

RESEARCH REPORT

Dermal, histological anomalies with variations in enzyme activities of the earthworms *Lampito mauritii* and *Drawida willsi* after short term exposure to organophosphate pesticides**S Samal^{1*}, C.S.K Mishra¹, S Sahoo²**¹Department of Zoology, College of Basic Science and Humanities, Odisha University of Agriculture and Technology, Bhubaneswar-751003, India²School of Life Sciences, Sambalpur University, Jyoti Vihar, Burla-768019, India

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Abstract

Monocrotophos and glyphosate are two potent organophosphate pesticides used on agricultural farms in India to control insect pests and weeds, respectively. Consistent application of these chemicals poses a risk of residual soil contamination with possible adverse implications on non-target organisms, like earthworms. The present study evaluates the impacts of these pesticides on the skin, muscles and certain biochemical parameters such as protein content, lipid peroxidation (LPX) level, activities of lactate dehydrogenase (LDH), acetylcholinesterase (AChE) and catalase (CAT) of two tropical earthworms *Drawida willsi* and *Lampito mauritii*. Monocrotophos at 1.0, 2.0, 3.0 g/kg soil and glyphosate at 0.1, 0.15, 0.2 g/kg soil were used for the experiment. At high concentrations, both pesticides induced lesions and skin undulation in the earthworms. In *L. mauritii*, the postclitellar region indicated muscle disorganization with high concentrations of monocrotophos. The lowest protein level was recorded in *D.willsi* and *L. mauritii* with high concentrations of monocrotophos. *L. mauritii* exhibited maximum LPX at high concentrations of glyphosate. Both the earthworms indicated the least LDH activity with high pesticide concentration. Minimal AChE activity in *L. mauritii* was observed with a high concentration of glyphosate. A high concentration of monocrotophos inhibited CAT activity in *L. mauritii*. The variable response of the selected morpho-histological and biochemical parameters in the earthworms to different pesticide concentrations could be useful early warning biomarkers to evaluate soil residual toxicity.

Key Words: earthworm; glyphosate; monocrotophos; soil contamination; biomarker

Introduction

Pesticide application has consistently increased over the years on a global scale to control pest and weed populations in agricultural fields. Residual chemical toxicity from pesticides on several non-target organisms have previously been reported (Santos *et al.*, 2010; Walker *et al.*, 2010). Earthworms are abundantly found in agro-ecosystems and perform vital ecological functions such as facilitating the decomposition of organics and the mineralization of nutrients (Bartlett *et al.*, 2010; Tiwari *et al.*, 2016). Earthworms are extremely sensitive to environmental changes, can uptake toxic substances through their permeable

skin and those substances can accumulate in the body; therefore, earthworms are used as important indicators of ecosystem perturbations (Hartenstein *et al.*, 1981; Kavitha *et al.*, 2008; Samal *et al.*, 2017; Nayak *et al.*, 2018; Mishra *et al.*, 2019; Samal *et al.*, 2019a; Singh *et al.*, 2019; Mishra *et al.*, 2020). However, an increase in soil pesticide concentration is likely to deleteriously impact these animals (Gobi *et al.*, 2004; Garcia *et al.*, 2011; Santos *et al.*, 2011; Mohan and Sajayan, 2015; Nayak *et al.*, 2018; Samal *et al.*, 2019a).

Monocrotophos and glyphosate are potent organophosphorus pesticides used in India's agricultural fields to control insect pests and weeds, respectively. The recommended agricultural doses (RAD) of monocrotophos and glyphosate in India are 2.0 g/kg soil and 0.15 g/kg soil, respectively (Agronika, 2005). Due to manual pesticide application practised in India, there is always a high risk of overdose beyond the recommended dose, which enhances the risk of residual soil toxicity.

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Bioaccumulation of pesticides in earthworms could have a deleterious impact on these animals (Hackenberger *et al.*, 2008; Van Gestel *et al.*, 2011). Several studies have substantiated the adverse effects of pesticides on earthworms. Alterations in ureotelic and ammoniotelic activity were noticed in *Lamipito mauritii* exposed to monocrotophos (Patnaik and Dash, 1991). A minimal concentration of monocrotophos is toxic enough to induce dermal and histological alterations in earthworm (Abbiramy *et al.*, 2018). Reports are also available on ways herbicides could adversely impact the growth and reproduction of earthworms (Helling *et al.*, 2000; Zhou *et al.*, 2007; Correia and Moreira, 2010; Chen *et al.*, 2018; Niemar *et al.*, 2018). The negative impact of glyphosate on earthworms, *Octodrilus complanatus*, *Lumbricus terrestris* and *Aporrectodea caliginosa* in vineyards in Italy's northeast region has been reported (Stellin *et al.*, 2018). Significant histopathological and enzymatic changes in the earthworms *Glyphidrilus tuberosus* and *Eudrilus eugeniae* due to high concentration of phosphogypsum and pesticides in soil, respectively, have also been reported (Nayak *et al.*, 2018, Samal *et al.*, 2017, 2019a,b).

Drawida willsi (Epigeic) and *L. mauritii* (Anecic) are abundantly found in India's crop fields and are likely to be exposed to variable concentrations of pesticides in soil. Information about the impact of organophosphates on these earthworms are not adequate. Therefore, this study was undertaken to observe the possible morphological, histopathological changes and alterations in certain biochemical parameters in these earthworms with 24h exposure to three different concentrations of monocrotophos and glyphosate in soil under laboratory conditions.

