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Vol. 12(9), pp. 706-715, 2 March, 2017
DOI: 10.5897/AJAR2016.11844
Article Number: 20BF3B062991
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Effect of pesticides and micro-organisms on earthworm *Eisenia fetida* (Savigny, 1826)

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Received 18 October, 2016; Accepted 30 November, 2016

Earthworms might be limited in their activities on soil by pesticides used at important rates in agriculture and human pathogenic micro-organisms introduced in soil by excreta. This study using a modified toxicity filter paper contact test from Organisation for Economic Co-operation and Development (OECD), aimed at assessing the toxicity of six pesticides formulations and six micro-organisms on the earthworm *Eisenia fetida.* The study performed over 7 months at the laboratory QAlEA (University of Caen Normandie, France), analysed the mortality every 24 h for 96 h and at 96 h of *E. fetida* when exposing to pesticides and microbial suspensions. The statistical test used is the Student's t-test at the significant level of 0.05. No mortality of earthworms was observed when testing these pesticides at their recommended agricultural concentrations. Toxicity order from the highest to the lowest, based on LC_{50}, was Capiscol, Stratos Ultra Jardin, Polyvalent, Roundup GT Plus, Polyflor and KB Limace. Among tested micro-organisms, only *Enterobacter cloacae* (culture broth) and *Listeria monocytogenes* (culture broth and supernatant) generate mortalities of *E. fetida*. Finally, all these tested pesticides do not lead to *E. fetida* mortality if they are used at their recommended agricultural concentrations. Earthworms species *E. fetida* are also stressed by some micro-organisms. Furthermore, the filter paper contact test OECD might be used as a tool to evaluate the response of *E. fetida* to abiotic and biotic stresses.

Key words: Stress, toxicity, pesticides, micro-organisms.

INTRODUCTION

Among soil inhabiting organisms, earthworms are largely represented, counting more than 80% of soil

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invertebrate's biomass in tropical and temperate ecosystems. They can be divided into categories of epigeic, endogeic and anecic according to their burrowing abilities, feeding preferences and sizes (Mariana et al., 2001; Felten et al., 2009; Lalthanzara et al., 2011). Generally, they participate in soil aeration and water infiltration by creating burrows, increasing the nutrients content of the soil by incorporating litter into the soil, mixing soil minerals with organic material and producing on soil surface, their castings. These castings containing readily available and enriched form nutrients are from the large amount of the decomposed litter, manure and others organic matters they ingest. Regarding earthworms' capacity of affecting positively soil functioning through different mechanisms, they have been recognized as ecosystem engineers (Jones et al., 1994; Bartlett et al., 2010; Decëans et al., 2001). Thus, their abundance in soil is an indicator of the soil health and their activities are of great importance for agro-ecosystem sustainability.

However, these soil invertebrates of great interest are frequently facing different abiotic and biotic stresses in their living environment. They are threatened by biotic stresses (prey for platyhelminthes, amphibians, birds, mammals) and by abiotic stresses such as urbanism and intensive agriculture (Taboga, 1981; Carvalho, 2006; Fiore et al., 2004). Of all cases of threats to earthworms, intensive agriculture receives more attention because it requires important amounts of inputs including among others chemical pesticides.

Pesticides get to the soil either by direct application on the soil or runoff from foliar spraying, all in accordance with the manufacturer's instructions. Chemical stress caused by pesticides on earthworms are located at all biological levels such as physiology, biochemistry and genetic (Pelosi et al., 2014; Correia and Moreira, 2010). Beyond their impacts on earthworms, pesticides adversely affect humans, animals and soil organisms by contaminating environments such as air, soil and groundwater (Hussain et al., 2009; Bolognesi and Merlo, 2011; Barnhoorn et al., 2015). In a bid to guaranty food security for a growing earth's population estimated in 2050 to about 9 billion people (FAO, 2011), new formulations of pesticides and other chemical agricultural inputs are continuously manufactured for controlling diseases of comestible plants. Therefore, increasing information focusing on the effect of the pesticides formulations on earthworms is of great importance in the protection of soil biodiversity.

Besides the presence of pesticides in the soil, micro-organisms responsible for many severe diseases may also contaminate the soil through infected excreta of animals and humans and therefore, be in earthworm's surroundings. The biological interactions in soil between human pathogen micro-organisms and earthworms are indirectly elucidated by assessing the antibacterial properties of earthworms' coelomic fluid or its extract (Bhattacharjee and Ghosh, 2015; Li et al., 2011). On the other hand, earthworms usually take part in different interactions, soil living organisms have with one another (Pimm, 1982; Russell et al., 1985). Better knowledge of the earthworms/micro-organisms interactions is useful in environmental protection (e.g. use of earthworms in biological control of pests and in sewage treatment).

