POTENTIAL SIDE-EFFECTS OF A FERTILIZER ON GROWTH, BIOCHEMICAL COMPOSITION AND BIOMARKER RESPONSES OF THE GREY WORM (*APORRECTODEA CALIGINOSA* SAVIGNY, 1826)

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Abstract. Earthworms are a major component of soil fauna communities in most ecosystems and comprise a large proportion of macro fauna biomass. They play multiple roles in soil heath. Earthworms represent one of the most important biological indicators in the terrestrial environment. However, they are exposed to agrochemicals used to increase crop yield. The present study was conducted to assess the potential hazards of a complete fertilizer (NPK), commonly used in agriculture, on juveniles of *Aporrectodea caliginosa* Savigny, 1826 an abundant species in the region of Tebessa (Northeast Algeria). The fertilizer was tested at different doses ranging between 400 and 1200 mg on juvenile earthworm under laboratory conditions. Growth, biochemical composition (lipid, protein, and carbohydrate content) and four selected biomarkers of oxidative stress (glutathione peroxidase, lactate dehydrogenase, glutathione S-Transferase and glutathione) were examined weekly during an exposure of four weeks. The earthworm mortality was monitored after 4 weeks of exposure. The LD_{25}, LD_{50} and LC_{90} values were 518.9, 644.9 and 996.4 mg, respectively. The tested fertilizer (LD_{25} and LD_{50}) showed inhibitory effects on the growth of *A. caliginosa*. Moreover, it resulted in a significant reduction in the energy reserves as evidenced by a reduction in carbohydrate, lipid and protein amounts. Lastly, enzymatic assays revealed a stimulation of the detoxification system traduced by an increase in glutathione peroxidase, lactate dehydrogenase and glutathione S- Transferase activities and a decrease in glutathione rate compared to control series. Overall results indicate that application of the fertilizer tested could have harmful effects on earthworms.
Keywords: *ecosystem engineers, NPK fertilizer, growth rate, energy reserves, detoxification system, oxidative stress*

Introduction

In order to increase crop yield, pesticides and fertilizers are now being used in higher quantities than in the past (Gill and Garg, 2014; Băcanu et al., 2019). When these agrochemicals enter the soil, they may disturb the soil ecosystems by impairing the physical, chemical and biological components specially the non-target beneficial microorganisms and earthworms (Edwards and Bohlen, 1992). Indiscriminate use of these products has led to many negative effects such as loss of top soil due to pollution by fertilizers, weedicides and pesticides, as well as magnification of chemicals in food chains and food webs (Shruthi et al., 2017). They are also responsible for extensive soil air and water pollution, and affect not only the target organisms, but also the non-targeted species such as earthworms. They constitute more than 80% of the invertebrate biomass in most of the agroecosystems of the world (Yasmin and D'Souza, 2010) and contribute to a number of soil properties, such as soil structure, porosity, water

retention, cationic exchange and pH buffering capacity (Lal, 2004). Due to their beneficial role in the agroecosystem, earthworms are used as indicator species for monitoring the impact of pollutants, changes in soil structure and agricultural practices (Yasmin and D'Souza, 2010). Earthworms recognized as 'ecosystem engineers' are naturally in contact with the solid and aqueous phases of the soil, ingest large amounts of soil and are therefore directly exposed to contaminants, industrial activities and atmospheric deposition. However, their diversity, density and biomass are strongly influenced by soil management (Gill and Garg, 2014). Pelosi et al. (2014) reported that earthworms are affected at all levels of the organism; changed individual behaviors, disrupted metabolism and enzyme activities, increased individual mortality, reduced fertility, retardation of growth and reproduction. Several studies have been carried out on the toxic impact of some pesticides on soil organisms, especially earthworms, although little research has been done on the toxicity of different fertilizers to earthworms in a soil profile (Yahyaabadi et al., 2018). A number of studies have found the positive effects of fertilizers on earthworms and their populations (Estevez et al., 1996; Curry et al., 2008). Few researchers also emphasized the negative effects of chemical fertilizers on earthworms (Tindaon et al., 2011; Bhattacharya and Sahu, 2014). Both beneficial (Callaham et al., 2003) and harmful effects (Marhan and Scheu, 2005) of inorganic fertilizers on earthworm populations have been reported from different agroecosystems. Several studies were conducted on the toxicity of NPK using earthworms as test animal by Paper contact method (Abbiramy and Ross, 2013a, b; Bhattacharya and Sahu, 2016). But very few studies were conducted to test the toxicity of NPK on the soil ecosystem. The use of biochemical biomarkers to investigate the contaminant toxicity, metabolization, and detoxification in earthworms is nowadays becoming a current practice (Denoyelle, 2007; Reinecke and Reinecke, 2007). The importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses by scavenging of reactive oxygen species (ROS) (Lijun et al., 2005). 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.

Our objective was to assess the potential hazards of a balanced fertilizer (NPK), widely used in agriculture, against *Aporrectodea caliginosa* an abundant earthworm species in Tébessa area (Northeast Algeria) (Bouazdia and Habes, 2017) and used as bioindicator of soil contamination. Thus, the fertilizer was tested on survival, growth, biochemical composition of the whole body and also on two selected environmental stress biomarkers (GPx and LDH). The purpose of this study was to obtain more comprehensive understanding of the effects of the fertilizers on earthworm, and to provide more information about the potential ecological risk agrochemicals on the soil ecosystem particularly beneficial and non-target fauna. The information obtained from this study is considered critical, as it will provide a foundation for risk assessment.

