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Original Articles

Biochemical response and vermiremediation assessment of three earthworm species (*Alma millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus*) in soil contaminated with a glyphosate-based herbicide

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ABSTRACT

The global use of glyphosate-based herbicides (GBHs) and release of glyphosate residues in soil environment have over the years been a major concern. In this study, we aim to evaluate the biochemical response and vermiremediation potential of three indigenous earthworm species; namely *Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceus* in GBH treated soils. Study design: Three weed plants (*Tridax procumbense*, *Ludwigia pasturis* and *Pannicum maximum*) were transplanted into 140 plastic pots and 20 adult individual earthworms of each species were later introduced into 60 pots. In total, each earthworm species treatments representing 20 pots and 20 pots without earthworm were sprayed with 115.49 mL/m^2 (equivalent to 83.2 g a.i/m^2) of Roundup® Alphée. The remaining 60 pots with earthworm species were left unsprayed. Activities of glutathione-S-transferase (GST), lactate dehydrogenase (LDH), metallothionine (MT), acetylcholinesterase (AChE), antioxidant defense system and lipid peroxidation (MDA) were monitored at the 1st, 2nd, 4th, 6th and 8th weeks Post Herbicide Application (PHA) using standard methods. Glyphosate residues in the soil and earthworm species were quantified with a High-Performance Liquid Chromatography (HPLC) with fluorescence detector. Bioaccumulation Factor (BAF) was also calculated. Results: Higher activities of GST and LDH and reduction in MT activities were observed in the three earthworm species exposed to GBH compared to the unexposed while AChE activity was insensitive to the herbicide. The antioxidant defence system was able to protect *E. eugeniae* and *L. violaceus* against oxidative stress. The presence of earthworms reduced glyphosate residues in the soil. *E. eugeniae* and *L. violaceus* were bioaccumalators and biomagnifiers of glyphosate as indicated by the BAF (> 1) obtained after 8th week PHA. Relationships exist between glyphosate BAF in *E. eugeniae* and *L. violaceus* and exposure duration as well as between glyphosate residues in tissues of the earthworm species and their biochemical parameters. Conclusions: Alterations in the enzymatic activities and antioxidant defence of the earthworm species could be an index for GBH contaminated soil monitoring and assessment; both *E. eugeniae* and *L. violaceus* showed potential to vermiremediate soils contaminated with GBH.

1. Introduction

The global use of agrochemicals such as herbicides by farmers in agricultural system is increasing and one notable herbicide used is the glyphosate-based herbicides (GBHs).

GBHs, which contains glyphosate (N-phosphonomethyl glycine) as its active ingredient and other unspecified surfactant for easy absorption into plants, is a leading post-emergent, organophosphate systemic, broad-spectrum and non-selective herbicides for the control of weeds (Antoniou et al., 2012; Mesnage et al., 2015). The increased usage of GBHs have been widely reported in many countries (Garthwaite, 2010; Gianessi and Williams, 2011; Steinmann et al., 2012) including Nigeria (Chikoye et al., 2007). Apart from its use as a weed control agent, it is also used as a desiccant on variety of food products (Monsanto, 2010) and consequently leads to its reported residue in foodstuffs and, animal and human hence exposure to the herbicide (Bohn et al., 2013).

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Although GBHs have been proven to be effective in the control of weeds from agricultural and non-agricultural soils, residues of glyphosate in the soil system have over the years, been a major concern (NASS, 2013). This anthropogenic release of herbicides into the soil environment advocates the urgent need to monitor the ecotoxicological risk assessment of the herbicides and mitigate their potential impacts. A major reliable method involved the use of the soil organisms such as earthworms.

Earthworms are important soil invertebrate that are directly in contact with soil particles. Leachates in soil may permeate their skin as well as alimentary tract (Drake and Horn, 2007) thereby causing toxicological effects. Owing to their ecological importance, high biomass and sensitivity to environmental pollution, earthworms have been reported to sentinel species for soil monitoring and ecotoxicological risks assessment of pollutants in terrestrial ecosystems (Landrum et al., 2006; Reinecke and Reinecke, 2007). To this effect, earthworms have been used in ecotoxicological risks monitoring and as bio-indicator of lethal and non-lethal pollutants including heavy metals in environments (Georgescu et al., 2011), mine site (Honsi et al., 2003) and sawmill soil (Bamidele et al., 2015). In our previous studies, we used earthworms to monitor the level of pollution in abattoir soil (Owagboriaye et al., 2016; Dedeke et al., 2016). Earthworms have been used to evaluate organophosphates pollution in soil (O'Halloran et al., 1999).

One method often used in environmental risk monitoring of chemical toxicity is the assessment of biomarkers (McCarthy and Shugart, 1990) which has been reported to provide information on the negative effects of pollutants as well as acting as early warning signals of the impending environmental damage (Lynn and Kathryn, 2001).

The most studied earthworm biomarkers in ecotoxicological assessment are acetylcholinesterase (AChE) which plays a major role in neurotransmission and muscular activities (Wang et al., 2015; Hackenberger et al., 2018), glutathione S-transferase (GST) which helps in the detoxification and neutralization of pollutants and lactate dehydroginase (LDH) which is an enzymatic biomarker of stress exposure in earthworms (Shekari et al., 2008). In addition, alterations in activities of enzymatic antioxidants (catalase, GPx, SOD) and non-enzymatic antioxidant (GSH) defense system, which protect animal cells from oxidative damage (Pastore et al., 2003), are also valuable biomarkers of pollution monitoring in soil environments (Ribera et al., 2001; Schreck et al., 2008; Wiegand et al., 2007). Most of the biomarkers studies on earthworm have been mainly focused on metal pollution assessment, while only a few studies have been focused on biomarker responses in herbicides assessment (Sanchez-Hernandez, 2006).

Moreover, most studies on ecotoxicological assessment conducted have used earthworm species *Eisenia fetida* as a representative organism of soil invertebrates (Ribera et al., 2001; Verrell and Van Buskirk, 2004; Yasmin and D'Souza 2007; Correia and Moreira, 2010; Buch et al., 2013; Piola et al., 2013). Although the International Organization for Standardization (ISO) (ISO, 1993; 1998) and Organization for Economic Co-Operation and Development (OECD) (OECD 1984; 2004) recommended *Eisenia fetida* as the standard test organism to be used in terrestrial ecotoxicological monitoring and assessment mainly due to its ability to be bred easily and its susceptibility to pollutants which mimics that of true soil organisms. However, *Eisenia* species can only thrive on compost matters and cannot be found in agroecosystems (Yasmin and D'Souza, 2007; Correia and Moreira, 2010; Buch et al., 2013; Piola et al., 2013). In addition, *Eisenia* species have been reported to be less sensitive compared to other earthworm species (Ma and Bodt, 1993; Kula 1995; Fitzgerald et al., 1996). These therefore, questioned the credibility of *Eisenia* species as the sentinel organism in environmental health monitoring and assessment. This necessitates the need for more suitable earthworm species as bioindicator.

Remediation of contaminated soil has been proposed to be a reliable technique that can mitigate the impact of contaminant in the environment. Conventional remediation focused on chemical treatments or physical removal of contaminant from the environment, but bioaugmentation, biostimulation or phytoremediation has also been established due to its safety effect on the environmental (Hamdi et al., 2007; Juwarkar et al., 2010). Vermiremediation involving the use of earthworms to remove contaminants from soil has been indicated as an alternative method of remediation in ecotoxicological monitoring (Sinha et al., 2008). This knowledge has been demonstrated in the use of earthworms to remove contaminants such PAH (Eijsackers et al., 2009), PCBs (Singer et al., 2011), oils (Fernández et al., 2011) and heavy metals (Udovic and Lestan, 2010) from soil. However, it remains unknown if earthworm species can remediate a GBH from the soil.

In an effort to monitor the ecotoxicological risk assessment of a GBH in a contaminated soil, we designed this study to evaluate the biochemical responses of three indigenous earthworm species (*Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceus*) in GBH contaminated soils using a panel of biomarker. In addition, we also assessed the vermiremediation potential of the earthworm species in the contaminated soil in order to mitigate the potential impact of the herbicide. These earthworm species are native to Africa and commonly found in the soils of agricultural, industrial and residential areas in Nigeria and have been proven to be a good indicator of environmental pollution in sawmills (Bamidele et al., 2015; 2016) and abattoir area (Owagboriaye et al., 2016; Dedeke et al., 2016).

