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Research Article

Toxicity Assessment of Buprofezin, Lufenuron, and Triflumuron to the Earthworm *Aporrectodea caliginosa*

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Earthworms are particularly important soil macroinvertebrates and are often used in assessing the general impact of pesticide pollution in soil. The present study was conducted in order to investigate the toxicity of three insect growth regulators (IGRs) buprofezin, lufenuron, and triflumuron, at different application rates and exposure times toward mature earthworms *Aporrectodea caliginosa*. The effects of these pesticides on the growth rate in relation to the activities of acetylcholinesterase (AChE) and glutathione S-transferase (GST) as biochemical indicators were evaluated to elucidate the mechanisms of action. Toxicity studies indicated that lufenuron was the most harmful pesticide to mature earthworms, followed in descending order by buprofezin and triflumuron. A reduction in growth rate in all pesticide-treated worms was dose-dependent over the 28-day exposure period, which was accompanied by a decrease in AChE and GST activities. Relationships between growth rate, AChE, and GST provided strong evidence for the involvement of pesticidal contamination in the biochemical changes in earthworms, which can be used as a bioindicator of soil contamination by pesticides.

1. Introduction

Insect growth regulators (IGRs) as third-generation insecticides have a specific mode of action on insects and a lower toxicity against vertebrates than the conventional insecticides [1–4]. However, heavy applications of such pesticides in agricultural areas may also impact not only on the target species but also on nontarget organisms in and adjacent to the target areas. The concern specifically addressed in the present study relates to the possible effects of pesticides on nontarget soil organisms such as earthworms. Earthworms were chosen as example of nontarget soil organisms due to their beneficial role in the soil and their very low abundance in the orchards and in the adjacent areas [5]. Earthworms are particularly important soil macroinvertebrates and are often used in assessing the general impact of heavy metal and pesticide pollution in soil [6, 7]. Macroinvertebrates have the ability to preserve and contribute to the overall productivity of the soil ecosystem by maintaining soil structure and by regulating the turnover of organic matter [8]. Earthworms feed, cast, and burrow in soil and are exposed to soil contaminants via their intestine or skin [9]. It is also easy to quantify life-cycle parameters of earthworms, accumulation and excretion of pollutants, and biochemical responses [10].

Although the toxicity of pesticides on earthworms in contaminated soil has been evaluated from a variety of points of view, such as avoidance, survival, growth, reproduction and protein content [11–15], the responses of the test organisms to IGRs have not been fully investigated. IGRs have adverse effects on vertebrate and invertebrate organisms and are known to be acutely toxic to some organisms. The level of toxicity to invertebrates, however, does not seem to be similar to that of vertebrates and needs further investigation [2]. As it is difficult and expensive to detect the presence and exact concentrations of these insecticides in the environment, due to their relatively short half-lives, it may be more effective to use biomarkers to assess exposure. A number of researchers have used biomarkers effectively to determine pesticide effects on earthworms [16–18]. Biomarkers, measuring effects on suborganism levels, can provide links between a chemical and its toxic effect and are generally more sensitive than the more traditional measures of contamination, such as mortality and abundance [19–22].

Biochemical reaction studies mostly focus on the evaluation of possible adverse effects of chemicals on aquatic organisms [19, 22]. The severity of soil contamination caused by persistent organic pollutants is commonly assessed with standard acute and reproduction tests. These standard tests, however, are unable to provide accurate evaluation of the biochemical responses of selected organisms to a given chemical exposure. The biochemical responses, generally called early warning signals, can provide valuable information in assessing the potential risk factors of soil contamination [23]. Therefore, the main aim of this ecotoxicology study was to determine the toxic effects of three IGRs—buprofezin, lufenuron, and triflumuron—on the earthworm *Aporrectodea caliginosa*. The acute and subchronic toxic effects of these pesticides on two biomarker responses (acetylcholinesterase (AChE) and glutathione S-transferase (GST)) were also examined to evaluate the impact on and risk to soil organisms and ecosystems.

2. Materials and Methods

2.1. The Insecticides and Chemicals. Formulated buprofezin (Applaud, 25% SC) was obtained from Nihon Nohyaku Co., Japan, lufenuron (Match, 5% EC) from Syngenta, and triflumuron (Alsystin, 48% SC) from Bayer CropScience. The stock solutions of each compound were prepared in water on the day of the experiments and were used immediately. Bovine serum albumin (BSA), Folin-Ciocalteu phenol reagent, acetylthiocholine iodide (ATChI), 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), glutathione, and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich Chemical Co., USA. All chemicals were used without further purification.

2.2. Earthworms and Assay Conditions. Earthworms used in this study belonged to species commonly found in Egypt (*Aporrectodea caliginosa*). Individual worms were collected from dunghills around Alexandria Governorate and reared in artificial soil in large plastic containers ($38 \times 60 \times 10$ cm) covered with muslin cloth to reduce water evaporation, as described by Heimbach [24]. The worms were maintained in this artificial soil at $23 \pm 2^\circ$C for one month before the experiments. Earthworms used in this study were adults with well-developed clitella. As earthworms are hermaphrodite, no sexual differences were taken into account. The adults were removed from the artificial soil 24 h before use and stored in Petri dishes on damp filter paper (in the dark at $23 \pm 2^\circ$C) to void gut contents.

