ACUTE TOXICITY OF TWO COMMERCIAL PREPARATIONS OF ACETAMIPRID TO *APORRECTODEA TRAPEZOIDES* (DUGÈS, 1828) USING CONTACT FILTER PAPER TEST

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Abstract. Since the commercial formula is the product applied in the environment, the use of commercial preparation makes it possible to determine both the effects of the active principles and those of the adjuvants. The use of pesticides presents a burden for soil and has negative effects on earthworms, which are indicators of soil quality. This research aimed to assess the effect of two commercial Acetamiprid preparations on the cosmopolitan earthworm *A. trapezoides* using the filter paper contact test. The results showed that Aceplan and Rustilan were supertoxic with LC_{50} after 48-h of exposure of 0.064 and 0.086 μg(ai).cm^{-2}, respectively. It appears that the tested commercial preparations can be as toxic as their active ingredient. In addition to mortality, direct contact with insecticides caused morphological, behavioral and physiological changes in the tested worms. This emphasizes the importance of further ecotoxicological evaluation of commercial insecticide preparations so as not to underestimate their effects on other earthworm species and on soil-dwelling organisms.
Keywords: *earthworm, acetamiprid, pesticide, toxicity, LC_{50}*

Introduction

Acetamiprid is a systemic insecticide from neonicotinoids group. This group represents the most widely used insecticides in the world, with 25% of the global insecticide market (Bass et al., 2015) due to their excellent insecticidal activity (Yamada et al., 1999). It is the second active ingredient of the first generation of neonicotinoids after imidacloprid. It was synthesized in 1989 and put on the market in 1995 by Nippon Soda Co., Ltd. (Yamada et al., 1999). Since 1995, the use of acetamiprid has increased rapidly, like other neonicotinoids, due to changes in application techniques through the increased use of coated seeds and with increasing insect resistance and/or concern over the high mammalian toxicity of other insecticides (Tomizawa and Casida, 2005; Jeschkeet al., 2011; Douglas and Tooker, 2015). Acetamiprid acts as an agonist to nicotinic acetylcholine receptors in insects and causes insect paralysis and death (Mohamed et al., 2009). Like all pesticides, it is applied in the form of commercial preparations containing, in addition to the active ingredient, solvents and/or compounds

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which improve absorption or dispersion. These components are generally considered inactive constituents, and their toxicity is often not included in the debate about possible adverse effects. According to Lackmann et al. (2021), commercial preparations can be more toxic compared to their active ingredients. Skandrani et al. (2006) demonstrated that they are more toxic than the pure active components, with an even 150 times higher toxicity for bifenthrin preparations. More authors emphasize the importance of a more integrated ecotoxicological assessment of commercial pesticide preparations, not to underestimate their effects on the environment (Lackmann et al., 2021).

In Algeria, due to the interest in maximizing the crop productivity and economic return, farmers overuse this insecticide (under different commercial formulations) beyond the recommended dosage. This practice will have a potential adverse impact on soil communities, especially earthworms. These non-target organisms are one the most common soil organism, also called as soil ecosystem engineers (Eisenhauer, 2010), which play an important role in the functioning of this ecosystem (Spurgeon et al., 2003). Earthworms improve the biophysicochemical characteristics of soil and thus increase its fertility (Rida and Bouché, 1997). They are particularly susceptible to pesticides because they are continuously exposed through skin contact or by feeding on contaminated litter in soil (Jager et al., 2003; Rodríguez-Castellanos and Sanchez-Hernandez, 2007). Pesticides can affect earthworms by killing them or by causing temporary or permanent impairments or by altering their activities and behavior (Stanley and Preetha, 2016) which will certainly have consequences on the functioning of soil ecosystem. Therefore, earthworms can be used to provide safety thresholds for pesticide applications. According to the European Commission (EC, 2002), pesticides risk assessment in soil ecosystem is mainly based on earthworms.

