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Toxicological and biochemical effects of selective and non-selective herbicides on non-target organisms - periwinkles (*Tympanotonus Fuscatus*) and earthworms (*Lumbricus Terrestris*)

Doris Fovwe Ogeleka*, Duke Okoro, Onome Oghenefejiro Udubrae
Department of Chemistry, Federal University of Petroleum Resources, Effurun, Delta, Nigeria

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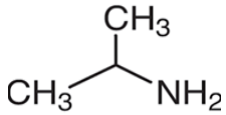
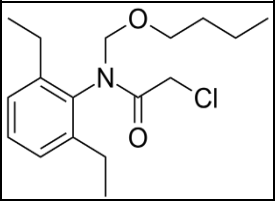
Abstract
In this study *Tympanotonus fuscatus* and *Lumbricus Terrestris* were exposed to Cotchlor® and Gobara®, a selective and non-selective commonly used herbicides in order to determine the toxicological and biochemical effects. The acute toxicity of Cotchlor® and Gobara® were determined using the Organization for Economic Development and Cooperation (OECD) #218 and 207 protocol respectively. Similarly, the anti-defensive mechanisms [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] as well as oxidant activity – lipid peroxidation indicated as malondialdehyde were used to assess the herbicides' harmful effects. The effective concentration (EC_{50}) of Cotchlor® for periwinkle *(Tympanotonus fuscatus)* was 25 ± 1.44 mg/L while Gobara® was 250 ± 1.5 mg/L. Similarly, the EC_{50} for earthworm for Cotchlor® and Gobara® was 25 ± 1.55 mg/L and 50 ± 1.72 mg/L respectively. As the concentrations of exposure in *Tympanotonus fuscatus* increased, the antioxidant activity namely SOD, CAT and GPx decreased significantly at a level of $P<0.05$. The study concluded that the test herbicides produced reactive oxygen species (ROS) resulting in increasing levels of lipid peroxidation, which may have altered the activities of SOD, CAT and GPx causing oxidative stress in *Tympanotonus fuscatus*. Due to their hazardous potentials, the release of the test chemicals and other similar herbicides into the environment may result in a significant loss and biochemical alterations of the non-target test species - periwinkles and earthworms.

Keywords: effect concentration (EC_{50}), herbicides, oxidative stress, periwinkle, reactive oxygen species (ROS)

Introduction
The increase in population across the world has over the years had a tremendous effect on the environment due to the relative increase in food demand against production. As a result, the use of pesticides (herbicides) has become necessary for the production of food which has also resulted in some detrimental consequences on the environment. Farmers all around the world, particularly in Nigeria have been forced to increase agricultural production, which has resulted in a high frequency of insects, plant diseases, and weeds. In order to address these issues, farmers resort to use agrochemicals such as pesticides and herbicides. Herbicides are used to control weeds, however some of them are harmful to non-target organisms, the environment and humans (Dutta and Dutta, 2016; Ogeleka et al., 2017) [4, 16].
Herbicide's role in weed management cannot be overstated however, during application a significant amount of herbicides are washed and carried away by rains and floods into recipient water bodies, thereby altering the physico-chemical qualities of the water (Bhalchandra et al., 2001) [2]. The continuous flow of agricultural effluents into fresh water frequently results in pollution accumulation in these bodies of water (Mason et al., 1991) [10].
In addition, herbicides used in agriculture to combat weeds are extremely hazardous to soil biota (MacFarlane et al., 2013) [11]. These herbicides are thought to be hazardous to nematodes, earthworms, snails and other biological organism residing in the soil. In most terrestrial habitats, earthworms play a significant role in soil formation and organic matter breakdown and they have long been regarded as useful soil barometers as well as indicators of soil pollution and fertility (Ogeleka et al., 2017) [16]. Earthworms are a common prey of many terrestrial vertebrate species, such as birds and small mammals, hence they play an essential part in the food chain (Lanno et al., 2004; Oni and Hassan, 2013) [8, 18]. Similarly, periwinkles are edible species that provide protein for higher organisms along the food chain including humans and may also be affected by herbicide applications since they are non-target species.
This study was aimed to assess the toxicological and biochemical impacts of a selective and non-selective herbicides - Cotchlor® and Gobara® on non-target environmental receptors namely periwinkles (Tympanotonus Fuscatus) and earthworms (Lumbricus Terrestris).

