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Acute toxicity of two dry glyphosate-based herbicide formulations (Water Dispersible Granules and Solubles Granules) on the earthworm *Eudrilus eugeniae,* KINBERG, 1867 (Oligochaeta, Eudrilidae)

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ABSTRACT: The dry formulations of agrochemicals reduce the difficulties related to transport and exposure of users. Nevertheless, these products do not exclude drawbacks and their use requires a deeper knowledge of their toxicological and ecotoxicological effects. The purpose of this work was to study the acute toxicity and reproductive toxicity of glyphosate-based RAPID MAX 750 WG and TAKO-KELE 757 SG on the earthworm *Eudrilus eugeniae,* ubiquitous in tropical area. Lethality tests were conducted according to EPS1/RM/43, MA.500-VTL1.0, and OECD guideline 207 Methods. LC50 of RAPID MAX were (1687.56±50.53 mg of glyphosate (a.e.) /kg of soil (DW) for adult earthworms and 1416,99±53,06 mg of glyphosate (a.e.) /kg of soil (DW) for juvenile earthworms. LC50 of TAKO-KELE 757 SG were 2215.66±61.83 mg of glyphosate (a.e.) /kg of soil (DW) and 1658.62±38.72 mg of glyphosate (a.e.) /kg of soil (DW), respectively for adult and juvenile earthworms. Juvenile and adult earthworms were more sensitive to RAPID MAX 750 WG compared with TAKO-KELE 757 SG, as LC50 related to RAPID MAX 750 WG were lower than those of SG TAKO-KELE 757 SG. The acute toxicity (lethality) of both herbicides was more severe on juvenile earthworms.

KEYWORDS: Glyphosate-based herbicide, Dry formulations, lethality, *Eudrilus eugeniae,* Earthworm.

1 INTRODUCTION

Glyphosate-based herbicides (GBH) owe their reputation to their effectiveness on most monocotyledonous and dicotyledonous weed species. Their active ingredient, glyphosate, is systemic and inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the synthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) that are essential in the metabolism and natural defense of plants against abiotic and biotic stressors [1,2]. The efficacy of glyphosate herbicides coupled with the expiration of MONSANTO's patent in 2000 as well as the development of glyphosate-resistant crops have made glyphosate the most widely used herbicide in the world [3-7]. Like other pesticides, the use of GBH requires knowledge of their deleterious effects on non-target soil organisms such as termites, millipedes, dung beetles, earthworms, bacteria that are the engineers of the soil [8-10]. The health risk assessment of pesticides in the European Union and the United States is substantially focused on the active ingredients because access to the complete composition of these products is almost impossible due to the confidentiality imposed by the commerce law [12]. In most countries, pesticide toxicity tests follow the guidelines of the Organization for Economic Cooperation and Development (OECD), in order to standardize and make pesticides more profitable, to facilitate international trade, and to limit the number of animals subjected to toxicity tests. This situation leads to the underestimation of the real toxicity of plant protection products, which, apart from the active ingredient, contain many adjuvants such as activators and spray modifiers that are all potentially harmful [11-15]. Therefore, in the case of GBH several studies agree that the main surfactant of GBH, polyoxyethylene tallowamine (POEA) [16-19]. In addition to the inherent toxicity, these adjuvants might interact with each other or with the active ingredient to generate more toxic effects in the case of synergy or potentiation.

In view of the toxicity of co-formulants, current global trends in formulation technology attempt to eliminate petroleum-based solvents when possible, to develop water-based formulations such as suspension concentrates (SC) and oil-in-water (EW) emulsions, and to replace dusty powders, and sometimes liquids with water dispersible (WG) and water-soluble granules (SG). These changes eliminate the problem of flammability during transport and storage and in many cases, reduce the dermal toxicity of the product during handling [20, 21]. Nevertheless, these formulations do not exclude environmental hazards; hence, their toxicity deserves to be evaluated to avoid ecological imbalance and erosion of soil fertility [22, 16]. For this purpose, toxic endpoints can include mortality, behavior, reproductive status or physiological and biochemical changes. In addition, as environmental factors such as temperature and type of soil are likely to impact of pesticides toxicity, toxicological tests must be performed under environmental conditions close to those of the areas these agrochemicals are intended for [23-25].

