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Original Article

The Toxicity of The Antiparasitic Amitraz in Tropical Soils on The Reproduction of Edaphic Organisms

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Abstract

The objective was to evaluate the effect of environmental toxicity in soils (tropical) with increasing doses of the antiparasitic amitraz on the survival and reproduction of two edaphic organisms, *Folsomia candida* and *Enchytraeus crypticus*. Soils contaminated with increasing doses of amitraz caused a significant reduction in the survival and reproduction of the population of adults and juveniles of *F. candida*, in the natural soil tested, although, for TAS, the reduction was significant only for juvenile individuals. However, the species *E. crypticus* did not show any sensitivity to exposure to amitraz in all tested soils. We concluded that the addition of increasing doses of this antiparasitic substance can cause imbalance on the terrestrial ecosystem, since it is harmful to at least one member (and perhaps more) of the springtail family, an important edaphic species, whose members play many relevant functions for the maintenance of soil fertility and quality.

Keywords: Environmental toxicology; Soil contamination; Terrestrial invertebrate ecotoxicology.

INTRODUCTION

The antiparasitic amitraz has been used since the 70s (Jonsson et al., 2010)measurable level of resistance to amitraz. Standard counts of all ticks between 4.5 and 8.0. mm diameter on one side of each animal were made each week and treatment was applied when tick numbers exceeded a threshold of 25 engorged adults per side. The three treatments were: 1, spinosad spray whenever tick numbers exceeded the threshold; 2, amitraz spray whenever tick numbers exceeded the threshold; 3, spinosad whenever tick numbers exceeded the threshold for the first 2 months, then amitraz for 2 months, with alternation every subsequent 2 months. Engorged adult female ticks were collected from each treatment group on 10 or 11 occasions during the study and tested using the larval packet test bioassay (LPT. In addition, this product is low cost, which facilitates its use in different locations and the control of parasites.

The mode of action of this pesticide on invertebrates is not well understood (Jonsson, *et al.*, 2018), but it is described as a formimidin, which controls ectoparasites such as ticks, inhibiting ovulation (Kanapadinchareveetil *et al.*, 2019). The main routes of environmental contamination are through animal waste as a source of organic fertilizer that are medicated with chemical compounds that excrete

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waste and metabolites via feces/or urine (Kim *et al.*, 2018) environmental water contamination resulting from veterinary antibiotics has not been studied extensively. In this work, we developed an analytical method for the simultaneous determination of multiple classes of veterinary antibiotic residues in environmental water using on-line solid-phase extraction (SPE).

Causing negative effects on the environment (Sarmah *et al*., 2006) the majority is excreted unchanged in faeces and urine. Given that land application of animal waste as a supplement to fertilizer is often a common practice in many countries, there is a growing international concern about the potential impact of antibiotic residues on the environment. Frequent use of antibiotics has also raised concerns about increased antibiotic resistance of microorganisms. We have attempted in this paper to summarize the latest information available in the literature on the use, sales, exposure pathways, environmental occurrence, fate and effects of veterinary antibiotics in animal agriculture. The review has focused on four important groups of antibiotics (tylosin, tetracycline, sulfonamides and, to a lesser extent, bacitracin. Through surface water it can also produce long-term contamination (Jaffrézic *et al*., 2017), consecutively affecting non-target organisms (Zortéa *et al*., 2017). Arthropod and annelid bioindicators are ecologically relevant (Bandeira *et al*., 2020, 2021), collembolas are one of the most abundant organisms in soils, play roles in the decomposition of litter and are sensitive to anthropogenic changes (Gruss *et al*., 2023). Enchytreids are important because they tolerate acidic soils, other annelid species express greater sensitivity and act in soil bioturbation and are sensitive to chemical substances (Camargo Carniel *et al.*, 2019; Castro-Ferreira *et al.*, 2012).

Due to the lack of information about the dangerousness of the antiparasitic amitraz in the edaphic community, ecotoxicological tests are necessary to verify the real effect of this chemical compound after its release into the environment, and consecutively negative effects on environmental compartments like many other veterinary drugs (Serafini *et al.,* 2019; Testa *et al*., 2020; Zortéa *et al.,* 2018). Thus, the relevance of these studies should not be discarded, since after exposure to chemical substances in the environment, they can cause deleterious effects in these individuals (Serafini *et al*., 2019) which, after a few descendant generations, can be perceived when directing generations (Guimarães *et al.*, 2019) and they are usually exposed only during a fraction of their life-cycle. This approach is very important but does not cover the potential effects of multigenerational (MG directly affecting the balance of the terrestrial ecosystem.