Eisenia fetida is a favorite worm species for composting and is frequently used as a biological monitor for testing the effects of contaminants on soil biota (Das Gupta et al., 2011; OECD, 1984; Garg et al., 2006). This study is cast within the framework of protecting earthworms that are important members of soil biodiversity and exploiting their ecosystem services for the environmental protection and repression of phytopathogenic micro-organisms. Its objective is to give an overview of stresses related to pesticide formulations and micro-organisms, that earthworms are facing in their living environment. Specifically, this study permitted assessment of the toxicity of six pesticides formulations (Round-up GT Plus, Stratos Ultra Jardin, Capiscol, Polyvalent, Polyflor and KB Limace) and six micro-organisms (*Geotrichum candidum* ATCC 203407, *Escherichia coli* UCMA 10579, *Enterobacter cloacae* UCMA 10580, *Listeria monocytogenes* UCMA 6115, *Salmonella typhi* UCMA 10598 and *Staphylococcus aureus* UCMA 6834) on *E. fetida*.

MATERIALS AND METHODS

Earthworms

Earthworms used in this study belong to the epigeic species *E. fetida*. They were purchased from Vers La Terre (Pezanas, France) and breeded at the analysis laboratory (University of Caen Normandie, France) with the help of a vermicomposting system (Worm Café). The vermicomposting was started with 1 kg of earthworms and carried out according to the manufacturer's instructions (VerlaTerre, 2016). At the composting set up, the vermicomposting system possessed two plates with one (the work plate) containing earthworms and coconut fibre. The coconut fibre was used as bedding for earthworms which was obtained by fully soaking a coconut fibre block (from Ceylon Garden Coir) in 6 to 7 L of tap water. Two handfuls of kitchen wastes (vegetable products, eggshells and coffee grounds) were placed three times every week on the coconut fibre. Moistened paper towel was put every week in the work plate to provide earthworms with fibres. The vermicomposting system was kept at room temperature (18 ± 2°C). From the third month of composting, adult earthworms (weighing between 300 and 600 mg) with well-developed clitella, were taken from the composting system and kept in fast for 3 h in the dark, at room temperature before use for toxicological assessment (OECD, 1984).

Pesticides

Six pesticides formulations Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace from four different chemical classes were tested (Table 1). Their active substances are involved in widely used pesticides in agriculture field (Anonymous 1).

Table 1. Characteristics of used pesticides.

Pesticide	Nature	Active substance	Recommended agricultural dose	Corresponding area (m^2)	Manufacturing company
Round-up GT plus	Herbicide	Salt of glyphosate isopropylamin (607 g/L) equivalent to glyphosate acid (450 g/L)	20 to 40 mL/3L	80	Monsanto
Stratos Ultra Jardin	Herbicide	Cycloxydim (100 g/L)	2 to 4 mL/L	10	Fertiligène
Polyflor	Fungicide	Propiconazole (5 g/L)	10 mL/L	10	Syngenta Agro SAS
Capiscol	Fungicide	Azoxystrobin (250 g/L)	0.8 to 1 mL/L	10	Syngenta Agro SAS
Polyvalent	Insecticide	Deltamethrin (15 g/L)	2.5 to 4 mL/5 L	50	Bayer SAS
KB Limace Appat Granulé	Anti-slug	Metaldehyde (5%)	7 g	10	Fertiligène

Micro-organisms and their culture conditions

The fungus, *G. candidum* ATCC 203407 and bacteria *E. coli* UCMA 10579, *E. cloacae* UCMA 10580, *L. monocytogenes* UCMA 6115, *S. typhi* UCMA 10598 and *S. aureus* UCMA 6834 used in this study have been provided by the CONOBIAL (Conservatoire Normand de la microBiodiversité Alimentaire, Université de Caen Normandie, France). *G. candidum* ATCC 203407 was grown on Malt Extract Broth (MEB) at 25°C for 48 h (Naz et al., 2013) and bacterial strains were cultivated on liquid medium Luria-Bertani (LB) at 37°C for 48 h according to Rahman et al. (2012) with some modifications. These micro-organisms were incubated with shaking (120 rpm).