2. Materials and methods

2.1. Experimental design

This study was conducted at the research section of the Animal House of the Department of Zoology and Environmental Biology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria. The study set-up was in accordance to Gaupp-Berghausen et al. (2015) with little modifications. A total of 140 plastic pots (diameter: 25 cm, length: 30 cm) perforated at their bases (for drainage) and filled with soil (PH 5.56; sand 49.38; silt 35.27%; clay 15.35%; organic carbon 19.05%; organic matter 32.94%; Nitrogen 0.11%; Phosphorus 0.32%) collected from an arable field of the University research farm (with no history of herbicide application) were used for this study. Each pot was planted with three weeds; *Tridax procumbense*, *Ludwigia pasturis* and *Pannicum maximum* in a similar pattern (distance between plants in each pot was maintained at 4.8 cm). Weeds at 5 cm height were collected from the University research farm and transplanted into the pots. Each pot was irrigated daily throughout the experimental period. Two weeks after transplanting, 3 earthworm treatment species (*A. millsoni*, *E. eugeniae*, *L. violaceus*) were separately introduced into the pots. The earthworms were collected from natural soils around Ago-Iwoye community and carefully brought to the laboratory with moist soil sample within an hour of collection. The earthworms were acclimatized for two weeks in a laboratory culture at 23 ± 2 °C as described by Heimbach (1984). Adult earthworms (*A. millsoni* 1.1 ± 0.08 g; *E. eugeniae* 1.1 ± 0.02 g; *L. violaceus* 0.9 ± 0.06 g fresh weight) with well-developed clitella were removed from the culture at 24 h prior to the commencement of the pot experiments and placed on moist filter paper to rid the earthworms of their gut contents. The earthworms were later rinsed with distilled water, dried with moist paper and placed in each pot. The earthworms burrowed immediately into the soil upon release. Ground hay was added to the pots to provide food for the earthworms. Twenty pots (20 replicates) were used per earthworm species; each pot (replicate) was seeded with 20 earthworms, making a total of 60 treatment pots (to be exposed to/sprayed with GBH). The number of earthworms seeded was based on the report of Edwards and Bohlen (1996) that a population density of 400 to 800 earthworms occupies a square meter of land. After 3 weeks of the weed transplant (when *T. procumbens* has attained height 14 cm, *L. pasturis* was 22 cm and *P. maximum* was 28 cm), each pot was sprayed with 7.2 mL of Roundup® Alphée which contains glyphosate concentration of 720 g/L (Scotts Celaflor, Mainz, Germany) for two consecutive days (exposed). Another set of 20 pots

grown with the weeds but devoid of earthworms were also sprayed with herbicide (positive control). In total, each earthworm species treatments representing 20 pots and the 20 pots without any earthworm species were sprayed with 115.49 mL/m^2 (83.2 g a.i/m^2) of Roundup® Alphée which is 87% lower than the recommended dose of 800 plants /L (Monsanto, St. Louis/Missouri, USA). A similar set up of each species of the earthworm in 60 pots without herbicide application, but sprayed with water (control/unexposed) were also established making a total number of 140 pots set up.

2.2. Earthworm analysis

In order to determine the response and remediation potential of the earthworm species in GBH treated soils, a total of 3 individual earthworms were collected from the exposed and unexposed pots after 1st week, 2nd weeks, 4th weeks, 6th weeks and 8th weeks of herbicide application. The treated and control earthworms collected were carefully washed free of soil particles with distilled water and placed in Petri dishes containing moist filter paper to void out their gut contents. The earthworms were immobilized in 20% (V:V) ice-cold glycerol solution for 3 min and homogenised in 3 mL ice-cold potassium phosphate buffer (pH 7.1)/ 3 mL ice-cold Tris-HCl 25 mmol buffer (pH 7.2) using a Teflon Homogenizer. The homogenates were centrifuged at 8,000 rpm for 20 min at 4 °C and the supernatants were analysed for the activities of metallothionin (MT), lactate dehydrogenase (LDH), Cholinesterase (ChE) and Glutathione S-transferase (GST). Oxidative stress response of the earthworm species was determined by evaluating their antioxidant defense system including activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidise (GPx) and concentration of reduced glutathione as well as level of lipid peroxidation (MDA). Levels of glyphosate residue in the earthworm species and soil samples at 1st week, 2nd weeks, 4th weeks, 6th weeks and 8th weeks after herbicide application was also determined and bioaccumulation factor (BAF) was calculated.

2.2.1. Biochemical enzyme analysis

We used the rapid colorimetric method of Ellman et al. (1961), modified by Curry et al. (2008) for earthworms, to determine the activity of AChE. The substrate compound was acetylthiocholine iodide while a chromogen was 5,50dithiobis (2-nitrobenzoic) acid (DTNB) and read at 412 nm. The standard method of Habig et al. (1974) which involved the conjugation of 0.05 mL reduced glutathione (GSH) with 0.05 mL 1-chloro-2,4-dinitrobenzene (CDNB) was employed for the determination of GST activity. The GST activity was calculated from the extinction coefficient (GSH-CDNB) and expressed as nmol DNPG produced min/mg protein. For MT activity determination, we added 0.1 mmol phenylmethylsulfonyl fluoride (PMSF) as antiprotease agent and 0.5 mmol dithiothreitol (DTT) as the sulfydryl-protecting agent to prevent the MT from oxidation and 1 mL of the supernatant was heated at 100 °C for 10 min in order to precipitate non-MT protein and later cooled on ice for 5 min. We fractionated aliquots of the heat-treated homogenate by gel-permeation chromatography [Sephadex G 75 (26 mm by 60 cm)] and the MT concentration in the samples was estimated with the mercury-saturation assay described by Suriya et al. (2012). LDH activity was determined according to the method of Vassault (1983) where 2.5 mL of a TRIS/NaCl/NADH solution and 0.5 mL of pyruvate solution were added to the homogenate at 340 nm every 30 s for 3 min and the values were expressed as nmols of reduced pyruvate/min/mg protein. The protein content in earthworm sample was determined using the method of Bradford (1976) with bovine serum albumin as a standard

2.2.2. Antioxidant defense analysis

The rate of NADPH oxidation at 340 nm in 1 mL of reaction mixture was measured for the determination of GPx activity (Nusetti et al., 2001). Concentration of GSH was determined according to the GSH-400 method described by Blume et al. (1975). Lipid peroxidation was determined by measuring the lipid peroxidation end product, malondialdehyde (MDA) as thiobarbituric acid reactive substances (TBARS) which was measured fluorometrically at 535 nm as described by Gagné (2014). The production of hydrogen peroxide (H_2O_2) at 240 nm was measured for the determination of catalase activity (Saint-Denis et al. 1998). The activity of SOD was measured in soluble fraction according to the colorimetric assay method of xanthine/xanthine oxidase activity at 450 nm described by Valeska et al. (2009)

2.2.3. Glyphosate residue analysis in earthworm and soil sample

Glyphosate residue in the earthworm tissue and soil that were sampled simultaneously at each interval of the weeks was analysed with high-performance liquid chromatographic (HPLC) technique according to Sancho (1994) modified by Mardiana-Jansar and Ismail (2014). The soil samples (0–5 cm depth) were air-dried at room temperature and allowed to pass through a 2 mm sieve after which 5 g was taken. The samples were extracted with 10 mL of 0.6 potassium hydroxide (KOH) by shaking on a shaker at 120 oscillations/minute for 30 min and later centrifuge at 3500 rpm for 30 min. The supernatants were filtered and the filtrates (3 mL) were derivatized through the addition of 0.5 mL of 0.05 M borate buffer and 1.5 mL of 1000 ppm 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl) at room temperature for1 hour. The samples were washed with 2 mL diethyl-ether to eliminate excess derivatization reagent and the aqueous phase was recovered for HPLC injection. The homogenised earthworm sample was extracted with 25 mL of chloroform and 0.1 N of hydrochloric acid (HCl). Aqueous extract fraction of the extract was cleaned-up by elution through ion exchange resin in Fe^{3+} form. Elution of the compound from resin was done with HCl and the Fe was removed with a cation exchange resin. HCl was removed by concentrating the sample to dryness and glyphosate concentration was determined by HPLC after post-column derivatisation (Pijanowski, 1988; DFG, 1992; Oppenhuizen and Schuette, 1995). The chromatographic analysis was performed with Agilent Technologies 1200 series high-performance liquid chromatography (HPLC) with fluorescence detector (Santa Clara, Califonia, USA). The column, Zorbax Eclipse XDB-C18 column (250 × 4.6 mm) (Thomas Scientific), was run in isocratic mode and the mobile phase consisted a mixture of 1.5%, phosphate buffer (pH 5.8) and acetonitrile in ratio 55:45. A flow-rate of 0.5 mL/minute under isocratic conditions was maintained for the column at 40 °C with an injection volume of 4 µL. A blank sample was spiked with prepared calibration standard glyphosate solutions and a calibration curve was obtained from the matrix-matched multilevel calibration solutions. Glyphosate measurement was done by comparing peak heights of samples to the standard calibration curve.