Among the candidate species, earthworms play key role, on the one hand because of their function as ecosystem and because of their great sensitivity to polluting substances. Indeed, earthworms contribute to soil fertilization stimulating the bacteria involved in the degradation of the soil lignin generated by plants. They accelerate the degradation of organic matter and the bioavailability of minerals necessary for plant development [26, 27]. Earthworms also improve the permeability and aeration of the soil through the multiple galleries excavated by these invertebrates so that the biomass and the intensity of earthworms' activity in the drilosphere is an indication of the soil health [28]. The aim of this work was to evaluate the acute and reproductive toxicity of two dry glyphosate-based herbicide (GBH) formulations (WG and SG) on "African nightcrawler" *Eudrilus eugeniae,* an epigeous earthworm that is ubiquitous in tropical areas [29-31]. The study focused on the effect of the whole formulations (active ingredients + adjuvants) instead of the ingredients alone (glyphosate). The outcome of this study could broaden our knowledge of the effects of GBH, and help in the choice of the less harmful type of formulation in a context of the soil quality preservation.

2 Material And Methods

2.1 Experimental Site

The experiments were completed in the locality of Azaguié-Ahoua, located in the forest zone 40 km northeast of Abidjan (5°38'21" N and 4°3'20" W). The climate of the area is a sub-equatorial characterized by the abundance of rainfall and a relatively constant temperature around 27°C [32, 33]. Meteorological data from 1996 to 2013 indicate that the climate regime is characterized by the existence of 4 seasons during the year, including two dry seasons and two rainy seasons. The long dry season lasts 3 to 5 months covering december, january and february. The short dry season cover september and november. The area belongs to a region of high agricultural activity. Cash crops such as rubber, banana, cocoa and mangosteen are abundant and pesticides are used for crops protection. However, this use of pesticides is very limited due to the low income of the farmers.

2.2 Herbicidal Products

Water dispersible granules (WG) and water soluble granules (SG) formulations were used. For the WG formulation, the commercial product was RAPID MAX 750 WG (75% ammonium salt of glyphosate, GBH-A) while for the SG formulation, the agrochemical was TAKO-KELE 757 SG (75.7% ammonium salt of glyphosate, GBH-B). Both products were registered in Côte d'Ivoire under the registration numbers 111053 He (GBH-A) and 161834 He (GBH-B). These herbicides were previously submitted to Central Laboratory of Agrochemistry and Ecotoxicology for formulation control test. The two formulations were stable and their glyphosate contents were in accordance with that declared on the packaging.

2.3 Determination Of The Physico-Chemical Parameters Of The Test Media

The soil used in the preparation of the test media was taken at the depth of 0-10 cm, on fallow parcel. After drying, the physico-chemical properties of the soil were determined before incorporation into the test media. Soil organic matter content was determined by using Nabertherm muffle furnace (Lilienthal, Germany), according to the method MA. 1010-PAF 1.0 [34,35]. Soil pH (water) was determined following the method MA. 205-pH 1.0 [36], using Hach HQ40d multimeter (Loveland, USA). The water retention capacity of the soil was determined according to ISO 11268-2: 1998 [37]. Conductivity was measured using the diluted extract method [38]. Soil particle size was determined through washing and sieving using an electronic sieve Retsch AS200 (Eragny, France). Temperature and hygrometry of the test media were monitored using BOECO thermo-hygrometer (Hamburg, Germany).