Acute toxicity test

The contact filter paper test of OECD (1984) was, with some modifications, used to assess the acute toxicity of pesticides, micro-organisms suspensions and their supernatants on *E. fetida*. Decimal dilutions were firstly performed for each type of product, in order to determine a range of concentrations in which a 0–100% mortality of the earthworms was obtained. The testing suspensions were also assessed at non-diluted concentrations. Concerning pesticides of which dilutions covered the Recommended Agricultural Dose (RAD), close dilutions (20, 40, 60 and 80% ratios of decimal dilutions) inside the 0–100% mortality interval were then carried out. The RAD of the used pesticides correspond to the dilutions and concentrations based on their active substances, respectively 6.6×10^{-3} to 1.3×10^{-2} and 2.97 to 5.85 g/L of glyphosate acid for Round-up GT Plus, (2 to 4) x 10^{-3} and 0.2 to 0.4 g/L of cycloxidim for Stratos Ultra Jardin, 10^{-2} and 0.05 g/L of propiconazole for Polyfor, 8×10^{-4} to 10^{-3} and 0.2 to 0.25 g/L of azoxystrobin for Capiscol, (5 to 8) x 10^{-4} and 0.0075 to 0.012 g/L of deltamethrin for Polyvalent and 10^{0} and 5% of metaldehyde for KB Limace. For micro-organisms suspensions and their supernatants, close dilutions were at 50% ratio of decimal dilutions. All dilutions were performed using peptone water (PW) composed of 1 g/L of peptone (polypeptone AES). PW and the growth medium LB were used as control. Micro-organisms suspensions included (i) stationary phase micro-organisms grown in liquid medium LB (culture broth) and, (ii) their washed cells. Washed cells were obtained by washing twice with peptone water. Firstly, culture of stationary phase micro-organisms was vortexed and centrifuged for 10 min at 7000 g. The supernatant was discarded and the pellet was retained for the washing steps. For the first washing, 10 mL of peptone water were added to the early stage pellet. The mix was vortexed and centrifuged for 10 min at 7000 g. The supernatant was discarded and the pellet was retained. Washing process was run again with the pellet from first washing. After this step (second washing), the pellet was suspended in 10 mL of peptone water by vortexing and this last mix was used as washed cells. Supernatants were obtained by centrifuging cultures of stationary phase micro-organisms at 7000 g for 10 min.

As a modified protocol of OECD (1984), a 10 cm diameter circular piece of filter paper (Fioroni, Paris, France) was placed in a 9-cm Petri dish and moistened either with 970 µL of each concentration of pesticides or 990 µL for micro-organisms suspensions and their supernatants. One earthworm was placed on this Petri dish in the contact with the moistened filter and a 10 µL quantity of testing suspension was spread inside the lid of dish. The dish was incubated in the dark at room temperature (18 ± 2°C) for 96 h and earthworm status (alive or dead) was recorded every 24 h. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end. The alive and dead statuses of earthworms are designated by the numbers 1 and 0, respectively. The toxicity test on *E. fetida* of each dilution of tested suspensions was carried out in triplicate (three living earthworms used) per experiment. At the beginning of the experiment (t = 0 h), the 3 living earthworms correspond to the number 3 and account for 100% of *E. fetida* survival. The percentage of *E. fetida* survival every 24 h is determined as follows: Percentage of *E. fetida* survival at time t = 100 x N at time t / N at time 0 h, with N representing the number of living *E. fetida*. Experiments were repeated at least 6 times for each dilution or concentration of tested suspensions and control suspensions LB and PW. Finally, 18 earthworms were used for assessing the toxicity of each concentration of tested suspensions (pesticides and micro-organisms) and control suspensions (LB and PW).

Statistical analyses

All statistical analyses were performed using Statisca 7 (StatSoft Inc, Tulsa, USA). The comparison between, (i) the effects average on *E. fetida* of each micro-organism suspension and its supernatant and, (ii) that of the control LB was carried out by a Student's t-test at the significant level of 0.05. On the basis of their lethal median concentration (LC_{50}) value, the toxicity levels of pesticides were compared by Anova ($p < 0.05$) and these pesticides were classified as being supertoxic (< 1 µg cm^{-2}), extremely toxic (1 - 10 µg cm^{-2}), very toxic (10 - 100 µg cm^{-2}), moderately toxic (100 - 1000 µg cm^{-2}) or relatively nontoxic (>1000 µg cm^{-2}) (Roberts and Dorough, 1984).

RESULTS AND DISCUSSION

Toxicity of pesticides

The toxicity to *E. fetida* of agricultural applications of pesticides Round-up GT plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace is

