive discharge of heavy metals by agriculture, industry and mining.

Bioavailability of pollutants in soil largely depends on several factors that include pH, organic matter and clay content of soil. Polluted soils may contain several classes of toxicants such as inorganic ions, organic compounds, organometallic compounds and radioactive isotopes. As such, toxicity of soil

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pollutants to living organisms may be a result of synergistic and/or antagonistic effects of the pollutants. Several studies have shown that exposure of soil organisms to mixtures of pollutants in soil may cause oxidative stress.

Oxidative stress entails upsetting the pro-oxidant and antioxidant balance in favour of the former, resulting in the damage of lipids, proteins and DNA by reactive oxygen species. Reactive oxygen species include the Hydroxyl radical (•OH) Superoxide anion (•$O2^-$) and Hydrogen Peroxide (H_2O_2). Reactive oxygen species may be produced from normal endogenous metabolism or through free radical interactions associated with organic xenobiotics, metabolites and heavy metals. To combat the effects of reactive oxygen species, living organisms have evolved antioxidant defenses such as the induction of antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and DT-diaphorase. The use of different animal species to study antioxidant enzyme systems as indicators of environmental pollution has grown. There is growing interest in the use of native species as indicators for assessing environmental pollution. In the current study, the land snail, *Achatina fulica*, was used because of its availability, sedentary nature and contact with the soil [1-7].

MATERIALS AND METHODS

Chemicals

All chemicals, enzymes, and substrates were bought from Sigma-Aldrich chemical company, Germany, or Cole-Palmer. All other laboratory reagents, used in this study, were of analytical grade.

Soil Sampling

Figure 1 shows the sampling sites at a coal mining area. Site A is 4 km North-East of coal fired power plant and near the main road whilst site B is about 8 km South-West of the coal fired power plant. Site C is 6 km North-West of coal fired power plant and site D is 1 km from the coal fired power plant.

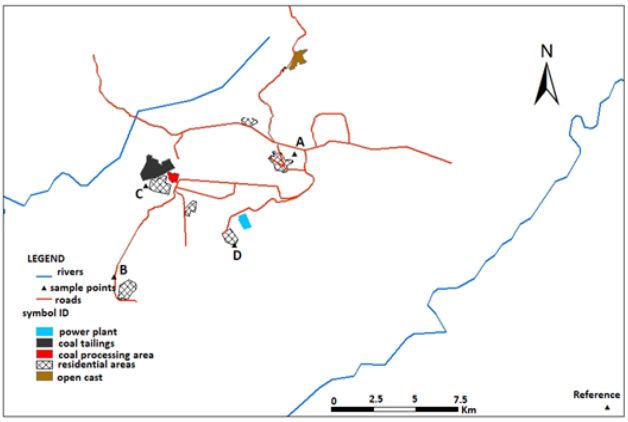

Figure 1: Sampling points at a coal mining area, Zimbabwe.

Fifteen samples of soil were randomly taken from an area measuring 90 m × 50 m at a depth of 10 cm-15 cm. Similarly, samples from a reference site located 50 km away from the coal mining area were collected. The samples from each area were thoroughly mixed and divided into 3 composite samples. Some of the soil samples were immediately refrigerated at -20°C pending analysis. Soils intended for exposures were kept at room temperature and soil samples meant for metal analysis were ground in a mortar and sieved through a 2 mm screen according to the national environment protection council protocol [8-11].

Exposures

Snails, *Achatina fulica*, were collected around the National University of Science and Technology (NUST) campus in Bulawayo, Zimbabwe and reared in the laboratory for 1 year. This area was considered relatively unpolluted compared to the study area. The snails were fed on lettuce twice per week. Fifteen glass tanks (21 cm × 19 cm × 19 cm) were set up in triplicates. Two kilograms of soil obtained from the 5 different sites were put into each tank. Thereafter approximately 300 ml of distilled water was added to soil in each tank in order to moisten the soil. Twelve snails, of approximately 12 g in weight, were placed in each tank and exposed for 45 days. The tanks were cleaned twice per week during the exposure period through removing Faecal wastes, slime and decaying food left over. At 15 day intervals, 4 snails from each tank were sacrificed and dissected and digestive glands were pooled. Snail tissues intended for antioxidant biochemical tests were rapidly homogenized while tissues for analysing MT levels were refrigerated at -80°C until used. All exposure experiments were done in triplicates [12-15].

