Heavy metal accumulation and biochemical evaluation of earthworms from sawmills in Abeokuta, South-Western Nigeria

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Abstract: Over the years, sawmilling industries have shown a high growth in the rain forest areas of Nigeria, releasing several wastes into the environment. This study aims at using earthworms (*Eudrilus eugeniae, Libyodrilus violaceous* and *Hyperiodrilus africanus*) of sawmill origin as bio-indicators of metal pollution in sawmills. Four major sawmills located in Abeokuta (7º9'11''44" N - 3º19'35'' E), namely Lafenwa, Sapon, Isale-Ake and Kotopo sawmills were used for this study. The arboretum of the Federal University of Agriculture, Abeokuta was used as control site. Earthworms, plant and soil samples were collected each month for three months (March to May, 2013), randomly from different points at each of the locations. Protein analysis was conducted on the earthworms using gel electrophoresis while the activities of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) were done spectrophotometrically. Heavy metal analysis was also conducted on soil, plant and earthworm samples using Atomic Absorption Spectrophotometer. Gel electrophoresis results revealed the presence of nine protein bands in *E. eugeniae* from Sapon and Kotopo sawmills, as compared to six protein bands of *E. eugeniae* from the control site. Seven protein bands were observed in *L. violaceous* from Lafenwa and *H. africanus* from Isale-Ake sawmills. Levels of SOD, GPx and CAT activities were significantly higher ($P < 0.05$) in *E. eugeniae* from Sapon sawmill than those of Kotopo sawmill and the control site. This study also revealed that Pb and Cd concentrations were higher in the earthworms, plants and soil from the sawmills than those of the control site. Sapon sawmill recorded significantly higher ($P < 0.05$) levels of Cd and Cu in plants as well as Pb and Cd in soil samples than those of the other locations. The concentrations of Cu, Co and Ni were higher in the soil of the control site than in the sawmill soils. Stronger relationship exists in the metal concentrations between the earthworms and soils ($R= 0.602$) than between the plants and soil ($R= 0.405$). Sawmilling therefore posses potential risks on sawmill soil and soil fauna, especially earthworm species. Rev. Biol. Trop. 63 (4): 1213-1221. Epub 2015 December 01.

Key words: stress enzymes, Bio-indicator, heavy metals, sawmilling, pollution, earthworms.

Anthropogenic activities have constantly polluted the environment, rendering it increasingly unsafe for the habitation of human and other organisms. Recently, there has been high increase in the establishment of sawmills located in different areas in Nigeria (Abulude, 2006). Sawmilling is a major enterprise providing direct and indirect employment for thousands of people in the tropical rain forest region of Nigeria, where there is abundance of trees (Ihekwaba, Nwafor, & Adienbo, 2009). These sawmills generate a lot of wastes which include sawdust, wood off-cuts, wood backs, plain shavings, wood rejects among others (Dosunmu & Ajayi, 2002). Sawdust is the major residue of sawmilling operations and a

by-product of wood processing and is generally regarded as waste (Lennox, Abriba, Alabi, & Akubuenyi, 2010).

Nwajei and Iwegbue (2007) recorded some trace elements in sawdust particles in the vicinity of sawmill in Sapele, Nigeria. Abulude (2006) also reported some levels of heavy metals in suspended air particles in four sawmills. The concentration of some of these metals in soil as well as their accumulation in the biotic components such as earthworms and plants of the sawmills need to be properly evaluated.