2.2.4. Bioaccumulation factor

The bioaccumulation factor (BAF) which quantified the accumulation of glyphosate by earthworms was determined through the ratio of the concentrations of glyphosate in earthworms and the soil substrate (OECD, 2010).

$$\text{Bioaccumulation factor (BAF)} = \frac{\text{Concentration of glyphosate in earthworm tissue}}{\text{Concentration of glyphosate in soil substrate}}$$

2.3. Statistical methods

Data obtained were subjected to statistical analyses using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp., 2011). Mean values were compared between the experimental weeks using the Analysis of Variance (ANOVA). Post hoc test was done using the Student-Newman-Keuls (SNK). Mean values of parameters were compared between the glyphosate exposed earthworm group (Exposed)

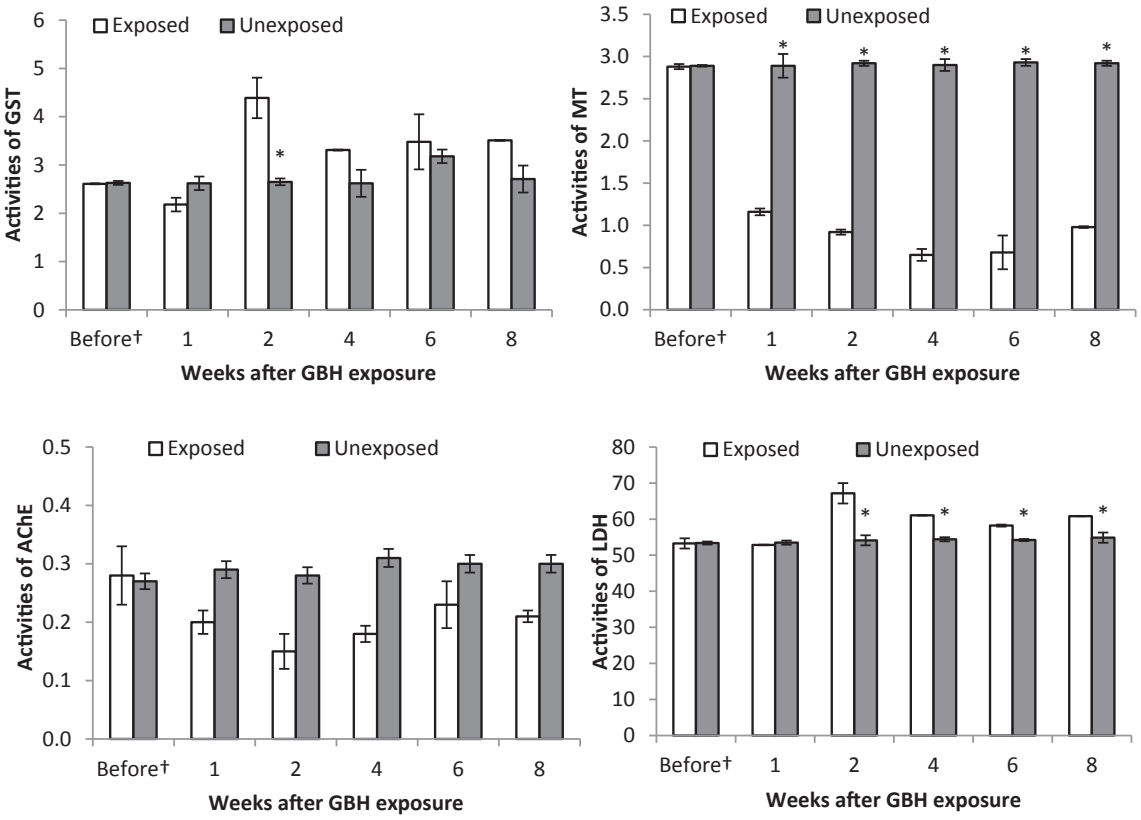

Fig. 1. Enzyme activity in *Alma millsoni* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit = GST-nmol DNPG/mg protein/min.; MT- mmol/mg protein/min.; AChE-nmol/mmg protein/min.; LDH-nmol/mg protein/min.

and the non-exposed group (Unexposed) using the independent sample T-test. Results were presented as Mean ± Standard deviation. Pearson correlation was used to check the relationship between residues of glyphosate residues in earthworm tissue and their body biochemical parameters. P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Biochemical enzyme activity in Alma millsoni after experimental week of exposure

There was no significant difference ($p > 0.05$) in the activities of GST, MT, ChE and LDH recorded in *A. millsoni* unexposed to GBH throughout the experimental weeks (Fig. 1). Activity of GST was observed to be significantly highest ($p < 0.05$) in *A. millsoni* after 2nd weeks of GBH exposure compared to the unexposed. Activity of MT was not significantly different ($p > 0.05$) in the exposed and unexposed groups before GBH exposure but significantly lower ($p < 0.05$) in the exposed groups at 1st, 2nd, 4th, 6th and 8th weeks after GBH exposure compared to the unexposed. LDH activity was not significantly different ($p > 0.05$) in the exposed and unexposed groups before exposure and 1st week after exposure, but significantly higher ($p < 0.05$) at 2nd, 4th, 6th and 8th weeks after GBH exposure compared to the unexposed group. However, AChE was not significantly different ($p > 0.05$) between the exposed and unexposed groups throughout the experimental period.

3.2. Biochemical enzyme activity in Libyodrilus violaceus after experimental week of exposure

Activities of GST and LDH in *L. violaceus* were not significantly different ($p > 0.05$) between the exposed and unexposed groups before exposure (Fig. 2). However, after 1st, 2nd, 4th, 6th and 8th weeks of GBH exposure, activities of GST and LDH were significantly higher ($p < 0.05$) in the exposed group compared to the unexposed. Also, before GBH exposure and 1st week after exposure, there was no significant difference ($p > 0.05$) in the activity of MT between the exposed and unexposed groups but MT activity was significantly lower ($p < 0.05$) in the exposed group at 2nd, 4th, 6th and 8th weeks after GBH exposure. Activity of AChE was not significantly different ($p > 0.05$) between the exposed and unexposed groups throughout the experimental weeks.

3.3. Biochemical enzyme activity in Eudrilus eugeniae after experimental week of exposure

Before GBH exposure, there was no significant difference ($p > 0.05$) in the activities of GST, MT, AChE and LDH recorded in *E. eugeniae* between the exposed and unexposed groups (Fig. 3). In addition, AChE activity was not significantly different ($p > 0.05$) between the exposed and unexposed groups from week 1 to 8th weeks after GBH exposure. Meanwhile, GST and LDH activities were significantly higher ($p < 0.05$) in the exposed group after GBH exposure compared to the unexposed. MT activity was significantly lower ($p < 0.05$) in the exposed group after GBH exposure at all the experimental weeks compared to the unexposed.