Although many toxicity studies have been conducted, the fact remains that only a few earthworm species have been tested (Venkateswara Rao et al., 2003). In standardized acute tests, *Eisenia fetida* (Savigny, 1826) and *E. andrei* (Bouché, 1972) are used because they are relatively easy to breed and have a short generation time (OECD, 1984). Despite these advantages, these species generally do not inhabit mineral soils (Lowe and Butt, 2007) and therefore are rarely found in cultivated fields where pesticides are applied. Other species have also been advocated for ecotoxicological tests, such as *Lumbricus rubellus*, *L. terrestris*, *Dendrodrilus rubidus*, *Eudrilus eugeniae*, *Octolasion cyaneum*, … (Reinecke et al., 1997; Giggleman et al., 1998; Langdon et al., 2001; Lowe and Butt, 2007). However, these species are rarely found in cultivated soils or not common in all cultivated (Sims and Gerard, 1999). According to the European Food Safety Authority (EFSA et al., 2017), it has been a move to use environmentally relevant species (*i.e.* soil dwelling and representative of the ecosystem to protect), such as *Aporrectodea trapezoides* (Dugès, 1828). This cosmopolitan earthworm is highly representative of agricultural soils. It is ubiquitous and the most distributed in all soils and in different climates (Blakemore, 2009; Fernández et al., 2010). It displays high ecological plasticity and adaptability in agroecosystems (Sims and Gerard, 1999). Additionally, this species is parthenogenetic which makes it preferable for ecotoxicological tests due to its low genetic heterogeneity (Lowe and Butt, 2007). For laboratory culture, *A. trapezoides* seems to be a suitable species with its high percentage of adult and hatchling survival, and high cocoon viability (Fernández et al., 2010). Due to its habitat in the topsoil, it is vulnerable to surface-applied pesticides. This increases its ecological relevance compared with standardized species, which makes it an ideal candidate for assessment of the potential impact of chemical applications in the field.

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The objective of the present research was therefore to assess the effects of commercial preparation of Acetamiprid on the earthworm *A. trapezoides* by applying the filter paper contact test. Since the commercial formula is the final product applied in the environment, the use of commercial preparation makes it possible to determine both the effects of the active principles and those of the adjuvants. To investigate, whether there is a discrepancy between the products, acute toxicity was assessed for two commercial preparations.

Material and methods

Earthworms

The cosmopolitan earthworm species, *A. trapezoides* (Dugès, 1826), was adopted as the test species. Earthworms were collected by manual digging and hand-sorting soil from Constantine Department (northeastern Algeria) and kept in aerated plastic containers with natural soil. All worms were washed briefly with deionized water, and were kept on moist filter paper for 24-h at 20 ± 2 °C in the dark to devoid their gut contents, after which they were rinsed again with deionized water, blotted on the filter paper and placed in a test vial. Only adult worms (those with well-developed clitellum) were used in these experiments with individual wet weights (after void of the gut content) of 1.20 ± 0.23 g.

Test chemicals and solutions

The test substance used in the tests was acetamiprid ($C_{10}H_{11}ClN_4$, CAS 135410-20-7) of two commercial insecticide formulations marketed in Algeria under the names: Rustilan (Nanjing Zonechem Co., Ltd., 20 SP) and Aceplan (Rivale, 20 SP). These preparations contain 20% of active ingredient (ai) in soluble powder form. Test solutions were freshly prepared before the beginning of the tests using distilled water. The concentrations of insecticides were prepared in mg(ai).mL^{-1} and the toxicity was measured in μg(ai).cm^{-2}. The results are presented considering the concentration of active ingredient for each formulation of insecticide.

Acute toxicity test

A modified contact filter paper test described in the OECD guideline 207 (OECD, 1984) was performed according to Wang et al. (2012). The test vial was a Petri dish of 10 cm in diameter and 2 cm in height. Round filter paper (Whatman No. 1) was cut to the suitable size, placed in such a way that sides were lined with filter paper, and treated with the commercial formulations. For each treatment, ten replicates were used, each consisting of one adult worm per vial. The test was set using experimental concentrations of 0.02, 0.04, 0.08, 0.16 and 0.32 μg(a.i.).cm^{-2}. 5 ml of test solution per vial were evenly distributed onto the filter paper. Control groups were also run in parallel, with 5 mL of distilled water only. After evaporated, the filter paper was rewetted with 5 mL of distilled water. An earthworm was introduced per vial. The dish was incubated in the dark at 20 ± 2 °C and 80–85% relative humidity for 48-h. A preliminary test was conducted to determine a concentration range for the chemical in which a 0–100% mortality of the earthworms was obtained. After 24 and 48-h, the worms were observed for mortality and other indications of toxicity (inflamed clitellus, expulsion of coelomic or bloody fluid, etc.). The earthworm was monitored for mortality by a gentle mechanical stimulus to the front part (prostomium). An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end.