Thus, our objective was to evaluate the effect of environmental toxicity in (tropical) soils with increasing doses of the antiparasitic amitraz on the survival and reproduction of two edaphic organisms, *Folsomia candida* and *Enchytraeus crypticus*.

MATERIAL AND METHODS

Soils

For the experiment, an Arenosol (WRB-FAO, 2015) with yellow characteristics, tropical climate, natural from a pasture area, located in the state of São Paulo, SP, Brazil, was used. Taking as reference an artificial soil, recommended by ISO 11268-2 (ISO, 1998), called (TAS), which consists of a mixture of 75% fine industrial sand, 20% kaolin and 5% coconut fiber, which replaces the tundra material used in countries with a temperate climate, making this soil closer to Brazilian tropical realities (Garcia, 2004). The physical and chemical characteristics of both soils are presented in Table 1. The natural soil was subjected to three cycles of freezing (24 h) and drying in an oven at 65 ºC to eliminate the edaphic fauna found in the samples (Segat *et al*., 2015). Soil moisture was corrected to 65% of the maximum water holding capacity (CAA). which replaces the tundra with coconut fiber.

Antiparasitic test

The doses were based on the recommendations for use in the field, according to the manufacturer (10 mL of the product for 5 L of water – applied through pulverization) (AMITRAZ 12.5% MitraNox-NOXON®, s.d.). We prepared six doses of the compound for each soil. For the Arenosol (0; 0.35; 1.75, 3.50; 17, 50 and 35.00 mg kg^{-1}) and TAS (0; 0.37; 1.87, 3.75, 18.75 and 37.50 mg kg^{-1}).

The chemical compound amitraz in its commercial form is insoluble in water since it is of oily nature. Thus, it was necessary to perform dilutions in acetone, to promote a homogeneous mixture in the soil, using a volume of 25 mL, for each 100 g of soil. After being dissolved in acetone with the correction for each dose tested and applied to the two types of soil, the doses were transferred, for a period of 16 h, to an exhaust chamber so that the acetone could evaporate before adding the organisms (Jensen *et al.,* 2003). After removing the exhaust chamber, we applied the necessary water to complete the total required moisture, and subsequently the organisms for each test.

Ecotoxicological tests

Two methodologies standardized by the ISO and adjusted according to each species were used, ISO 11267 (1999) for *F. candida*, and ISO 16387 (ISO, 2004) for *E. crypticus*. Were carried out survival and reproduction tests for both species. *F. candida* was submitted to acute and chronic tests, and each replicate received 10 individuals, 10 - 12 days old, in a plastic flask (height: 11.5 cm; diameter 3.5 cm) filled with

30 g of soil, previously treated with the test substance in the respective doses. The feed provided was biological yeast (*Saccharomyces cerevisiae*). The flasks were opened weekly for aeration and moisture correction. 28 days after the start of the test, we counted the number of surviving adults and generated juveniles. For this purpose, the individuals were transferred to another flask and water and black ink were added to facilitate visualization and counting, using the *ImageTool* software (University of Texas Health Science Center, 2002).

For *E. crypticus,* 10 individuals with apparent clitella were introduced into a plastic flask (height: 11.5 cm; diameter: 3.5 cm), filled with 30 g of soil spiked with the test substance in the respective doses. *E. crypticus* were fed with ground and sterilized oat flakes, split in two times, at the beginning of the experiment and after 14 days. After the 28th day, the content of the flask was wet sieved (75 µ mesh) and colored with ethyl alcohol with Bengal rose (1% solution with ethanol), to facilitate visualization and counting.

Statistical analyses

The data complied with normality (Shapiro-Wilk test) and homogeneity (Levene test). They were subjected to analysis of variance (ANOVA-*one-way*) and the means were compared with Dunnet's test ($P < 0.05$). Based on the ANOVA, we calculated the values of LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration).

The EC_{50} values (estimated dose for affecting 50% of the organisms) and for the reproduction test were calculated using non-linear regression models (logistic model). The LC_{50} values (dose estimated to cause 50% mortality) in the survival tests were estimated from the PriProbit® 1.63 Software (Sakuma, 1998).