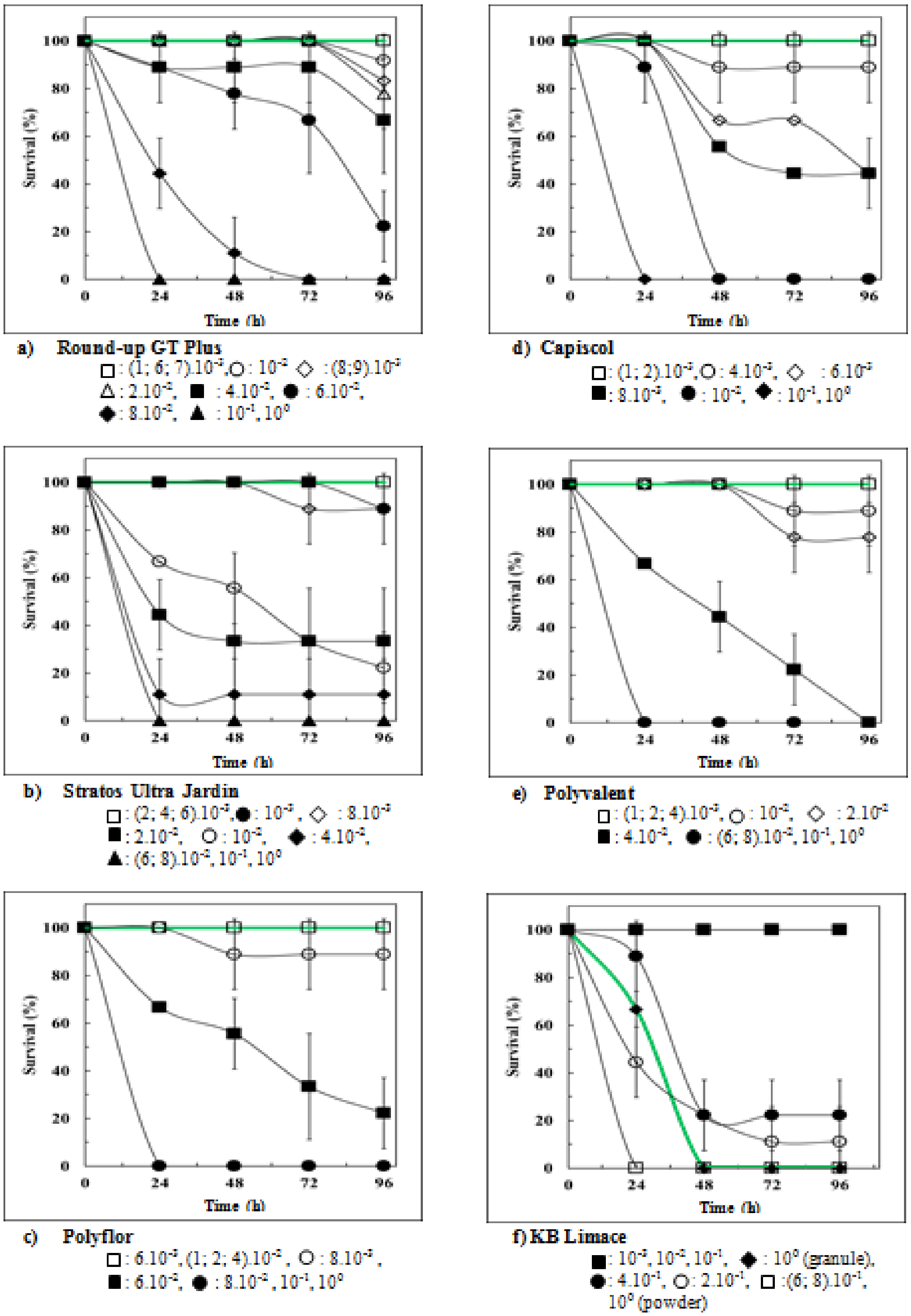

Figure 1. Survival of *Eisenia fetida* as function of time at different concentrations of pesticides (v/v: Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol and Polyvalent; w/v: KB Limace; green curve: RAD).

presented in Figures 1, 2 and 3. The percentage of *E. fetida* survival decrease during time with increasing of overall pesticides concentrations (Figure 1). For KB Limace particularly, this proportionality principle is not