Materials and methods

Experimental setup

Monocrotophos (Monokem) was obtained from Sumimoto Chemical (India) Pvt. Ltd and glyphosate (Shriram Dart Plus 71) from Shreeji Pesticides Pvt. Ltd. Both the soil and earthworms were selectively sampled from an organic field of the University research farm to ensure that both soil and the test animals were not previously exposed to chemicals. The earthworms were then allowed to acclimatize in laboratory conditions for 15 days in an earthen culture pot with 5 kg field soil. For the present study, RAD of the chemicals was taken as the medium concentration along with one low and one high concentration. Thus, three concentrations of monocrotophos—1.0 g/kg soil, low (T1); 2.0 g/kg soil, medium (T2); 3.0 g/kg soil, high (T3)—and three concentrations of glyphosate—0.1 g/kg soil, low (T1); 0.15 g/kg soil, medium (T2); 0.2 g/kg soil, high (T3)—along with a control (C) were used in triplicate for the study. The chemicals were applied to soil in treatment pots (T1, T2, T3) and mixed thoroughly. Soil moisture was maintained at 50 % in all the pots. Ten clitellated earthworms of approximately equal size (*D. willsi*: 56 ± 3 mm; *L. mauritii*: 152 ± 4 mm) and weight (*D. willsi*: 1.5 ± 0.4 g; *L. mauritii*: 2.09 ± 0.3 g) were transferred into the control and experimental pots for 24h exposure.

Three earthworms from each pot were collected randomly and the experiment was repeated 3 times. The pots were covered with cotton nets to protect the experimental animals.

Dermal and muscular study

The earthworms were sampled randomly after 24h of exposure for morphological and histological studies. The animals were thoroughly washed with distilled water and then transferred to a wet filter paper to remove gut contents and then sacrificed. Sections from postclitellar segment were taken for scanning electron microscopic studies after processing with 2.5 % glutaraldehyde and upgrading (30 %, 50 %, 70 %, 90 %, 100 %) ethanol. Air-dried sections were observed under the scanning electron microscope and photographed.

For histopathological studies, sections of earthworms from different regions (pre clitellar, clitellar, post clitellar) were taken with the help of a sharp-edged stainless steel blade. The sections thus obtained were washed with distilled water and fixed in Bouin's fluid for 24 h. The dehydration of the sections was carried out by upgrading ethanol followed by xylene exposure. Sections were embedded in paraffin wax at 60 °C to prepare wax blocks. Transverse paraffin sections of 5 µm thickness were obtained using a rotary semi-automated microtome (Leica). Deparaffinisation of stretched sections was done using xylene and downgrades of ethanol. The slides were stained in Delafield's haematoxylin and eosin. Stained slides were mounted using DPX and were observed under a binocular microscope at 100X magnification and photographed using a digital camera (Nikon).

Determination of protein, LPX and enzyme activity

Fresh tissue samples were cleaned and weighed quickly to avoid post-mortal tissue degradation. Potassium phosphate buffer (0.05 M, pH 7.4) was used for tissue homogenization. Homogenates were centrifuged for 15 minutes at 10,000 rpm with the help of a cooling tabletop centrifuge. Aliquots of supernatant were collected in 1.5 mL plastic tubes and stored at -20 °C in a deep freezer.

Tissue protein content was measured by Folin–Ciocalteu method as per Lowry *et al.* (1951) at 700 nm. Bovine serum albumin was taken as a known standard to compare the amount of proteins present in the sample. For Lipid peroxidation (LPX) estimation, an aliquot of 100 µL was added to the reaction mixture (900 µL) of 0.8 % of TBA, 20 % of acetic acid, 8.1 % SDS and 2 % BHT. The mixtures were allowed to heat at 95 °C for 45 min before taking a reading at 532 nm. The thiobarbituric acid-reactive substances (TBARS) were measured to determine the concentration of MDA following Ohkawa *et al.* (1979) and expressed in nmol/mg protein. Lactate dehydrogenase (LDH) estimation was done by adding samples in a reaction mixture containing 0.05 M phosphate buffer, 7.5 mM NADH and 30 mM sodium pyruvate, and the assay quantified the amount of enzyme catalyzing the conversion of 1

μmol of NADH per 1 min at 340 nm (Cabaud and Wróblewski, 1958). The Ellmann *et al.* (1961) procedure was followed to measure acetylcholinesterase (AChE) activity at 412 nm, taking 2 mM DTNB and 1 mM Acetylthiocholine iodide. A sample (25 μL) was added to the reaction mixture (1.975 μL) of phosphate buffer and hydrogen peroxide to initiate the catalase assay as per Cohen *et al.* (1970). The enzyme was estimated by observing the decrease in hydrogen peroxide concentration per unit time at 340 nm. The enzymes were expressed in U per mg protein.

Statistical analysis of data for one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) was done with SPSS-20.0 software to determine the significance of variations in

biochemical parameters. The significance level was set at 5%.

Results

Electron microscopic images (Fig. 1) indicated that both the untreated (control) earthworm species have smooth dermal layers. No distinct anomaly was noticed in the worms at low and medium concentrations of the pesticides. However, high concentration of both test chemicals caused dermal undulations in the earthworms. The morphological alterations were seen in each treated species at high concentrations of test chemicals, which was 97.5 % of the total number for *L. mauritii* and 99 % for *D. willsi*.