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Data analysis

Data were presented as mean ± SD (standard deviation). The R software (version 3.3.2) (R Core Team, 2016) was used to perform all statistical analyses. Mortality calculations were based on the number of observed dead earthworms after the 24 and 48-h exposure period (*Formula 1*). The corrected mortality was calculated after determining the percent mortality using Abbott's *Formula 2* (WHO, 2009), and the data collected during the experiment was subjected to Probit analysis (Finney, 1971) as suggested by the standard paper contact toxicity procedure.

$$\text{Percent mortality} = \frac{\text{total number of earthworms died}}{\text{total number of earthworms released initially}} \times 100 \quad \text{(Eq.1)}$$

$$\text{Corrected mortality} = \frac{\text{Mortality in treatment} - \text{Mortality in control}}{100 - \text{Mortality in control}} \times 100 \quad \text{(Eq.2)}$$

The logit procedure of the "drc" package (version 3.0-1) (Ritz and Strebig, 2016) within the R software environment was used for the determination of lethal concentrations (LC_{10}, LC_{50}, LC_{90}). The classification of pesticide toxicity was done according to Roberts and Wyman Dorough classification (Roberts and Wyman Dorough, 1984) by considering LC_{50} values after 48-h exposure to studied commercial formulations: 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 non-toxic (> 1000 µg.cm^{-2}).

Results

Mortality

The mortality results of *A. trapezoides* exposed to the studied commercial formulations of acetamiprid at 24 and 48-h are shown in *Figure 1*. There were no mortalities found in the control and 0.02 µg(ai).cm-2 after 24-h, however it is recorded at all test concentrations after 24 and 48-h of exposure. The lethal rates showed a clear concentration-mortality relationship for both commercial formulations (Fig. 1). Maximum mortality was observed for Aceplan preparation at concentration 0.32 µg(ai).cm^{-2} after 48-h of exposure, for which immediate mortality was recorded. Only the 0.02 µg(ai).cm^{-2} concentration was non-toxic to earthworm for 24-h.

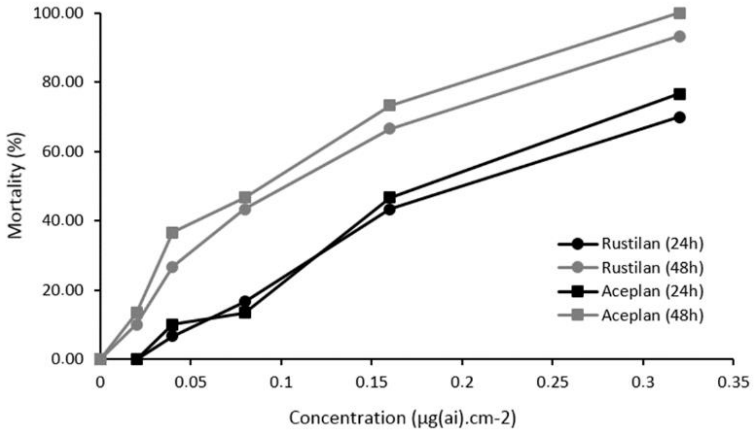

Figure 1. *Mortality rate recorded after 24 and 48-h for the two commercial formulations*

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LC_{50}

The statistical analysis of these results is done by the probit method. Thus, the acute contact toxicity (LC_{50}) values at 24 and 48-h are noted in *Table 1*. It appears that the lethal concentration decreases with the duration of the exposure. After 24-h, the LC_{50} values were 0.192 µg(ai).cm^{-2} for Rustulan and 0.164 µg(ai).cm^{-2} for Aceplan. These values are increased after 48-h: 0.086 µg(ai).cm^{-2} and 0.064 µg(ai).cm^{-2} for Rustilan and Aceplan, respectively. According to these results, studied commercial preparations are categorized as "supertoxic" to *A. trapezoides* (Roberts and Wyman Dorough, 1984).

