



## Evaluation of The Toxic Effect of Sarolaner on Earthworms (*Eisenia foetida*): A Pilot Study

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**Abstract** | There is a new class of insecticides/acaricides—*isoxazolines*—that shows good efficacy against several ectoparasites. Sarolaner belongs to this family of molecules, and its potency, safety and pharmacokinetics have been optimised for use in dogs and cats. Furthermore, it presents significant selectivity for insect neurons. Nevertheless, its negative effects on susceptible non-target microorganisms like earthworms (which can be indirectly affected by insecticides) are unknown. The present study examined the effect of sarolaner on earthworms via two methods. In the first experiment, faeces were collected from 24 rabbits, 18 of which were medicated with sarolaner. The faeces were applied to a compost with earthworms. In the second experiment, sarolaner was applied directly to the earthworms. For both experiments, the number of earthworms was evaluated on days 1, 15 and 30 post-treatment. There were no significant differences between the sarolaner-treated and control groups at 15 and 30 days. Thus, sarolaner exhibited no negative effects with regards to earthworm survival.

**Keywords** | Earthworm, Evaluation, Sarolaner, Toxicity

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## INTRODUCTION

Since 2013, the use of isoxazolines in the veterinary market has increased. Recent studies demonstrated the efficacy of these molecules against several ectoparasites, including fleas, mites (*Demodex* sp., *Sarcoptes scabiei*, *Otodectes cynotis*, *Caparinia tripilis* and *Psoroptes cuniculi*), and ticks (McTier et al., 2016; Romero et al., 2016; Romero et al., 2017; Sheinberg et al., 2017). The  $\gamma$ -aminobutyric acid (GABA)-gated chloride channel and GABA receptor (GABA-R) are the targets for an expanding number and variety of insecticides, including the polychlorocycloalkanes

(e.g.,  $\alpha$ -endosulfan), fiproles (e.g., fipronil), and avermectins. Like these, isoxazolines (the latest addition with outstanding potency on pests) exhibit insecticidal and miticidal activities by targeting arthropod chloride ions channels linked to GABA or glutamate receptors (Shoop et al., 2014; Zhao and Casida, 2014). They present significant selectivity for insect neurons (Kilp et al., 2016). However, the effect of these molecules on ecosystem functions is unknown. Isoxazolines can potentially accumulate in the soil and may affect the survival of key soil ecosystem microorganisms. Toxicological studies are highly important and essential for the evaluation of

environmental effects. Several organisms may be involved in toxicity tests: terrestrial organisms, vertebrates, aquatic organisms and plants (Kolar and Erzen, 2006).

Non-target sensitive microorganisms, like earthworms, may be undesirably affected by insecticides (Cang et al., 2017). Earthworms are a terrestrial invertebrate widely distributed in the soil environment (Zhang et al., 2020). They can absorb organic compounds through skin diffusion, as well as by ingesting soil particles (Miao et al., 2018). *Eisenia foetida*, also known as the red Californian earthworm, represents 60–80% of the total animal biomass of soils (Durán and Henríquez, 2009; Jouquet et al., 2010). This species is responsible for organic matter biotransformation, improves the soil structure and nutrient cycle and plays a key role in the evaluation of terrestrial ecotoxicity (Domínguez et al., 2009; Araneda, 2016; Zapata et al., 2016). *Eisenia foetida* has been recommended by the OECD (Organization for Economic Cooperation and Development) as a biomarker for toxicity studies with soil organisms due to its ease of cultivation, low cost and high sensitivity to environmental contaminants (Iturbe-Requena, et al., 2019).

Wang et al. (2012) showed 24 different insecticides cause toxicity in *E. foetida*. The highest toxicity is from neonicotinoids. Compared with antibiotics, carbamates and organophosphates, pyrethroids are less toxic. Similarly, ethyl carbamate causes *E. foetida* mortality and toxicity, data that reflect the importance of knowing the risks of ecotoxicity with different insecticide products (Iturbe, 2019). The effect of one of the isoxazolines has already been evaluated in non-target organisms such as fish and bees, showing from low toxicity to notable risk depending on the organism evaluated (Jia et al., 2018; Sheng et al., 2019). However, there are no studies on possible toxicity from sarolaner, a prominent isoxazoline compound. The objective of this study was to evaluate the impact of sarolaner on earthworm (*E. foetida*) survival.