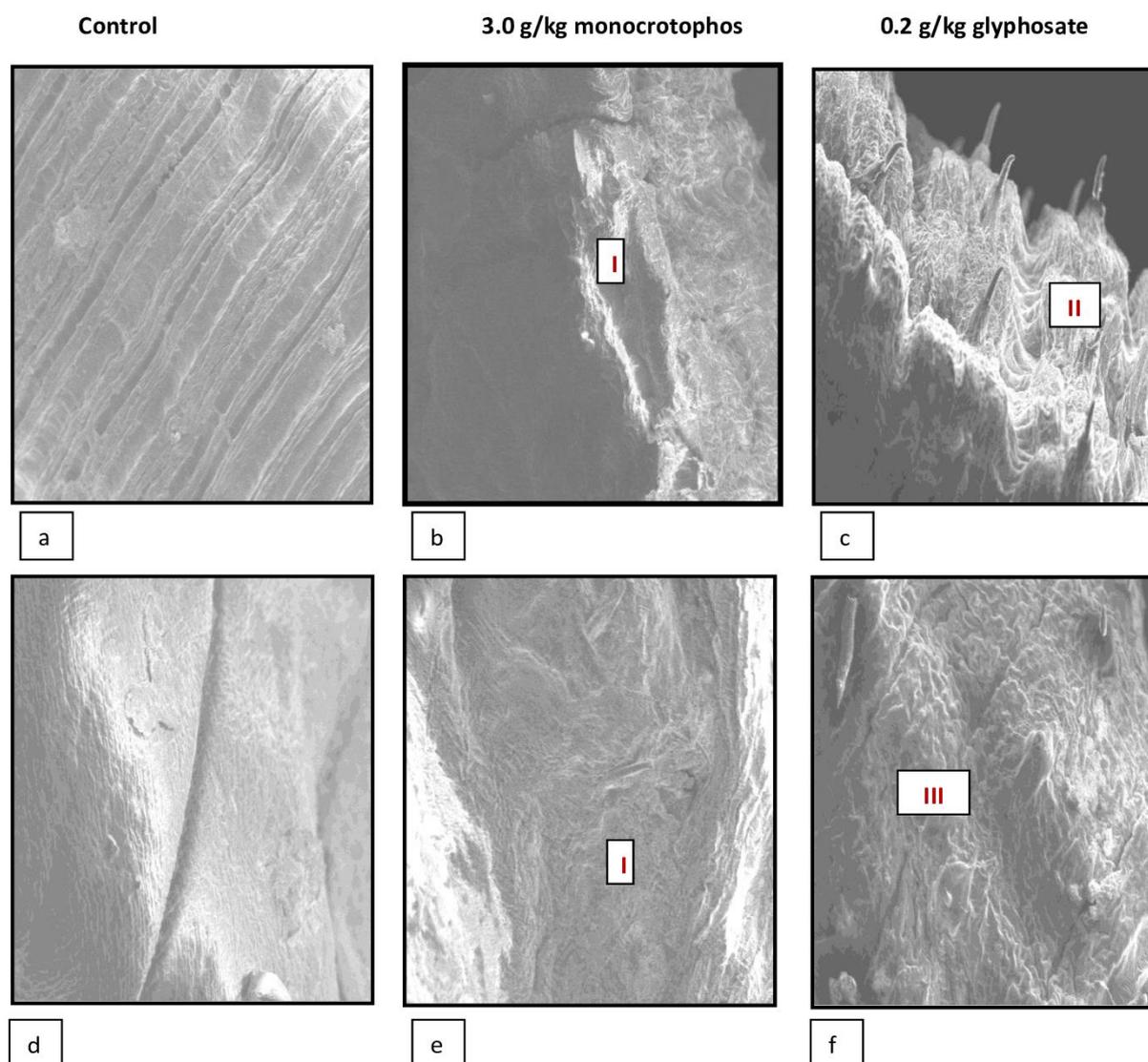


Fig. 1 Scanning electron micrographs (5.00 Kv, 200x magnification) showing dermal anomalies of earthworms treated with monocrotophos and glyphosate. a-c *D. willsi*, d-f *L. mauritii*. I- skin lesion, II- Folding of dermal area, III- roughness on dermal surface