Materials and methods

Earthworm collection and soil properties

Earthworm populations were sampled at the end of autumn, which is known to be a period of great earthworm activity (Bouché, 1972). Population samples of the endogenic juvenile's earthworm's *A. caliginosa* L. were collected by hand from the region of El

Merdja, located at 4.5 Km northeast of the city of Tebessa (northeastern Algeria) (35°25'N, 8°10'E; elevation: 830 m a.s.l.). 40-cm-depth block of soil was excavated and earthworms were hand-sorted according to the method of Bouché (1972) from an upland non-irrigated paddy field which had no record of input of agrochemicals. After collection, the earthworms were cultured for 7 days of adaptation (Mekahlia et al., 2016) at their native soil. The experiment was maintained at $60 \pm 2\%$ soil moisture and 25 ± 2 °C soil temperature. The physical and chemical properties of the soil are shown in *Table 1*.

Table 1. *Physical and chemical properties of the soil*

Properties	Results
Texture	clay
pH	8.68 ± 0.01
Electronic conductivity	335.5 ± 3.5 µsm/cm
Organic matter	$11.86 \pm 0.4\%$
CaCO3	$24.25 \pm 0.75\%$

Then, they were categorized into three age classes (juveniles, immature and adults) on the basis of size and presence or absence of genital papilla and clitellium (Bhattacharya and Sahu, 2016). Earthworms are considered sub-adult if they have a full tubercula pubertatis but no clitellum and adult if they are clitellate (Sims and Gerard, 1999). They are considered juvenile if they have neither tubercula pubertatis nor clitellum (Pelosi, 2008).

Fertilizer and treatment

Inorganic fertilizer was supplied from Agricultural Services Directorate of Tebessa area. Mixtures containing all the three principal nutrients (N, P and K) are termed complete fertilizers. NPK fertilizer contains: 15% N $+ 15\%$ P_2O_5 $+ 15\%$ K_2O. The studies carried out in plastic containers of $20 \times 10 \times 7$ cm (length $\times$ width $\times$ depth), were set up each with 500 gm of native soil. Before the initiation of the exposure test, earthworms were rinsed in tap water and moved to Petri dishes on moist filter paper to void their gut contents.

Serial amounts of the dry powder (400 to 1200 mg) were prepared (*Fig. 1A*). These were added to the soil surface and then mixed thoroughly with enough water to ensure a homogeneous mixture (*Fig. 1B*). For the control treatment, distilled water was used. The test was carried out with 4 replicates each containing 10 earthworms per dose. The tested fertilizer was replaced weekly using the same concentration. The mortality was monitored by counting the number of dead earthworms at the end of different time periods (1st, 2nd, 3rd, and 4th week) and Finney's probit method was followed to calculate LD_{25}, LD_{50} and LD_{90} values.

Earthworms were considered dead if they showed any of the following statuses: disappeared, lacking movement and no response to a definite tactile stimulus (Zheng et al., 2016). The choice of doses was made according to studies of Shruthi et al. (2017) and on the basis of the high mortality rate observed after treatment with the recommended agricultural dose (RAD corresponding to LD_{90}).
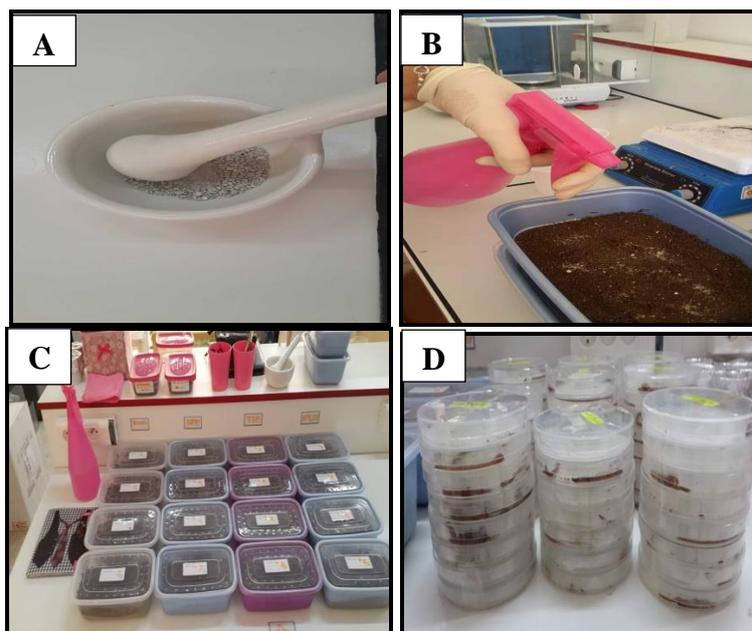
Figure 1. Experimental protocol. (A) Preparation of fertilizer (powder). (B) Treatment of the soil. (C) Earthworm rearing and treatment. (D) Voiding of the earthworm

Performance test

Ten individual earthworms were added to each plastic container and the sets were subdivided as follows; five plastic containers were applied for a control test with only water and 10 others were treated with two doses of NPK, corresponding to the LD_{25} and LD_{50} respectively (*Fig. 1C*). During the test period, earthworms were separated from the test substrate, counted, cleaned with deionized water and blotted with filter paper. Then, they were weighed on weeks 1, 2, 3, and 4, using an electro-balance. All the replicates were examined on each sampling date and the worms then replaced in the containers.