Heavy Metal Analyses

Metal analysis in soil: Soil was digested using the aqua regia method of EPA-ROC. The filtered aqua regia extracts were diluted to 100 ml with distilled water and the solutions assayed for heavy metals using an atomic absorption spectrometer (XplorAA Dual, GBC, and Australia).

Metal analysis in snail issue: One gram of dry snail tissue was digested in 30 ml of a mixture of HCl and HNO_3 in the ratio of 3:1 for 5 hours on a hot plate. The digest was left on the hot plate to evaporate to about 1 ml and removed to cool after which it was quantitatively washed with distilled water and filtered using an ashless Whatman filter paper No. 42 into a 25 ml volumetric flask. The solutions were assayed for heavy metals using an atomic absorption spectrometer (XplorAA Dual, GBC, and Australia)

Biochemical Analyses

For the determination of antioxidant enzyme activity after the exposure time, digestive glands of four snails were pooled and homogenized in five volumes of 50 mM phosphate buffer and centrifuged at 4°C for ten minutes at 10000X g. The protein content in the supernatant (post mitochondrial fraction) was determined using the method. The post mitochondrial fraction was aliquoted and stored at -80°C until analysis.

Superoxide dismutase activity: Superoxide dismutase activity was measured following the method described by Sun et al. Xanthine and Xanthine oxidase were used to generate superoxide anion radicals which reacted with Nitroblue Tetrazolium (NBT) to form a chromophore. The reaction mixture contained 1 mg/ml post mitochondrial fraction or standard superoxide dismutase solution (0 ng/tube-300 ng/tube) and superoxide dismutase assay reagent containing 0.3 mm xanthine, 0.6 mm Ethylene Diamine Tetra Acetic Acid (EDTA), 150 µM nitroblue tetrazolium, 400 mm sodium carbonate and 0.1% w/v Bovine Serum Albumin (BSA) in the ratio 4:2:2:1:0.6 respectively. The reaction was started by adding 50 µl of 167 U/L xanthine oxides and allowed to occur for 30 minutes in a water bath at 25°C. Thereafter, absorbance was measured at 560 nm. One enzyme unit superoxide is defined as the amount which inhibits NBT reaction by 50%. Specific activity was given as units/mg protein.

Catalase activity: Catalase activity was determined following the method as described by Clairborne which measures the decrease of hydrogen peroxide concentration with time. A reaction mixture containing 100 µl of 1 mg/ml tissue extract, 50 mm potassium phosphate buffer pH 7 and 19 mm H_2O_2 in the phosphate buffer was made in a quazte cuvette. The mixture was incubated at 25°C and the change in absorbance at 240 nm was monitored for 30 seconds using Shimadzu UV-1800 UV/Visible Spectrophotometer. Catalase activity was calculated using the extinction coefficient for H_2O_2 (Ɛ=43.6 $M^{-1}cm^{-1}$). Specific activity was given as units/mg protein.

Glutathione peroxidase activity: Glutathione peroxidase was measured using a method as described by Flohe and Gunzler but modified in our laboratory for the microplate reader, using hydrogen peroxide as the substrate. The reaction mixture contained 50 mm potassium phosphate buffer, pH 7.0, 10 mM reduced glutathione, 20 mm sodium azide, 2.0 mm NADPH in 0.1% $NaHCO_3$, 10 U/ml glutathione reductase, homogenate (1 mg/ml protein) and lastly 1.5 mm hydrogen peroxide in order to initiate the reaction. The decrease in absorbance was monitored using a Spectramax 340 pc 384 microplate reader at 340 nm at 25°C for 5 minutes. The activity of GPx was expressed as µmoles of NADPH oxidised per minute. Specific activity was given as units/mg protein [16-19].

DT-diaphoresis activity: DT-diaphoresis activity was measured using the method originally described by Lind et al. and modified by Siwela et al. Twenty micro-litres of the 1 mg/ml post mitochondrial fraction were added to a reaction mixture containing 50 mM tris/HCl buffer (pH 7.5), 0.8% Triton X-100 and 5 mM NADH. Reduction of 400 µM 2, 6 dichlorophenol indophenol was followed at 600 nm (ε=21 $mM^{-1}cm^{-1}$) over 5 minutes at 25°C. Results were reported as nmol/min/mg protein.