## MATERIALS AND METHODS

The protocol used in this study was approved by the ethics committee of the UAEM Amecameca University Center, Mexico (CBE/CUA/2016/05). A compost was prepared with *E. foetida* according to OECD guideline 207 (1984). During the first 2 weeks, the worms were fed with organic matter (a mixture of fruits, vegetables, cereals and eggs; no citrus fruits, oils and meats). In the first experiment, 80% of the organic matter was then replaced with rabbit faeces. To collect faeces, 24 rabbits (13 males and 11 females) were randomly chosen. None of the rabbits were sterilized, and the females were not pregnant. Each rabbit was housed individually in separate cages to avoid excreta

mixing; they were fed commercial feed. They were divided into four groups of 6 individuals each. Three groups were treated with 2.5 mg/kg sarolaner (Simparica®; Zoetis), and the fourth group was not treated with sarolaner (control group). Stool was collected for each rabbit group (with and without treatment). Stool administration was performed in each of the vermicompost sections: three compartments received sarolaner-treated rabbit faeces and one section received the control rabbit faeces. The morphology and number of earthworms in each section on days 1, 15 and 30 were compared.

In the second experiment, four more groups of 100 worms each were selected and fed with organic matter for 2 weeks. Subsequently, organic matter with 120 mg sarolaner was added to three groups; the fourth group served as the control and did not receive sarolaner. The number of worms was evaluated on days 1, 15 and 30 post-treatment by separating the worms from the organic matter.

## STATISTICAL ANALYSIS

The number of worms cultivated with rabbit faeces and the number of worms to which sarolaner was directly applied were analysed using a general linear model (Tukey's standardised range test) with  $\alpha = 0.05$ . Fisher's exact test was used for statistical comparisons. Three replicates were performed for the sarolaner treatment in each experiment.

## RESULTS

For the earthworms exposed to rabbit faeces, there were no significant differences in the number of worms between sarolaner-treated and control groups at 15 and 30 days; there was an increase in the number of earthworms in all groups. These data indicate that sarolaner did not negatively affect the number of worms cultivated (Table 1). Table 2 shows the comparison of the control group and the average of the three sarolaner replicates. There were no significant differences at 15 and 30 days, during which the control and treatment groups exhibited similar behaviour.

For the second experiment, sarolaner was directly applied to the worms in culture. There were no significant differences in earthworm numbers at day 15 or 30 post-treatment between the control and the sarolaner groups (Table 3). These data again revealed that sarolaner treatment did not negatively affect the worms. The comparison between worms treated directly with sarolaner and the control (Table 4) showed no differences at 15 and 30 days post-treatment.

**Table 1:** Comparison of the number (N) of worms fed with faeces from sarolaner-treated rabbits (replicates I, II and III) and faeces from untreated rabbits (control) on days 15 and 30 post-treatment.

Treatments	Initial (N)	Day 15 (N)	P	Day 30 (N)	P
Control	100	128		200	
Sarolaner I	100	120	0.73	187	0.69
Sarolaner II	100	134	0.80	250	0.18
Sarolaner III	100	171	0.11	195	0.88

P= > 0.05, determined using Fisher's exact test.

**Table 2:** Comparison of the number of worms grown with faeces from sarolaner-treated rabbits and worms grown with faeces from untreated rabbits (control) on days 15 and 30 post-treatment.

Day 15 Number of worms	Day 15 Number of worms	Day 30 Number of worms
128 <sup>a</sup>	128 <sup>a</sup>	200 <sup>a</sup>
141.63 <sup>a</sup>	141.63 <sup>a</sup>	210 <sup>a</sup>
694.3	694.3	1176.3

Statistical analysis performed with GLM Tukey's staged range test; columns with the different letter were significantly different. \* mean of the 3 replicates

**Table 3:** Comparison of the number (N) of worms treated directly with Sarolaner (replicates I, II and III) and worms without treatment (control) on days 15 and 30 post-treatment.