Materials and Methods
Test Chemicals
The details of the test chemicals used in this assessment are indicated in Table 1.

Table 1: Herbicides and their active ingredients

S/N	Trade Name	Active Components	Class / Application	Formulation	Structure
1	Gobara®	Isopropyl amine salt	Non-selective, systemic, post emergent broadleaf herbicide - that effectively burns down and controls annual and perennial grass	360 mg/L of Isopropyl amine salt	
2	Cotchlor®	Butachlor (N-(butoxy methyl)-2-chloro-N-(2, 6-diethylphenyl)	Selective, systemic, pre-emergent and post-emergent herbicides that is used to kill weeds and control broadleaf and grassy weeds in a variety of commercial crops	50% butachlor	

Methods

Periwinkle Toxicity Bioassay

Healthy periwinkles (*Tympanotonus Fuscatus*) were used for the 10-day experimental procedure for sediment toxicity adopting the Organization for Economic Cooperation and Development (OECD) #218 protocols (OECD, 2004) [15]. The periwinkles were acclimated for seven days prior to the commencement of the test in clean sediment. The experiment started with a range finding test to establish a working range for the concentration to be used in the definitive (actual) test. From the prepared stocked solution, a range finding test using concentrations in the range of 1, 10, and 100 mg/L was done for the test chemicals - Cotchlor® and Gobara®. From the results of the range-finding, concentrations of 6.25, 12.5, 25, 50 and 100 mg/L was prepared for the definitive test for Cotchlor® while 31.25, 62.5, 125, 250 and 500 mg/L was prepared for Gobara®.

For each test concentration, 1000 g (1 kg) of sediment from the organisms' habitat was placed into the test vessels and 2000 mL of the test solution was introduced. Five (5) g of cellulose was added as food for the organisms to ensure they were not starved during the experimental period. After three hours of attenuation of the test chemicals, ten (10) healthy organisms were cleaned, weighed and carefully transferred into each test containers in triplicates. The control setup was prepared alongside with the test chemicals but without the test chemicals.

Earthworm Toxicity Bioassay

The earthworms (*Lumbricus Terrestris*) were collected by gentle hand sorting at a coordinate of latitude $5^0$33˙59.6" N and longitude $5^0$49'59.8" E. This process was carried out using the Organization for Economic Cooperation and Development (OECD) protocol #207 (OECD, 1984; Sandoval *et al*., 2001) [14, 24]**.** Seven days prior to the commencement of the test, the earthworms were acclimatized on soils from the earthworms' natural habitat. A range finding test started the experiment and from the prepared stocked solution, a concentrations of 1, 10, and 100 mg/L were done for the test chemicals - Cotchlor®® and Gobara®. Having obtained the results of the range-finding a concentration of 6.25, 12.5, 25, 50, and 100 mg/L was prepared for the definitive test into triplicates of five concentrations for Cotchlor® while 6.25, 12.5, 25, 50, and 100 mg/L was prepared for Gobara®. For each of the test concentration 1000 g (1 kg) of natural soil of organism habitats was placed into the test tank and 70 mL of each test chemicals was introduced. Five (5) g of cellulose was added to the test tank as food for the organisms to prevent starvation. After attenuation of about 3 hours, ten (10) healthy organisms were cleaned, weighed and carefully transferred into each test containers. There was no test chemical in the control setup prepared alongside with the test chemicals.

Assessment of Response

Mortality was evaluated on the 10^{th} and 14^{th} day for the lethal test for the periwinkle and earthworm respectively. Physical changes (morphology) and behavioral responses were also noted. The organisms were considered dead if there was no movement when prodded with a metal rod or if there is no activity after 5 min of placing the periwinkle or earthworm on a white paper.