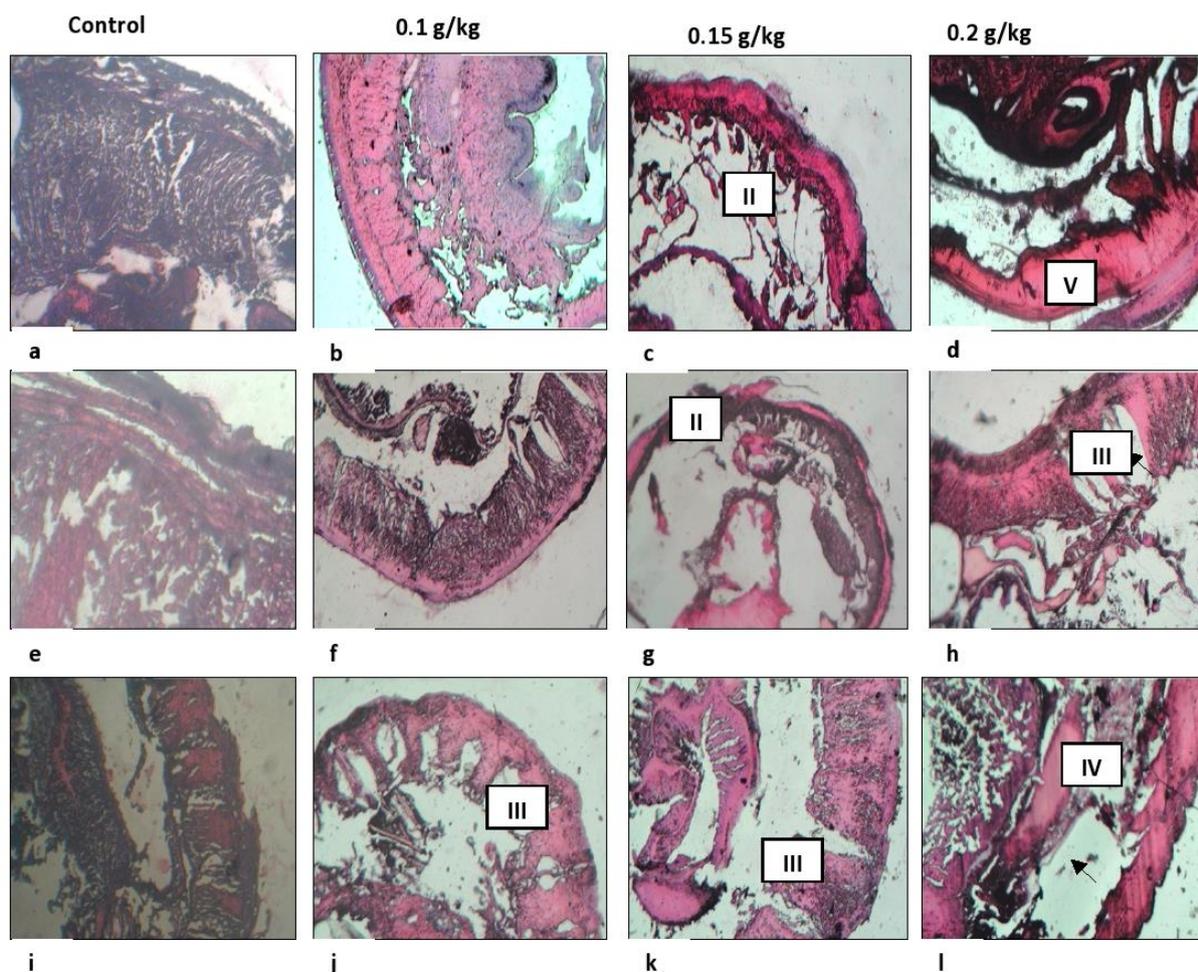


Fig. 3 Photomicrograph showing transverse section (100X) of *D. wilsii* stained with Hematoxylin and Eosin stain after exposure to 24 h of glyphosate treatment from low to high concentration respectively passing through (a-d)- Preclitellar region, (e-h)- Clitellar region (i-l)- postclitellar region. II- disintegrated muscle layer, III- vacuolated muscle, IV- Necrosis, V- merged circular and longitudinal muscle layer

noticed. A transverse section of *L. mauritii* through the postclitellar region exposed to a high concentration of the pesticide indicated damage to the muscles as well as epithelial layers (Fig. 4 i). Minor displacement of circular and longitudinal muscle layers was observed at a low concentration of glyphosate in the preclitellar region of *L. mauritii* (Fig. 5 b). In the postclitellar region, the epithelial layers were intact. However, no visible anomaly in the intestine (Fig. 5 j) was noticed. At a high concentration, epithelial layers were found to be thin and muscle layers were ruptured (Fig. 5 i). A considerable amount of cell debris was also noticed. The clitellar region indicated a damaged epithelial layer and indistinct muscle layer.

Tissue protein levels were found to be at a minimum (80.7 ± 2.71 ; 83.52 ± 6.14 mg/g tissue) in *D. wilsii* and *L. mauritii* respectively, with exposure to high concentrations of monocrotophos. The protein level variation in *D. wilsii* in response to different concentrations of monocrotophos was found to be significant ($p < 0.05$). The highest

protein level (59.31 ± 3.72 mg/g tissue) was found in *D. wilsii* at high concentration of glyphosate. The protein level in *L. mauritii* increased when exposed to low and medium concentrations of glyphosate, but indicated a lower value at high concentration. However, the protein level in earthworms at all treatments were higher than in the control (Fig. 6 a). The protein level variation in *L. mauritii* exposed to different concentrations of glyphosate was found to be significant ($p < 0.05$).

LPX level increased consistently with the low and medium concentrations of pesticides, but indicated minimum values (0.095 ± 0.007 ; 0.192 ± 0.06 nmol/mg protein) at high concentration in *D. wilsii* exposed to monocrotophos and glyphosate, respectively (Fig. 6 b). In *L. mauritii*, the LPX level indicated an increasing trend with low and medium concentrations of monocrotophos, but the difference was not significant. The LPX level was found to be the highest (0.132 ± 0.018 nmol/mg protein) in *L. mauritii* exposed to a high concentration of glyphosate. The LPX level variation in *L. mauritii* was