2.3. Toxicity and Growth-Inhibitory Bioassay against Earthworm A. caliginosa. Tests were conducted to determine the toxicity of the selected pesticides to mature earthworms. Procedures used were based on those described by Heimbach [24]. Buprofezin and triflumuron were tested at 50, 100, 150, 200, and 300 mg active ingredient, a.i/kg artificial soil, while lufenuron was tested at 0.5, 1, 5, 10, and 25 mg/kg soil because it caused 100% mortality at concentration of 50 mg/kg soil. The artificial soil was prepared using 70% sand, 20% kaolin clay, and 10% sphagnum peat moss, and the pH was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$. The moisture content was adjusted to 35% of the final weight. Four buckets were used per dosage of each pesticide, and four controls were used per pesticide. Mature individuals weighting between 0.6 and 0.7 g were selected. Ten prewashed and ventilated mature earthworms were then introduced into each container and placed in an incubation chamber at a temperature of $23 \pm 2^\circ$C with a 12 : 12 photoperiod. The controls were prepared in a similar way except that only water was added to the soil. Lost moisture was replaced during the 48 h assessment on a lost weight basis. The buckets were weighted after each assessment, and the lost weight was replaced with distilled water. Mortality was counted every week by washing away the artificial soil, and toxicity was calculated for all tested pesticides. Earthworms were regarded as dead when they did not react to a mild mechanical stimulus. Individuals who died during earlier assessments were also regarded as dead for the assessments thereafter. The weights of each earthworm was determined after 1, 2, 3, and 4 weeks of exposure and compared with that of parallel controls. Such weight determination was performed using four replicates for each treatment and parallel control, with the other replicates being used for biochemical studies. The weight of earthworms in each dose group reported from the various exposure periods were then used to compute the growth inhibition as follows:

$$\text{growth inhibition (\%)} = \left(\frac{C_L - T_L}{C_L}\right) \times 100, \quad (1)$$

where C_L is the mean larval weight (g) in the control and T_L is the mean larval weight (g) in the treatment.

2.4. Biochemical Studies. Two grams of treated or untreated worms was removed from the artificial soil, stored for 3 h in Petri dishes on damp filter paper, and placed in the dark at $23 \pm 2^\circ$C to void gut contents. Definite weight of larvae was homogenized in 3 mL of potassium phosphate buffer (pH 7.0) using a glass/Teflon homogenizer on ice. The homogenates were centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was used in protein determination and as the crude enzyme extract. Biochemical constituents were determined after 2 and 4 weeks of exposure. The final supernatant was subjected to protein, acetylcholinesterase (AChE), and glutathione S-transferase (GST) assays. All of the experiments were done in triplicate. Protein concentrations were determined by the Lowry method [25]. Activity of acetylcholinesterase (AChE) was determined by the colorimetric method of Ellman et al. [26], using ATChI (0.075 M) as substrate. The specific activity of AChE was expressed as $\Delta OD_{412} \cdot min^{-1} \cdot mg\ protein^{-1}$. Glutathione S-transferase (GST) activity was measured as described by Saint-Denis et al. [27], using 1-chloro-2,4-dinitrobenzene