The weights of earthworms in each concentration reported from the various exposure periods were then used to calculate the growth inhibition as follows Shi et al. (2007):

$$GIn = (W_0 - W_t/W_0) \times 100 \tag{Eq.1}$$

where *GIn* is the growth inhibition for dose group *n*, W_0 is the weight on day 0 and W_t is the weight after *t* days of exposure.

Specific growth rates for a specific interval (SGR) were calculated by the formula:

$$SGRn = (\ln Wj - \ln Wi)/(tj - ti) \tag{Eq.2}$$

where SGRn is the specific growth rate for a specific interval for dose group n from day i to j, Wi is the weight on day i and Wj is the weight after j days of exposure (OECD, 2006).

Determination of biochemical composition

Specimens of both treated (LD_{25} and LD_{50}) and control earthworms removed from the soil, were cleaned with deionized water, and moved to Petri dishes on moist filter

paper to void their gut contents (*Fig. 1D*). One earthworm was selected from each container to determine biochemical composition at the tested period (i.e., at 1st, 2nd, 3rd, and 4th week).

The main components (proteins, carbohydrates and lipids) were extracted following the procedure of Shibko et al. (1967). Fragments of the body (50 mg) were extracted in 1 ml of TCA (20%). In brief, quantification of proteins was carried following the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (1976) with bovine serum albumin as a standard. The absorbance was measured at 595 nm. Carbohydrates were determined as described by Duchateau and Florkin (1959) using anthrone as reagent and glucose as standard. Lipids were measured by the vanillin method of Goldsworthy (1972) and the table oil (99% triglycerides) used as a standard. Data were expressed in µg per mg of fresh tissue and assays conducted with 3 replicates per treatment.

Glutathione peroxidase assay (GPx)

The same sample preparation protocol was followed for the biomarkers assay. GPx assay was performed according to Flohe and Gunzler (1984). Fragments of the body (50 mg) of control and treated earthworm (LD_{25} and LD_{50}) were homogenized in 1 ml of phosphate buffer (pH 7.8). The homogenate was centrifuged (3000 rpm for 10 min), and the supernatant was recovered as an enzyme source. The assay was performed on an aliquot of 200 µl of supernatant added to 400 µl of the GSH solution (0.2 mM, pH 10). Absorbance reading was performed after 5 min at 412 nm. Data were expressed in µM per min per mg of protein and assays conducted with 3 replicates per treatment.

Lactate dehydrogenase assay (LDH)

The determination was based on the conversion of lactate to pyruvate or pyruvate to lactate. The assay of LDH was conducted according to the method of Hill and Levi (1954) as previously described (Sifi and Soltani, 2019) using NAD (nicotinamide adenine dinucleotide) as substrate. Fragments of the body (50 mg) of control and treated earthworm (LD_{25} and LD_{50}) were homogenized in 1 ml of Tris/HCl (0.1 M, pH 7.2). The homogenate was centrifuged (3000 rpm for 5 min) and then the supernatant recovered for use as enzyme source. The assay was performed with 50 µl of supernatant added to 675 µl of substrate buffer (0.2 M, pH 10) and 50 µl of NAD solution. The absorbance reading was done every minute for 5 min at 340 nm. Data was expressed in µM per min per mg of protein and assays conducted with 3 replicates per treatment.

Glutathione S-transferase assay (GST)

The assay of GST was carried out according to Habig et al. (1974) previously described (Dris et al., 2017) with use of GSH (5 mM). Fragments of the body (50 mg) of control and treated earthworm (LD_{25} and LD_{50}) were homogenized in 1 ml phosphate buffer (0.1 M, pH 6). The homogenate was centrifuged (14000 rpm for 30 min). 200 µl of the resulting supernatant was added to 1.2 ml of the mixture GSH-CDNB in phosphate buffer (0.1, pH 7). Changes in absorbance were measured at 340 nm every minute for a period of 5 min. Data was expressed in nM per min per mg of protein and assays conducted with 3 replicates per treatment.

Glutathione assay (GSH)

The assay of GSH was conducted according to the method of Weckberker and Cory (1988) previously used (Maiza et al., 2013). Fragments of the body (50 mg) of control and treated earthworm (LD_{25} and LD_{50}) were homogenized in 1 ml of EDTA (0.02 M, pH 6). The homogenate was subjected to a deproteinization with sulfosalysilic acid (SSA) at 0.25%. The optical density was measured at 412 nm. Data were expressed in µM per mg of protein and assays conducted with 3 replicates per treatment.

Statistical analysis

Data are presented as a mean value ± SEM (standard error mean) in each treatment group. The normality of data was verified using the Kolmogorov-Smirnov test, and the homogeneity of variances was checked by Levene's test. Comparison of the experimental groups was tested by analysis of variance (ANOVA), and means were tested for statistical significance by a post hoc Tukey's honestly significant difference test. The statistical tests were performed using GraphPad Prism, version 7.00 (GraphPad Software, San Diego, CA, USA), where $p < 0.05$ indicates a statistically significant difference.

Results

Effect on juvenile earthworm

Different doses of NPK fertilizer: 400, 500, 600, and 1200 mg were applied on juvenile earthworm of *A. caliginosa* exhibited toxicity. The mortality was scored at 4weeks after treatment. There was a wide variation in toxicity of NPK on juvenile earthworm with respect to dose conducted in five replicates. About 6% mortality of earthworms were recorded when they were exposed to 400 mg of NPK in all the five replicates. The mortality increased to 24% when they were exposed to 500 mg of NPK followed by 40% at 600 mg, and 96% of the juvenile earthworms died at 1200 mg of NPK. Lethal doses (LD_{25}, LD_{50} and LD_{90}) values with 95% confidence limit for juvenile earthworm were of 518.9, 644.9 and 996.4 mg respectively (*Table 2*). No mortality was reported in control series.