Table 1. *Lethal concentrations (µg(ai).cm^{-2}) and 95% confidence levels of the two commercial formulations*

	Rustilan			**Aceplan**		
	LC_{10}	**LC_{50}**	**LC_{90}**	**LC_{10}**	**LC_{50}**	**LC_{90}**
After 24-h	0.050 ± 0.017 (0.011 – 0.083)	0.192 ± 0.050 (0.122 – 0.351)	0.732 ± 0.309 (0.350 – 1.361)	0.037 ± 0.011 (0.023 – 0.079)	0.164 ± 0.044 (0.102 – 0.356)	0.614 ± 0.292 (0.356 – 0.814)
After 48-h	0.020 ± 0.009 (0.004 – 0.037)	0.086 ± 0.021 (0.052 – 0.148)	0.371 ± 0.169 (0.230 – 0.758)	0.019 ± 0.007 (0.005 – 0.032)	0.064 ± 0.013 (0.040 – 0.097)	0.211 ± 0.072 (0.128 – 0.676)

LC_{50} ± SD; between brackets: 95% of confidence limits

Behavioral and body changes

In comparison to the control which displayed leisurely motions, the two commercial formulas applications led to different changes in tested earthworms at morphological (body lifting, coiling, swelling, etc.), behavioral (reduction of activity, reduction of movement, etc.) and physiological (bleeding, lesions, etc.) levels. At lower concentrations, 0.02 and 0.04 µg(ai).cm^{-2}, earthworms exhibited progressive signs, and symptoms like coiling, curling and mucus secretion with sluggish movements were observed. At the concentration of 0.080 µg(ai).cm^{-2}, we also observed extremely sluggish mobility, excessive mucus secretion and swelling (*Fig. 2a*). Increasing insecticide concentrations leads to increased damage to earthworm bodies (*Fig. 2b,c*): swelling, constriction, lesion, blooding and ultimately unresponsiveness to stimuli. These changes started to appear from 0.16 µg(ai).cm^{-2} in the anterior region as of the first 24-h of exposure. In the highest insecticide concentrations 0.32 µg(ai).cm^{-2}, a lack of response to stimuli was noted from first hours. Worms were not able to move after a few hours of exposure; neural retention starts and the body parts separate, leading to death. Thus, the deleterious effects of this concentration were lesions, inflammations and separation of the posterior body parts (*Fig. 2c,d*). About fifty percent of the worms exposed to this higher concentration detached their posterior parts in 48-h of exposure. Just after the death of the worms, their bodies become solid and change color (darker).

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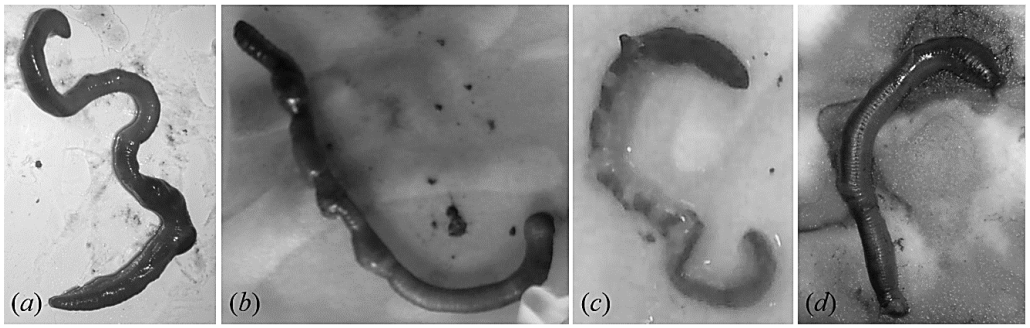

Figure 2. *Detrimental effects of body parts observed on A. trapezoides exposed to different concentrations of the two commercial formulations. a. Coiling, curling, and mucous secretion at 0.080 µg(ai).cm^{-2}, b. Swelling, rupture of the cuticle and bloody lesions at 0.160 µg(ai).cm^{-2}; c. Hyper oozing of coelomic fluid, multiple ruptures at body length and degeneration occurred on posterior end at 0.032 µg(ai).cm^{-2}; d. Fragmentation of posterior parts and dark pigmentation at 0.032 µg(ai).cm^{-2}*