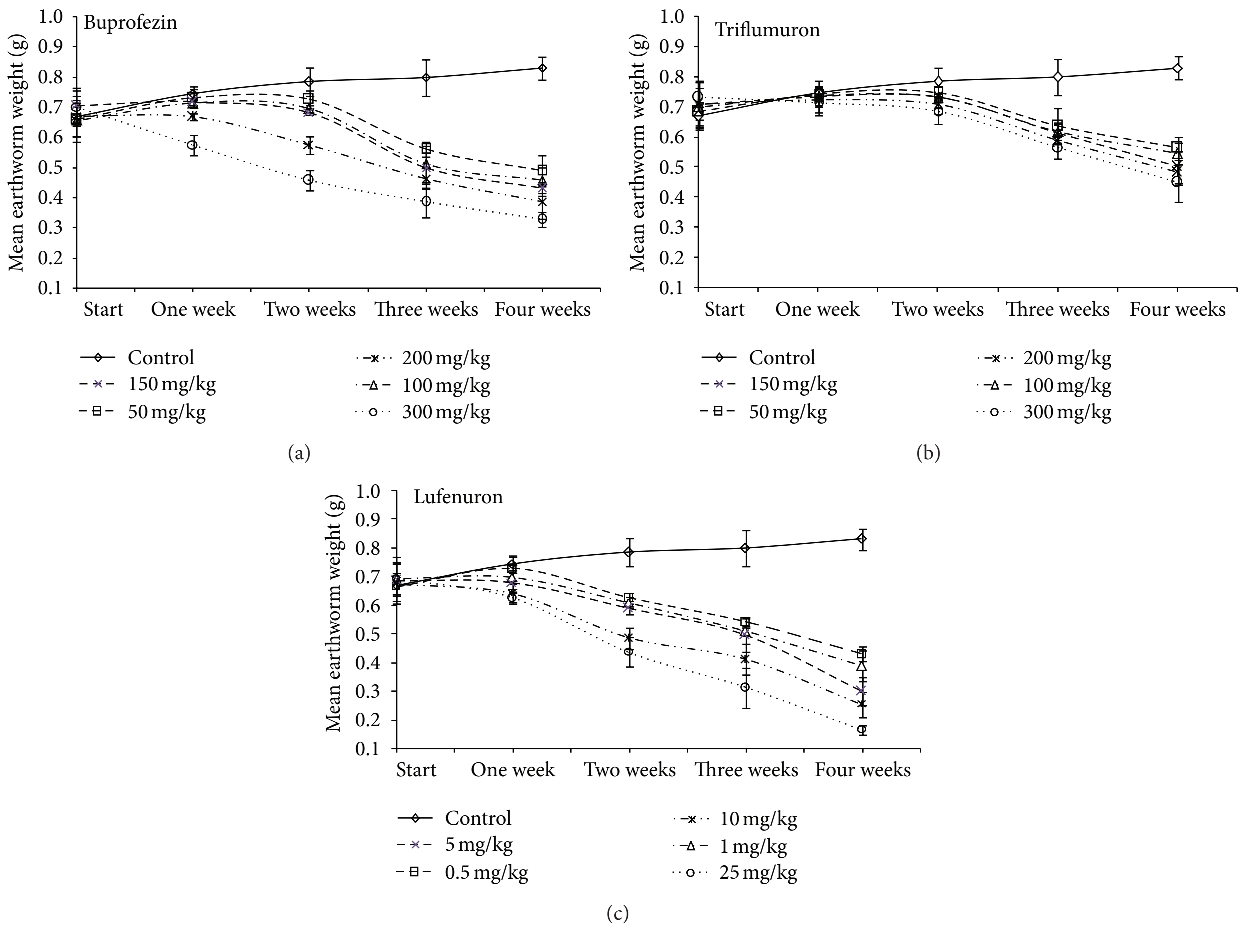

Figure 1: Mean weight ± SD of earthworms *A. caliginosa* in artificial soil treated with buprofezin, triflumuron, and lufenuron at different application rates in the laboratory. Growth weight measured when experiment started and 1, 2, 3, and 4 weeks after treatment.

(CDNB) as substrate. The specific activity was expressed as $OD_{340}\cdot min^{-1}\cdot mg\ protein^{-1}$. I_{50}, the concentration producing 50% inhibition of enzyme activity was calculated according to probit analysis [28].

2.5. Statistical Analysis. All quantitative estimations of toxicity parameters were based on four replicates, and the values were expressed as mean ± standard error. The probit analysis [28] was conducted using SPSS 12.0 software (Statistical Package for Social Sciences, USA). A regression was made of mortality against concentration and the lethal concentration caused 50% mortality (LC_{50}) was obtained. The log dose-response curves also allowed determination of the I_{50} values for the enzyme activity. The 95% confidence limits for the range of LC_{50} and I_{50} values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration. The data on growth weight and enzyme activity were each subjected to one-way analysis of variance (ANOVA) using Duncan's test post hoc pairwise multiple comparison procedure for contrast of the differences among treatment means. The results were expressed as means ± SE. Results with $P < 0.05$ were considered significant with all statistical significance. Univariate analyses were performed to determine the individual contribution of each response to the global response. When a significant effect was observed, post hoc comparison (LSD test) was carried out to test for differences between the exposed and control groups.

3. Results

3.1. Effect of Buprofezin, Triflumuron, and Lufenuron on Growth Changes of Earthworm A. caliginosa. A comparative analysis of weight growth change in the earthworms *A. caliginosa* exposed to 0, 50, 100, 150, 200, and 300 mg a.i of buprofezin and triflumuron/kg artificial soil and 0, 0.5, 1, 5, 10, and 25 mg of lufenuron/kg in relation to different exposure times (1, 2, 3, and 4 weeks) is presented in Figure 1. Under the experimental conditions used here, the mean weight of the individual worms in control increased significantly from 0.670 to 0.830 g after 4 weeks of exposure. However, the earthworms exposed to the artificial soil treated with insecticides decreased in their mean weight. The general tendency of the results showed that the decrease in weight in all pesticide-treated worms was dose-dependent over the 28-day exposure period with increasing concentration. The most prominent decrease was observed after two weeks of the exposure with the three IGRs while lufenuron was the

Table 1: ANOVA results on the weight growth change responses of mature earthworms (*A. caliginosa*) exposed to buprofezin, triflumuron, and lufenuron contaminated artificial soil.