The roles of earthworms in the soil ecosystem have been well documented (Owa, Dedeke, & Yeye, 2002; Renu, Pandey, Bisht, Kandpal, & Kaushal, 2006; Olayinka et al., 2011). Being an important and large soil organic matter decomposer, earthworms have gained acceptance for use in tests to assess the effects and accumulation of chemicals in soil organisms. It is widely used to analyze toxicity of pure chemicals in standardized soil (Haimi, 2000). Among terrestrial invertebrates, earthworms are choice test organisms for soil contamination surveys (Reinecke & Reinecke, 2004; Ricketts, Morgan, Spurgeon, & Kille, 2004) since they are easy to handle, widespread in their terrestrial distribution and have the capacity to accumulate and concentrate large quantities of inorganic and organic pollutants (Booth, Heppelthwaite, & McGlinchy, 2000; Rao, Surya, & Madhavendra, 2003) with acquired adaptive resistance to the toxicity of pollutants including heavy metals (Spurgeon & Hopkin, 1999).

Several studies have shown that metal trace elements are, at cellular level, often involved in oxidative stress, which results from the production of reactive oxygen species (ROS) (Lijun, Xuemei, Yaping, & Enbo, 2005). ROS, which includes the superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) affect mainly lipids, proteins, carbohydrates and nucleic acid (Damien et al., 2004). Lijun et al. (2005) explained that the antioxidant system comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPx). Superoxide radicals that are generated are converted to H_2O_2 by the action of SOD, and the accumulation of H_2O_2 is prevented in the cell by CAT and GPx.

Electrophoretic techniques allow rapid and accurate identification of organisms (Hamdan & Magdy, 2010) and are useful in clarifying relationships at sub specific and population levels when examining individual genetic variant (Hotchkin & Kaya, 1984). Snider (1973) reported that electrophoretic analysis of whole cell proteins by one dimensional pattern provides a rough measure of the number and physicochemical properties of gene products. With the degradation role of earthworms on the organic matter of sawmill soils as reported by Bamidele, Idowu, Ademolu, & Atayese (2014), there is the need to monitor the effect of metal pollution on the protein banding pattern of the earthworms.

This study therefore aims at evaluating the level of some heavy metals in soil, plants and earthworms of sawmill vicinities as well as investigate changes in the body protein banding pattern and the concentration of some antioxidative stress enzymes (SOD, GPx and CAT) in the earthworms obtained from the sawmills.

MATERIALS AND METHODS

Experimental site: Four sawmills located in Abeokuta, Southwestern Nigeria namely Lafenwa (07º09'44''12" N-03º19'35'' E), Sapon (07º09'11''44" N - 03º20'49'' E), Isale-Ake (07º09'48''03" N - 03º21'23'' E) and Kotopo (07º11'05''10" N-03º25'39'' E) were used for this study. They supply most of the processed wood and wood products used in Abeokuta and neighbouring towns. Earthworms, plant and soil samples were collected from each of the study sawmill respectively. The arboretum of the Federal University of Agriculture, Alabata, Abeokuta (07º10'00''00" N - 03º02'00'' W) was used as control.

Earthworm collection: Earthworms were collected according to the method described by Owa, Olowoparija, Aladesida, & Dedeke

(2013). The soil was carefully turned using a spade while the earthworms were handpicked into containers and transported to the laboratory where they were washed with distilled water. The worms were refrigerated for four hours to kill and allow them to purge the soil in their gut in order to avoid the effect of gut contents on heavy metal levels of the earthworms, after which the worms were rinsed with distilled water.

The earthworm species were identified and authenticated by Dr. A. A. Aladesida, an earthworm taxonomy specialist of the Department of Pure and Applied Zoology, Federal University of Agriculture, Abeokuta. The earthworm species used are *Libyodrilus violaceous* (Beddard, 1891), *Hyperiodrilus africanus,* (Beddard, 1890) and *Eudrilus eugeniae,* (Kinberg, 1866). These earthworms as used according to location are as follows:

Control site: *Eudrilus eugeniae*
Kotopo sawmill: *Eudrilus eugeniae*
Sapon sawmill: *Eudrilus eugeniae*
Lafenwa sawmill: *Libyodrilus violaceous*
Isale Ake sawmill: *Hyperiodrilus africanus*

The variation in the earthworm species used became inevitable because the distribution of earthworm species among the locations were not the same all through. The earthworm specimens were kept in the museum of the Biological Sciences Laboratory, Federal University of Agriculture, Abeokuta, Nigeria.