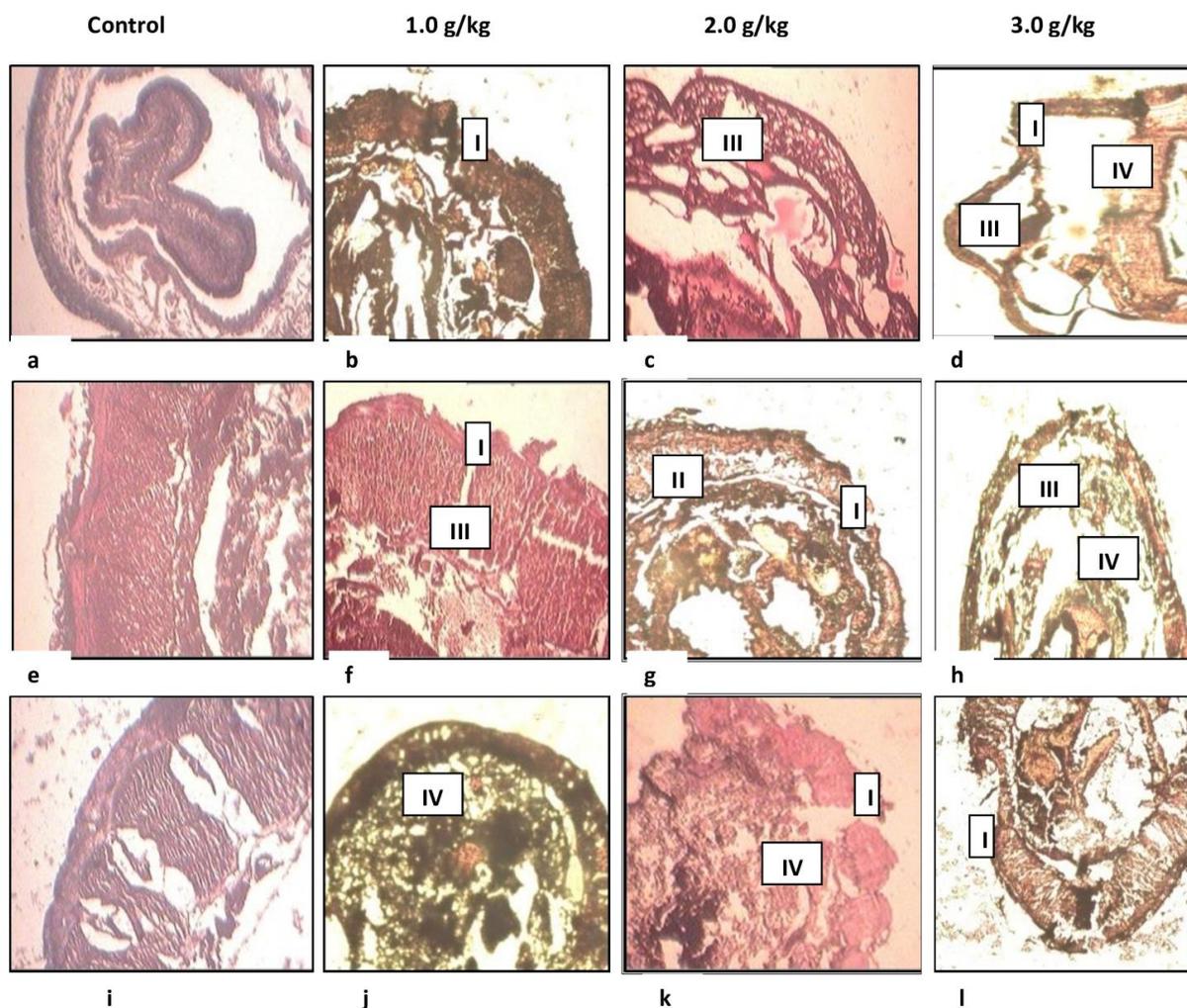


Fig. 4 Photomicrograph showing transverse section (100X) of *L. mauritii* stained with Hematoxylin and Eosin stain after exposure to 24h of monocrotophos treatment from low to high concentration respectively passing through (a-d)- Preclitellar region, (e-h)- Clitellar region (i-l)- postclitellar region. I- ruptured epidermal layer, II- disintegrated muscle layer, III- vacuolated muscle, IV- Necrosis

found to be significant between concentrations in response to glyphosate exposure ($p < 0.05$).

The lowest LDH activities (0.101 ± 0.009 ; 0.24 ± 0.03 U/mg protein) were observed in *D. willsi* exposed to a high concentration of monocrotophos and glyphosate, respectively. The variation of enzyme activity at high concentration of glyphosate was significant ($p < 0.05$). This enzyme activity was observed to be at the minimum (0.015 ± 0.007 ; 0.028 ± 0.005 U/mg protein) in *L. mauritii* in response to a high concentration of monocrotophos and glyphosate, respectively (Fig. 6 c).

AChE activity in *D. willsi* increased with low and medium concentrations of monocrotophos and declined at high concentration, but the activity was at the minimum (0.069 ± 0.006 U/mg protein) at a high concentration of glyphosate. In *L. mauritii*, the activity was the highest (0.103 ± 0.005 U/mg protein) at a high concentration of monocrotophos,

but subsequently decreased with low and medium concentrations of glyphosate (Fig. 6 d). The variations in this enzyme activity in both the earthworms exposed to the pesticides were not significant.

CAT activity in *D. willsi* decreased with a low concentration of monocrotophos but subsequently increased with medium and high concentrations. The minimum activity (0.265 ± 0.04 U/mg protein) of this enzyme was observed at a high concentration of glyphosate. In *L. mauritii*, the minimum (0.125 ± 0.004 U/mg protein) enzyme activity was observed at high concentration of monocrotophos. However, the enzyme activity was at the maximum (0.301 ± 0.06 U/mg protein) at a high concentration of glyphosate (Fig. 6 e). The variation in the enzyme activity in response to monocrotophos was not significant in *D. willsi*. However, significant variation in the enzyme activity was observed in *L. mauritii* at high concentration ($p < 0.05$).