Table 2. *Toxicity test for A. caliginosa exposed to different doses of NPK*

Doses (mg)	Mortality (%)	LD_{25} (95% FL)	LD_{50} (95% FL)	LD_{90} (95% FL)	HillSlope	R^2
400	6.00 ± 4.80 a					
500	24.00 ± 4.80 b	518.9	644.9	996.4		
600	40.00 ± 4.00 b	(479.1 to 557.3)	(598.6 to 710.8)	(801.2 to 1284)	0.507	0.99
1200	43.00 ± 9.08 c					

Effect on growth

Specific growth rate of earthworms is shown in *Figure 2A,* positive Specific growth rate means the weight of earthworms increased, and negative ones represent growth inhibition of earthworms at specific intervals. The SGR of the earthworms in both dose-treated during all the exposure intervals exposed to NPK were negative. With the time increase, the specific growth rates decreased at all tested periods: 0-1 week (F_2,

$_{12} = 46.53$; $p < 0.001$), 1-2 week ($F_{2,12} = 18.57$; $p < 0.001$), 2-3 week ($F_{2, 12} = 17.95$; $p < 0.001$) and 3-4 week ($F_{2, 12} = 103.3$; $p < 0.001$).
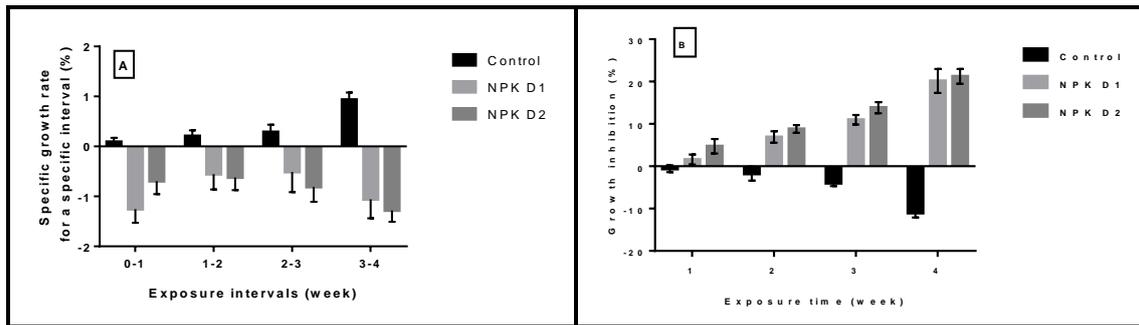

Figure 2. *Effects of fertilizer administrated at two doses on specific growth rate (%) (A) and growth inhibition (%) (B) in juvenile of A. caliginosa (mean ± SEM, n = 5 repeats, each containing 10 individuals)*

The growth inhibition of earthworms at 1, 2, 3, and 4 weeks for the two doses of fertilizer are shown in *Figure 2B*. Under the experimental conditions used here, the growth inhibition for the controls was negative. The fertilizer showed inhibitory effects on the gain of weight at the first ($F_{2,12} = 21.39$: $p < 0.001$), the second ($F_{2,12} = 88.64$: $p < 0.001$), the third ($F_{2,12} = 393.3$: $p < 0.001$: $p < 0.001$) and the fourth week ($F_{2,12} = 409.3$: $p < 0.001$) with a dose relationship at the first (Dose 1 vs Dose 2: $p = 0.0059$) and the third week (Dose 1 vs Dose 2: $p = 0.0032$).

Effects on biochemical components

Changes in main biochemical components (lipids, carbohydrates and proteins) were determined in whole body of *A. caliginosa* juveniles at different times of exposure (1st week, 2nd week, 3rd week and 4th week) to tested doses (500 and 600 mg) (*Figs. 3, 4* and *5*).

According to ANOVA test, results showed a significant decrease ($p < 0.001$) in the lipid contents in both treated series (Dose 1 and Dose 2) as compared to controls during all tested exposure time with a dose-response relationship: 1st week ($F_{2, 6} = 38.36$; $p < 0.001$), 2nd week ($F_{2, 6} = 363.1$; $p < 0.001$), 3rd week ($F_{2, 6} = 69.09$; $p < 0.001$) and 4th week ($F_{2, 6} = 4609$; $p < 0.001$).
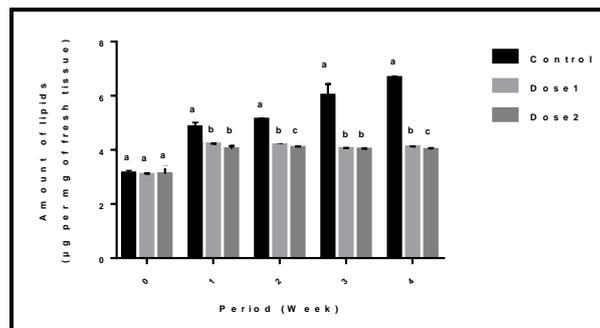