3.4. Level of antioxidant defense parameters in Alma millsoni after experimental week of exposure

The levels of antioxidant defense parameters (CAT, SOD, GSH, GPx) and lipid peroxidation (MDA) recorded in *A. millsonii* were not significantly different ($p > 0.05$) between the exposed and unexposed

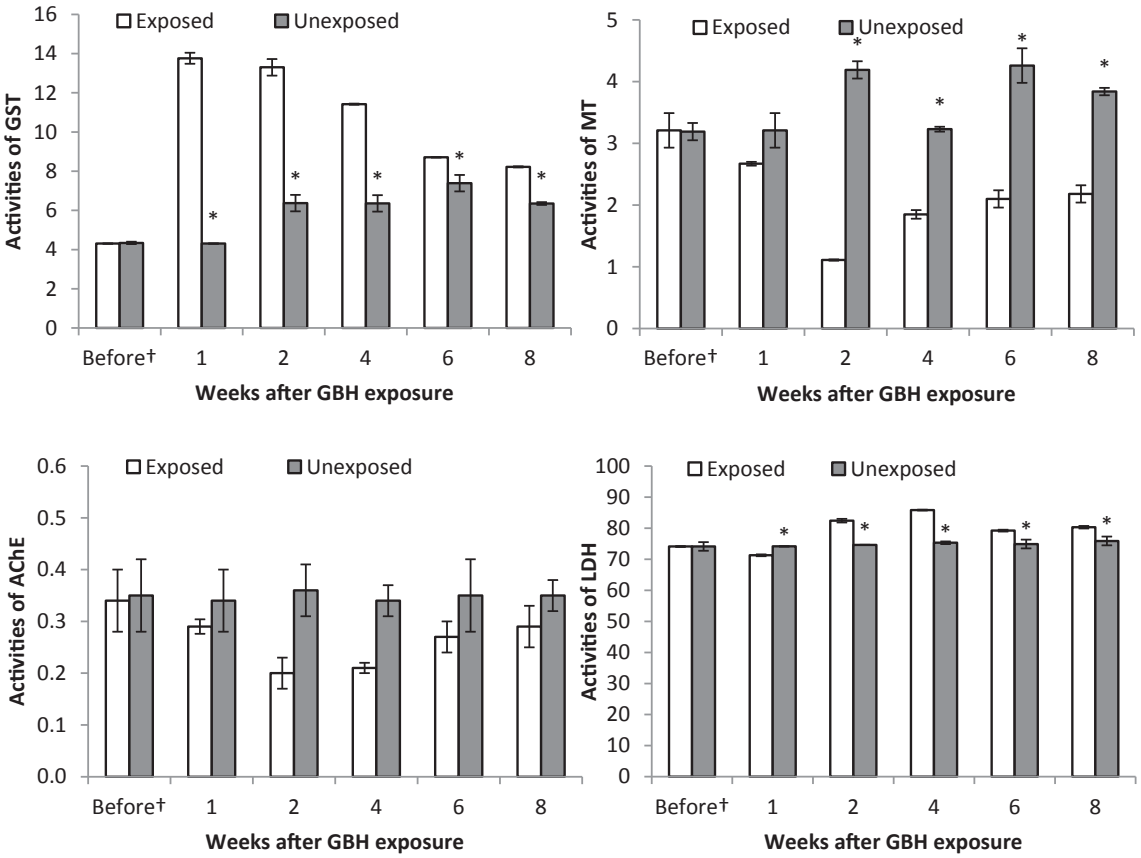

Fig. 2. Enzyme activity in *Libyodrilus violaceus* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit = GST-nmol DNPG/mg protein/min.; MT-mmol/mg protein/min.; AChE-nmol/mmg protein/min.; LDH-nmol/mg protein/min.

groups before GBH exposure (Fig. 4). Also, concentration of GSH was not significantly different ($p > 0.05$) between the exposed and unexposed groups throughout the experimental weeks. CAT activity was not significantly different ($p > 0.05$) between the exposed group after 1st week and 2nd weeks of GBH exposure compared to the unexposed. However, CAT activity became significantly higher ($p < 0.05$) in the exposed group after 4th, 6th and 8th weeks of GBH exposure. Activities of SOD and GPx were significantly lower ($p < 0.05$) in the GBH exposed groups throughout the experimental weeks compared to the unexposed. On the other hand, MDA was only significantly higher ($p < 0.05$) in the exposed group after 2nd weeks of GBH exposure but not significantly different ($p > 0.05$) between the exposed and unexposed groups during the other experimental weeks.

3.5. Level of antioxidant defense parameters in Libyodrilus violaceus after experimental week of exposure

The levels of the antioxidant enzymes and MDA recorded in *L. violaceus* were not significantly different ($p > 0.05$) between the exposed and unexposed groups before GBH exposure (Fig. 5). Also, activity of SOD and level of MDA were not significantly different ($p > 0.05$) between the exposed and unexposed groups throughout the experimental weeks. CAT and SOD activities were significantly higher ($p < 0.05$) in the exposed group compared to the unexposed throughout the experimental weeks. At 1st week and 2nd weeks after GBH exposure, GPx activity was observed to be significantly higher ($p < 0.05$) in the exposed group compared to the unexposed. This was however not significantly different ($p > 0.05$) between the exposed and unexposed groups after the 4th, 6th and 8th weeks of GBH exposure.

3.6. Level of antioxidant defense parameters in Eudrilus eugeniae after experimental week of exposure

Levels of the antioxidant enzymes and MDA recorded in *E. eugeniae* were not significantly different ($p > 0.05$) between the exposed and unexposed groups before GBH exposure (Fig. 6). Level of MDA was also not significantly different ($p > 0.05$) between the exposed and unexposed groups during the experimental weeks. However, activities of CAT and SOD were observed to be significantly higher ($p < 0.05$) in the exposed groups after the 1st 2nd 4th 6th and 8th weeks of GBH exposure compared to the unexposed. After 1st week of GBH exposure, there was no significant difference ($p > 0.05$) in the concentration of GSH between the exposed and unexposed groups but GSH concentration was significantly lower ($p < 0.05$) in the exposed group across the other experimental weeks compared to the unexposed. Also, activity of GPx was observed to be significantly higher ($p < 0.05$) in the exposed group after 1st and 2nd weeks of GBH exposure. GPx activity was however not significantly different ($p > 0.05$) between the exposed group after the 4th, 6th and 8th weeks of GBH exposure compared to unexposed group.

3.7. Concentration of glyphosate residue in soil and earthworm species after experimental week of exposure

The concentrations of glyphosate residue in the experimental soils and all the earthworm species are represented in Fig. 7. The concentration of glyphosate residue in the soils was observed to reduce significantly ($p < 0.05$) from the 1st week after glyphosate exposure to the 8th weeks. However, the rate of reduction in glyphosate residue was significantly higher ($p < 0.05$) between the 1st and 2nd weeks after