Test validation

For the results to be validated, the tests of *F. candida* and *E. crypticus* must be in accordance with validation criteria given by the respective guidelines of ISO 11267 (1999) and ISO 16387 (2004). They must present adult mortality below 20%, the number of juveniles must be greater than 100 organisms per replicate and the coefficient of variation (CV) must be less than 30% (*F. candida*) and 50% (*E. crypticus*) in the control treatment.

RESULTS

The tests performed with *F. candida* met the validation criteria in accordance with the guidelines of ISO 11267 (1999), for all tested soils. For the Arenosol, the average survival in the controls was 8 individuals, the juvenile average was 125, with a C.V. of 5.88%. In TAS, the average survival in the controls was 8 individuals, there was an average of 210 in reproduction, and the C.V. was 12.75%.

For *E. crypticus* met the validation criteria in accordance with ISO 16387 (2004). In the test, the Arenosol presented an average of 235 juvenile organisms and the C.V. of 30.42% and TAS had an average of 358 juveniles and a C.V. of 35.70%.

Folsomia candida

The results obtained in the tests of survival and reproduction in natural soil (Arenosol) expressed that the population of adult and juvenile organisms of *F. candida* suffered a significant reduction due to amitraz, even in smaller doses, below the recommended dose for the control of parasites (Figure 1A). However, in the reference artificial soil, called TAS, in which high mortality was evident only for young individuals, when exposed to amitraz (Figure 1B), this compound is evidently toxic to *Folsomia candida*.

The values stipulated for NOEC (non-observed effect concentration), LOEC (lowest observed effect concentration), and obtained for LC_{50} (concentration estimated to cause 50% mortality) and EC_{50} (estimated concentration capable of affecting 50% of organisms) with their respective confidence intervals are shown in Table 2 for both evaluated soils.

Enchytraeus crypticus

Amitraz did not cause a significant reduction in the juvenile population. The results obtained in the evaluation of amitraz toxicity in the reproduction of *E. crypticus* in natural and artificial soil (Arenosol and TAS) are present in Figures 2A-B.

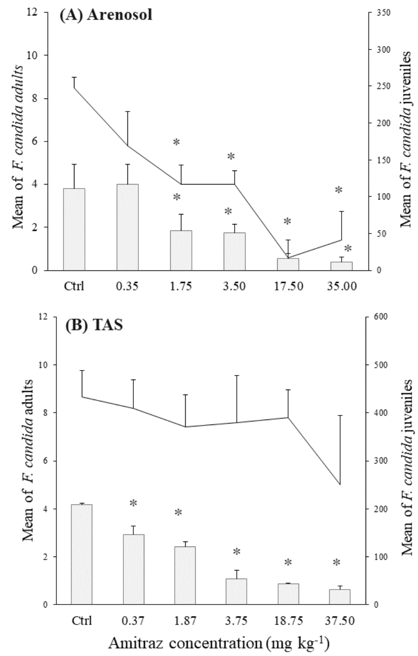

Figure 1 – Adults survival lines and juvenile bars of *F. candida* when exposed to increasing doses of amitraz tested in Arenosol (A) and Tropical Artificial Soil (TAS) (B). (┬) standard deviation. Asterisks (*) indicate a significant difference for the average number of individuals compared to the control (Ctrl) ($p < 0.05$; one-way ANOVA followed by Dunnett's test).

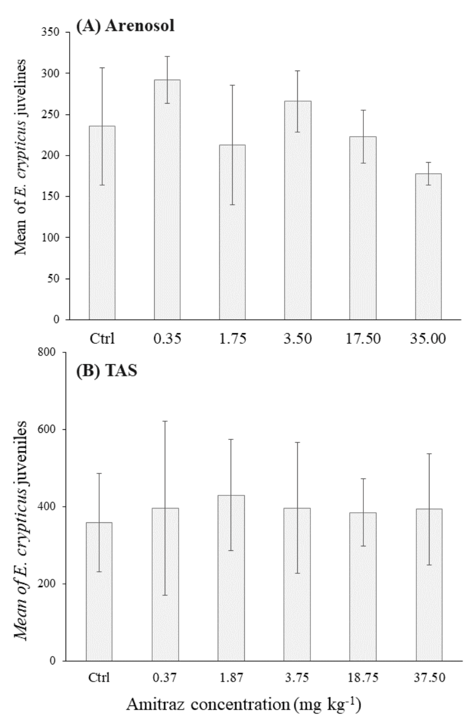

Figure 2 – Juvenile medium bars of *E. crypticus* when exposed to increasing doses of amitraz tested in Arenosol (A) and Tropical Artificial Soil (TAS) (B). (┬) standard deviation ($p < 0.05$; one-way ANOVA followed by Dunnett's test).