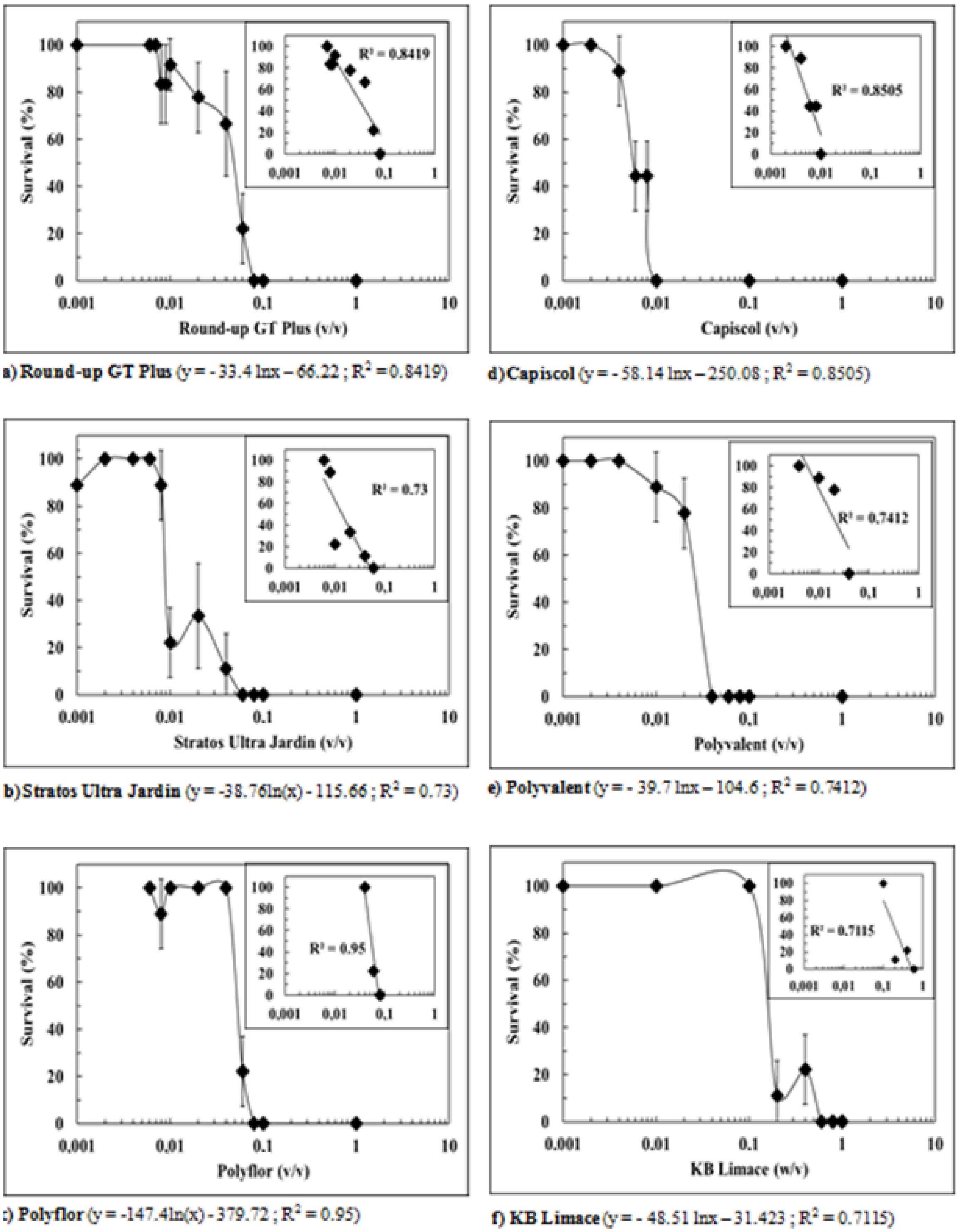

Figure 2. Survival of *Eisenia fetida* as a function of pesticides concentrations, over 96 h exposure.

always observed because of the granulated form of this pesticide formulation. In fact, this form does not enable a continuous contact between used earthworm and this pesticide, and consequently generate a biased estimation of its toxic effect. For all tested pesticides, no mortalities of earthworms were observed when exposed over 96 h at their Recommended Agricultural Dose (RAD) (Table 1, Figure 2). Concerning Round-up GT Plus, the toxicity result on *E. fetida* from its RAD value (2.97 to 5.85 g/L of glyphosate acid) to lower concentrations, is in accord with

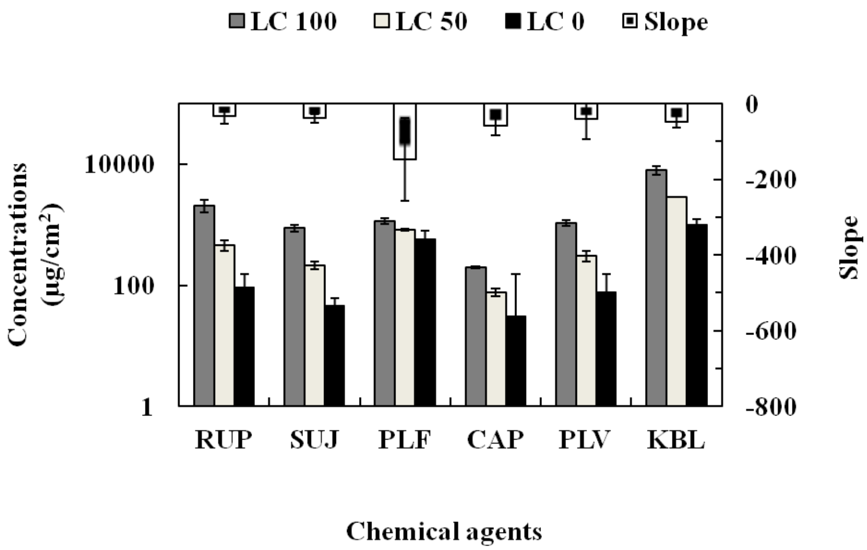

Figure 3. Pesticides toxicity on earthworm *Eisenia fetida* [RUP: Round-up GT Plus; SUJ: Stratos Ultra Jardin; PLF: Polyflor; CAP: Capiscol; PLV: Polyvalent; KBL: KB Limace; LC_{100}: Minimal concentration leading to 100% mortality; LC_{50}: Concentration leading to 50% mortality; LC_0: Maximal concentration leading to 0% mortality; Slope indicates the variation of earthworms survival (%) per dilution unit inside the [LC_0; LC_{100}] interval].