Biochemical assays

Following the exposure of the periwinkles to the test herbicides for the acute toxicity test, the organisms were removed, rinsed and used for the biochemical bioassay. Homogenates of the periwinkles were prepared by homogenizing 0.5 g of the tissues in ice-cold phosphate buffer at pH7.2. The homogenates were centrifuged at 4000 rpm for 10 minutes and the supernatant were used for the biochemical analysis.

Measurement of oxidant activities

a. Lipid peroxidation assay (indicated as Malondiadehyde - MDA)

Peroxidation was determined by adopting the method of Buge and Aust, (1978) [3] based on malondialdehyde assay. Malondiadehyde, a product of lipid peroxidation, when heated with 2-thiobarbituric under acid conditions

forms a pink coloured product which has a maximum absorbance at 532 nm. MDA content was expressed as mmol/MDA.

Measurement of antioxidant enzymes activities

a. Superoxide dismutase

The activity of superoxide dismutase (SOD) in the species was estimated spectrophotometrically using the method of Nishikimi *et al*., (1972) [13]. The assay of SOD is an indirect method based on the inhibitory effect of SOD in the initial rate of epinephrine (adrenaline) auto-oxidation. SOD was expressed in unit/mg protein tissue.

b. Catalase

The activity of catalase was determined in the tissue homogenates by the method of Ramos-Vasconcelos and Hermes-Lima (2003) [23]. It was based on the measurement of the rate of decomposition of hydrogen peroxide (H_2O_2) after the addition of the material containing the enzyme. Catalase was expressed in unit/mg protein tissue.

c. Glutathione peroxidase

Determination of glutathione peroxidase activity was carried out by the method of Ahmed *et al*., (2021). The samples were incubated with phosphate buffer with appropriate concentrations of glutathione and peroxide as substrates to determine the GPx activity. After sufficient incubation time, the CUPRAC reagent (Cu $(Nc)_2^{2+}$) was added to stop the enzyme's reaction. The unreacted substrates act to reduce Cu(II)-neocuproine complex ($Cu(Nc)_2^{2+}$) to a strongly coloured Cu(I)-neocuproine complex ($Cu(Nc)_2^{+}$), which was measured spectrophotometric ally at 450 nm. The glutathione peroxidase activity was linked to a decrease in the absorbance of the coloured Cu (I)-neocuproine complex (Cu $(Nc)_2^{+}$). Glutathione peroxidase was expressed in unit/mg protein tissue.

Statistical Analysis

The results from the various experiments and analysis were expressed as mean ± standard deviation (mean ± SD). The data were analyzed using Student's t test in Analysis of Variance (ANOVA) to indicate statistical difference between the exposed and control groups. Values of $P \leq 0.05$ were considered statistically significant.

Results

Acute Toxicity

The results obtained from the acute toxicity of periwinkle exposed to different concentrations of Cotchlor® and Gobara® are represented in Figures 1 - 2 while earthworm data are shown in Figures 3 - 4.