Table 1. Physical and chemical parameters of the typical Arenosol and Tropical Artificial Soil (TAS)

Soil	OM[1]	pH	CEC[2]	Clay	P	K	Ca	Mg	Hal	Cu	Zn	Fe
	(%)			(%)	(mg dm^{-3})		(cmol dm^{-3})			(mg dm^{-3})		
Arenosol	2.9	5.3	9.29	15	4	152.1	4.2	0.9	3.8	-	-	-
TAS	5.0	5.8	77.0	20.0	28.0	0.07	25.0	9.0	12.0	0.2	6.0	1.9

[1]OM - Organic matter.

[2]CEC - Cation exchange capacity at pH 7.0.

- not-analyzed

Table 2. Ecotoxicological parameters (NOEC, LOEC, LC_{50} and EC_{50}) calculated for *F. candida* exposed to amitraz.

Test	Parameter	Soils contaminated with amitraz (mg kg^{-1})	
		Arenosol	TAS
Acute	NOEC	0.35	VC
	LOEC	1.75	VC
	LC_{50}	1.70 [CIC]	VC
Chronic	NOEC	0.35	<0.37
	LOEC	1.75	0.37
	EC_{50}	1.78 (CI: 0.81-2.76)	1.58 (CI: 0.74-2.43)

CI: confidence intervals

CIC: Confidence interval not calculated, due to data conformation

VC: values not calculated

DISCUSSION

The toxicity effects at lower doses than recommended, and the correction of this for dry soil (10 mL of product per 5 L of water – pulverization application) (Bulla amitraz 12.5% MitraNox-NOXON®, sd), were more evident for the springtails than for the enchytraeids. The greater toxicity of amitraz to *F. candida* is due to this species belonging to the phylum Arthropoda (Fountain & Hopkin, 2005), since arthropods are the focus of the active principle of this pesticide (Hu *et al*., 2019). In contrast, *E. crypticus,* who belongs to the group of oligochaetes (Castro-Ferreira *et al.,* 2012), showed less sensitivity to the active principle because they belong to a different target group. Generally, exposure in springtails (arthropods) is through soil pore water, whereas in annelids exposure is by passive diffusion of water or via intestinal absorption of soil particles contaminated by pesticides (Bandeira *et al*., 2021).

Thus, the importance of studying different trophic groups for sensitivity to the same substance becomes evident (Camargo Carniel *et al*., 2019; Lin *et al.,* 2019), in order to verify the level of real toxicity of the compound to each edaphic organism. Generally, exposure in springtails (arthropods) is through soil pore water, whereas in annelids exposure is by passive diffusion of water or via intestinal absorption of soil particles contaminated by pesticides.

Little is known about the effect of amitraz to invertebrates in general (Jonsson, *et al.*, 2018), however research shows an expressive toxicity of formamidines in general to insects, being related to the substance's mode of action through the central nervous system (CNS) of the affected organism (dos Reis *et al.,* 2021). Formamidine can produce a result similar to octopamine, causing changes in the mechanisms mediated by G protein, resulting in tremors and seizures and thus causing paralysis or even the death of adult arthropods (Chen *et al*., 2007; Fular *et al*., 2022), which corroborates the results obtained in this work, as *F. candida* was significantly affected by amitraz.

However, despite of what we said above, it was not possible to find studies evaluating the toxicity of amitraz on different soil quality bioindicators previously. Nevertheless, the negative effects of formamidines on arthropods, such as mites of the species *Tetranychus urticae,* moquistoes of the species *Chironomus riparius* and flies of the species

Drosophila melanogaster, were observed by different authors, with deleterious impacts (Chang & Knowles, 1977; Dudai *et al.*, 1987; Monteiro *et al.*, 2019).