Compound	Dose			Duration			Dose × duration		
	df	F	P	df	F	P	df	F	P
Buprofezin (A)	5, 72	71.90	<0.0001	3, 72	73.89	<0.0001	15, 72	6.14	<0.0001
Triflumuron (B)	5, 72	20.63	<0.0001	3, 72	49.49	<0.0001	15, 72	4.08	<0.0001
Lufenuron (C)	5, 72	91.02	<0.0001	3, 72	107.81	<0.0001	15, 72	8.10	<0.0001
A ∗ B ∗ C	10, 216	87.85	<0.0001	3, 72	255.87	<0.0001	30, 72	8.89	<0.0001

df: degree of freedom; F: F-max of Hartley; $P < 0.05$ (significance of the F ratio).

Table 2: Toxicity of buprofezin, triflumuron, and lufenuron on mature earthworms (*A. caliginosa*) 28 days after treatment.

Compound	LC_{50}[a] (mg a.i/kg) ± SE	95% confidence limits (mg/kg)		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]	Toxicity index[e]
		Lower	Upper				
Buprofezin	421.41 ± 31.76	298.38	851.62	1.25 ± 0.24	−3.28 ± 0.53	1.53	0.44
Triflumuron	476.97 ± 50.61	353.56	822.11	1.78 ± 0.29	−4.77 ± 0.65	2.58	0.39
Lufenuron	1.87 ± 0.45	0.59	3.96	1.06 ± 0.10	−0.29 ± 0.0.08	9.08	100

[a]The concentration causing 50% mortality of the earthworm. Results of LC_{50} are expressed as mean of four replicates ± standard error.
[b]Slope of the concentration-mortality regression line ± standard error.
[c]Intercept of the regression line ± standard error.
[d]Chi-square value.
[e]Toxicity index = (LC_{50} level for the most effective pesticide/LC_{50} level for the other pesticide) × 100.

most active, followed in the descending order by buprofezin and then triflumuron. High inhibition of the earthworms growth was found with high concentrations of lufenuron and buprofezin. This was most prominent in the period between the second and fourth weeks of treatment. During the first week of exposure, there was no significant difference between the mean weights of the earthworms cultured in lufenuron-treated soil at all concentrations tested and the control. However, the mean weight was significantly decreased after two weeks of the treatment reaching to 42% inhibition at 25 mg a.i/kg soil. After four weeks of exposure, 0.327, 0.448 and 0.166 g/worm were found at the highest dose of buprofezin, triflumuron, and lufenuron, respectively. This decrease corresponded to 61, 46, and 81%, respectively, of the control value. Post hoc comparisons using Duncan's test showed that the effects of different dose rates of buprofezin, triflumuron, and lufenuron had a high significance difference ($P < 0.000$) compared to the untreated earthworms at time duration exposures (Table 1). In addition, there are high significant differences ($P < 0.0001$) in the statistical analysis by two-way interaction between dose and duration of the exposure with buprofezin (df = 15.72; F = 6.14); triflumuron, (df = 15.72; F = 4.08), and lufenuron (df = 15.72; F = 8.10).

The effect of pesticide exposure on mortality was investigated in order to evaluate which substance was the most hazardous to the earthworms. The LC_{50} values of the tested pesticides after four weeks of exposure are shown in Table 2 with the corresponding 95% confidence limits and slopes of the toxicity lines. Results showed that lufenuron was the most toxic pesticide (LC_{50} = 1.87 mg a.i/kg soil), followed in the descending order by buprofezin and triflumuron (LC_{50} = 421.41 and 476.97 mg a.i/kg soil, resp.). It can be noticed that buprofezin and triflumuron are not significantly different in their toxicity according to their 95% confidence intervals. From comparing the efficacy of lufenuron while the other two pesticides according to their toxicity indices at LC_{50}, it was found that lufenuron has toxicity index = 100, with buprofezin and triflumuron having toxicity indices of 0.44 and 0.39, respectively, compared to lufenuron.

3.2. Inhibitory Effects of Buprofezin, Triflumuron, and Lufenuron on AChE and GST Activities. An overall picture of the AChE activity of earthworms exposed to buprofezin, triflumuron, and lufenuron is given in Table 3, and the statistical analysis data are indicated in Table 4. The result revealed that the specific activity in the control was significantly increased with exposure time, whereas specific activities 10.23 and 39.83 were found after two and four weeks, respectively. However, significant differences ($P < 0.05$, ANOVA, Duncan's test) were found among the AChE activity of exposure two and four weeks at all tested concentrations compared to the control except with the lowest concentration (0.5 mg/kg artificial soil) of triflumuron that showed 9.53 compared to 10.23 in the untreated earthworms after two weeks of exposure (Table 3). The lowest AChE activity (1.14) was found at the highest concentration of buprofezin (300 mg/kg soil) after two weeks of treatment compared to 10.23 in the control. However, the lowest activity (0.71) was recorded at 25 mg/kg soil of lufenuron after four weeks of exposure compared to 39.83 in the control. Examining the duration of exposure, it can be noted that the enzyme activity among the treatments increased with the time except with lufenuron. AChE activity was strongly inhibited by lufenuron followed by buprofezin, and then triflumuron in descending order. In addition, the effect of the exposure duration showed significant effect. This finding was confirmed by calculating the concentration that caused 50% enzyme inhibition (I_{50}) yielding values of 15.24, 45.04, and 84.49 mg/kg soil for lufenuron, buprofezin,

Table 3: Mean AChE specific activities ± SE measured in mature earthworms (*A. caliginosa*) after exposure to different concentrations of buprofezin, triflumuron, and lufenuron for one, two, and four weeks.