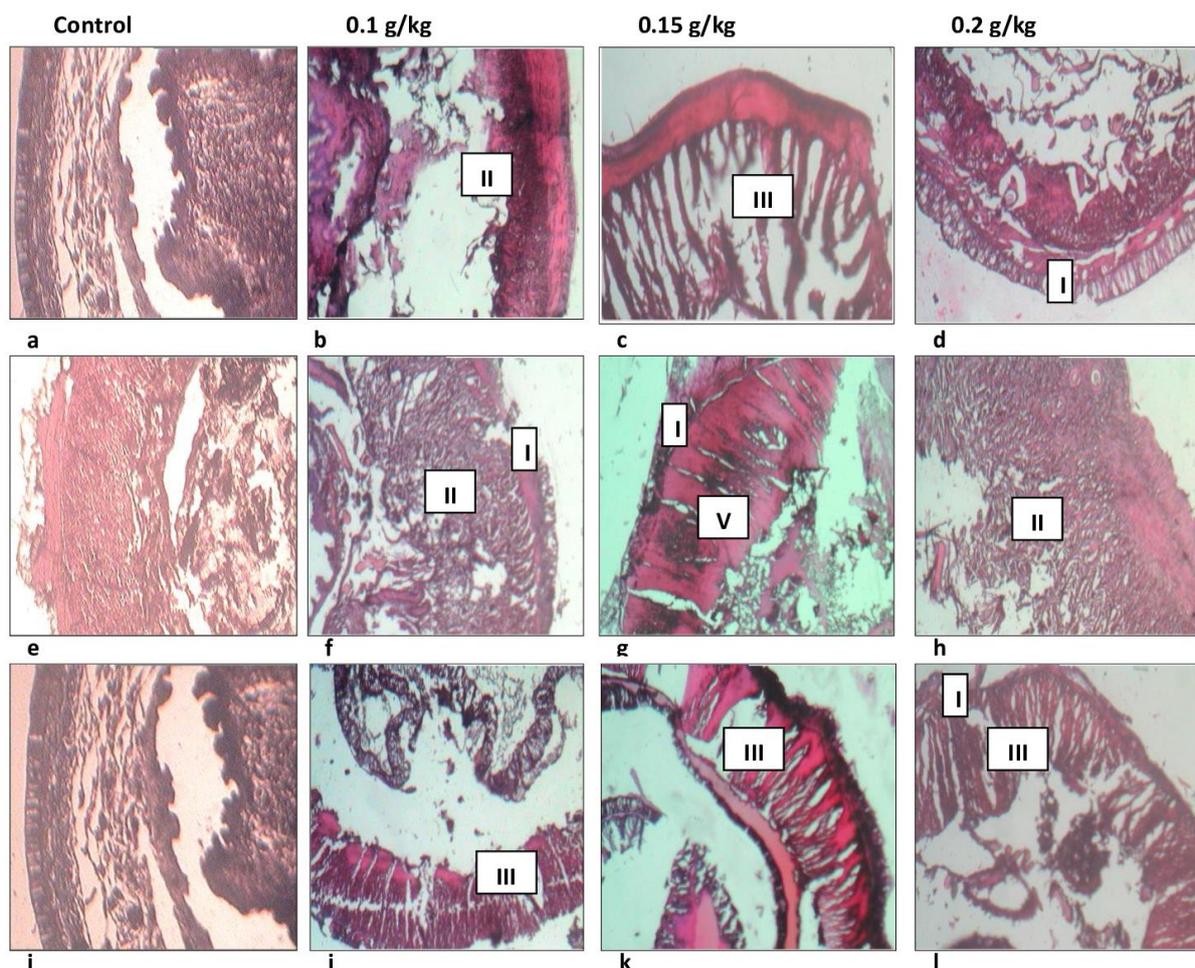


Fig. 5 Photomicrograph showing transverse section (100X) of *L. mauritii* stained with Hematoxylin and Eosin stain after exposure to 24 h of glyphosate treatment from low to high concentration respectively passing through (a-d)- Preclitellar region, (e-h)- Clitellar region (i-l)- postclitellar region. I-ruptured epidermal layer, II- disintegrated muscle layer, III- vacuolated muscle, V- merged circular and longitudinal muscle layer

Discussion

Soil toxicity can be evaluated using indicator organisms such as earthworms. In these animals, contaminants may be absorbed through the skin and then transported throughout the body (Saxe *et al.*, 2001; Jager *et al.*, 2003; Vijver *et al.*, 2003). Various workers have noticed morphological aberrations such as swelling, lesions and skin discolouration in earthworms due to pesticide and heavy metal toxicity (Rao *et al.*, 2003; Youn, 2005; Yvan *et al.*, 2005; Reddy and Rao, 2008; Singh *et al.*, 2019). Reddy and Rao (2008) reported morphological and histological alterations in *Eisenia fetida* exposed to the organophosphate profenofos. Identical effects have been observed in *E. fetida* with the herbicide glyphosate and 2,4-D (Correia and Moreira, 2010). Mukherjee and Parida (2015) have reported lindane induced toxicity and significant biomass loss in *E. eugeniae*. Nunes *et al.* (2016) studied the effect of abamectin on *Eisenia andrei* and observed thinning and discolouration of

the skin, constriction of the body and fragmentation of the posterior segment. Singh *et al.* (2019) reported variable morphological changes in the earthworm *E. eugeniae* with sublethal concentrations of triazophos.