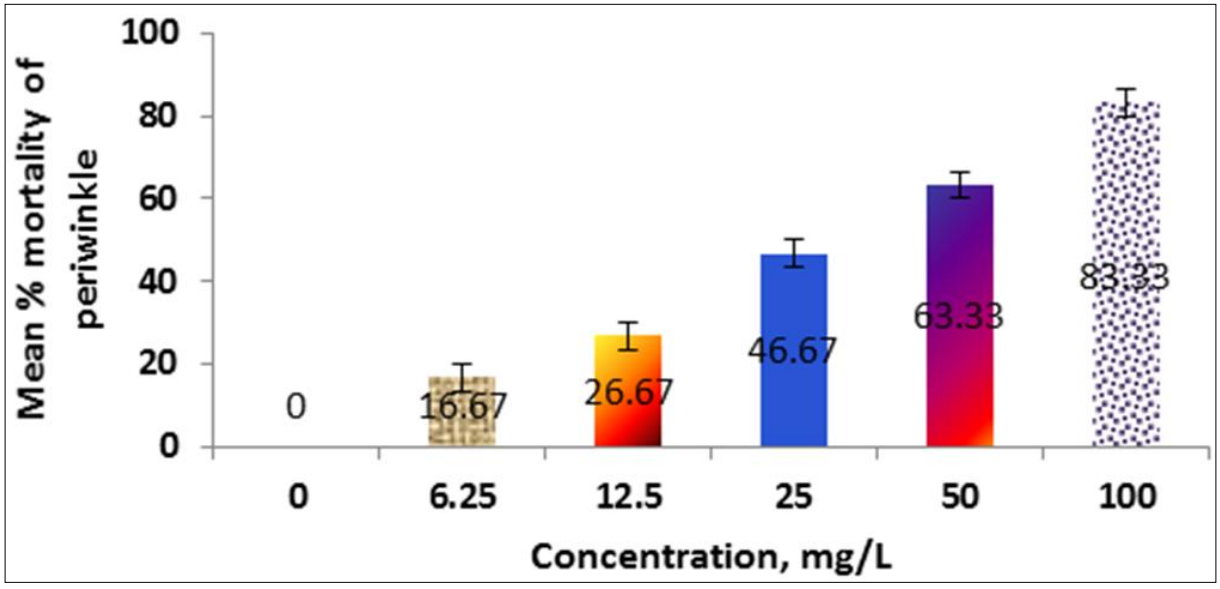


Fig 1: Mean percentage mortality for Cotchlor® exposed to periwinkle (*Tympanotonus fuscatus*)

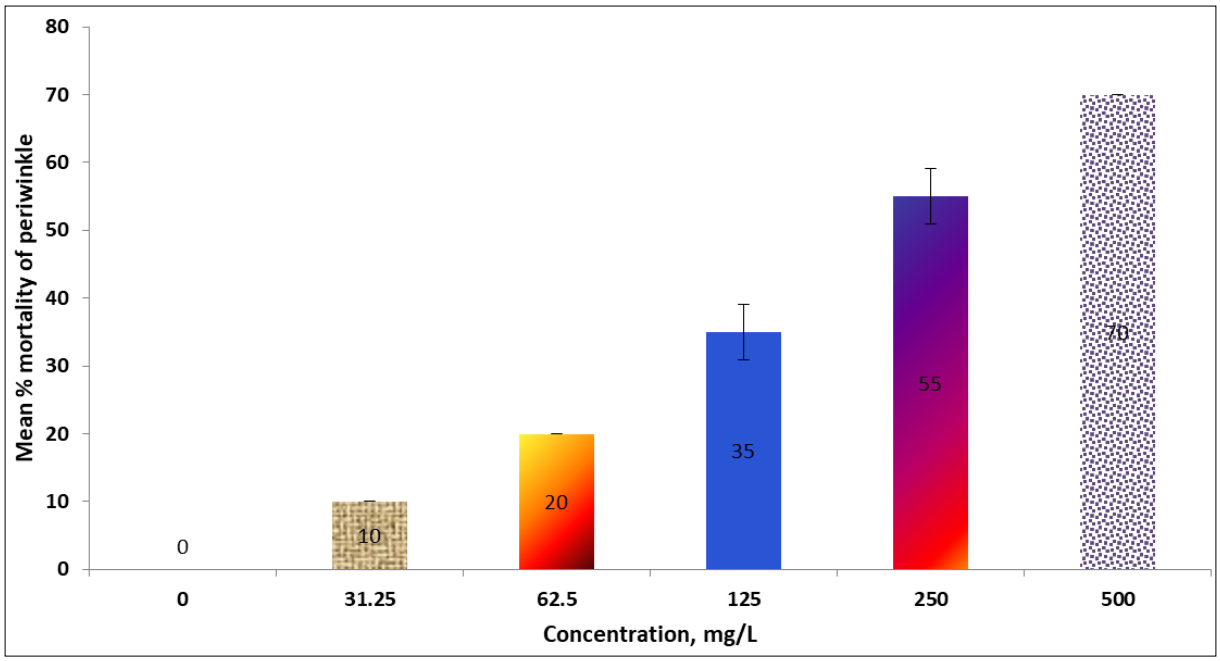


Fig 2: Mean ± SE mortality of Periwinkle *(Tympanotonus fuscatus)* exposed to Gobara®