Plant and soil collection: Soil and plant samples were also collected in the same location from where the earthworms were collected. Soil samples were collected according to the method described by Agbaire & Emoyan (2012). The surface litter was removed from the top soil and the soil dug to a depth of 15 cm was collected into a polyethene labelled bag. Plants were collected using secateurs. Combined samples were collected at locations where there were more than one plant species (Idowu, Wewe, & Amusan, 2007).

Protein analysis (Gel electrophoresis): This was done using ProteoLadder 150 kit (catalogue number 12710) of the Norgen Biotek Corp, Canada.

Protein extraction: Adult earthworms were selected and homogenized for protein extraction. An amount of 0.3 g of earthworm sample was weighed into an eppendorf tube and 500 µL of 0.1 M Tris-HCl pH 7.6 was added. It was homogenized and later spun at 10 000 rpm for 10 minutes. The supernatant was taken into a fresh tube and kept in the refrigerator for SDS-PAGE gel electrophoresis.

SDS-Page gel electrophoresis: Polyacrylamide gel of 12 % was prepared which is separation gel and stacking gel of 3 % was prepared as well. A mixture of 5 µL of the sample extract and 5 µL of the loading buffer was boiled at 95 ^{o}C for 5 minutes and loaded on the gel. The sample was run for 60 minutes at 150 V.

SDS-Page gel staining: The plate was dismantled and the gel removed, it was stained in 0.1 M Coomassie blue solution for 1 hour and later removed and distained in several rinses of Ethanol/Acetic acid solution until the gel became clear for viewing. Picture of the gel was later taken for documentation.

Stress enzymes analysis: The earthworm samples were homogenized with 0.1 M phosphate buffer (pH 7.4), using a ceramic mortar and pestle in ice. The homogenized samples were then centrifuged and the supernatant frozen for further analyses. Total protein content was determined using Cypress Diagnostics Kit, Langdorp Belgium. The catalase activity was determined according to the method of Sinha (1972).

Glutathione peroxidase activity was according to Rotruck et al. (1973). To 500 µL of homogenized sample, phosphate buffer (500 µL), NaN_3 (100 µL), GSH (200 µL), H_2O_2 (100 µL), and 600 µL of distilled water were added. The whole reaction mixture was incubated at 37 ^{o}C for 3 minutes after which 0.5 mL of

TCA was added and thereafter centrifuged at 3 000 rpm for 5 minutes. To 1 mL of each of the supernatants, 2 mL of K_2HPO_4 and 1 mL of DTNB was added and the absorbance was read at 412 nm against a blank.

The level of SOD activity was determined by the method of Misra & Fridovich (1972). An amount of 1 mL sample was diluted in 9 mL of distilled water to make a 1 in 10 dilution. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 mL buffer, 0.3 mL adrenaline and 0.2 mL of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Heavy metal analysis: Heavy metal content was determined according to the method of A.O.A.C. (2006). Plant and earthworm samples were oven-dried at 60 ^oC for 48 hours. Soil samples were dried at 80 ^oC for 48 hours. Dry matter of each of the plants, earthworms and soil samples were weighed and crushed to powder. To 1 g each of the powdered samples, 7 mL of concentrated hydrochloric acid and 21 mL of nitric acid (69 % AnalaR grade) was added and boiled on heating mantle until the colour turned colourless. The mixture was allowed to cool, filtered, and then diluted with distilled water to 100 mL. An Atomic Absorption Spectrophotometer (AAS Buck 210VGP System) was used to determine the concentration of Lead, Copper, Cadmium, Nickel and Cobalt in the digested samples.