Our observations on dermal anomalies in *D. willsi* and *L. mauritii* corroborate the earlier reports above on different earthworm species, likely due to trauma caused by pesticide exposure, which induced damage in the muscles and dermis to variable degrees, depending on the concentration.

Reports are available on the effects of diverse groups of chemicals on the histology of earthworms, which are more or less similar to the results obtained in this study. The earthworms *Perionyx sansibaricus* and *E. fetida* suffered from muscle degeneration and cuticular rupture when exposed to the weedicide burachlor (Muthukaruppan *et al.*, 2005; Reddy and Rao, 2008; Gobi and Gunasekaran, 2009). Significant muscular aberrations were reported in *Nsukkadriilus mbae* after exposure to sublethal doses of atrazine (Oluah

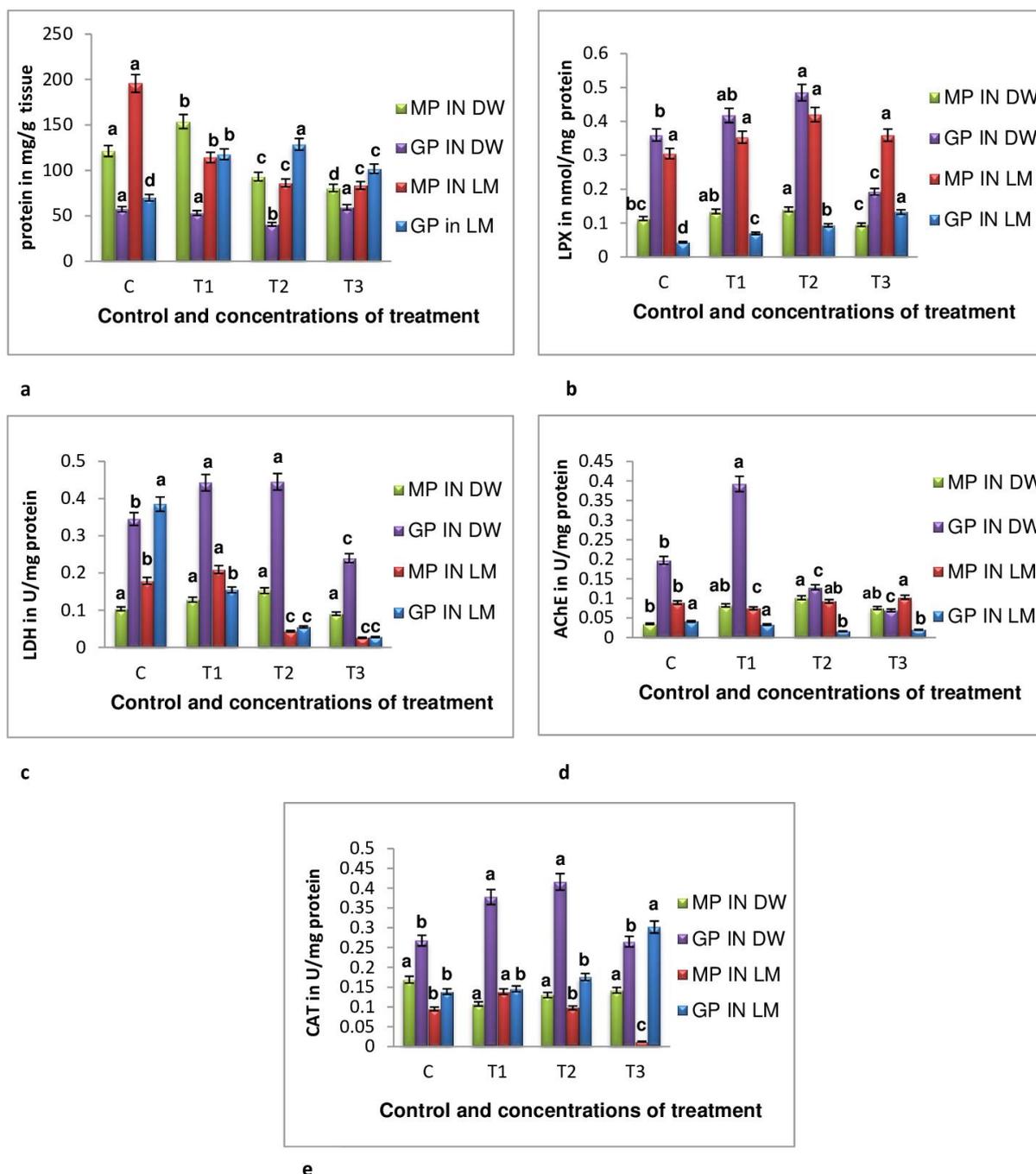


Fig. 6 Changes in certain biochemical parameters in *D. willsi* and *L. mauritii* exposed to monocrotophos and glyphosate. a- Protein content, b - LPX level, c- LDH activity, d- AChE activity and e- CAT activity. MP- Monocrotophos, GP- Glyphosate, DW- *D.willsi*, LM-*L.mauritii*. Different letters above the same colour bar indicate significant differences between control and pesticide concentrations at $p < 0.05$

et al., 2010). Effects of various weedicides, heavy metals and hydrocarbons on different earthworms have been tested, which indicated muscle anomalies (Lourenço *et al.*, 2011; Sharma and Satyanarayan, 2011; Canbek *et al.*, 2012; Eseiğbe *et al.*, 2013; Bangarusamy *et al.*, 2014; Enuneku *et al.*, 2014; Oluah and Ochulor, 2014). High

concentrations of pesticides could cause significant damage to muscle and intestinal epithelium of tropical earthworms *G. tuberosus* and *E. eugeniae* (Nayak *et al.*, 2018; Samal *et al.*, 2019a).