Correia and Moreira (2010) indicating no mortality for the same earthworm specie exposed to 1-1,000 mg/kg of glyphosate in soil. These data obtained after exposure to RAD concentrations from the filter paper contact toxicity test (described as an initial screen toxicity) showing no mortality of *E. fetida* after a 96 h exposure to pesticides, indicate that these pesticides generate mild or moderate stress on earthworms in soil (OECD, 1984). However, their use at important rates (e.g. in intensification of agriculture or for controlling pest in small scale or handicraft agriculture in undeveloped countries) could lead, through a runoff process from treated agricultural fields, to an acute and chronic poisoning in terrestrial and aquatic organisms. For instance, concerning Capiscol, the active substance azoxystrobin generates a risk for water quality and is recognized to be toxic for aquatic organisms (Rodrigues et al., 2013; Olsvik et al., 2010). Indeed, its RAD assessed in this study, equivalent to 0.2 to 0.25 g/L of azoxystrobin is several folds higher than 0.026 µg/L which is an environmental concentration value of azoxystrobin that induces a decrease of population in the cladoceran specie *Daphnia magna* after exposure (Warming et al., 2009). Other works showed that propiconazole (active substance of the formulation Polyflor) has low mobility and high adsorption in soil rich in organic matter so that it accumulates in top layers of soil (Thorstensen et al., 2001; Khairatul et al., 2013; Kim et al., 2002). Especially, some earthworms preferred as food by a wide range of animal, could be potential sources of pesticides intoxication by accumulating these chemical compounds (Hankard et al., 2004). This may result through food chain in pesticides bioaccumulation in terrestrial and aquatic organisms (Senthilkumar et al., 2001). On the other hand, the use at important rates of pesticides in agricultural fields could have long-term effects on non-target organisms (earthworms and others soil inhabiting organisms). These effects not only resulting in earthworms death, could disrupt or modify their activities and metabolisms (genes expression, physiology, behavior, population density) (Pelosi et al., 2011; Correia and Moreira, 2010; Contardo-Jara et al., 2009). A 96 h exposure of *E. fetida* to tested pesticides (Figure 2) showed that the range (LC_0 to LC_{100}) of highest and lowest pesticides concentrations causing respectively no mortality and 100% mortality of the studied earthworms population were 91.56 to 2090.62 µg/cm^2, 45.78 to 885.08 µg/cm^2, 579.88 to 1159.76 µg/cm^2, 30.52 to 198.38 µg/cm^2, 76.3 to 1083.46 µg/cm^2, 1007.16 to 7980.98 µg/cm^2 for Round-up, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace, respectively. The RAD values ranges of Round-up GT Plus (91.56 to 198.38 µg/cm^2) and Stratos Ultra Jardin (30.52 to 61.04 µg/cm^2) being close or including their respective LC_0 values, reinforce the thesis of non-use at important rates of these pesticides. These data constitute helpful information for database about chemical toxicity and agro-industry pesticides.

Toxicological profile of the pesticides

Unlike KB Limace, the LC_{50} after a 96 h exposure of the

Table 2. Survival percentage during time of *E. fetida* exposed to micro-organisms suspensions and their supernatant

Controls	Dilutions	Time (h)					
		0		24		48	
		CM	**CM**	**CM**	**CM**	**CM**	**CM**
PW		100	100	100	100	100	100
LB							
	10^0	100	100	77.77 ± 22.22 [a]	77.77 ± 22.22 [a]	0	0
	5.10^{-1}	100	100	100 [a]	100 [a]	33.33 ± 23.56 [a]	33.33 ± 23.56 [a]
Micro-organisms		**CB**	**S**	**CB**	**S**	**CB**	**S**
G. candidum	10^0	100	100	100	100	100	100
E. coli	10^0	100	100	44.44 ± 19.24 [a]	77.77 ± 19.24 [a]	0	0
	5.10^{-1}	100	100	88.88 ± 19.24 [a]	100 [a]	44.44 ± 19.24 [a]	0 [a]
E. cloacae	10^0	100	100	11.11 ± 19.24 [b]	11.11 ± 19.24 [b]	0	0
	5.10^{-1}	100	100	88.88 ± 19.24 [a]	77.77 ± 19.24 [b]	11.11 ± 19.24 [a]	0 [a]
L. monocytogenes	10^0	100	100	11.11 ± 19.24 [b]	100 [a]	0	0
	5.10^{-1}	100	100	77.77 ± 19.24 [b]	100 [a]	11.11 ± 19.24 [a]	0 [a]
S. typhi	10^0	100	100	66.66 ± 57.73 [a]	77.77 ± 38.49 [a]	0	0
	5.10^{-1}	100	100	100 [a]	100 [a]	11.11 ± 38.49 [a]	0 [a]
S. aureus	10^0	100	100	44.44 ± 19.24 [a]	33.33 ± 33.33 [a]	0	0
	5.10^{-1}	100	100	100 [a]	88.88 ± 19.24 [a]	22.22 ± 33.33 [a]	0 [a]

CB: Culture Broth; CM: Culture Medium; S: Supernatant; LB: Luria-Bertani; PW: Peptone Water. For same dilution between the control suspension and a given tested microbial suspension, values with the same letter in the same column are statistically ($p < 0.05$) equivalent.