Treatments	Initiation (N)	Day 15 (N)	P	Day 30 (N)	P
Control	100	141		198	
Sarolaner I	100	133	0.75	187	0.74
Sarolaner II	100	126	0.54	160	0.22
Sarolaner III	100	136	0.84	201	0.93

P= > 0.05, determined using Fisher's exact test.

**Table 4:** Comparison of the number (N) of worms treated directly with sarolaner and worms without treatment (control) on days 15 and 30 post-treatment.

	Day 15 (N)	Day 30 (N)
Control	141.0 <sup>a</sup>	198.0 <sup>a</sup>
Sarolaner*	131.6 <sup>a</sup>	182.6 <sup>a</sup>
EE	26.3	434.3

Statistical analysis performed with GLM Tukey's staged range test; columns with the different letter were significantly different. \* mean of the 3 replicates

## DISCUSSION

Earthworms are dominant fauna in most temperate terrestrial ecosystems (Shi et al., 2017). They play a crucial role in modifying soil structure and accelerating the breakdown of organic matter and nutrient recycling (Domínguez et al., 2009). Thus, their abundance in soil is an indicator of the soil health (Ouina et al., 2016). With the emergence of increased resistance of insects to insecticides, the application of higher doses of these can increase adverse effects for non-target organisms. The effects of toxic substances on

reproduction, growth rates and behavioral traits of *E. fetida* have been reported. Therefore, it is important to assess the ecological risks of insecticides for ecosystems (Wang et al., 2015).

Isoxazolines (sarolaner, fluralaner, afoxolaner and lotilaner) are a new class of oral ectoparasiticides for pets. These compounds have demonstrated remarkable activity against the most common dog ectoparasites, namely fleas and ticks. They have several advantages over first-generation oral parasiticides, which have a limited ability to effective-

ly kill both fleas and ticks in dogs (Beugnet and Franc, 2012). The sarolaner mechanism of action in insects and mites involves blocking chloride channels linked to GABA and glutamate receptors. This blockage prevents chloride ion entry into nerve cells and consequently increases nerve stimulation and leads to the death of the parasites. Sarolaner was shown to exhibit a greater potential for blocking insect receptors compared to the mammalian receptors (McTier et al., 2016). Furthermore, it does not interact with insecticidal nicotinic receptors or other GABAergic insecticidal receptors like other insecticide classes, including neonicotinoids, fiproles, milbemycins, avermectins and cyclodienes (Shoop, 2014). However, there are no reports of the effect of isoxazolines, including sarolaner, on other microorganisms, including those that belong to the Lumbricidae family.

In dogs, sarolaner has been shown to provide rapid killing of existing fleas, as well as newly infested fleas, for 35 days (Six et al., 2016a). Reduction of 94% was seen in 8 hours after oral administration, and fleas were eliminated from all dogs during the 35-day assessment period (Six et al., 2016a). Sarolaner has been reported to be effective against 5 species of ticks infesting dogs in United States America (Six et al., 2016b), and 4 species infecting dogs in Europe (Geurden et al., 2016). Newer publications show efficacy against the Australian paralysis tick (Packianathan et al., 2017) and the Asian long horn tick (Oda et al., 2019). For multiple tick species, killing of > 90% of ticks in 24 hours persists for 28 days. By contrast, in this study, neither the feces of rabbits treated with sarolaner nor the direct administration killed earthworms at 15 and 30 days post-treatment. This data is consistent with a previous observation that direct administration to earthworms did not cause harmful effects (Shoop, 2014).