Figure 3. *Effects of fertilizer applied at two doses on amounts (µg per mg of fresh tissue) of lipids in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 repeats each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)*
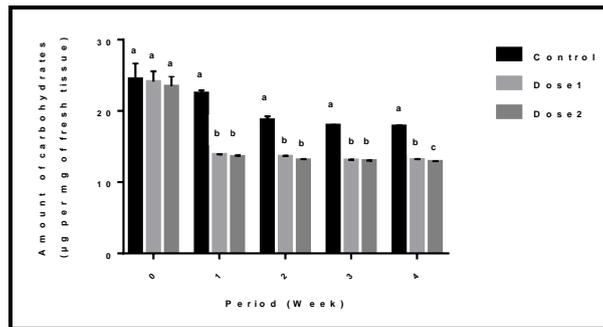
Figure 4. Effects of fertilizer applied at two doses on amounts (µg per mg of fresh tissue) of carbohydrates in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 pools each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)
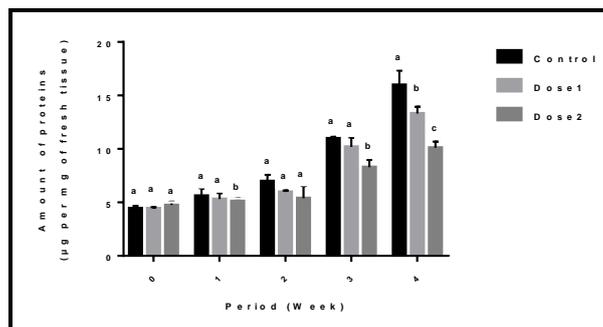

Figure 5. Effects of fertilizer applied at two doses on amounts (µg per mg of fresh tissue) of proteins in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 repeats each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)

The carbohydrate contents decreased significantly at the 1st ($F_{2, 6} = 1247$, $p < 0.001$), the 2nd ($F_{2,6} = 324.3$; $p < 0.001$) and the 3rd week ($F_{2, 6} = 28509$; $p < 0.001$) without dose-response relationship and at the 4th week ($F_{2, 6} = 4218$; $p < 0.001$) with a dose-response relationship (Dose 1 vs Dose 2 : $p = 0.035$).

Lastly, concerning the protein rates, results showed a significant decrease at 1st week ($F_{2, 6} = 5.23$, $p = 0.048$) and 3rd week ($F_{2, 6} = 13.48$; $p = 0.006$) with the highest dose (Dose 2) and at 4th week ($F_{2, 6} = 29.16$; $p < 0.001$) with a dose-response relationship (Dose 1 vs Dose 2; $p = 0.014$).

Effects on biomarkers

Results of the specific activities of antioxidant defense enzyme (GPx, LDH and GST) and a cofactor GSH in *A. caliginosa* are summarized in *Figures 6, 7, 8* and *9*. They show that values are very similar in different tested periods. GPx activity increased significantly with the two doses at the first (Control vs Dose 1: $p = 0.038$; Control vs Dose 2: $p = 0.017$) and the fourth week (Control vs Dose 1: $p = 0.033$; Control vs Dose 2: $p = 0.013$) without dose-response relationship. At the third week, an activation of GPx was observed with the highest dose (Dose 2) ($p = 0.024$). No effect of the NPK was reported with the two doses applied ($P > 0.05$) at the second week.

Concerning LDH activity, a significant increase was observed in first, second, third and fourth week with the highest dose compared to controls ($p = 0.002$; 0.001; 0.002 and 0.005 respectively). No effect of the product was reported with the lowest applied dose ($P > 0.05$) in all tested periods.
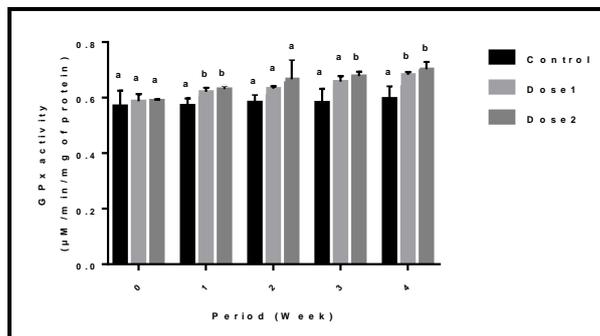

Figure 6. Effects of fertilizer applied at two doses on GPx activity (µm/min/mg of protein) in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 repeats each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)

Figure 7. Effects of fertilizer applied at two doses on LDH activity (µM/min/mg of protein) in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 pools each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)
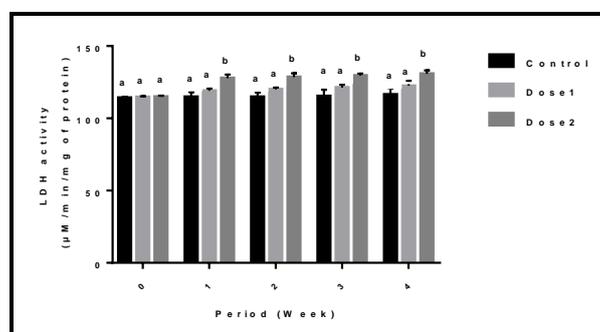