Data collected were subjected to statistical analyses using the Statistical Package for Social Sciences (SPSS) version 16.0. ANOVA was conducted to determine significant differences in the values of heavy metals and stress enzymes in *Eudrilus eugeniae* earthworms, as well as heavy metal levels in plant and soil samples between locations. Post Hoc test was also done using LSD (Least Significant different). Linear regression was conducted to check the relationship between the metal concentrations in earthworms, plants and soils. P-value was set at 0.05. Protein bands were scored depending on their presence (1) or absence (0).

RESULTS

Heavy metals: The result of metal concentrations in earthworms, plant and soil samples from the five study locations is presented in Table 1. Concentrations of Pb, Cd and Cu were significantly higher ($P < 0.05$) in the *E. eugeniae* collected from Sapon sawmill than those of Kotopo sawmill and the control site. Also, Sapon sawmill recorded significantly higher ($P < 0.05$) levels of Cd and Cu in plants as well as Pb and Cd in soil samples than those of the other sites. Pb and Cd concentrations were higher in the earthworms, plants and soil from the sawmills than those of the control site (Table 1).

Concentrations of Cu, Co, and Ni were higher in the soil samples from the control site than those from the sawmills (Table 1). The average concentrations of Pb, Cu, and Co in the earthworm, plant and soil from all the study locations followed the trend, soil > earthworm > plant.

Linear regression revealed that the relationship between metal concentrations in earthworms and plants with that of the soils was significant ($P < 0.001$) (Fig. 1). However, a stronger relationship exist between the metal concentration in the soil with those of earthworms ($R = 0.602$) than those of the plants ($R = 0.405$).

Protein analysis (Gel electrophoresis): The Gel electrophoresis results revealed the presence of nine protein bands in *Eudrilus eugeniae* from Sapon and Kotopo sawmills compared to six protein bands of *E. eugeniae* from the control site. Seven protein bands were observed in *Libyorilus violaceous* from Lafenwa and *Hyperiodrilus africanus* Isale-Ake sawmills (Fig. 2). The protein bands of earthworms from

TABLE 1
Heavy metal concentration (ppm) in earthworms, plant and soil from five sawmills in Abeokuta, South-Western Nigeria

	Location	Earthworm species	Pb	Cd	Cu	Co	Ni
EARTHWORM	Control	*E. eugeniae*	0.2322*	0.0078*	0.4400*	0.3660*	0.3474*
	Kotopo	*E. eugeniae*	0.2695*	0.0101*	0.3420*	0.4340*	0.3446
	Sapon	*E. eugeniae*	0.6766*	0.0548*	0.7040*	0.2720*	0.1596*
	Isale ake	*H. africanus*	0.3160	0.0326	0.2180	0.1160	0.1320
	Lafenwa	*L. violaceous*	0.4633	0.0244	0.5580	0.3100	0.3479
PLANT	Control		0.1181*	0.0404*	0.1220*	0.3160*	0.0735*
	Kotopo		0.2724*	0.0953*	0.5880*	0.3240	0.0493*
	Sapon		0.1580*	0.1505*	0.7800*	0.2420*	0.1227*
	Isale ake		0.2060	0.0457	0.4080*	0.2320*	0.0814*
	Lafenwa		0.2031	0.0483	0.2500*	0.3180	0.0511*
SOIL	Control		0.1452*	0.0611*	0.7140	0.4720*	0.1057*
	Kotopo		0.2430*	0.0877*	0.2660*	0.3960*	0.0400
	Sapon		0.7276*	0.1038*	0.3740*	0.3560*	0.0808*
	Isale ake		0.4430*	0.0621*	0.7200	0.3320*	0.0498
	Lafenwa		0.6097*	0.0777*	0.3940*	0.1620*	0.0212

*The mean difference is significant at $P < 0.05$ (for earthworm [*E. eugeniae*], plant and soil respectively between the study locations.