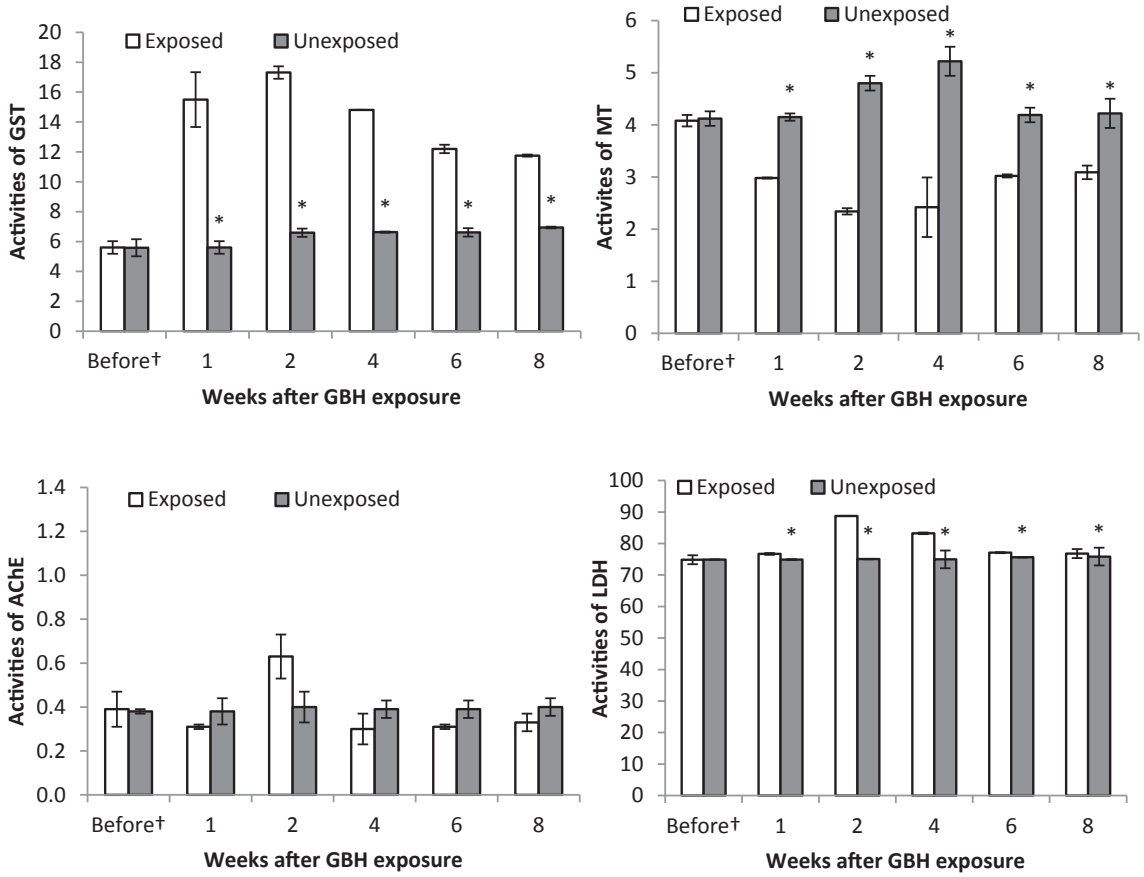

Fig. 3. Enzyme activity in *Eudrilus eugeniae* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit = GST-nmol DNPG/mg protein/min.; MT-mmol/mg protein/min.; AChE-nmol/mmg protein/min.; LDH-nmol/mg protein/min.

exposure in the soil containing *A. millsoni* than those of *E. eugeniae* and *L. violaceus*. However, the concentration of glyphosate residue in the soil without earthworm species was observed to remain higher and significant ($p < 0.05$) after 1st 2nd and 4th weeks of GBH application with reductions only after the 6th and 8th weeks (Fig. 7D). On the other hand, all the three earthworm species had the lowest level of glyphosate residue at the 1st week after glyphosate exposure. Glyphosate residue was observed to be highest ($p < 0.05$) at the 2nd week after exposure in *A. millsoni* and *L. violaceus*. In *E. eugeniae* however, glyphosate residue was highest ($p < 0.05$) at the 4th week after exposure.

3.8. Bioaccumulation factor of glyphosate in earthworm species after experimental week of exposure

The BAF of glyphosate in *A. millsoni* was less than 1 throughout the experimental weeks (Table 1). Also, BAF of glyphosate in *E. eugeniae* and *L. violaceus* were less than 1 from week 1 to week 6 after exposure. However, the BAF was greater than 1 in these earthworms (*E. eugeniae* and *L. violaceus*) at the 8th week after exposure. Linear relationship between the days of GBH exposure and glyphosate BAF in *E. eugeniae* ($R2 = 0.790$) and *L. violaceus* ($R2 = 0.941$) was very strong and significant ($p < 0.05$) (Fig. 8). This was weak and not significant in *A. millsoni* ($R2 = 0.342$, $p > 0.05$).

3.9. Relationship between glypgosate residues in earthworm tissue and its biochemical parameters measured.

A significantly strong positive correlation ($r = 0.719$, $p < 0.05$) was recorded between the residues of glyphosate in *A. millsoni* and its body level of MDA. The residues of glyphosate in *L. violaceus* showed significantly strong positive relationships with the body levels of GST, catalase, SOD and GPx. In a similar trend, the residues of glyphosate in *E. eugeniae* recorded significantly positive correlation with the body levels of GST, LDH, SOD and GPx (See Table 2).

4. Discussion

The assessment of biomarkers in organism is a reliable tool in environmental pollution studies. It has become a major component of, and alternative to environmental monitoring worldwide (Salvio et al., 2016). In this study, we observed that activities of GST and LDH increased in the three earthworm species exposed to GBH relative to the unexposed. GST is a cytosolic enzyme which performs a crucial role in the detoxification, biotransformation and neutralization of several pesticides and endogenous metabolic by-products in the body (Hayes et al., 2005; Tiwari et al., 2016), and LDH, being a major glycolytic enzyme present in earthworms tissue (Shekari et al., 2008), is an enzymatic indicator of stress exposure which provide energy to the organism within a short period of time (Tripathi et al., 2011). Therefore, the increase in the activities of GST and LDH in the earthworm species exposed to GBH as recorded in this present study could be linked to the physiological adjustment of the body to cope with likely oxidative stress and some other biochemical adjustments imposed by the herbicide. However, it is also very important to note that the increased in the activities of GST and LDH in the exposed earthworm species was highest at the 1st and 2nd weeks of exposure and later decreased. These biphasic activities of GST and LDH in the earthworm species is similar to a process referred to as hormesis, a term often used to describe a biphasic dose-response of cells or organisms to a toxic agent. This hermetic response in organisms has been reported to induce an

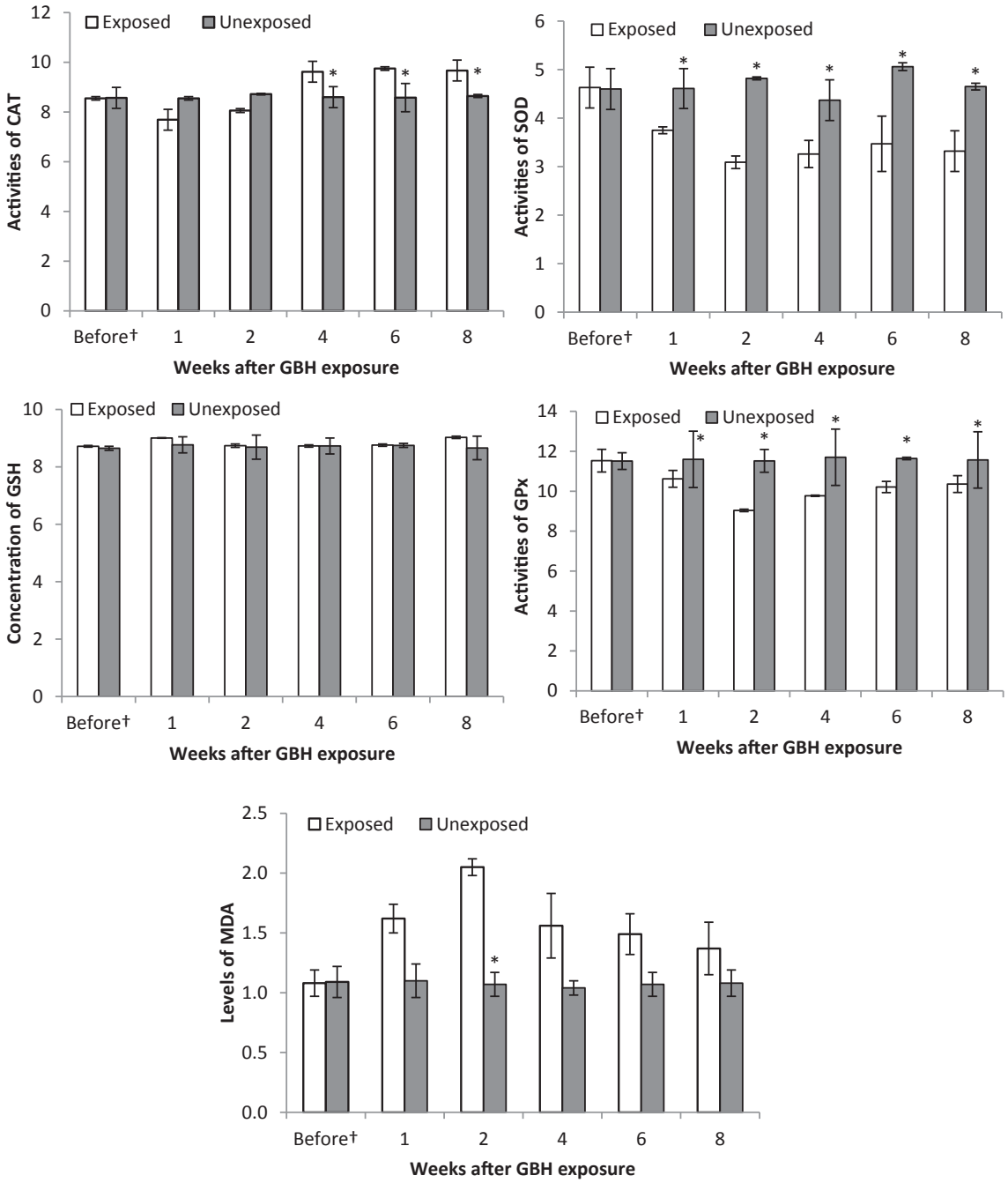

Fig. 4. Oxidative stress markers in *Alma millsoni* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit: CAT-U/mg protein; SOD-U/mg protein; GSH-U/g tissue; GPx-U/mg protein; MDA-nmol/mg protein.