Figure 8. Effects of fertilizer applied at two doses on GST activity (nM/min/mg of protein) in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 repeats each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)
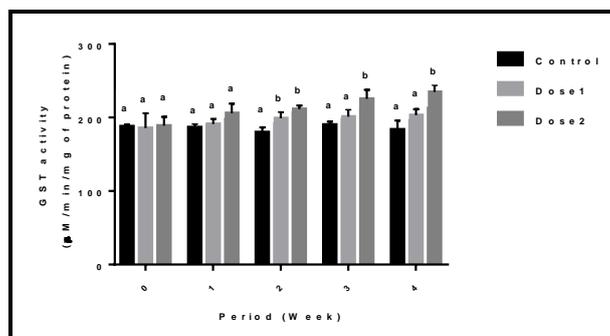
Figure 9. *Effects of fertilizer applied at two doses on GSH rate (µM/mg of protein) in juvenile of A. caliginosa during the exposure period (mean ± SEM, n = 3 pools each containing 50 mg of fresh tissue). The different lowercase letters indicate significant differences at the same time based on Tukey's HSD test (p < 0.05)*
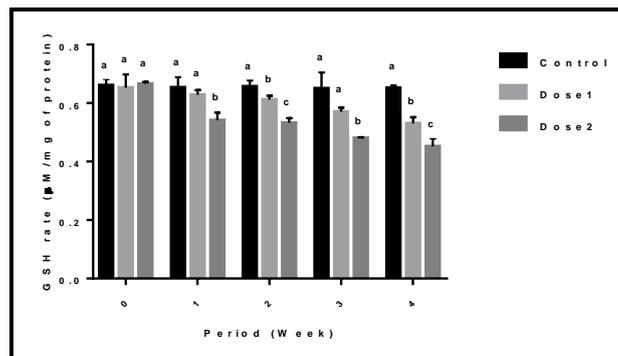
Results of the specific activity of GST in *A. caliginosa* exposed to two doses of NPK fertilizer showed a significant increase in GST activity at the second month with two tested doses (Control vs Dose 1: $p = 0.038$; Control vs Dose 2: $p = 0.0039$) and the third (Control vs Dose 2: $p = 0.009$; Dose 1 vs Dose 2: $p = 0.046$) and the fourth month with the highest dose (Control vs Dose 2: $p = 0.002$; Dose 1 vs Dose 2: $p = 0.023$) respectively as compared to control series.

Finally, our results showed a significant decrease in the rate of GSH in the treated series at the first (Control vs Dose 2: $p = 0.005$; Dose 1 vs Dose 2: $p = 0.01$) and the third month with the highest dose (Control vs Dose 2: $p = 0.001$; Dose 1 vs Dose 2: $p = 0.037$) as compared to control series respectively. In addition, we note a significant decrease of GSH rate at the second (Control vs Dose 1: $p = 0.041$; Control vs Dose 2: $p = 0.0003$; Dose 1 vs Dose 2: $p = 0.003$) and the fourth month (Control vs Dose 1: $p = 0.0009$; Control vs Dose 2: $p < 0.0001$; Dose 1 vs Dose 2: $p = 0.008$) with the two tested doses as compared to control series.

Discussion

Effects on juvenile earthworm

Mortality, growth inhibition and biochemical responses are typical indicators for assessing the toxic effects of chemicals on earthworms (Shi et al., 2007; Wu et al., 2011). Study found that the juvenile, immature and adult worms of *Drawida willsi* survived in soil containing up to 250, 370 and 490 mg/kg of NPK respectively. But 100% mortality of juveniles were recorded when worms were exposed to concentration of 490 mg/kg of NPK. However, at a concentration of 640 mg/kg and 730 mg/kg, 96% of immature and 92% of adult mortality was observed respectively. Gradually the mortality increased with the increase of doses (Bhattacharya and Sahu, 2016).

The result of this study further demonstrates that the inorganic mineral fertilizer can also be toxic to earthworms when contacted directly (Abbiramy et al., 2013a). The results of Shruthi et al. (2017) revealed that, when the food material was incorporated with various inorganic fertilizers, there was wide variation among the treatments with respect to mortality of adult and juvenile *Eudrilus eugeniae* earthworms ranging from

0.00 to 100 per cent. Little information has been generated on the toxicity of inorganic fertilizers to earthworms in sugarcane and agricultural eco-system (Shruthi et al., 2017).

Effects on growth

Earthworms provide key soil functions that favor many positive ecosystem services. These services are important for agroecosystem sustainability but can be degraded by intensive cultural practices (Pelosi et al., 2014). The biggest threat to soil health is pesticides and synthetic chemicals including fertilizers (Miglani and Bisht, 2019). The presence of contaminants in soil may cause stress to the individual which can divert energy from reproduction, burrowing activity and growth (Pelosi et al., 2014). The use of body mass change as a biomarker is thought to be ecologically relevant, as high losses in body mass are thought to lead to negative effects on survival and reproduction (Dittbrenner et al., 2010). These biomass changes could be a good indicator of chemical stress and link chemical effects to energy dynamics and, ultimately, to growth inhibition (Shi et al., 2007; Wu et al., 2012).

Several studies demonstrated that the application of chemical fertilizers as pulverization or powder can have negative effect on earthworm populations (Reinecke and Reineke, 2004; Rai et al., 2014). Weight loss appears to be a valuable indicator of physiological stress, related to the degree of intoxication and time of exposure (Van Gestel et al., 1995; Frampton et al., 2006). Our results showed an inhibitory effect of fertilizer on the gain of weight of *A. caliginosa* juvenile. Some studies have shown that growth of earthworms appeared to be more severely affected at juvenile stage than at adult stage (Zhou et al., 2008). It is reported that the use of inorganic fertilizer influences the biomass of earthworms (Lalthanzara and Ramanujam, 2010). The harmful effect of inorganic fertilizers has been reported to be due to its strong acidic and toxic effect of ammonia on earthworms (Donahue, 2001). It can be interpreted as indication of general health (Olvera-Velona et al., 2008). Robert (2008) reported that nitrogen containing fertilizers like NPK fertilizer are repellant to snails and insects. According to him, the fertilizer act as a repellant by inhibiting feeding and disrupting growth. El-Deeb et al. (2017), showed that three types of NPK fertilizers (high nitrogen, high phosphorus and balanced) treatment can influence *Biomphalaria alexandrina* snail growth, while Ragab and Shoukry (2006) reported that sublethal concentrations of urea had a stronger effect on the growth rate of the same species than sublethal concentrations of ammonium nitrate. Meanwhile, Abdel-Hamid et al. (1998) observed that urea and ammonium nitrate reduced the growth of juvenile *B. alexandrina* snails. Wu et al. (2020) reported that soil pH due to the increasing N fertilizer applications had significant inhibitory effects on the growth of *E. foetida* earthworms. Specific growth and growth inhibition rates in all BDE-47-treated groups of *E. foetida* were significantly different from those of the controls (Xu et al., 2015).