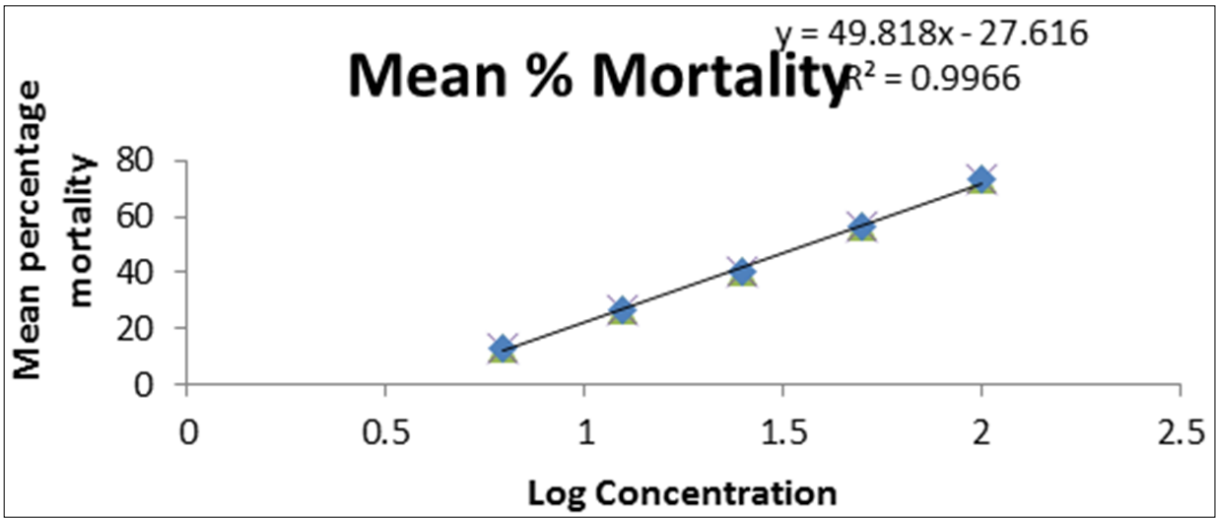


Fig 3: Mean percentage mortality of earthworm *(Lumbricus Terrestris)* exposed to Cotchlor®