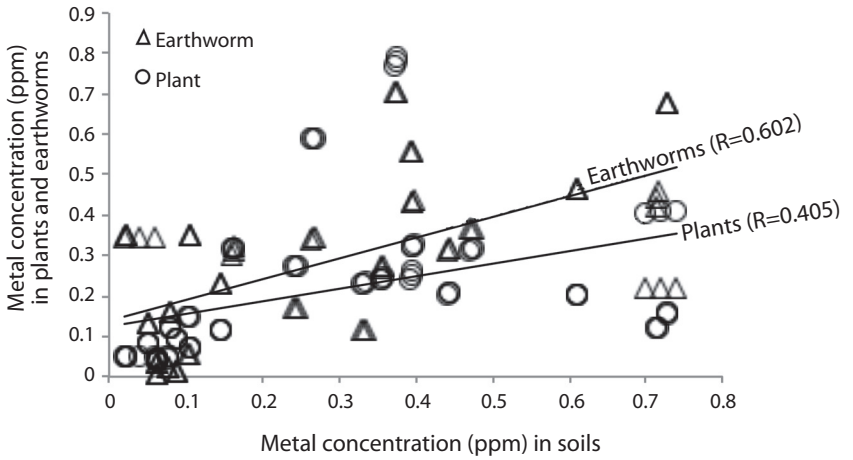

Fig. 1. Linear relationship between metal concentrations in earthworms and plants with metal concentrations in the soils ($P < 0.001$) from five sawmills in Abeokuta, South-Western Nigeria.

the control site ranged between 14.7 kiloDalton (kDa) and 100 kDa while those from the sawmills ranged from 14.7 to 150 kDa.

Stress enzyme: The result of the stress enzymes examined is shown in Table 2. There were significant differences ($P < 0.05$) in the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in *E. eugeniae* from Kotopo sawmill, Sapon sawmill and the Control site (Table 2). Superoxide dismutase, glutathione peroxidise and catalase activities in *E. eugeniae* followed the trend Sapon sawmill > Control site > Kotopo sawmill.

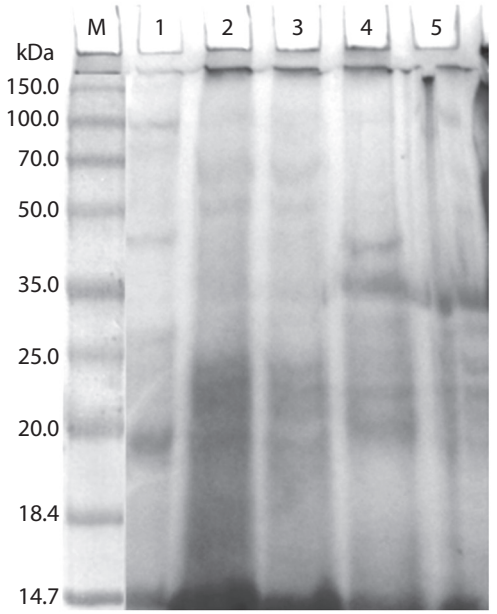

Fig. 2. SDS Gel for the earthworm samples from five sawmills in Abeokuta, South-western Nigeria. 1- Control; 2- Sapon, 3- Kotopo, 4- Lafenwa and 5- Isale Ake sawmills.

DISCUSSION

Higher concentrations of Pb and Cd in *E. eugeniae*, plants and soils from the sawmills than the control could have resulted from human activities. Awofolu (2005) reported that the levels of Pb and Cd in the environment have increased tremendously in the past decades as a result of human activities. Most of the sawmilling machines engines run on fuel (diesel or petrol) while others depend on standby power generating sets at various locations within the sawmills. Fuel spills from the generating sets as well as improper disposal of spent oil, coupled with pollution from wood preservatives and vehicular exhaust could be responsible for the high concentration of Pb and Cd in the samples from the sawmills.