Pesticide	Concentration (mg a.i/kg soil)	Specific activity ± SE ($\Delta OD_{412}\cdot min^{-1}\cdot$mg protein^{-1}) 2 weeks	4 weeks	I_{50} with 95% confidence limits (mg a.i/kg soil) 2 weeks	4 weeks
	0 (Control)	$10.23^{a} \pm 0.337$	$39.83^{a} \pm 0.481$		
Buprofezin	50	$4.71^{d} \pm 0.190$	$13.22^{c} \pm 0.789$	45.04 (25.24–61.70)	24.74 (10.17–53.05)
	100	$3.28^{e} \pm 0.128$	$12.34^{cde} \pm 1.487$		
	150	$2.85^{e} \pm 0.076$	$10.15^{efg} \pm 0.164$		
	200	$2.08^{f} \pm 0.146$	$7.91^{gh} \pm 0.828$		
	300	$1.14^{g} \pm 0.038$	$3.40^{ik} \pm 1.684$		
Triflumuron	50	$6.51^{c} \pm 0.321$	$16.62^{b} \pm 0.248$	84.49 (57.01–107.76)	28.77 (5.68–50.76)
	100	$4.49^{d} \pm 0.219$	$12.65^{cd} \pm 0.273$		
	150	$3.57^{e} \pm 0.286$	$10.81^{def} \pm 0.093$		
	200	$3.39^{e} \pm 0.177$	$9.68^{fg} \pm 0.283$		
	300	$3.13^{e} \pm 0.448$	$7.33^{h} \pm 1.172$		
Lufenuron	0.5	$9.53^{a} \pm 0.326$	$10.44^{def} \pm 1.375$	15.24 (10.37–25.98)	0.077 (0.015–0.184)
	1	$8.19^{b} \pm 0.413$	$6.30^{h} \pm 0.641$		
	5	$6.44^{c} \pm 0.167$	$3.70^{i} \pm 0.741$		
	10	$5.27^{d} \pm 0.235$	$1.20^{kl} \pm 0.171$		
	25	$4.97^{d} \pm 0.426$	$0.71^{l} \pm 0.499$		

Results are expressed as mean ± standard error ($n = 3$). Values followed by the same letter within a column are not significantly different ($P \leq 0.05$) according to Duncan's test. a.i. is active ingredient of the tested pesticides.

Table 4: ANOVA results on the acetylcholinesterase (AChE) responses of mature earthworms (*A. caliginosa*) exposed to buprofezin, triflumuron, and lufenuron contaminated artificial soil.

Compound	Dose df	Dose F	Dose P	Duration df	Duration F	Duration P	Dose × duration df	Dose × duration F	Dose × duration P
Buprofezin (A)	5, 24	229.63	<0.0001	1, 24	575.95	<0.0001	5, 24	82.99	<0.0001
Triflumuron (B)	5, 24	538.65	<0.0001	1, 24	1787.17	<0.0001	5, 24	217.88	<0.0001
Lufenuron (C)	5, 24	415.78	<0.0001	1, 24	76.78	<0.0001	5, 24	260.55	<0.0001
A ∗ B ∗ C	10, 72	511.72	<0.0001	1, 72	967.46	<0.0001	10, 72	241.744	<0.0001

df: degree of freedom; F: F-max of Hartley; $P < 0.05$ (significance of the F ratio).

and triflumuron, respectively, after two weeks of exposure. These values were significantly decreased after four weeks of exposure reaching 0.077, 24.74, and 28.77 mg/kg soil for lufenuron, buprofezin, and triflumuron, respectively. Post hoc comparisons using Duncan's test showed that the inhibitory effects of different rates of the three IGRs were highly significant compared to the control at two duration exposures (Table 4), that is, buprofezin ($F = 575.95$; $P < 0.0001$), triflumuron ($F = 1787.17$; $P < 0.0001$), and lufenuron ($F = 76.78$; $P < 0.0001$). Moreover, there are highly significant differences in the statistical analysis by two-way interaction between dose and duration of the exposure with the three IGRs (buprofezin, $F = 82.99$; $P < 0.0001$; triflumuron, $F = 217.88$; $P < 0.0001$; and lufenuron $F = 260.55$; $P < 0.0001$).