2.4 Acquisition Of Test Earthworms

Earthworms *Eudrilus eugeniae* were captured on the test site (Azaguié-Ahoua) through traps made of rabbit droppings. Individuals directly collected from the traps served as broodstock and were purified to the second generation before the tests. For this purpose, the earthworm rearing method was based on the guideline for chemical testing developed by OECD and the Centre d'Expertise en Analyse Environnementale du Québec [39, 40]. These protocols were adapted to our working conditions. The vivariums were made using transparent plastic tank (42 cm x 32 cm x 22 cm) to which dry sieved fallow soil and ground rabbit droppings were added. The bottoms and the covers of the tanks were finely perforated to allow aeration of the medium and drainage of excess water. Each vivarium contained 8 kg of dry soil at the bottom with 2 kg of rabbit droppings above the soil.

2.5 Lethality Tests

The lethality test method derived from those proposed by OECD guideline for testing chemicals, Environnement Canada (EPS1/RM/43 method) and the Centre d'Expertise en Analyse Environnementale du Québec (MA.500-VTL1.0 method) [39,41, 42]. Each of the test media was made of transparent plastic box (500 ml) to which 300 g of soil (dry fallow, sorted and sieved using 2 mm mesh sieves) and 27 g of dry and sieved crushed rabbit droppings, were added. Fine perforations were made on the covers to allow aeration of the medium. Tests were realized on 35 days old adult earthworms (1.15 ± 0.21 g body weight) and 7 days old juvenile earthworms (0.042 ± 0.001 g of body weight). Test related to GBH-A used 8 solutions of 1.34; 2.02; 2.69, 3.36; 5.38; 6.72; 10.09 and 13.45 g of glyphosate (a.e.) /l. Eight glyphosate (GBH-B) solutions of 1.82; 2.73; 3.63; 4.54; 7.27; 9.08; 13.63 and 18.17 g of glyphosate (acid equivalent) /l. The solutions were tested separately with 5 replicates for each solution and 3 earthworms per medium. For these two herbicides and for each category of earthworms (juvenile and adult), negative controls (medium without herbicide) were also constituted in 5 replicates with 3 earthworms per medium to evaluate the inherent toxicity of the medium. The volume of solution added to each medium during all tests was 150 ml; this corresponds approximately to the water retention capacity of the soil. Surviving earthworms were counted 14 days later. Each of these tests was repeated three times to obtain average values of lethal concentrations. The glyphosate (acid equivalent) content of the tested solutions and the soil were determined following equations 1 and 2.

$$[\mathrm{Gly_{a.e.}}]_1=[\mathrm{Gly_{am}}]\times(\mathrm{M_{ae}}/\mathrm{M_{am}}) \quad (1)$$

$$[\mathrm{Gly_{a.e.}}]_2=1000\times[\mathrm{Gly_{a.e.}}]_1\times \mathrm{V/m} \quad (2)$$

$[Gly_{a.e.}]_1$ is glyphosate (acid equivalent) content of test solution (g of glyphosate (a.e.) /l); $[Gly_{am}]$ is glyphosate (ammonium salt) of test solution (g/l), this concentration was obtained by diluting the GBH in water; M_{ae} is the molar weight of glyphosate (acid form) (g/mol); M_{am} is the molar weight of glyphosate (ammonium salt) (g/mol); $[Gly_{a.e.}]_2$ is glyphosate (acid equivalent) theoretical content of spiked substrate (mg of glyphosate (a.e.) /kg of soil (DW); V is the volume of solution used for spiking (l); 1000 is gram to milligram conversion factor and m is test substrate weight (kg).

2.6 Statistical Analysis

The Probit logistic regression model was used to analyze the acute toxicity data. Indeed, this model made possible the prediction of the percentages of mortality that the herbicide caused on the subjects as well as the concentration that could generate an x percentage of death. This logistic model was constructed using Xlstat 2019.4.2 software (Addinsoft). Concentration – response curves were drawn using Quest Graph™ LC50 Calculator [43]. For all tests statistical significance was set at $p<0.05$.