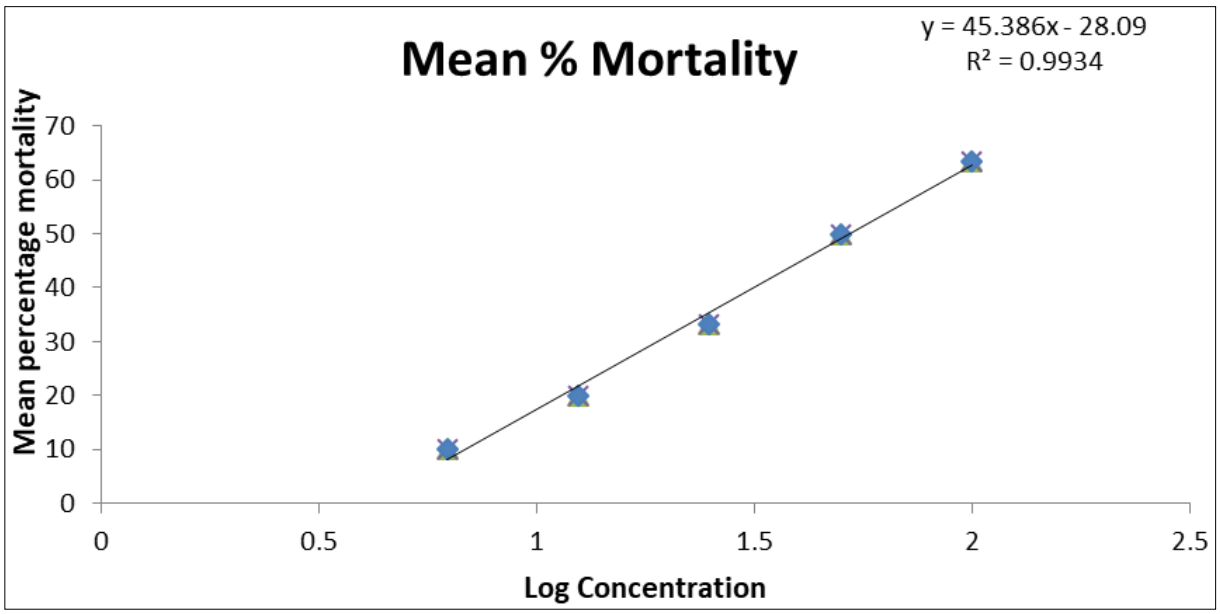


Fig 4: Mean percentage mortality of earthworm *(Lumbricus Terrestris)* exposed to Gobara®

Effective concentration (EC_{50})

The effective concentration (EC_{50}) of Cotchlor® for periwinkle *(Tympanotonus fuscatus)* was 25 ± 1.44 mg/L while Gobara® was 250 ± 1.5 mg/L. Similarly, the EC_{50} for earthworm for Cotchlor® and Gobara® was 25 ± 1.55 mg/L and 50 ± 1.72 mg/L respectively (Table 2).

Table 2: Effective concentration (EC_{50}) for test herbicides exposed to *Tympanotonus fuscatus* and *Lumbricus Terrestris*

Test Chemical	Organism	Line equation (Y – value)	EC_{50}	Rating
Gobara®	*Tympanotonus fuscatus*	y = 47.9x – 64.842	250. 0 ± 1.5	Practically non-toxic
	Lumbricus Terrestris	y = 45.386x + 28.09	50.0 ± 1.72	Slightly toxic
Cotchlor®	*Tympanotonus fuscatus*	y = 56.451x – 31.551	25.0 ± 1.44	Slightly toxic
	Lumbricus Terrestris	y = 49.818x + 27.616	25.0 ± 1.55	Slightly toxic

Data were processed and expressed as mean ± SD based on three replicates

Mean Results for Biochemical indices for *Tympanotonus fuscatus* exposed to Cotchlor®

The results for biochemical indices for *Tympanotonus fuscatus* exposed to Cotchlor® are given in Table 3. The assay was done to assess the impact of the test chemicals on the organisms' biochemical system using the lowest and highest exposure concentrations in comparison with the control groups. From the results obtained, it showed that there were significant differences between the activities of the anti-defensive activities in the organisms exposed to the test herbicides and the control at levels of $P < 0.05$.

Table 3: Mean values ± standard deviation of biochemical assay for *Tympanotonus fuscatus* exposed to Cotchlor®

Concentration (mg/L)	MDA (mmol/MDA)	SOD ($Unitsg^{-1}$tissue)	CAT ($Unitsg^{-1}$tissue)	GPx ($Unitsg^{-1}$tissue)
Control	$1.06±0.18^{a}$	$46.1±2.07^{a}$	$31.9±3.11^{a}$	$40.3±1.79^{a}$
6.25	$1.96±0.16^{b}$	$41.1±2.47^{b}$	$27.6±2.41^{a}$	$33.0±3.20^{b}$
100	$2.45±0.19^{c}$	$28.9±7.01^{b}$	$24.7±1.68^{b}$	$32.6±1.65^{b}$

Values are means ± standard deviations of triplicate determinations. Values not sharing a common superscript on the same column differ significantly ($p<0.05$). CAT = Catalase, SOD = Superoxide dismutase, GPx = Glutathione peroxidase and MDA = Malondialdehyde.

Discussion

Contamination of the environment is usually caused by indiscriminate discharge of industrial chemicals / substances into water and soil. In recent years, the vast majority of contaminations have been caused by the intentional and unintentional application of herbicides, particularly in agricultural management. However, the herbicides do not particularly target the weed and so they cause harm to non-target plants and animals during application. Farmers and non-farmers alike have used herbicides inappropriately for decades, with uncontrolled application in the user's favour without consideration of the resultant effects the herbicides would impact on plants, crops and soil-dwelling organisms. The inability of some of these recalcitrant chemicals to biodegrade quickly in the environment could cause a myriad of harm, discomfort and death in organisms that live and thrive in the soil. Although herbicides help to increase the food supply and boost the economy, they also contribute to environmental pollution and cause detrimental effects on non-target species and consequently humans. Persistent herbicides can remain active in the environment for long periods of time, potentially causing soil and water contamination and adverse effects to non-target organisms instead of the target weed. In some cases, compounds that result from herbicide degradation may continue to be significantly toxic in the environment (Ogeleka et al., 2020) [17].