The SGR of this earthworm in almost all the treatments during all the exposure intervals exposed to perfluorooctane sulphonate and perfluorooctanoic acid were negative (Zheng et al., 2016). Growth inhibition of earthworms exposed to lindane and deltamethrin treated soil were significantly higher than those of the controls appeared to be time-dependent, with longer exposure duration resulting in higher inhibition (Shi et al., 2007). Choo and Baker (1998) found that endosulfan and fenamiphos significantly reduced the weight of *Aporrectodea trapezoids* juvenile. Zhou et al. (2006) have reported that the weight of the earthworms was a more sensitive index compared to the mortality in indicating toxic effects of acetochlor and methamidophos. Helling et al.

(2000) tested in laboratory the effect of copperoxychloride, while Yasmin and D'Souza (2007) investigated the impact of three pesticides, carbendazim, glyphosate and dimethoate on *Eisenia fetida* and found a significant reduction in the earthworm growth in a dose-dependent manner.

Effects on biochemical composition

Edwards (1985) reported that the dry matter of an earthworm body contains 60 to 70% protein, 7 to 10% fat, 8 to 20% carbohydrate, 2.3% minerals and variety of vitamins. Biochemical parameters in organisms exposed to toxic contaminants have been used as biomarkers and may be considered an important diagnostic tool to assess the exposure and effects of xenobiotics (McLoughlin et al., 2000). Changes in energy reserves and/or energy consumption have been used as biomarkers of toxic stress (Świątek and Bednarska, 2019). During stress, organisms need more energy to detoxify, biotransform and excrete the toxicants with the view of minimizing the toxic effects (Khalil, 2016). Very little work has been done to test the effect of fertilizers on the biochemical profile of earthworms.

Lipids play extremely important roles in the normal function of a cell. They not only serve as highly reduced storage form of energy, but they also play an intimate role in the structure of cell membranes and the organelles found in the cell (Kandil et al., 2009). Indeed, lipids are the preferred energy fuel offered to tissues when needed after carbohydrates. During periods of chronic stress, they also constitute another source of energy (Padmaja and Rao, 1994). In our study, a decrease in total lipids was noted in *A. caliginosa* earthworm treated with NPK fertilizer compared to control earthworm.

Carbohydrates are the first energy source mobilized by organisms under toxic stress (Moolman et al., 2007). Thus, it could be suggested that the body fat of earthworms contains the enzyme system necessary to convert glucose into fatty acid and glycerol and that these in turn would be incorporated into neutral phospholipids, triglycerides and phosphatidylcholines which are involved in the growth of the organism (Mosleh et al., 2003). In our research, the tested fertilizer, NPK caused a significant reduction in the energy reserves as evidenced by a reduction in carbohydrate rates. Hence, the adverse effect of atrazine on glucose levels of earthworms may contribute to the reduction of their growth rate (Mosleh et al., 2003). It should be noted that the evolution of glucose level from chlorfluazuron over the test period could be a result of activation of phosphorylase and glucose-6-phosphatase, as reported by Koundinya and Ramamurthi (1979). The exposure of *E. andrei* earthworm to Zn caused an increase in energy consumption at the cellular level, reflecting the relatively high energy demand of responding to toxic stress, but no effect was observed at the whole body level (Świątek and Bednarska, 2019). Some factors such as contamination by metals and soil characteristics influence glycogen, protein and lipid content in *A. caliginosa* with a 50% depletion of energy reserves (Beaumelle et al., 2014).

Protein content is considered to be the building material and involved in the alteration of almost every physiological function. It is always proportional to the growth of worm (Shankerappa, 2013). The protein content of earthworms shows considerable variability between different species and between different experimental treatments in the same species, possibly due to variability in the extent of gut inclusion (Sun and Jiang, 2017). Our experiment showed a significant decrease in protein rates in *A. caliginosa* treated with NPK fertilizer. A lower concentration of soluble protein in treated worms with pesticides suggests that physiological compensatory mechanisms were activated to

provide intermediates for deriving energy (Mosleh et al., 2003). Because it appears that the lower growth rate of worms may be a result of lower food intake, the reduction in protein content may be due to a catabolism of protein in response to worm energy demand, as suggested for an isopod in response to parathion (Ribeiro et al., 2001). Other studies have looked at the decrease in protein content and enzyme activities in response to agrochemicals (Ismail et al., 1997). In contrast, protein content increased significantly after exposure with TSP and the mixture in *L. terrestris* and no effect of sekator was reported (Mekahlia et al., 2016). This increase might indicate physiological adaptability to compensate stress and the development of cellular defenses induced by the pesticide and phosphate fertilizer impact. Furthermore, protein accumulation could be necessary to restore enzymes or lost in tissue necrosis induced by sekator or TSP exposure (Mosleh et al., 2006). Similarly, Mosleh et al. (2006) observed changes in protein content among aquatic worms *Tubifex tubifex* exposed to copper.