3 Results And Discussion

3.1 Physico-Chemical Parameters Of The Test Media

The initial soil organic matter content and pH were 5.31± 0.05 % and 5.67 ± 0.07 respectively. The water retention capacity of the soil was 42.09 ± 0.60 % and the conductivity was 116 ± 8.74 µS/cm. It was loamy sand with 72.17 ± 1.02 of sand, 26.05 ± 1.04 % of silt and 1.78 ± 0.11 % of clay. Average temperature and relative humidity were 27.85 ± 0.21 °C and 86.59 ± 0.69 %, respectively.

3.2 ACUTE TOXICITY OF GBHS ON ADULT EARTHWORMS

The number of the earthworms killed increased significantly when glyphosate concentration increased. The value of Chi-2 associated with -2Log less than 5 % (p<0.0001) indicates that the explanatory variable (glyphosate concentration) brought significantly information to the earthworm's mortality variability. Moreover, Chi-2 (p > Chi-2) probabilities associated with base 10 logarithm of glyphosate concentration (g/l) have been less than 5% (p < 0.0001). Thus, the base 10 logarithm of glyphosate concentration provides a significant amount of information on earthworm's mortality. Furthermore, the theoretical mortality (0 %) generated by the model has been inferior or equal to the mortality observed during all trials (0 %). Therefore, the death of the earthworms was only due to glyphosate-based herbicides. The base 10 logarithm of glyphosate concentration bring significant quantity of information to the binary variable (earthworms' death). The dose-response curves from the various trials reliably indicate that earthworm mortality was a function of the concentrations administered, with a 95 % confidence. **Figure 1** points out dose-response curves.

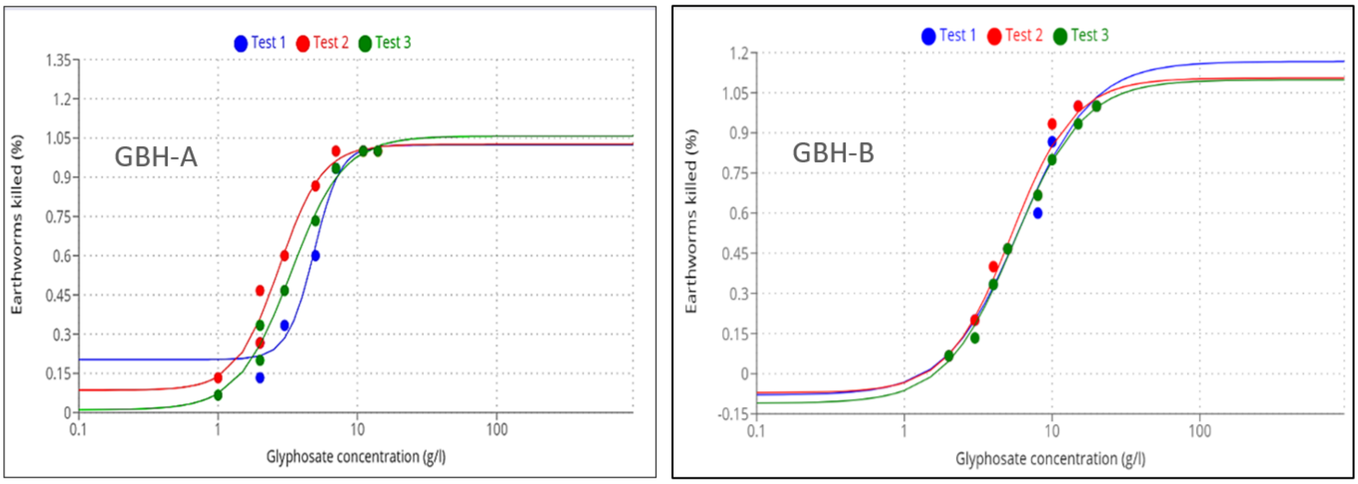

Fig. 1. Concentration-response curves of GBHs on adult earthworms

The lethal concentration (LC50) of GBH-A was 1687.56 ± 50.53 mg of glyphosate (a.e.) /kg of soil (DW) until lethal concentration GBH-B was 2215.66 ± 61.83 mg of glyphosate (a.e.) /kg of soil (DW) (**Table 1).**