adaptive beneficial effect against a toxic agent at higher dose (Calabrese and Blain, 2005; Calabrese, 2005). Therefore, the observed biphasic activities of GST and LDH in the exposed earthworm species could be an adaptive stress response or mechanisms against the toxicity of GBH. Rodríguez-Seijo et al. (2018) earlier recorded a similar increase in LDH and GST activities in earthworms exposed to high level of low-density polyethylene microplastics and associated this to cellular damages through lipid peroxidation, energy consumption and imposed oxidative stress. On the other hand, a lower activity of MT in the exposed earthworm species may suggest ability of the earthworm species to adapt and survive in the soil containing this herbicide with lower oxidative stress. MTs are important metal binding proteins in the cells of organisms (Amiard et al., 2006) and studies have measured the activity of MT in earthworm as a potentially useful biomarker of metal pollution in soil (Ma et al., 2008; Wang et al., 2013; Dedeke et al., 2016). To the best of our knowledge, there is paucity of available information on the use of MT activity as a biomarker of GBH ecotoxicity assessment. Therefore, we recommend further studies exploring the potential of this enzyme.

Although organophosphate pesticides have been reported to inhibit the activity of AChE in animals by covalently phosphorylating the serine residue within its active site (Tiwari et al., 2016), but our study has shown that exposure to GBH has no significant effect on the activity of AChE in all the exposed earthworm species on a temporal basis. AChE is an enzyme of great importance in neurotransmission and muscular activities. Koelle (2013) described AChE as an enzyme which plays an important role in the breaking down of the neurotransmitter acetylcholine, converting it to choline and acetic acid. This study

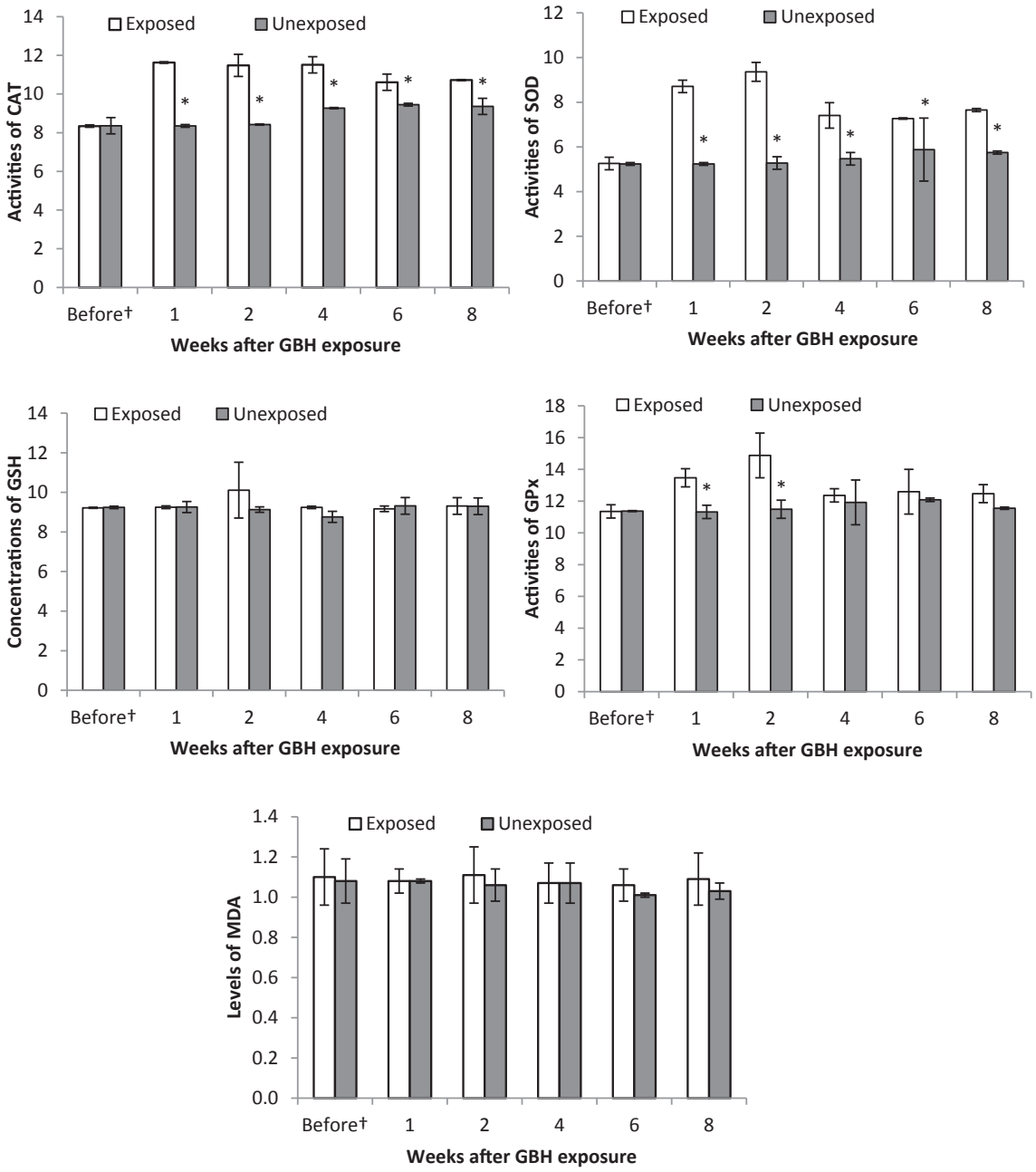

Fig. 5. Oxidative stress markers in *Libyodrilus violaceus* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit: CAT-U/mg protein; SOD-U/mg protein; GSH-U/g tissue; GPx-U/mg protein; MDA-nmol/mg protein.

therefore suggests that exposure of the earthworms to GBH in the soil does not significantly affect the neuronal and muscular activities in the earthworms' tissue. In addition, the mechanism underlying the biocidal effects of organophosphorus pesticide in animals is known to be through inhibition of AChE enzymes which result in neurotransmission abnormalities and subsequent paralysis and death of the animal (Lynn and Katheryn, 2001). Therefore, the ability of the earthworm species to cope and survive the potential toxic effect of GBH could be attributed to non effects of the chemical on the activity of the earthworms' AChE enzyme. This finding contradict Denoyelle et al. (2007) who reported a significant inhibition of AChE activity in earthworms species *Allolobophora chlorotica* exposed to organophosphorus and carbamate pesticides. The variations in AChE inhibition could be due to factors, such as the nature, quantities and the period of the sprayed pesticides (Rault et al. 2007), the earthworm species (Rault et al., 2008) and the soil properties (Floch et al. 2009). Our finding however agrees with Schreck et al. (2012) who observed no effect of a pesticide on the AChE activity in the exposed *Aporrectodea nocturna*.