Fipronil, a phenylpyrazole insecticide, has been widely used for insect control and plant protection; it is effective against a wide range of insect pests (Qin et al., 2014). Fipronil is a chronic pesticide that binds with organic matter and is detrimental to earthworms in shallow soil (Yu et al., 2013). It can reduce the earthworm reproduction rate, as does imidacloprid, a neonicotinoid insecticide (Alves et al., 2013). Although fipronil does not promote acute toxicity in earthworms (Mostert et al., 2002), earthworms can accumulate fipronil and its soil metabolites. These compounds can then be transferred and/or bioaccumulate in predatory organisms that feed on earthworms (Qin et al., 2014).

Although organophosphates and carbamates have different structures, both inhibit acetylcholinesterase (AChE) activity (Carmo et al., 2010). The toxicity of organophosphates for earthworms depends on the evaluated parameter. These chemicals will certainly show a very high toxicity if AChE

inhibition and the consequent physiological damage are evaluated (Reddy and Rao, 2008).

Neonicotinoids are among the most effective insecticides for pest control. They act as competitive inhibitors of nicotinic acetylcholine receptors (nAChR) in the central nervous system (Elbert et al., 2008). These compounds can endanger soil organisms, including earthworms (Ishaaya and Degheele, 1998). In a study by Wang et al. (2012), among the different chemical classes evaluated, neonicotinoids, such as imidacloprid and nitenpyram, show the highest toxicity towards *E. foetida* through direct contact. Soil toxicity was also tested, namely by assessing mortality at 7 and 14 days post-treatment. The neonicotinoids again exhibit the highest toxicity. Even a more recent study, in addition to affirming the toxicity on physiological and biochemical indicators of earthworms, indicated possibilities of damage at the genetic level after application of nitenpyram (Zhang et al., 2020).

In contrast, the current study showed that soil-level and direct sarolaner administration to the vermicompost had no effect on earthworm reproduction. Imidacloprid, in comparison with carbaryl, cyfluthrin, chlorpyrifos and fipronil, exhibits a negative effect on the earthworm (Mostert et al., 2002) that was not observed with sarolaner. Luo et al. (1999) also found adverse effects and developed the sperm deformity test to detect possible adverse influences of pesticides on earthworm reproduction. These deficits occur for imidacloprid concentrations greater than 0.5 mg/kg dry soil.

Pyrethroid insecticides bind to a distinct receptor site in the sodium channel and are relatively less toxic to mammals because they are rapidly metabolised in those animals as well as ecosystems (Narahashi, 2000). Most of these compounds are not toxic to earthworms in soil toxicity tests (Inglesfield, 1984). These results are similar to the current study with regards to the lack of sarolaner toxic effects.

The adverse effect of subchronic or chronic exposure is also important in ecological risk assessments (Liu et al., 2011). Isoxazoline toxicity has been examined in zebrafish and honeybee. Fluralaner exhibits low toxicity in this fish, but it can rapidly bioaccumulate (Jia et al., 2017). Instead, after exposure of fluralaner to the honeybee, high toxicity risk was reported (Sheng et al., 2019); other isoxazolines have not been tested in this model.

The growth and reproduction of earthworms are important measures used in environmental ecotoxicity (Wu et al., 2011). Thus, the relative lack of a toxic sarolaner effect on earthworms provides crucial information about the potential environmental safety of this compound.

In the present investigation, sarolaner was not toxic to earthworms (*E. foetida*) through either indirect exposure (through the faeces of rabbits medicated with this isoxazoline) or direct administration of sarolaner to the vermicompost. Sarolaner did not affect the reproduction and growth of earthworms up to 30 days post-administration.

## CONFLICT OF INTEREST

There are no conflict of interests.

## AUTHORS CONTRIBUTION

Camilo Romero Núñez: Conceptualization, Methodology, Software, Validation, Formal Analysis, Data Curation, Writing – Original Draft, Supervision, Project Administration. Rafael Heredia Cárdenas: Resources, Software, Validation, Formal Analysis, Data Curation, Writing – Review & Editing. Laura Miranda Contreras: Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. Galia Sheinberg Waisburd: Resources, Methodology, Review & Editing, Visualization. Alberto Martin Cordero: Resources, Methodology, Review & Editing, Visualization.

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