In the present study, we have noticed that monocrotophos at each concentration and glyphosate at high concentration could bring about

severe muscle and cuticular disintegration. Disorganization of the circular and longitudinal muscles, along with an accumulation of cell debris in the earthworms, could seriously impair their ecological functions. Clitellar damage could impair these animals' cocoon production and other reproductive functions.

We also noticed significant alterations in the biochemical parameters in both the earthworms, which corroborate the findings of several workers. Significant protein reduction in the earthworm *Lumbricus terrestris* has been reported after exposure to high concentrations of the insecticides endosulfan and aldicarb (Mosleh *et al.*, 2003). The reduction in the animal's protein level was significant between treatment concentrations. The identical result had been obtained by Ismail *et al.* (1997) with a consistent reduction in the total protein content in the earthworm *Aporrectodea caliginosa* in response to chlorfluazuron exposure. Another study indicated that phorate, an organophosphate pesticide, could cause significant protein reduction in earthworms *Perionyx sansibaricus*, *L. mauritii* and *Metaphire posthuma* (Tripathi *et al.*, 2009). In our study, the lowest protein levels in both the earthworms studied were observed in animals exposed to a high concentration of monocrotophos, which is in agreement with the findings on other species of earthworms. It appears that these pesticides at high concentrations adversely impact protein synthesis and accumulation in *D. willsi* and *L. mauritii*. *L. mauritii*, when exposed to low and medium concentrations of glyphosate, exhibited higher protein content which was reduced in response to a high chemical concentration. This is likely due to possible accumulation of stress proteins in response to low and medium concentrations and hyper toxicity at high chemical concentration.

Peroxidation of lipids in animals under physiological stress is conventionally used as a marker to evaluate oxidative stress (Paulina *et al.*, 2011). Lipid peroxidation is usually determined from the carbonylic compounds called malondialdehyde (MDA). The MDA is used as an index of lipid peroxidation (Khesiba *et al.*, 2001). The earthworm *E. eugeniae* indicated significantly high levels of MDA after exposure to hydrocarbons (Eseigbe *et al.*, 2013). In the present study, both the earthworms exposed to the pesticides indicated increased lipid peroxidation at low and medium concentrations. Interestingly, at high concentration of glyphosate, *D. willsi* indicated lower and *L. mauritii* showed higher lipid peroxidation levels. Animals, including earthworms, have protective mechanisms to scavenge peroxides generated due to lipid peroxidation, which is likely to vary in different species. The lower level of lipid peroxides at high concentration of these chemicals in *D. willsi* is likely due to the superior adaptive mechanisms in this worm to counter cell damage due to peroxides.

LDH accelerates the anaerobic energy production process by catalysing the conversion of pyruvate to lactate. Environmental changes could induce LDH activity. It is reported that the LDH activity declined with rising environmental temperature in the earthworms *M. posthuma* and *E.*

eugeniae (Tripathi *et al.*, 2011; Mishra *et al.*, 2018). A consistent decline in LDH activity with increasing concentration of the organophosphate pesticide phorate has been reported (Tripathi *et al.*, 2009). Our observations of the enzyme activity in both the earthworms are in agreement with these earlier reports, and it appears that high concentrations of both chemicals negatively influence LDH activity. However, Rico *et al.* (2016) postulated significantly increased LDH activity in *E. fetida* after exposure to tebuconazole, this finding contradicts our results.

Depleted AChE activity is impaired with altered neural conduction in earthworms due to pesticide toxicity (Patnaik and Dash, 1992; Pradhan and Mishra, 1998; Reddy and Rao, 2008; Samal *et al.*, 2017; Nayak *et al.*, 2018; Samal *et al.*, 2019a). Earlier studies by Rao *et al.* (2003) indicated that chlorpyrifos had an anticholinergic effect in the earthworm *E. fetida*. In our study, worms exposed to glyphosate indicated a suppressed level of AChE relative to the control, which is in agreement with these earlier reports. Identical results have also been obtained by Singh *et al.* (2019), who reported that the pesticide triazophos significantly inhibited AChE activity in *E. eugeniae*. However, contradictory results were obtained in worms exposed to monocrotophos, which indicated higher enzyme activity relative to the control. This was possibly due to the animals' short duration (24h) of exposure to this pesticide.

Animals produce CAT under enhanced physiological stress to scavenge free radicals. In all aerobic organisms, this is used as a preventive against cytotoxicity, mutagenicity or carcinogenicity, which might result due to a reduction in the level of antioxidants (Mates, 2000). In *L. mauritii*, high levels of this enzyme at all concentrations of glyphosate relative to control indicate that this species can counter the physiological stress more efficiently in comparison to *D. willsi*, where the enzyme activity was lower.

Therefore, we conclude that both monocrotophos and glyphosate are toxic to the earthworms. It is also evident that these dermal, histological and biochemical changes due to pesticide exposure are concentration-dependent and therefore could be used as potential markers to evaluate the degree of residual soil contamination. Further, the exposed earthworms could significantly lose their locomotory ability and ecological functions.

Acknowledgement

The authors are thankful to the Science and Technology Department, Government of Odisha, India for financial support (Grant number- ST-Bio-15/2014/1191) and Central Instrumentation Facility of O.U.A.T for scanning electron microscopy.

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