The specific activity of glutathione S-transferase (GST) in earthworms exposed to different concentrations of buprofezin, triflumuron, and lufenuron for 4 weeks is presented in Table 5 and the statistical analysis data are shown in Table 6. Generally, a clear dose-response relationship was obtained with statistically significant differences ($P < 0.05$, ANOVA, and Duncan's test) between all data points and the control after two and four weeks of exposure. The result revealed that the GST activity increased with the time of exposure with untreated value of 6.19 and 8.38 found in after two and four weeks of experiment, respectively. It can be noted that the high inhibition was found at high concentration after four weeks of exposure. This decrease is corresponded to 74.94, 68.22, and 90.69% inhibition for buprofezin, triflumuron (at 300 mg·kg soil), and lufenuron (25 mg·kg soil), respectively. Stronger inhibition was achieved in the earthworms exposed to lufenuron compared to buprofezin and triflumuron. This was confirmed by calculating the I_{50} values that were found to be 3.65, 105.22, and 155.28 mg/kg soil for lufenuron, buprofezin, and triflumuron, respectively, after two-week exposure, whereas $I_{50} = 0.36$, 60.77, and 86.27 mg/kg soil, respectively after four weeks (Table 5). Post hoc comparisons using Duncan's test showed that the inhibitory effects of

Table 5: *In vivo* effect of different application rates of buprofezin, triflumuron, and lufenuron on GST activity in earthworm after 2, 4, weeks of treatment.

Pesticide	Concentration (mg a.i/kg soil)	Specific activity ± SE ($OD_{340}\cdot min^{-1}\cdot mg\ protein^{-1}$)		I_{50} with 95% confidence limits (mg a.i/kg soil)	
		2 weeks	4 weeks	2 weeks	4 weeks
	0 (Control)	$6.19^{a} \pm 0.38$	$8.38^{a} \pm 0.07$		
Buprofezin	50	$3.75^{c} \pm 0.21$	$4.36^{c} \pm 0.08$	105.22 (72.82–136.45)	60.77 (27.25–86.43)
	100	$3.17^{cde} \pm 0.17$	$3.57^{d} \pm 0.15$		
	150	$2.89^{def} \pm 0.05$	$3.18^{def} \pm 0.05$		
	200	$2.58^{efg} \pm 0.17$	$2.77^{ef} \pm 0.15$		
	300	$1.79^{h} \pm 0.26$	$2.10^{g} \pm 0.18$		
Triflumuron	50	$5.13^{b} \pm 0.31$	$5.26^{b} \pm 0.19$	155.28 (132.04–185.630	86.27 (55.34–112.22)
	100	$3.59^{cd} \pm 0.38$	$3.63^{d} \pm 0.14$		
	150	$2.86^{def} \pm 0.14$	$3.24^{de} \pm 0.27$		
	200	$2.60^{efg} \pm 0.14$	$2.95^{ef} \pm 0.26$		
	300	$2.30^{fgh} \pm 0.33$	$2.66^{f} \pm 0.08$		
Lufenuron	0.5	$4.84^{b} \pm 0.25$	$4.12^{c} \pm 0.31$	3.65 (1.15–11.40)	0.36 (0.15–0.64)
	1	$3.50^{cd} \pm 0.13$	$2.85^{ef} \pm 0.15$		
	5	$2.70^{efg} \pm 0.04$	$1.65^{gh} \pm 0.20$		
	10	$2.46^{efgh} \pm 0.06$	$1.45^{h} \pm 0.28$		
	25	$1.96^{gh} \pm 0.31$	$0.78^{i} \pm 0.06$		

Results are expressed as mean ± standard error ($n = 3$). Values followed by the same letter within a column are not significantly different ($P \leq 0.05$) according to Duncan's test.

Table 6: ANOVA results on the glutathione S-transferase (GST) responses of mature earthworms (*A. caliginosa*) exposed to buprofezin, triflumuron, and lufenuron contaminated artificial soil.

Compound	Dose			Duration			Dose × duration		
	df	F	P	df	F	P	df	F	P
Buprofezin (A)	5, 24	201.60	<0.0001	1, 24	38.01	<0.0001	5, 24	8.304	<0.0001
Triflumuron (B)	5, 24	107.96	<0.0001	1, 24	15.72	0.001	5, 24	5.15	0.002
Lufenuron (C)	5, 24	197.91	<0.0001	1, 24	9.94	0.004	5, 24	17.10	<0.0001
A ∗ B ∗ C	10, 72	238.08	<0.0001	1, 72	2.28	0.135	10, 72	14.61	<0.0001

df: degree of freedom; F: F-max of Hartley; $P < 0.05$ (significance of the F ratio).

different rates of the three IGRs were significant compared to the untreated earthworms at two duration exposures (Table 6); that is, buprofezin ($F = 38.01$; $P < 0.0001$), triflumuron ($F = 15.72$; $P < 0.0001$), and lufenuron ($F = 9.94$; $P < 0.0001$). The effect of the type of pesticide was highly significant with the three IGRs, suggesting again the exposure to pesticides for four weeks resulted in significant decrease of the GST activity.