Sawdust was also reported by Nwajei & Iwegbue (2007) to contain some concentrations of Pb, Cd and some other metals. Over the years, sawdust become degraded and is incorporated into the soil. Concentrations of these metals in the earthworms could have resulted from uptake of soils into the tissue of the earthworms through their soil engineering activities on the sawmill soils.

The concentrations of these heavy metals (Pb and Cd) were significantly higher in both the soil and earthworm samples from Sapon sawmill than those from the other locations. Sapon sawmill is situated at the centre of commercial activities in Abeokuta with a heavy load of vehicular traffic passing through the two major roads very close to the sawmill. It is possible that the high level of Pb recorded in the earthworm and plant samples from Sapon sawmill is a result of soil contamination by vehicular emission in the busy roads, coupled with several other inputs from the surrounding commercial activities. Concentrations of these heavy metals were however lower than regulated limits on heavy metals applied to soils (Pb - 420 ppm and Cd - 85 ppm) (USDA, 2000).

Cu, Co and Ni concentrations were higher in the soils of the control site than those collected from the sawmills. However, the values were lower than the regulated limits for agricultural

TABLE 2
Stress enzyme activities in the earthworms from five sawmills in Abeokuta, South-western Nigeria

	Earthworm species	SOD (unit/mg protein)	GPx (μg/mL/mg protein)	CAT (μmole/mg protein)
Control site	*E. eugeniae*	3.21*	377.24*	1 117.64*
Kotopo sawmill	E. eugeniae	2.9*	338.85*	984.09*
Sapon sawmill	E. eugeniae	7.16*	852.07*	2 345.14*
Isale ake sawmill	H. africanus	7.9*	909.33*	2 655.26*
Lafenwa sawmill	L. violaceous	2.55*	300.29*	878.78*

*The mean difference is significant at $P < 0.05$.

soils (USDA, 2000). These elements (Cu, Co and Ni) were described as microelements (Sosorova, Merkusheva, Gyninova, & Ubugunov, 2012) and trace elements (Holmgren, Meyer, & Daniels, 1993) for plants, provided they are less than the regulated limits. It is possible that the activities on the sawmills have the potential to deplete some important microelements in the sawmill soils.

Average concentrations of Pb, Cu and Co from the locations followed the order soil > earthworm > plant. The lower concentrations of the metals in the plant samples compared to the earthworms as recorded in this study agrees with the report of Holmgren et al. (1993) that crop plants has little tendency of metal accumulation. Spiegel (2002) also recorded higher concentrations of metals in the earthworms than the plant samples used as bioindicator of emissions around industrial facilities. Higher metal concentration in the earthworms than in the plants also supports earlier reports that earthworms have high rate of heavy metal accumulation from the soil into their body tissues (Agbaire & Emoyan, 2012; Elaigwu, Ajibola, & Folaranmi, 2007; Hobbelen, Koolhaas, & Gestel, 2006). Stronger relationship existing between metal concentration in soil and earthworms (R = 0.602) than between the soil and plants (R = 0.405) as observed in this study also supports this.

Heavy metal concentrations of soil, plants and earthworms in this study were lower than those recorded for plant and earthworm used as bioindicator of trace elements along industrial facilities (Spiegel, 2002), sawdust particles of Sapele, Nigeria (Nwajei & Iwegbue, 2007), adult *Lumbricus rubellus*, adult *Aporrectodea caliginosa* and leaves of *Urtica dioica* (Hobbelen et al., 2006).

Heavy metal contamination of soils in the sawmills could have affected the body protein banding pattern of the earthworms from the sawmills. *Eudrilus eugeniae* from the sawmills expressed more protein bands than those of the control site. The expression of more protein bands in *E. eugeniae* could have resulted from the physiological adaptations of the earthworms to the pollutants including heavy metals from sawmilling activities. Hamdan & Magdy (2010) reported that observed changes in protein banding patterns in animal species could be as a result of gene mutation. This appearance of more protein bands as expressed in earthworms from the sawmills could be explained by the submission of Kordafshari, Hosseine, Meshgi, & Youssefi (2010) that the appearance of new protein bands is based on a mutational event at the regulatory system of unexpressed gene(s) that activate them.