Feng et al. (2015) noted that pollution monitoring in soil environments involves the measurements of induced biomarkers responses in earthworms as well as alterations in enzymatic antioxidants (such as catalase, GPx, SOD) and non-enzymatic antioxidant (GSH) which are used as index of unbalanced reactive oxygen species (ROS) generations and oxidative stress in animal physiological systems. We have been able to prove that an imbalance between free radicals production and antioxidant defences in rats exposed to a GBH and gasoline fume resulted to oxidative stress and damage (Owagboriaye et al., 2017; Dedeke et al., 2018; Owagboriaye et al., 2018). However, antioxidant defense system is known to protect animal cells from oxidative damage (Pastore et al., 2003) by catalyzing the decomposition of hydrogen peroxide to water

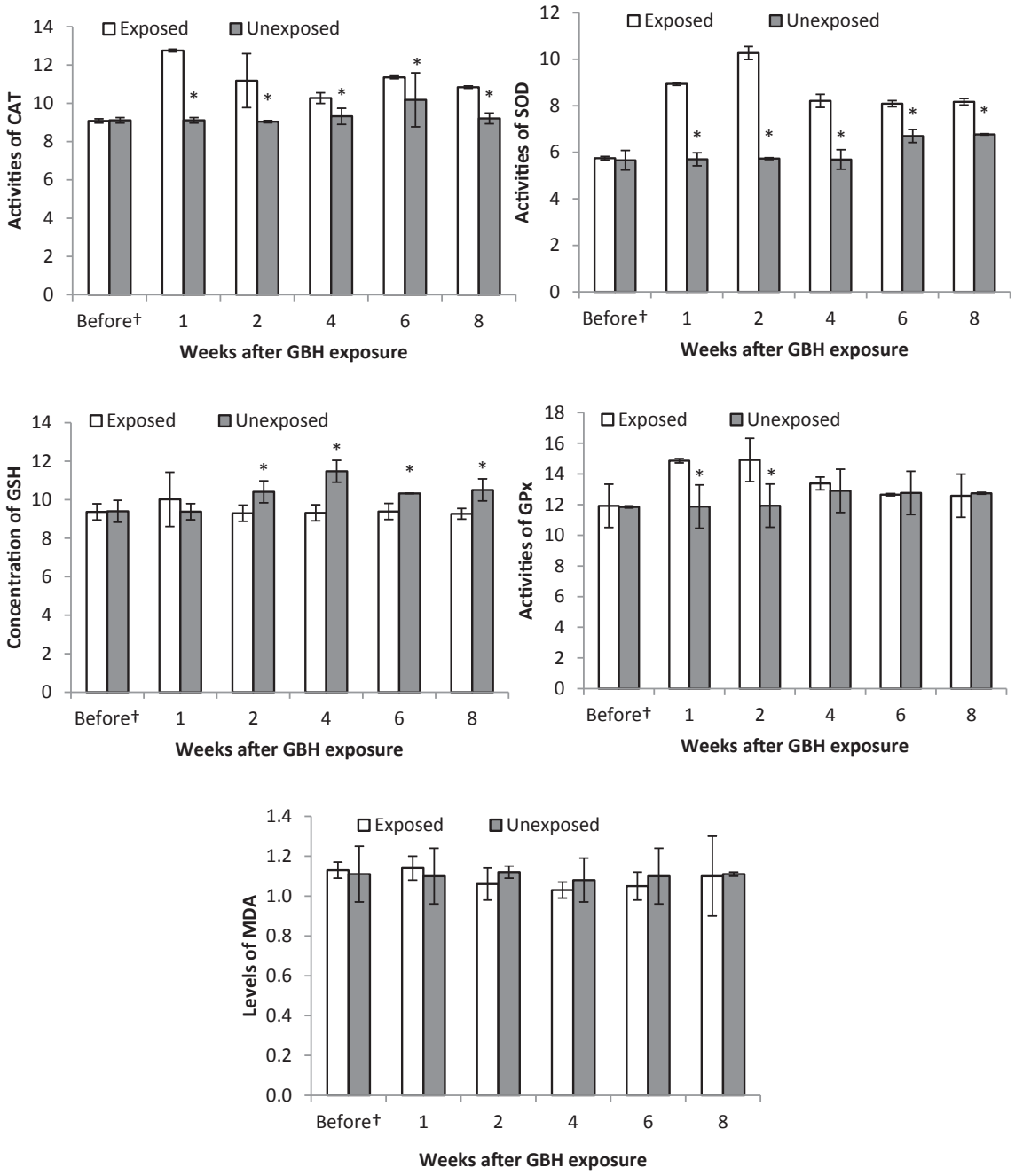

Fig. 6. Oxidative stress markers in *Eudrilus eugeniae* exposed to glyphosate-based herbicide; *Mean significantly different between the exposed and unexposed group (T-test; $p < 0.05$); Before† = 3 weeks before GBH exposure; Unit: CAT-U/mg protein; SOD-U/mg protein; GSH-U/g tissue; GPx-U/mg protein; MDA-nmol/mg protein.

and oxygen (Chelikani et al., 2004). This study shows that antioxidant defences in the tissue of earthworms were strong enough to protect the cells of the earthworm species exposed to GBH against oxidative stress and damage. This is evidenced in the higher activities of CAT, GPx and SOD and the insignificant level of MDA in the tissue of the exposed earthworm species compared to control. According to Odewabi et al. (2014), lipid peroxidation results from the release of free radicals which can cause tissue damage by reacting with polyunsaturated fatty acids in cellular membranes to form MDA whose level is used to show the degree of oxidative stress in animals (Liu et al., 2010). Liu et al. (2010) associated the increase in MDA level of *E. fetida* to high level of oxidative stress. In addition, oxidative stress and change in the intracellular ion homeostasis have been associated with increased activity of AChE (Mrdaković et al., 2016; Hackenberger et al., 2018) and since GBH has no effect on the AChE activity of the exposed earthworm species in this study, hence, may not induce oxidative stress.

Although oxidative stress was observed in *A. milsoni* only after the 2nd weeks of GBH exposure probably due to the reductions in the activities of SOD and GPx in its tissue, but a higher activity of CAT and concentration of GSH maintained throughout the experimental weeks may explain the absence of oxidative stress after the 4th, 6th and 8th weeks of GBH exposure. Meanwhile, it is of interest to note that concentration of GSH, which plays a crucial role in xenobiotic detoxification, removal of free radicals and hydroperoxides (Hermes-Lima, 2004), was maintained in the tissue of the earthworm species throughout the experimental weeks despite the fact that it's being consumed in metabolic reactions involving GST and GPx. Considering the higher activities of these enzymes, one would expect a reduction in the concentration of

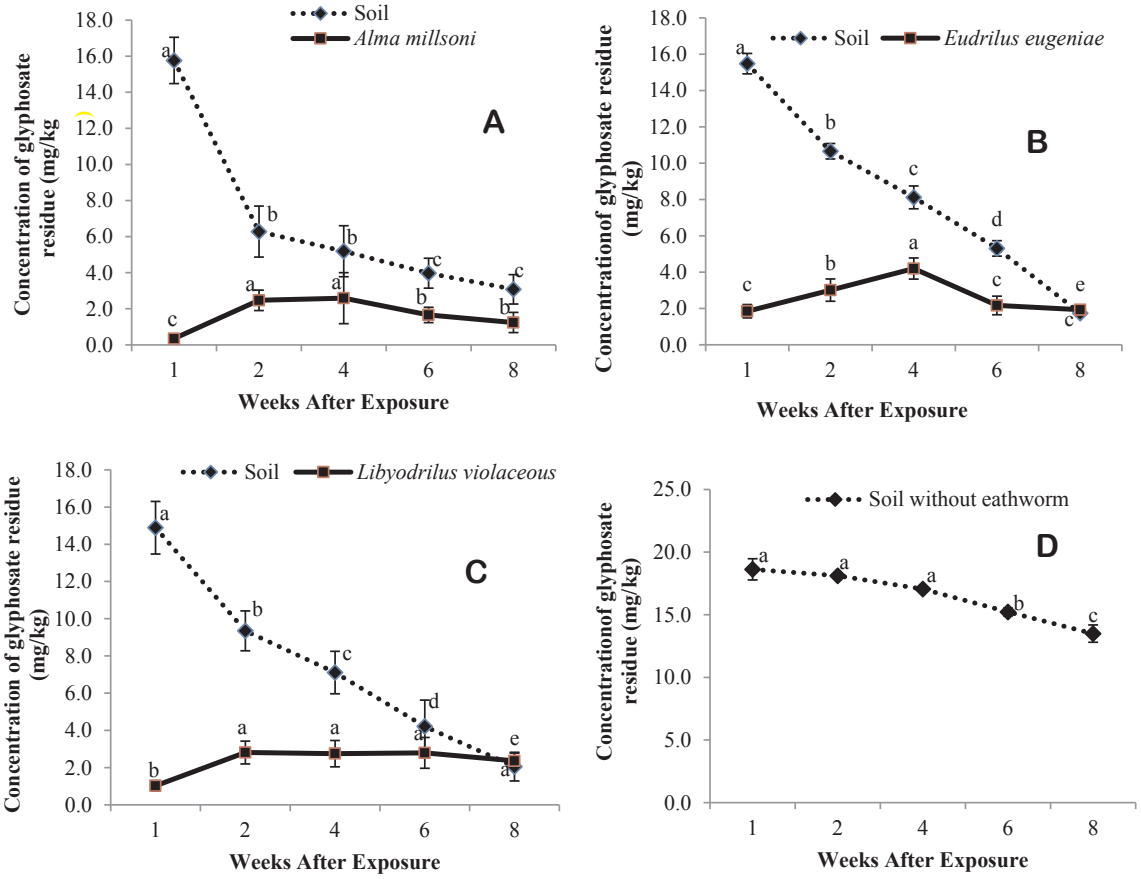

Fig. 7. Glyphosate residue in the soil and earthworm species after GBH application (A) Soil and *Alma millsoni* introduced; (B) Soil and *Eudrilus eugeniae* introduced; (C) Soil and *Libyodrilus violaceus* introduced; (D) Soil with no earthworm species.