Metallothionein levels: Snail tissue was homogenized in 3 volumes of 10 mM Tris-HCl buffer pH 7. The homogenate was heated in a water bath at 100°C for 5 minutes. The homogenate was cooled and then centrifuged at 10000X g for 20 minutes at 4°C. The supernatant was mixed with 50 mg/L cadmium chloride in 10% trichloroacetic acid and incubated for 10 min at room temperature. After incubation, 1 mg/ml bovine serum albumin was added and incubated at room temperature for 10 minutes followed by centrifugation at 20000X g for 20 minutes at 4°C. Thereafter the supernatant was digested with 55% nitric acid for 15 minutes. The digest was diluted to 25 ml with distilled water. Cadmium concentration was finally measured using an atomic absorption spectrometer (XplarAA Dual, GBC, and Australia). Metallothionein concentration was expressed as number of moles of Cd binding sites/mg protein.

Statistical Analysis

Biomarkers and the concentration of contaminants were expressed as mean ± Standard Deviation (SD). A one way analysis of variance was used to analyse metal concentrations. A two way Analysis of Variance (ANOVA) was conducted so as to test the effect of sites, duration of exposure and their interaction on the biomarkers. When significant differences were found, post hoc comparison tests for means were done using the Tukey's test. Significance of results was determined at $p<0.05$ [20].

RESULTS

The mean concentrations of heavy metals in soils collected from the 5 sites are reported in **Table 1**. Soil samples from the coal mining area had a significantly higher concentration of heavy metals compared to soils from the reference site ($p< 0.05$).

Table 1: Heavy metal concentration in soils collected from 5 different sites at a coal mining area in Zimbabwe.

Site	Heavy metal concentration (mg/kg)				
	Cd	Zn	Pb	Ni	Cr
Reference	$0.03^{a} \pm 0.01$	$50.37^{a} \pm 1.62$	$11.97^{a} \pm 0.67$	$2.27^{a} \pm 0.15$	$6.07^{a} \pm 0.65$
Site A	$0.67^{b} \pm 0.04$	$223.07^{b} \pm 10.22$	$17.77^{d} \pm 0.55$	$13.17^{c} \pm 0.71$	$14.10^{b} \pm 0.20$
Site B	$0.91^{c} \pm 0.04$	$70.73^{a} \pm 3.82$	$12.90^{ab} \pm 0.20$	$8.27^{b} \pm 0.50$	$24.33^{d} \pm 0.93$
Site C	$0.87^{c} \pm 0.04$	$277.97^{c} \pm 10.96$	$13.83^{bc} \pm 0.40$	$14.27^{c} \pm 0.35$	$19.13^{c} \pm 0.31$

Site D	1.2^{d} ± 0.09	202.27^{b} ± 8.59	22.47^{e} ± 0.60	17.37^{d} ± 1.11	34.23^{e} ± 0.91
*WHO	0.8	50	85	35	100

Values are means ± standard deviations of three replicates (n=3). For each metal, values with different letters in a column are significantly different (p<0.05) from each other. **WHO–World Health Organisation desirable maximum levels of elements in unpolluted soils.

Values are means ± standard deviations of three determinations (n=3). For each metal, values with different letters in a column are significantly different (p<0.05) from each other.

The total concentration of metals in soil from the coal mining area was found to be 0.92 ± 0.21 mg/kg cadmium, 193.50 ± 79.83 mg/kg zinc, 16.74 ± 3.95 mg/kg lead, 13.2713.27 ± 3.47 mg/kg nickel and 22.95 ± 7.80 mg/kg chromium. The order of metal concentrations was Zn>Cr>Pb>Ni>Cd. Soil samples collected from site D (1 km from coal fired power plant) contained the highest concentrations of cadmium, lead, nickel and chromium. Soils from the site B (8 km South-West of coal fired power plant) had the lowest concentration of nickel, zinc and lead at 8.27 mg/kg, 70.73 mg/kg and 12.9 mg/kg respectively compared to other sites at the coal mining area. Soil samples collected from site A (4 km North-East of coal fired power plant) had the lowest total cadmium and chromium concentration of 0.67 mg/kg and 14.10 mg/kg respectively compared to other sites at the coal mining area.