tested pesticides are all higher than their RAD. According to the toxicological classification of chemicals using their LC_{50} values (Roberts and Dorough, 1984), the toxicological profile of these pesticides used on *E. fetida* are the following ones from the highest to the lowest. Capiscol (76.3 µg/cm^2) is classified as a very toxic chemical, Stratos Ultra Jardin (213.64 µg/cm^2), Polyvalent (305.2 µg/cm^2), Round-up GT Plus (457.8 µg/cm^2) and Polyflor (824.04 µg/cm^2) are considered as moderately toxic chemicals and KB Limace (2828.36 µg/cm^2) is in the range of relatively nontoxic chemicals (Figure 3). This comparative of chemical stress from a 96 h exposure to *E. fetida* shows that the toxicity of a given pesticide is not related to its nature (be insecticide, fungicide, herbicide or anti-slug). The 96 h LC_{50} value of Polyvalent (15 g/L or 1.5% of deltamethrin), is equal to the 48 h LC_{50} value of deltamethrin 98.0% purity (327.8 µg/cm^2) on the earthworm specie *E. fetida* (Kim et al., 2002). This indicates the existence in the mixture Polyvalent, of many stressors on earthworms. Also, Wang et al. (2012) found 566.1 µg/cm^2 as LC_{50} value when assessing the acute toxicity of glyphosate (85% purity) on *E. fetida.* This value being greater than the one of Round-up GT Plus, suggests that Round-up GT Plus is more toxic than the glyphosate (85%). Indeed, the Round-up GT Plus used in this study containing 45% of glyphosate acid, its toxicity on *E. fetida* may be greatly caused by the chemical additives or the synergetic effects of the chemical mixture in the formulation. Similar effects on reproduction in Zebrafish (*Danio rerio*) have been demonstrated with glyphosate (analytical grade) and its formulation Round-up GC liquid (120 g/L of glyphosate acid) (Webster et al., 2014). Works by Tsui and Chu on different organisms (bacteria, microalgae, protozoa and crustaceans) indicated higher toxicity for the polyoxyethylene amine (POEA: surfactant included in the Round-up formulation) than the Round-up (formulation) and followed by the glyphosate acid and glyphosate isopropylamine (active substances) (Tsui and Chu, 2003). It is observed that Polyvalent and Round-up GT Plus present more ecotoxicological relevances than their active substances (in comparison to others works) and regarding the increasing use of mixed pesticides in agriculture, due to their high efficiency, there is a need for a better ecotoxicological risk assessment of manufactured pesticides, to do not focus only on the toxicity of the active substance. Hence, earthworms having all their integrity could act properly for the well-being of environment.

Toxicity of micro-organisms

Data from the toxicity test on *E. fetida* with micro-organisms suspensions over 96 h are presented in the Table 2. These data indicated no effect of *G. candidum* culture broth, washed cells of all tested bacteria and peptone water (control) on *E. fetida* survival. It was observed for the culture medium LB (control) and bacterial culture broths and their supernatants, a decrease of *E. fetida* survival, reaching 0% at 48 h for the non-diluted suspensions (10^0) and after 48 h for the 5.10^{-1}-diluted suspensions. The percentage of *E. fetida* survival recorded at 24 h for the 10^0 and 5.10^{-1}-diluted suspensions, respectively, was different ($p < 0.05$) between LB (79.99 and 100%) and culture broths of *E. cloacae* (11.11%) and *L. monocytogenes* (11.11 and 77.77%) but no difference ($p < 0.05$) was observed with *E. coli* (44.44 and 88.88%), *S. typhi* (66.66 and 100%) and *S. aureus* (44.44 and 100%). Concerning the bacterial supernatants, difference ($p < 0.05$) compared to the control LB was observed only with *E. cloacae* (11.11 and 77.77%). At 48 h, the percentage of *E. fetida* survival was no significantly different between LB and each of the tested bacteria for all dilutions at both broth and supernatant state. Overall, 40% of tested culture broths exhibit an effect on *E. fetida* compared to 20% of supernatants.

The observation about *G. candidum* culture broth may be the fact that this micro-organism is not pathogen for earthworms. Also, its belonging to the fungal reign might make it an appreciated food for *E. fetida.* Indeed, various species of earthworms have their feeding preferences towards fungi (Bonkowski et al., 2000). For example, according to Zirbes et al. (2012), the earthworm *Lumbricus terrestris* exhibits a preference for food substrates colonized by soil fungi *Mucor hiemalis* and *Penicillium* sp*.* The no observed effect of *G. candidum* culture broth on *E. fetida* in our study may suggest that earthworms are likely not stressed by the presence of fungi in their living environment. Earthworms due to their olfactory are rather attracted by fungi which synthesize chemical signals or volatile compounds, and feed them (Zirbes et al., 2011). So, the feeding mode of earthworms related to fungi could be used for biocontrolling the pathogen fungi in soil.