Table 1
Bioaccumulation factor of glyphosate in the experimental earthworm species.

	Alma millsoni	*Eudrilus eugeniae*	*Libyodrilus violaceus*
1 wk AE	0.02	0.12	0.07
2 wks AE	0.39	0.28	0.30
4 wks AE	0.50	0.52	0.39
6 wks AE	0.42	0.41	0.66
8 wks AE	0.40	1.11	1.15

wk(s) AE = week(s) after exposure.

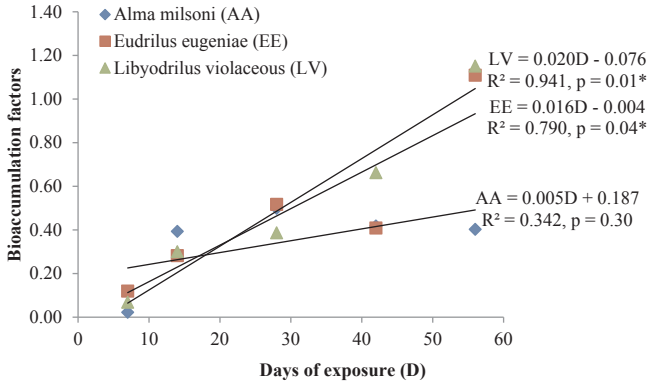

Fig. 8. Linear relationship between glyphosate-based herbicide bioaccumulation factors of the earthworm species and the days of exposure; *Relationship significant at $p < 0.05$.

Table 2
Relationship between glyphosate residues in earthworm tissue and its biochemical parameters.

Parameter	*Alma millsoni*	*Libyodrilus violaceus*	*Eudrilus eugeniae*
GST	0.338	0.789**	0.733*
MT	−0.544	−0.549	−0.486
ChE	−0.095	−0.087	0.510
LDH	0.481	0.344	0.671*
CAT	−0.338	0.840**	0.434
SOD	−0.504	0.761*	0.762*
GSH	0.532	0.217	0.429
GPx	−0.547	0.648*	0.740*
MDA	0.719*	0.180	0.175

Values represent the Pearson correlation (r – value); *Correlation significant at $p < 0.05$; **Correlation significant at $p < 0.01$.

GSH. We suspect a possible reduction in oxidized glutathione (GSSG), a product of GPx and GST catalytic activities, by glutathione reductase (GR), therefore maintaining the concentration of GSH in the earthworm's tissue (Tiwari et al., 2016).

The result of this study also showed higher bioaccumulation of glyphosate in *L. violaceus* and *E. eugeniae* than *A. millsoni*. Hobbelen et al. (2006) noted a variation in the accumulation of pollutants such as metals in earthworms due to individual differences in species and habits. *E. eugeniae* commonly referred to as 'African night crawler' is gregarious by nature with higher rate of activity in the soil. *L. violaceus* is a limicolous species that rarely migrates from its habitat or burrow except during feeding and cast deposit at the soil surface. Although the mechanism responsible for higher bioaccumulation of the glyphosate in *E. eugeniae* and *L. violaceus* is not clear, it may however be related to their higher activity rate in the soil. Furthermore, Swati and Vikram

(2011) had earlier suggested that higher accumulation of trace elements in the tissue of *E. eugeniae* and *Eisenia fetida* was as a result of chemical and microbial changes that ingested substrates undergo in the gut of the earthworm. It was noted that a large proportion of the organic fraction including the chelated metal fractions, is converted into soluble forms that are more available to organisms hence, their accumulation in the tissues of the earthworms. In a similar trend, report has shown that gut secretions may enhance the uptake of chemicals containing surfactant from the gut contents of earthworms. Various surfactants including Triton X-100 and Tween-80 have been reported to significantly increase the concentration of dichlorodiphenyldichloroethylene (DDE) taken up and bioaccumulated in the tissues of *E. fetida* and *L. terrestris* (White et al., 2007). Since a GBH is known to contain surfactants required for easy penetration of the herbicide into plants, we can also suggest a similar mechanism for the glyphosate accumulation in this present study.

In a similar trend, the BAF of glyphosate in *A. millsoni* was less than one throughout the experimental weeks while the BAFs of glyphosate in *E. eugeniae* and *L. violaceus* were greater than one. Lukkari et al. (2009) have reported that a difference in BAF for a heavy metal in earthworms could be related to their differences in specific metabolism and regulating mechanisms for the metal. Thus, we can reasonably suggest a similar principle in the earthworm species for glyphosate in this study. This may explain the accelerated removal of glyphosate residue from the soil containing these earthworm species. Meanwhile, level of glyphosate in the soil without the earthworm species was observed to remain higher with no significant reduction. Hence, *E. eugeniae* and *L. violaceus* may be a better bio-indicator and vermiremediator of glyphosate in soil, being able to rapidly remove the herbicide from the soil as well as bio-magnify the herbicide in their tissues. However, this activity may pose a danger of potential transfer of glyphosate into the food chain in future. Schreck et al. (2008) documented the potential of earthworm *Aporrectodea caliginosa* on the increased removal of herbicides such as gamma-Cyalothrin, Chlorpyrifos-ethyl and Folpet. In addition, Kersanté et al. (2006) demonstrated accelerated removal of atrazine from contaminated soil containing the earthworms' *A. caliginosa* and *Lumbricus terrestris*. However, Gevao et al. (2001) observed a slow removal rate of atrazine, isoproturon and dicamba from contaminated soils containing the earthworm *A. Longa*.

5. Conclusions

This study has demonstrated alterations in the enzymatic activities and antioxidant defense system in earthworm species (*A. millsoni, E. eugeniae* and *L. violaceus*) as a result of a GBH exposure. These alterations could be used to monitor the health of soil environment contaminated with GBH. The relationship between glyphosate residues in the three earthworm species and their biochemical parameters shows that GST (in *E. eugeniae* and *L. violaceus*) and LDH (specific for *E. eugeniae*) activities could be the most sensitive markers of ecotoxicological assessment while the activities of AChE and MT appear to be less sensitive. The relationship further showed that exposure of *E. eugeniae* and *L. violaceus* to GBH evoked GSH-dependent or antioxidant defence mechanisms as a compensatory response possibly to checkmate excessive production of free radicals. However, this molecular strategy was sufficed for prevention of oxidative stress and its consequent damage in the tissue of the earthworm species. This also suggests in this study that *E. eugeniae* could be the most sensitive and sentinel earthworm species for ecotoxicological risk assessment of GBH exposure, followed by *L. violaceus*, while *A. millsoni* appears to be the least.

This study also showed that all the earthworm species, at different points in time bioaccumulated certain amount of glyphosate in their tissues despite the hydrophilic nature of the herbicide, but *E. eugeniae* and *L. violaceus* was able to biomagnify the herbicide over a period of time. However, the increased rate of glyphosate removal from soil containing *E. eugeniae* and *L. violaceus* and the strong and significant relationship that exist between glyphosate BAF and exposure duration in the earthworm species suggested that both earthworm species may be used to vermiremediate soil contaminated with a GBH.

In this study, we have highlighted the importance of using *A. millsoni, E eugeniae* and *L. violaceus* as well as their biotransformation enzyme markers and antioxidant defense system in ecotoxicological risk assessments of soil contaminated with GBH. Our findings could advance knowledge in the area of ecological risk monitoring and indicator of soil contaminated with GBH. We recommend further studies on the kinetics and mechanisms underlying glyphosate accumulations and biomagnifications in these experimented earthworm species as well as possible ways by which the mechanisms, if known, could be employed in remediating glyphosate from the contaminated soil environment. Furthermore, there is need to measure the metabolites of glyphosate in the soil as well as changes in the activity of the earthworms gut microbiome in future studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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