4. Discussion

Growth inhibition can be a good indicator of chemical stress, which may link chemical effects to energy dynamics and ultimately inhibit growth of the tested organisms. The slight increase of control earthworms' weight suggested that the soil nutrients were just sufficient to sustain the survival of earthworms, but insufficient to allow for additional growth. The effect of buprofezin, lufenuron, and triflumuron on growth inhibition was in agreement with those reported on other pesticides. For example, a dose-dependent decrease was observed in the growth of *Eisenia fetida* exposed to dieldrin at several sublethal concentrations [14, 29]. A significant growth inhibition on the earthworm *Lumbricus rubellus* was also seen when exposed to PAH pyrene [30]. Both lindane and deltamethrin inhibited earthworm growth, and this is possibly correlated with an earthworm's strategy for natural survival: reducing food intake to avoid the toxins. This strategy is commonly used in earthworms to avoid poisoning not only with heavy metals [31] but also organic chemicals such as pesticides [7, 32]. Similar body adjustments were observed with the isopod *Porcellio dilatatus*, with reduction of consumption rates when exposed to high doses of endosulfan, significant effects on their other feeding parameters (food assimilation rates and efficiency), and finally inhibition of their growth [33].

Mosleh et al. [34] reported that chlorfluazuron at 107 mg/kg soil (LC_{25}) reduced the growth rate of mature *A. caliginosa* earthworms and showed a negative $\log(e) - 0.39$ after 4 weeks of exposure compared to the control that had a positive $\log(e) + 0.34$. It should also be noted that although buprofezin, triflumuron, and lufenuron were the pesticides least toxic to the earthworms, they caused the highest reduction in worm growth rate (Figure 1). This could be attributed to the high persistence of these pesticides in soil or to the slow degradation in the worms and subsequently less elimination of the metabolites. It also could indicate feeding inhibition with the earthworm regulating pesticide intake by reducing consumption and therefore probably affecting growth. This latter strategy was shown to be commonly used to avoid poisoning with heavy metals and pesticides. Martin [35] studied the toxic effect of certain herbicides (amitrole plus ammonium, 2,2-DPA, trifluralin, glyphosate, propazine, and simazine) at 1, 10, and 100 mg/kg artificial soil against earthworms, whereas mortality was observed only at 100 mg/kg soil of trifluralin. Surviving worms in the other treatments lost weight in 100 mg/kg artificial soil after 7 days.

Despite the low toxicity of the tested IGRs (Table 2) compared to the toxicity of other pesticides from the literature, buprofezin and lufenuron had high inhibition effect after 4 weeks of exposure. These results agree with those of Mosleh et al. [34], who found that the LC_{50} of chlorfluazuron as IGR against mature *A. caliginosa* earthworms = 140 mg/kg soil compared to other pesticide classes such as aldicarb (LC_{50} = 0.68 mg/kg soil), cypermethrin (LC_{50} = 73 mg/kg soil), and profenofos (LC_{50} = 127 mg/kg soil). However, they found that atrazine and metalaxyl were less toxic (LC_{50} = 381 and 518 mg/kg soil, resp.) to mature *A. caliginosa* earthworms than chlorfluazuron. In fact, earthworm mortality can be used as a reliable indicator of environmental pollution especially for pesticides. Kokta [36] reported that the pesticides with LC_{50} value higher than 1000 mg/kg were harmless to earthworms in the field. This finding also confirms that the LC_{50} values obtained in our study (1.87, 421, and 476 for lufenuron, buprofezin, and triflumuron, resp.) indicate that the three IGRs tested are harmful for microorganisms in soil. In addition, high doses of these insecticides can lead to a decrease in earthworm populations and to changes in the soil ecosystem. These data also indicated that such pesticides at their lower doses have very little effect on earthworm mortality. Wang et al. [15] investigated the contact filter paper and soil toxicity bioassays of 24 insecticides belonging to six chemical categories (neonicotinoids, antibiotics, IGRs, pyrethroids, carbamates, and organophosphates) on the earthworm *E. fetida*. The results showed a different pattern of toxicity except that neonicotinoids were the most toxic even under the soil toxicity bioassay system. The acute toxicity of neonicotinoids was higher than those of antibiotics, carbamates, IGRs, and organophosphates while in contrast, pyrethroids were the least toxic to the worms under the soil toxicity bioassay system.

The inhibition of AChE and GST in earthworms has been established for a number of species, including *A. caliginosa* [13, 20, 33, 37]. With some variation, most of the concentrations used in our study resulted in a time-related response of specific activity in the earthworms (Table 3). The differences between the experimental control and the treatments were statistically significant ($P < 0.05$) for all pesticides, indicating that the duration of exposure had an effect on the depression of enzyme activity, even at low concentrations. IGRs were used to control insects on different crops; therefore, these pesticides present in the soil after application and can reach and affect nontarget organisms such as earthworms. As the results of the present study, with a duration of four weeks, indicated a time-related effect of the pesticides, it seems reasonable to expect a chronic effect on these nontarget organisms in the field. It is also known that a low enzyme activity could be associated with abnormal behavior, which could influence feeding and growth rate as shown in Figure 1.

The effects on AChE and GST were also not reversed during the observation period, and it could be, as is known for higher organisms, that recovery of the enzyme activity requires days or even weeks [38]. The persistence of AChE inhibition in earthworms has been investigated before. Stenersen [39] observed a depression of activity by OP compounds, with a recovery to normal levels taking more than 50 days. In study by Booth and O'Halloran [19] who also found the enzyme to be continuously depressed with time and any recovery to be prolonged. It can be said that the toxicity test may be helpful for screening of pesticides especially sublethal effects on AChE and GST activities of earthworms and may aid in understanding ecotoxicological impacts on earthworms.