The concentration of zinc in soils from the reference site and site B did not vary significantly (p>0.05).

The mean concentrations of selected heavy metals in the digestive gland tissue of *A. fulica* exposed to soils from the 5 sites are shown in **Table 2**. Tissues of snails exposed to soil from all sites had significantly higher levels of cadmium, zinc and nickel compared to cadmium levels in snail tissue exposed to reference soils (p<0.05). Lead levels in snail tissue exposed to soil from site A and D were significantly higher than in snails exposed to reference soils (p<0.05). The total chromium concentration in snail tissue exposed to soil from site B, C and D was significantly higher than in snail tissue exposed to soil from reference soil (p<0.05). The highest concentrations of all the analysed heavy metals were observed in snails exposed to soil from site D.

Table 2: Heavy metal concentration in digestive glands of *A. fulica* exposed to soils collected from 5 different sites at a coal mining area in Zimbabwe.

Site	Heavy metal concentration (mg/kg)				
	Cd	Zn	Pb	Ni	Cr
Reference	0.13^{a} ± 0.04	165.25^{a} ± 10.08	2.52^{a} ± 0.33	0.35^{a} ± 0.06	1.52^{b} ± 0.02
Site A	2.69^{b} ± 0.13	832.80^{c} ± 10.02	4.46^{b} ± 0.30	1.54^{b} ± 0.14	0.80^{a} ± 0.08
Site B	2.68^{b} ± 0.19	274.26^{b} ± 7.96	3.08^{a} ± 0.14	2.30^{c} ± 0.15	1.78^{c} ± 0.08
Site C	3.35^{c} ±0.31	1419.82^{d} ± 50.58	2.53^{a} ± 0.31	2.47^{c} ± 0.29	1.61^{c} ± 0.06
Site D	5.48^{d} ± 0.21	904.75^{c} ± 7.09	5.36^{c} ± 0.27	2.55^{c} ± 0.12	3.13^{d} ± 0.09
**FAO/WHO limits	0.01	55	2	10	102

Values are means ± standard deviations of three replicates (n=3). For each metal, values with different letters in a column are significantly different (p<0.05) from each other. **FAO/WHO limits–Food and Agriculture Organisation/World Health Organisation permissible limits in molluscs.

Values are means ± standard deviations of three replicates (n=3). For each metal, values with different letters in a column are significantly different (p<0.05) from each other.

Figure 2 shows variation of antioxidant enzymes, superoxide dismutase (A), catalase (B), glutathione peroxides (C) and DT-diaphoresis (D) in *A. fulica* exposed to soils from a coal mining area over a 45 days period. The activities of SOD, CAT, GPx and DTD in snail tissue exposed to soil collected from all sites were significantly higher (p<0.05) on days 15, 30 and 45 than those of snail tissue exposed to reference soil.

The highest SOD activity was observed in snails exposed to soil collected from site C on day 30 whilst the whilst the highest CAT, DTD and GPx activities were observed in snail tissue exposed to soil from site D on day 30. The lowest CAT, DTD and GPx activities were observed in snails exposed to soil from site B on day 15. There was a significant decrease in GPx activity in snails exposed to soil from site C on day 45 (p<0.05) compared to the GPx activity in snails exposed to control soils.

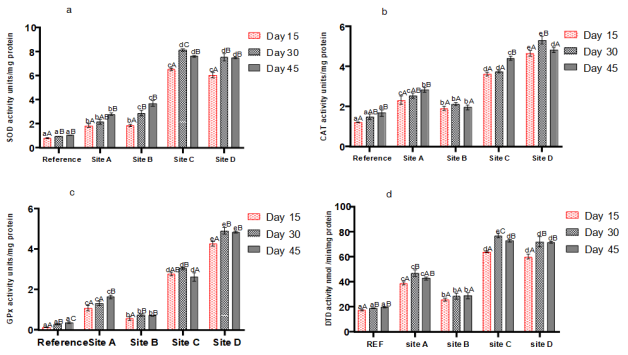

Figure 2: Variation of antioxidant enzymes, SOD (A), CAT (B), GPx (C) and DTD (D) in *A. fulica* exposed to soils from a coal mining area over a 45 day period (mean values ± standard deviations). For each exposure period, different lower case letters above bars indicate significant differences at different sites and different capital letters indicate significant differences between exposure periods at the same site based on the Tukey test ($p<0.05$). SOD-superoxide dismutase, CAT-catalase, GPx-selenium dependant glutathione peroxidases, DTD-DT-Diaphorase.