In contrast to culture broths of *E. cloacae* and *L. monocytogenes*, those of *E. coli*, *S. typhi* and *S. aureus* do not cause *E. fetida* mortality due to earthworm's antibacterial activity and immune system which may be discriminating. Indeed, to protect themselves or to mount their attack against soil organisms, earthworms produce the lysenin, a pore-forming toxin. Lysenin derived from coelomic fluid of *E. fetida* are particulary adapted to form pores in sphingomyelin-containing membrane (Sukumwang and Umezawa, 2013; Iacovache et al., 2008). Wang et al. (2006) reported that earthworms are infected by few micro-organisms although they live in an environment flocked with pathogens. The antibacterial barriers mainly include body wall, alimentary canal and parietal mucus.

After bacterial infection, lysozyme and antibacterial proteins (accounting for ones of responses of earthworms defense system) are enhanced and peaking at 4 h and 3 days, respectively (Hirigoyenberry et al., 1990). Besides, the *E. coli* strain used in this study (*E. coli* UCMA 10579 also called *E. coli* DH5α) is designed for laboratory use and is not a pathogen micro-organism (Chart et al., 2000; Jung et al., 2010). Therefore, one may consider *E. coli*, *S. typhi* and *S. aureus* as food for *E. fetida*. The results about culture broths of *E. coli* and *S. typhi* match with those of Eastman et *al.* showing that *E. fetida* eliminates human pathogens in domestic wastewater residuals (biosolids). There were for fecal coliforms and *Salmonella spp.* a 6.4-log and 8.6-log reduction in test samples (with earthworms) compared to the control (1.6-log and 4.9-log reduction), respectively (Eastman et al., 2001). Murry and Hinckley (1992) indicated the percentage decrease in the concentration of *Salmonella enteridis* cultured during 48 h in horse manure in presence of earthworms *E. fetida* compared to cultures without earthworms (8% versus 2%). In contrast with certain works about interactions *E. fetida* / *Enterobacteriaceae* showing *Enterobacter spp.* inhibited by earthworm's antibacterial activity or its digestive processes (Parthasarathi et al., 2007; Arslan-Aydoğdu and Çotuk, 2008), the *E. cloacae* strain used in this study reduces significantly *E. fetida* survival at both the culture broth level and the supernatant compared to control. This may be due to the antibacterial resistance and opportunist pathogen characteristics of this bacterium. In fact, *E. cloacae* is known as highly versatile and is capable of overproducing many antibiotic resistances such as AmpC β-lactamases, cephalosporinase that are able to render ineffective almost all antibiotic families (Davin-Reglis and Pages, 2015; Guérin, 2015). It is also able to form biofilm and to secrete various metabolites including cytotoxins (enterotoxins, hemolysins, pore-forming toxins) (Mezzatesta et al., 2012). Like *E. cloacae, L. monocytogenes* possesses enzymatic equipment that might inhibit the antibacterial activity of *E. fetida* and induce its pathogenicity.

Washed cells generate no mortality of *E. fetida* related to washing. Indeed, the washing of micro-organisms leading to washed cells (micro-organisms free from culture medium and metabolites) creates new conditions where these microbial cells or washed cells cannot properly express their virulence if needed. Then when possible, the stresses (washed cells) they cause are mild or moderate so that earthworms recover their steady state. By analogy to microbial stress during processing, Lado and Yousef (2002) reported that sub-lethal stress induces the expression of cell repair systems. These different effects (mortality or not) of tested micro-organisms under various status (culture broth, washed cell and supernatant) show that the filter paper contact toxicity test from OECD (1984) designed for early assessing the toxicity of chemicals in soil might be applied to micro-organisms. This constitutes a pilot study using the described OECD method for assessing the effect of micro-organisms on *E. fetida* and gives an overview of interactions earthworms / micro-organisms occurring in soil.

Conclusion

All these tested pesticides formulations (Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace) do not lead to *E. fetida* mortality when they are used at their recommended agricultural concentrations. At this concentration, they generate mild or moderate stress on *E. fetida*. Based on the LC_{50} values, the toxicological profile of these pesticide formulations used on *E. fetida* is the following one. Capiscol (76.3 µg/cm^2) is classified as a very toxic chemical, Stratos Ultra Jardin (213.64 µg/cm^2), Polyvalent (305.2 µg/cm^2), Round-up GT Plus (457.8 µg/cm^2) and Polyflor (824.04 µg/cm^2) are considered as moderately toxic chemicals and KB Limace (2828.36 µg/cm^2) is in the range of relatively nontoxic chemicals. Among all tested micro-organism suspensions, earthworms *E. fetida* were stressed by *E. cloacae* UCMA 10580 (culture broth) and *L. monocytogenes* UCMA 6115 (culture broth and supernatant). Furthermore, the filter paper contact test OECD might be used as a tool to evaluate the response of *E. fetida* to abiotic and biotic stresses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by the Ministère des Affaires Etrangères (France) and by the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (Côte d'Ivoire) through doctoral training fellowships.

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