Table 1. Statistical data regarding LC50 determination on adult earthworms

Type of formulation	Test	Chi-2		LC50 (mg of glyphosate (a.e.) /kg of soil (DW)	LC50 (mg of glyphosate (a.e.) /kg of soil (DW)
		-2 Log [Likelihood]	Log [Glyphosate]		
GBH-A	T1	< 0.0001	< 0.0001	1593.99	1687.56±50.53
	T2	< 0.0001	< 0.0001	1767.43	
	T3	< 0.0001	< 0.0001	1701.35	
GBH-B	T1	< 0.0001	< 0.0001	2279.56	2215.66±61.83
	T2	< 0.0001	< 0.0001	2092.03	
	T3	< 0.0001	< 0.0001	2275.39	

3.3 ACUTE TOXICITY GBHS ON JUVENILE EARTHWORMS

The number of the killed earthworms increased significantly, when the tested solutions content of glyphosate increased. In the uncontaminated media, the observed mortality has been 0 % during the three trials. The theoretical mortality (0 %) generated by the model was the same than which observed in the uncontaminated medium. **Figure 2** shows dose-response curves related to juvenile earthworms.

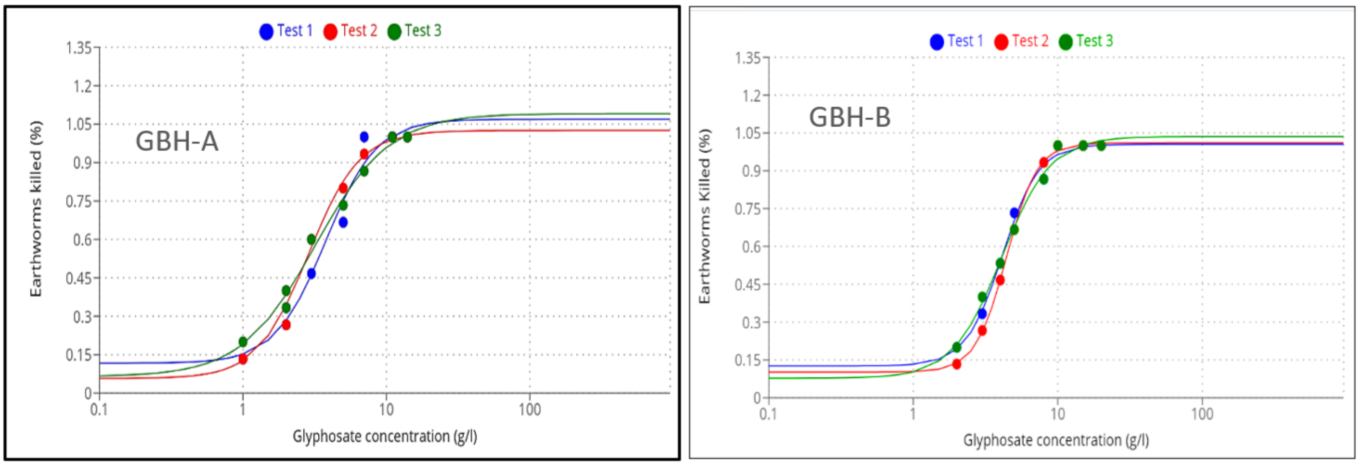

Fig. 2. Concentration-response curves of GBHs on juvenile earthworms

These results indicated that the juvenile earthworm's death in the contaminated medium is due to GBH alone. The LC50, the trials 1, 2, and 3 were respectively 1596.11 mg of glyphosate (a.e.) /kg of soil (DW), 1729.47 of glyphosate (a.e.) /kg of soil (DW) and 1650.28 mg glyphosate (a.e.) /kg of soil (DW). The lethal average concentration of glyphosate was 1658.62 ± 38.72 mg of glyphosate (a.e.) /kg soil (DW) (Table 2).