Discussion

In this study, the acute toxicity of two commercial preparations for the earthworm *A. trapezoides* was investigated by applying the contact filter paper test. The results showed that their effects were concentration dependent and the percentage of survival decreased with increasing concentration of insecticide time of exposure. According to Roberts and Wyman Dorough (1984), it appears that both insecticide formulas are classified as "supertoxic" to *A. trapezoides*. While there is no comparable data for the investigated insecticides on earthworm toxicity available from the literature, a study by Wang et al. (2012) investigated the comparative toxicity of their active ingredient, *i.e.* acetamiprid. It showed similar toxicity results from the filter paper test for the earthworm *Eisenia fetida* with the LC_{50} after a 48-h exposure period being 0.0088 µg.cm^{-2} and therefore also classified as supertoxic. This LC_{50} value is, approximately, seven to ten times lower than the obtained values for the commercial preparations of Aceplan and Rustilan, respectively. This indicates that both the insecticides can have adverse effects on key species of soil ecosystems at low concentrations.

In practice, the applied doses of these insecticides are 100-300 g.ha^{-1} (equivalent to 0.2–0.6 µg(ai).cm^{-2}), which could pose a real danger to earthworm populations if they reach the soil surface. The applied dose is much higher than obtained lethal doses. This can lead to earthworm toxicity through direct contact when they rise to the soil surface or indirectly through the ingestion of contaminated litter.

Overall, there is also no information on the toxicity mechanisms available for the investigated formulas on tested earthworm. However, several epidermal, morphological and behavioral changes following direct contact with insecticides have been recorded in studied species during the test. The same changes were reported by studies on *Eisenia andrei*, *E. fetida*, *Eudrilus Eugenia*e and *Perionyx excavates* for other insecticides (Venkateswara Rao et al., 2003; Gambi et al., 2007; An and Lee, 2008; Kumar and Singh, 2017a; Tiwari et al., 2019; Jeyaprakasam et al., 2021). The first exposure to insecticide caused morphological changes such as reduced body movements and swelling. Swelling is very common and represents the first response of tested worms to the presence of insecticides in the medium. Lesions and bleeding are observed only at high doses. According to Venkateswara Rao et al. (2003), worms became agitated and restless to

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overcome the toxic effects of acetamiprid and required huge amounts of energy that were obtained by autolysis of their own tissue from the posterior region (*Fig. 2d*). Similar kinds of autolysis from the same region were observed in *Polypheretima elongata* due to the toxic effects of textile dyes (Ramaswami and Subburam, 1992). According to Kumar and Singh (2017b), body fragmentation and cuticle shedding are similar to ecdysis in insects and snakes. Worms abruptly put in contact with acetamiprid curl, some parts of their bodies swell, afterwards bleeding is observed, and after a while the worms die. Just after death, their bodies become solid and change in color (usually darker).

Earthworms subjected to the greatest amounts of preparations develop constrictions, swelling in the clitellar area with many ruptures, and punctures in the body wall with many lesions, resulting in body fragmentation and cuticle shedding. These alterations due to the two formulations were also reported by Wang et al. (2012) in earthworm *E. fetida* after 48-h of exposure to their active ingredient. When insecticide comes into contact with the earthworm's skin, it enters the body's coelomic fluid, causing toxic symptoms. The epidermis and cuticle serve as a major barrier between the earthworm's body and the environment, allowing ion transfer and allowing or blocking xenobiotics (Clauss, 2001). Penetrating cells, the insecticide tears their membranes which causes the segments to swell.

All of these changes are not specific to *A. trapezoides* towards acetamiprid since they have been observed in several earthworm species exposed to other commercial insecticide formulations (Alves et al., 2013; Tiwari et al., 2019; Jeyaprakasam et al., 2021). This can present a huge hazard or risk to other features of the earthworm stand and all living organisms in the soils of pesticide-treated fields.

Conclusion

The current study focused on assessing the acute toxicity of two commercial preparations of Acetamiprid on the cosmopolitan earthworm *A. trapezoids* using contact filter paper test. Results showed that acetamiprid induced toxicity in studied species, which demonstrated elevated mortality in worms exposed to higher concentrations. Both insecticides could be classified as 'supertoxic' to this species with LC_{50} values of 0.064 and 0.086 $\mu g.cm^{-2}$ after 48-h for Aceplan and Rutilan, respectively. The insecticides will be absorbed through the skin and will thus provide a rapid response. In fact, they cause morphological, physiological and behavioral changes in the studied species, which makes them an important symbol for the ecotoxicology of this earthworm species. Future studies should evaluate their effects on soil (natural) to include soil matrix influences on insecticide availability and toxicity.

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