Based on the history of amitraz toxicity on insects, the results obtained in the current study proved to be alarming for edaphic organisms. Since amitraz is recommended for use directly in pastures, with the purpose of eradicating parasitic larvae in farm animals (Harrison & Palmer, 1981). Thus, having direct contact with the soil, causing continuous exposure to edaphic organisms and triggering generalized environmental toxicity, these chemical products should not be applied at sites where one does not wish to exterminate all insects (Thiele-bhn, 2003; Zortéa *et al.*, 2018).

Studies indicate that veterinary drugs are not completely metabolized by the animals, and chemical residues are released into the environment via excreta (urine and feces), later incorporated into the soil, as well as transported to surface waters and groundwater (Menz *et al*., 2019). Several factors influence the dynamics of chemical compounds in the soil, as soil texture, which means the proportion of clay, silt and sand and which defines the size of soil pores (Ding *et al.,* 2020), altering the capacity of the soil to retain contaminants. If the soil afterwards will be used to irrigate agricultural crops (Peijnenburg *et al.,* 2012), it is even more dangerous.

Considering the importance of the retention capacity of the different types of soil, the present study compared the results obtained in the EC_{50} between the two types of soils evaluated (natural and artificial). The toxicity differences were depending on the type of soil, since the artificial soil (TAS) showed higher toxicity when compared to Arenosol. There are circumstances when natural soils with higher clay contents have a high adsorption capacity, that is, causing a significant reduction in the toxicity of chemical compounds (Serafini *et al.*, 2019). The amount of water can also affect edaphic organisms, since the toxic effect on springtails can be potentiated when a chemical substance is made available in soil pore water the springtails absorb the water present in the medium (Peijnenburg *et al.*, 2012). This shows the relevance of the present study in analyzing soils with different textural characteristics and water retention capacities in order to verify whether the level of toxicity could be aggravated due to the physical properties of the tested soils.

In addition, amitraz is chemically unstable in acidic conditions (Jonsson, *et al.,* 2018), which may corroborate the toxic effects obtained in the tested natural soil, since no pH correction was performed. The natural Arenosol presented a pH of 5.3, while the TAS, during preparation, was corrected to pH 6.0, according to ISO 11267 (1998).

However, the soils with acidic pH (Arenosol, and TAS) analyzed in the present study may have been positive for the enchytraeidae due to their lower sensitivity to amitraz, when compared to the springtails. Enchytraeidae have greater resistance to acidic environments although their activity in soils maintains their dermis is in direct contact with water and soil (Castro-Ferreira *et al.,* 2012). It is worth mentioning that the United States Protection Agency classified the active ingredient amitraz as toxic (class III), and its main route of toxicity is through oral exposure (USEPA, 1996).

In the European Union, the use of this product is already restricted due to the high negative impacts that it brings to the bee communities. As a matter of fact residues of amitraz are still found in bee products, even though the use restriction started in 2005 (Rial-Otero *et al.*, 2007)scientist, beekeepers and chemical companies disagree about the reasons that have led to colony losses higher than 50% in some areas. This problem has become a public health issue due to the high honey worldwide consumption. The presence of pesticides in honey has been directly related to bees' mortality by some researchers through pesticide presence in (1. These effects are worrisome, since even the residual effects of amitraz being 7 to 10 days (Jonsson *et al*., 2018) the molecules can adsorb to soil and sediments and thus cause secondary effects to the ecosystem.

The present study is important, since it is known of the continuous use of the antiparasitic amitraz in production animals throughout Brazil today, even knowing the history of ineffectiveness of the compound in target organisms such as ticks (Klafke *et al.*, 2017). And, in this way, residues of amitraz and its metabolites can be found in Brazilian soils, a situation that is directly affecting organisms that are not the target of this active principle and the ecosystem.

Organisms that promote goods and services provided to men must be preserved so that there is noinestimable losses, as well as the decline of soil biodiversity, agricultural production and avoidance of environmental contamination.

CONCLUSIONS

Amitraz doses, lower than those indicated for use in the field, affect the survival of *F. candida* in the tested natural soil, unlike the Artificial Soil (TAS), where the effect was not toxic. However, the juvenile population of the collembolans decreased in all soils tested by exposure to this pesticide.

AUTHOR CONTRIBUTIONS

Reis TR, Segat JC conceived and designed the research; Reis TR and Segat JC performed data analysis; Baretta D and Cardoso EJBN made critical contribution to the discussion. All authors revised the manuscript. All authors read and approved the final manuscript.

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