Table 2. Statistical data regarding LC50 determination on juvenile earthworms

Type of Glyphosate-Based Herbicide	Test	Chi-2		LC50 (mg of glyphosate (a.e.) /kg of soil (DW)	LC50 (mg of glyphosate (a.e.) /kg of soil (DW) n=3
		-2 Log [Likelihood]	Log [Glyphosate]		
GBH-A	T1	< 0.0001	< 0.0001	1470.55	1416.99±53.06
	T2	< 0.0001	< 0.0001	1310.87	
	T3	< 0.0001	< 0.0001	1469.54	
GBH-B	T1	< 0.0001	< 0.0001	1596.11	1658.62±38.72
	T2	< 0.0001	< 0.0001	1729.47	
	T2	< 0.0001	< 0.0001	1650.28	

The acute toxicity study revealed a high sensitivity of adult and juvenile earthworms to GBH-A than GBH-B, as LC50 related to GBH-A were lower than those of GBH-B (**Figure 4**).

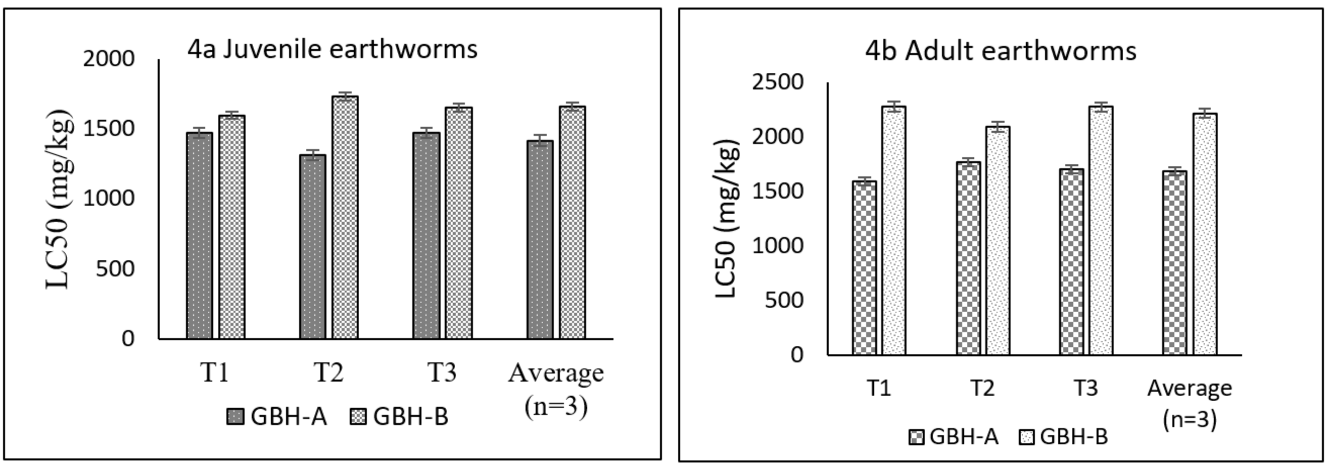

Fig. 3. Comparison histograms of lethal concentrations (LC50)

The juvenile earthworms were much more sensitive to the GBH-A and GBH-B formulation compared to adults because juvenile LC50 were lower than adult LC50 (**Figure 5**).