5. Conclusion

buprofezin, triflumuron, and lufenuron as IGRs were toxic or even lethal to earthworms under acute exposure. The toxic potential of lufenuron was greater than that of buprofezin and triflumuron after four weeks of exposure. All pesticides exerted significant effects on earthworm growth and enzymes activity. The reduction in the growth rate of pesticide-treated earthworms (*A. caliginosa*) was accompanied by a decrease in AChE and GST activities. These results suggest that changes in weight growth and enzyme activity during subtle exposure periods may be sensitive parameters in order to assess the extent of injury caused by pesticides, since they provide early warning responses at sublethal doses and are close related to the naturally occurring ones in the environment. Thus, the determination of growth rate and specific activities of certain enzyme systems in earthworms could result in their use as biomarkers for soil contaminated with pesticides. Earthworms are very beneficial to man and the environment; hence, the present observations on the ecophysiological toxicology may be helpful for protection and conservation of earthworm bioresources. In addition, the results obtained help to overcome a lack of ecotoxicological data on IGRs under tropical conditions, but more tests with different soil invertebrates are needed to improve pesticides risk analysis.

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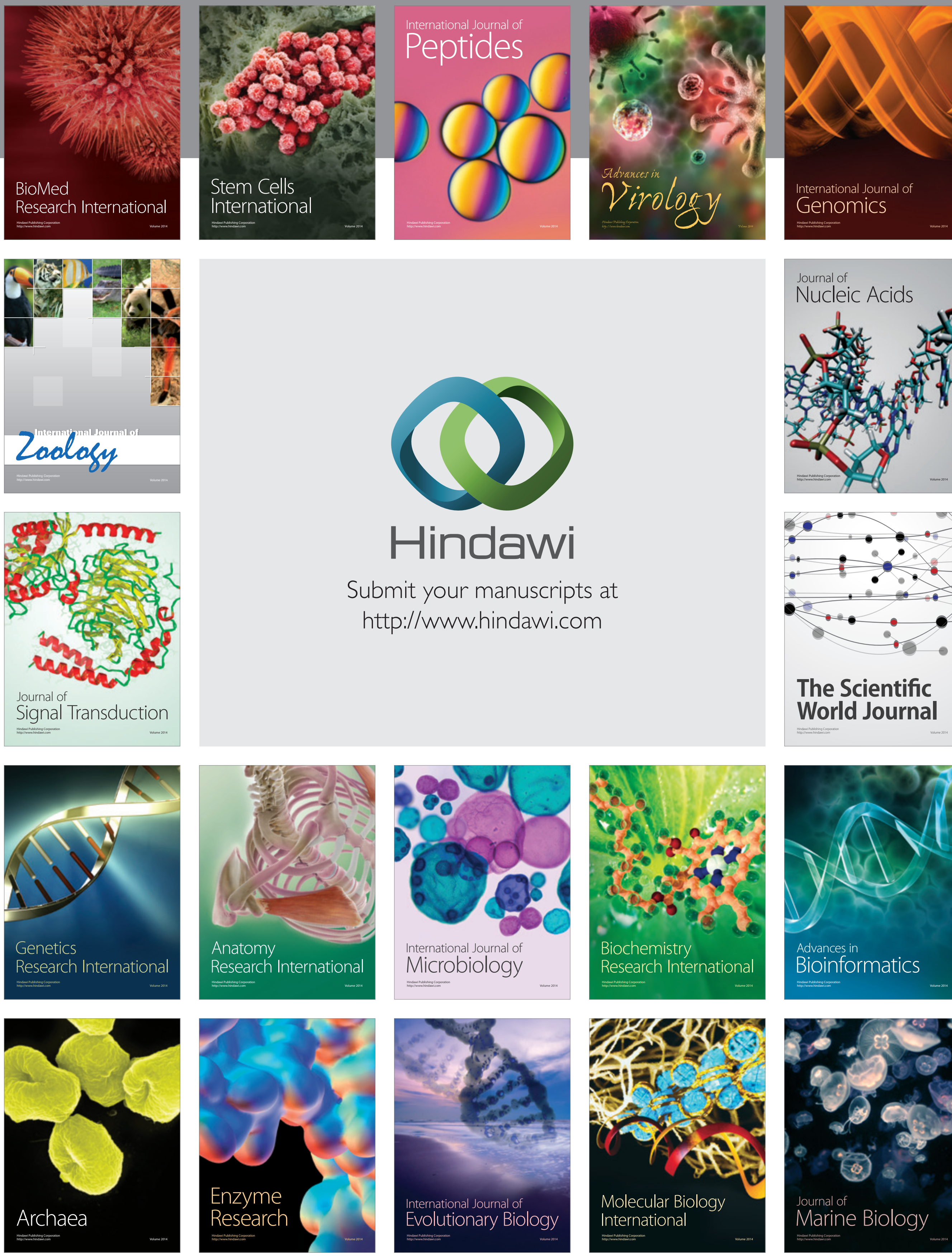
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