Effects on biomarker responses

The concept of a biomarker as a biological response to a chemical that indicates the degree of exposure or toxic effect is of considerable importance in environmental toxicology (Peakall, 1994). Although the activity of antioxidant enzymes may be increased or inhibited under chemical stress, there is, however, no general rule for the different enzymes (Nmaduka et al., 2018). Earthworm biomarkers have shown promise in various applications. Biomarker responses are of interest because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure contaminants (Arnaud et al., 2000).

Hence, biomarker responses (GPx and LDH activities) were used for evaluating the oxidative stress of earthworm *A. caliginosa* after short-term exposures to NPK.

LDH is involved in the metabolism of carbohydrates in cells and plays a key role in maintaining the balance between the catabolism and anabolism of carbohydrates (Chen et al., 2001). In a stressful environment, LDH converts pyruvate into lactate, which in turn leads to the enhanced concentration of carboxylic acid (lactic acid) in tissues and hemolymph. The results showed that LDH activity increased in response to increased concentrations of fertilizer. Such elevation of enzyme activity could be a result of the increased synthesis of particular enzymes to defend against insecticidal stress and to increase the sources of energy production through the breakdown of energy-rich nucleotides and amino acids (Mosleh et al., 2003). A similar result was obtained for lymphocytes in a report by Liu et al. (2001). The study of Mekahlia et al. (2016) revealed that sekator and triple superphosphate fertilizer, TSP induced the biomarker responses, which was proportional to exposure time and administered dose. A significant increase in the activity of LDH was observed in *Deroceras reticulatum* exposed to Caselio fertilizer (Abd-El Azeem and Sheir, 2018). In addition, a significant increase has been reported in the LDH activity in the land snails (*Eobania vermiculata*) exposed to methomyl (Khalil, 2016).

GPx is another enzyme that can remove H_2O_2 in organisms by using reduced glutathione as a hydrogen donor. The enzymes of the glutathione redox cycle, comprising GPx and GST, play a protective role against oxidative stress (Van der Oost et al., 2003). In our study, a significant increase in GPx activity was found in *A. caliginosa* exposed to NPK fertilizer. The increase in antioxidant enzyme GPx may be an important indicator of the detoxification capacity of the earthworm (Maity et al., 2018). Interestingly, same as our results, a previous study on earthworms (Wang et al.,

2016) also found that GPx activity was induced by naphthenic acids after 1 d but inhibited after 14 d of exposure. The reasons might be that (i) GPx activity was inhibited by excessive ROS in cells; and (ii) large amounts of glutathione (GSH) were consumed to remove ROS (Cao et al., 2017). While a slight inhibition was observed in GPx activity with high phosphorus fertilizer treatment, a marked induction was obtained in GPx activity after the second week with high nitrogen fertilizer treatment (El-Deeb et al., 2017). Elumalai et al. (2007) indicate that a combined effect of different trace elements induces GST and GPx activity in the whole body tissue of *A. caliginosa* for the increased demand of organisms to eliminate trace elements and manage peroxidative damage by reducing the level of peroxides.

Glutathione S-transferase (GST) is a phase II detoxification enzyme modulated by xenobiotics and thus has been suggested for use as a biomarker indicative of environmental contaminant exposure (Gunderson et al., 2016). GST activity is required for the maintenance of homeostasis of the internal environment (Maity et al., 2018). It aims to combine reduced glutathione (GSH) on electrophilic compounds to facilitate their elimination (Ketterer et al., 1983). By conjugation, GST neutralizes many xenobiotics and endogenous metabolites (Hayes et al., 2005), which seems to be a possible explanation for the reduced GSH content. Interestingly, the induction of GST and GPx activity that can be correlated to the reduction in the GSH level leads to depletion in cellular antioxidant status (Radu et al., 2010). GSH deficiency might be due to the faster rate of GSH consumption for scavenging free radicals non-enzymatically or in a reaction catalyzed by GPx through the oxidation of two molecules of GSH to a molecule of GSSG (oxidized glutathione) (Van der Oost et al., 2003).

In the study of Mekahlia et al. (2016), the biomarker responses (GSH, and GST activity) were used for evaluating the oxidative stress of earthworm *L. terrestris* after short-term exposures to TSP and sekator. The data indicated high sensitivity of biomarkers and the changes in the GST and GSH levels reflect the oxidative stress in the earthworm. Xue et al. (2009) and Maity et al. (2008) mentioned that increased levels of GST were seen in earthworms after exposure to tetrabromobisphenol and lead, respectively. Further, Aly and Schröder (2008) showed that the GST conjugation rate in *Eisenia foetida* varied using two different herbicides namely fenoxaprop and metolachlor.

Conclusion

Soil organisms are exposed to a wide variety of environmental pollutants. Earthworms are significantly influenced by environmental stress, and because of their sensitive metabolic and physiological changes. The results obtained showed that the NPK fertilizer inhibited the increase in weight of *A. caliginosa*, which could be due to the repulsion of the contaminated food. This fertilizer also reduced content of proteins, lipids and carbohydrates in the whole body and caused the activation of the system of detoxification, traduced by an increase of the specific activity of GPx, LDH and GST and a decrease of GSH. Earthworms are useful as test organisms to assess the toxicity of chemical stressors.

Further experiments are warranted to evaluate if these effects will occur in other organisms and can cause chronic effects after more prolonged exposure as well as on other resistance mechanisms mainly detoxification enzymes, histological changes, reproduction and residue accumulation in earthworms.

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