Figure 3 shows variation of metallothionein levels in *A. fulica* exposed for 45 days to soil collected from a coal mining area. The level of metallothionein was significantly higher ($p<0.05$) in snail tissue exposed to soil collected from all sites compared to snail tissue exposed to reference soil. A 6-fold increase in MT level was observed in snails exposed to soils from site D compared to snails exposed to reference soils. The lowest MT levels were measured in snail tissue exposed to soil from site A.

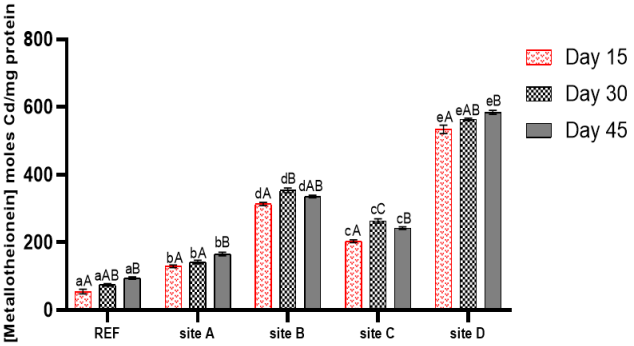

Figure 3: Variation of MT levels in *A. fulica* exposed to soil from a coal mining area over a 45 days period (mean values ± standard deviations). For each exposure period, different lower case letters above bars indicate significant differences at different sites and different capital letters indicate significant differences between exposure periods at the same site based on the Tukey test ($p<0.05$). MT-Metallothionein.

DISCUSSION

Heavy Metal Analysis

This study was done to determine the extent of heavy metal pollution in a coal mining area of Zimbabwe and also to determine the biological effects of pollutants from such soils using land snails, *Achatina fulica*, exposed to soils *ex situ*. Results revealed that soils from the coal mining area contain higher concentrations of Cd, Zn, Pb, Ni and Cr compared to reference soils. These results are in agreement with those by Bu et al., who recorded significantly higher total concentrations of heavy metals in surface soils of a coal mining city of Wuhui in China than background levels. Masto, et al., also made similar observations around Raniganj coalfield in India. The highest levels of total heavy metals found at site D (close to thermal power plant) are similar to those found by Okedeyi et al., in soils around three coal fired power plants in South Africa. Elevated total concentrations of heavy metals at the study area suggest that coal mining and related activities were the principal sources of heavy metals. The relative abundance of the total heavy metals was in the order: Zn>Cr>Pb> Ni>Cd. This finding is consistent with results by Bai et al., who found a similar trend in soils from several coal mines in China. Significant amounts of heavy metals we found probably emanated from coal fly ash, coal dust and vehicular emissions.

Site A had a significantly higher heavy metal concentration than site B probably because of the wind pattern and proximity to roads. On average a gentle breeze (16 km per hour) blows mostly from the easterly direction of the study area. Site A is also close to a dense road network as well as the coal fired power plant and coal processing plant. The close proximity to the major sources of pollution rather than wind pattern seems to be the main factor influencing the level of pollution at each sampling site. The built up areas shown on the map are high density residential areas. Sampling points were selected close to residential areas in order to relate findings to human settlement. We also presume that the relatively high lead concentration in soil observed at site A (close to a dense road network) was probably from vehicular emissions especially coal haulage trucks. Zimbabwe has been using leaded petrol for a long time.

As the environmental management authority of Zimbabwe does not have soil quality guidelines for heavy metals, the WHO desirable maximum levels were used to compare with our findings. The levels of cadmium in soil samples collected from sites B, C and D were greater than the WHO desirable maximum level of 0.8 mg/kg. The concentration of zinc in soil samples from all sites were above the WHO desirable maximum limit of 50 mg/kg. This suggests that soil organisms at the coal mining area were at risk of toxicity due to the aforementioned metals. The levels of cadmium and zinc in soil found in our study (0.92 mg/kg and 193.50 mg/kg respectively) are relatively higher than those reported by other workers.