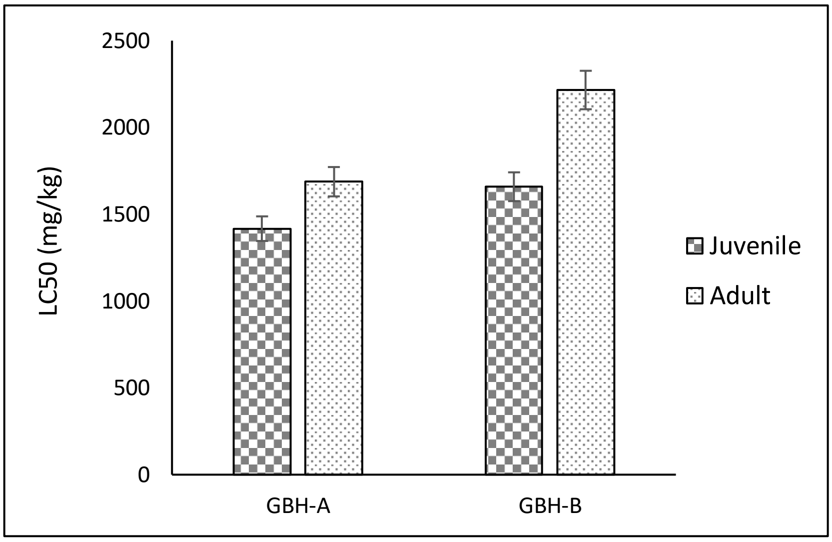

Fig. 4. Comparaison histogram of adult and juvenile earthworms using LC50

Our results corroborate those of Piola et al who demonstrated that two GBHs with the same active ingredient could have different degrees of lethality on earthworms [44]. According to these authors, Roundup FG (monoammonium salt, 72% acid equivalent) was 4.5 time more toxic than Mon 8750 (monoammonium salt, 85.4% acid equivalent), towards the earthworm *Eisenia andrei*.

The high toxicity of the GBH-A towards earthworms (juvenile and adult) might be due to the difference that may exist between the adjuvants two types of formulation, as these formulations have the same active ingredient, which is ammonium salt of glyphosate. In this context, two hypotheses are plausible. The first stipulates that GBH-A might contain adjuvants that might be absent in GBH-B. The second hypothesis suggests that if GBH-A and GBH-B contain the same adjuvants, but not the same level of adjuvants content. In this case, the contents of certain adjuvants might be higher in GBH-A than in GBH-B. Unfortunately, the manufacturers did not indicate the adjuvants involved in the agrochemicals because these ingredients are legally classified as confidential commercial information [12]. These results suggest that the toxicity of GBHs might be amplified by the adjuvants. This suggestion is comforted by Mesnage et al who demonstrated that GBHs (9 glyphosate-based formulations) were more toxic on human cell (hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines) than glyphosate alone [11]. Hence, the toxicity of agrochemicals might be underestimated when the adjuvants are not taken into account in the risk assessment related to human and environmental health. Despite efforts made by the agrochemical companies to replace toxic adjuvants such as POE-tallowamine by adjuvants with lower toxicity, the law authorizing the confidentiality of adjuvants must be revised in favor of the promulgation of inert ingredients for better toxicological and ecotoxicological studies [17].

4 CONCLUSION

The study demonstrated a difference of lethality between GBH-A (RAPID MAX 750 WG, 75% ammonium salt of glyphosate) and GBH-B (TAKO-KELE 757 SG, 75.7% ammonium salt of glyphosate, GBH-B) toward adult and juvenile earthworms (*E. eugeniae*). GBH-A was more toxic than GBH-B on adults and juvenile earthworms. Juvenile earthworms were more sensitive to both GBHs. As, the tow GBHs contains the same active ingredient (ammonium salt), we suggest that adjuvants might be responsible of difference of toxicity between GBH-A and GBH-B. The law authorizing the confidentiality of adjuvants in commercial phytosanitary products needs revision in favor of the promulgation of inert ingredients for better toxicological and ecotoxicological studies.

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