The activities of the stress enzymes (superoxide dismutase, glutathione peroxidise and catalase) in *E. eugeniae* followed the trend: Sapon sawmill > Control site > Kotopo sawmill. Sapon sawmill is the only sawmill surrounded by several residential and commercial buildings with very busy commercial activities. On the other hand, Kotopo sawmill is surrounded with more uncultivated land and few residential buildings. High level of the stress enzymes recorded in *E. eugeniae* from Sapon sawmill than those of Kotopo sawmill and the control site could therefore be as a result of the influence of pollutants entering the sawmill from the neighbourhood.

This study has shown that sawmilling posses potential risks on sawmill soil and soil fauna, especially earthworm species. There is therefore the need for a proper allocation, monitoring and management of land uses by these sawmills to mitigate soil contamination.

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RESUMEN

Acumulación de metales pesados y evaluación bioquímica de las lombrices de tierra de los aserraderos en Abeokuta, Sur-Occidente de Nigeria. A través de los años, la industria de aserraderos ha mostrado un alto

crecimiento en las áreas de bosque tropical de Nigeria, lanzando varios residuos al ambiente. Este estudio tiene como objetivo utilizar lombrices (*Eudrilus eugeniae*, *Libyodrilus violaceous* y *Hyperiodrilus africanus*) de origen aserradero como bioindicadores de contaminación por metales en los aserraderos. Se utilizaron cuatro grandes aserraderos ubicados en Abeokuta (7º9'11''44" N - 3º19'35'' E), a saber: Lafenwa, Sapon, Isale-Ake y Kotopo, para este estudio. El arboreto de la Universidad Federal de Agricultura, Abeokuta fue utilizado como sitio de control. Las lombrices de tierra, plantas y muestras de suelo se recogieron cada mes durante tres meses (marzo a mayo 2013), al azar en diferentes puntos en cada una de las localidades. El análisis de proteínas se llevó a cabo en las lombrices de tierra utilizando electroforesis en gel, mientras que las actividades de la superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx) se realizaron espectrofotométricamente. Análisis de metales pesados también se llevaron a cabo en muestras de suelo, plantas y lombrices de tierra utilizando espectrofotómetro de absorción atómica. Resultados de la electroforesis del gel reveló la presencia de nueve bandas de proteínas de *E. eugeniae* en los aserraderos de Sapon y Kotopo, en comparación con seis bandas de proteínas de *E. eugeniae* de el sitio control. Se observaron siete bandas de proteínas de *L. violaceous* de Lafenwa y *H. africanus* en los aserraderos Isale-Ake. Los niveles de SOD, GPx y actividades CAT fueron significativamente mayores ($P < 0.05$) en *E. eugeniae* del aserradero Sapon que las del aserradero Kotopo y el sitio control. El estudio también reveló que las concentraciones de Pb y Cd fueron mayores en las lombrices de tierra, plantas y el suelo de los aserraderos que los del sitio control. El aserradero Sapon mostró niveles significativamente mayores ($P < 0.05$) de Cd y Cu en las plantas, así como Pb y Cd en muestras de suelo que las de los otros lugares. Las concentraciones de Cu, Co y Ni fueron mayores en el suelo del sitio de control que en los suelos de los aserraderos. Existe relación más fuerte en las concentraciones de metales entre las lombrices de tierra y los suelos ($R = 0.602$) que entre las plantas y el suelo ($R = 0.405$). Por lo tanto, la actividad en los aserraderos posee riesgos potenciales en el suelo y su fauna, especialmente las especies de lombrices.

Palabras clave: enzimas de estrés, bio-indicadores, metales pesados, contaminación, lombrices de tierra, aserradero.

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