Total cadmium and zinc levels were approximately 4 fold higher in the digestive glands of snails compared to levels of these metals in the soil. These findings are comparable to those of Jonge, et al., who observed that land snails *Cepaea nemoralis* exposed to polluted soils had a 3 fold higher level of cadmium, and zinc compared to the snails exposed to reference soils. This is an indication of bioaccumulation of the

metals in snail tissues also noted that land snails *Cantareus apertus* are hyper accumulators of cadmium and lead. Land snails have been shown to reside in a broad range of habitats and to be hyper accumulators of heavy metals.

Heavy metals accumulate in snail tissue when they are taken up and stored faster than they are metabolized or excreted. The levels of cadmium, zinc and lead in the digestive gland tissues of snails exposed to soil from all sites were above FAO/WHO permissible limits, for molluscs. Living organisms that reside at the coal mining area and feed on land snails are at risk of exposure to high concentrations of cadmium, zinc and lead. On the other hand, the ability of land snails to concentrate heavy metals without experiencing serious damage makes them good candidates for use in monitoring terrestrial ecosystems.

Antioxidant Enzyme Analysis

Digestive glands of snails exposed to coal-fired power plant (site D) soils exhibited the highest SOD and CAT activities. Increased levels of SOD and CAT activities in the land snail (*Achatina achatina*) tissue collected from mining regions compared to snail tissue collected from a reference site have been reported also found increased levels of SOD and CAT in the land snail *Archachtina marinate* collected from polluted areas in Nigeria. Our findings were also in agreement with those who observed elevated CAT levels in land snails Helix asperse transplanted to soils around a steel plant for 12 weeks. The high activities of SOD and CAT in the snail tissues are due to high levels of reactive oxygen species as a result of snail exposure to metals. Superoxide dismutase and catalase are the first line of defense against reactive oxygen species. Superoxide dismutases catalyse the conversion of superoxide radical to hydrogen peroxide and oxygen. Hydrogen peroxide is subsequently broken down by catalase to water. The up-regulation of SOD and CAT activities is an adaptive response to prevent the build-up of superoxide anion and hydrogen peroxide. High activities of SOD and CAT observed throughout the 45 days exposure period indicate that the land snails were under oxidative stress.

Significantly high activity of GPx was recorded in snail tissue exposed to soil collected from site C and D. This is in agreement with findings who noted elevated GPx activities in tissues of the land snail *Theba pisana* collected from soils polluted by heavy metals. Thus the elevated GPx levels noted in our study implies that GPx operated optimally to counteract the effects of reactive oxygen species. In contrast, observed decreased activities of GPx in tissues of snails collected from polluted sites.

Metallothioneins, which are metal binding protein, were found to be significantly high in snails exposed to soils from at all sites compared to the reference site. This finding is similar to that who found elevated MT levels in the land snail *Eobania vermiculata* collected from heavy metal contaminated soils. Metallothioneins have been reported to not only sequestrate heavy metals and regulate essential metals but also directly scavenge reactive oxygen species through their cysteinyl thiolate groups.

CONCLUSION

Coal mining and related activities were shown to be major contributors of contaminants in the soil at the study area compared to background levels. The levels of metals in soil collected from the coal mining area exposes living organisms at the coal mining area to risk of oxidative stress as shown by elevated levels of biochemical markers.

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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Norah Basopo conceived, designed the study and conducted sampling. Donald Ndebele designed the study, conducted sampling and performed experiments, biochemical analysis and statistical analysis. Donald Ndebele wrote the manuscript. Norah Basopo and Andrew H. Siwela were supervisors of the project. They provided help in interpreting data as well as giving technical and editorial assistance.

ETHICS AND CONSENT TO PARTICIPATE

The study was approved by National University of Science and Technology Institutional Review Board (NUST/IRB/2017/22).

CONSENT TO PUBLISH

Authors agreed with the content and gave explicit consent to submit and full consent has been granted by national university of science and technology to submit this work.

AVAILABILITY OF DATA AND MATERIALS

The data sets used in/or analysed during the current study are available from the corresponding author on reasonable request.

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