Earthworms are as representative soil animals to be used for evaluating the use or abuse of field applications of herbicides (Rao et al., 2003; Schreck et al., 2008) [22, 25]. As non-target species, earthworm diversity and biomass are adversely affected by a variety of agricultural techniques, with indiscriminate herbicide use being one of the most damaging (Pelosi et al., 2014) [28]. Increased exposure time and dose can induce physiological harm to earthworms (cellular malfunction and protein catabolism) (Schreck et al., 2008) [25]. The results from this study revealed that the test herbicides had a significant effect on the exposed earthworms, implying that mortality was concentration depended, that is the higher the concentration, the greater the effect / impact.

According to Oliveira-Filho et al., (2005) [19], the longer periwinkles are exposed to chemicals, the greater the impacts on the digestive gland, foot, and mantle of the organisms. The mean percentage mortality as well as the EC50 value for the herbicides showed that Cotchlor® was slightly toxic to periwinkles. Gurvinder, (2019) [6] noted that several stress events generated upon the herbicide action can lead to oxidative dis-balance in various non-target species.

Recently, lipid peroxidation (LPO/MDA) has been gaining attention as a potential toxicological hazard and several herbicides have been shown to stimulate peroxidation of cellular membranes. In this assessment, there was significant increase in the level of lipid peroxidation indicated as MDA in the exposed species of Tympanotonus fuscatus to the test herbicides with respect to the control. The results concerning changes in the MDA levels indicated that the increase was concentration-dependent. Here, elevation in the MDA level and the

alteration in antioxidant enzyme activities (CAT, SOD and GPx) clearly indicated that the chemicals had the potential to cause / induce reactive oxidative stress (ROS) and related biochemical damages.

The enzyme superoxide dismutase aids in the defense of living organisms against ROS by catalyzing the dismutation of the most reactive and deadly free radicals, superoxide radicals (O2-), to less reactive molecular oxygen (O2) and hydrogen peroxide (H2O2). SOD activity was shown to be lower in this study compared to the control, which could be due to SOD's inability to scavenge the rapidly produced ROS (Liu et al., 2012) [9]. Catalase activities in periwinkle was lowered as a result of exposure to the test herbicides in this study, therefore reducing the balance in the level of antioxidant enzymes that mitigates oxidative stress by destroying cellular hydrogen peroxidase to produce oxygen and water. Deficiency or malfunction of catalase is expected to cause damage to the tissues of exposed organisms. Similarly, glutathione peroxidase (GPx) is an antioxidant enzyme with the capability to scavenge free radicals and this in turn helps to prevent lipid peroxidation and hence maintain adequate protection from oxidative breakdown.

In addition, the study recorded that indiscriminate release of test herbicides could cause lethal toxicity to periwinkles and earthworm species, leading to soil pollution, alteration in the food web and chain and subsequently death of vulnerable species. These observations are in accordance with reports by several researchers on herbicide-induced oxidative stress (OS) in different animal models (Gir´on-P´erez et al., 2006; Ural, 2013; Kaur and Jindal, 2017; Miladinovic et al., 2018; Ogeleka et al., 2020) [5, 26, 7, 12, 17].

Conclusion

Although, the use of herbicides in controlling weeds is important, however, its impact on non-target organisms should be considered for ecological safety. In this study, the results of acute toxicity showed that Cotchlor® and Gobara® have adverse effect on non-targeted organisms as indicated in the EC_{50} values. Similarly, the results for biochemical indices for the periwinkle exposure showed that Cotchlor® could cause oxidative stress through generation of ROS. The oxidative stress status was marked by an elevation in the level of MDA. Hence, strict regulations should be enforced for the application and disposal of these herbicides such that they are not released indiscriminately into the environment, thereby causing deleterious effects on non-target species.

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