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# Ecotoxicological effects of fipronil, neem cake and neem extract in edaphic organisms from tropical soil

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ABSTRACT

Veterinary medicines are widely applied for the treatment and prevention of animal diseases. Consequently, animal manure contains significant amounts of environmental pollutants that are potential sources of environmental pollution when inappropriately applied in soils. This work aimed to evaluate ecotoxicological effects of doses of commercial fipronil, neem cake and neem extract in the survival and reproduction of earthworms (*Eisenia andrei*), enchytraeidae (*Enchytraeus crypticus*) and springtails (*Folsomia candida*) in Oxisol and tropical artificial soil (TAS). Applications of fipronil, neem cake and extract in soil were carried out according to standardized ISO methodologies by using a random experimental design with five replicates. Toxic effects of fipronil for springtails in Oxisol and TAS were observed with $LC_{50}$ of 0.26 mg $kg^{-1}$ (0.18–0.35 mg $kg^{-1}$) and 0.29 mg $kg^{-1}$ (0.22–0.37 mg $kg^{-1}$), respectively. It was not observed significant toxic effects of fipronil for earthworms and enchytraeidae in both soils. However, significant amounts of juvenile earthworm and adult enchytraeidae decreased in fipronil doses higher than 10 mg $kg^{-1}$. Neem cake and extract were not toxic for earthworms and enchytraeidae but, significant amounts of juvenile springtails decreased in neem cake doses from 500 to 1000 mg $kg^{-1}$. It can be concluded that the use of veterinary medicines containing synthetic compounds for preventing diseases in animals needs to be controlled to avoid environmental pollution after applying manure in soil. Veterinary medicines containing natural compounds as neem cake and extract are eco-friendly and could be efficiently applied in soil in a sustainable way.

## 1. Introduction

Veterinary medicines have been widely applied for preventing diseases and improving the development indices of animals (Kummerer, 2010; Arnold et al., 2013). High concentrations of veterinary medicines applied in bovines for controlling parasites are excreted in faeces and urine (Jones et al., 2003; Tolls, 2001). Therefore, the soil fertilization by using bovine manure increases the concentration of certain compounds in soil and water sources (Förster et al., 2009; Zhang et al., 2016). As many chemical compounds present in veterinary formulations are considered emerging pollutants due to high toxicity for edaphic organisms, the use of animal manure as biofertilizers must be controlled and monitored (Santos et al., 2010; Di Nica et al., 2015). For instance, the fertilization of soil with animal manure containing synthetic veterinary formulations can affect the edaphic organism populations that are responsible by decomposition of organic matter and nutrient cycling (Beynon, 2012; Lumaret et al., 2012). Moreover, this can be decreasing the production indices of crops and increasing the environmental contamination problems (Bártíková et al., 2016).

According to current European legislation, the environmental impacts caused due to action of synthetic veterinary medicines in edaphic organisms of soil must be evaluated by considering effects and fate of each chemical compound in the environment (Liess and Beketov, 2011). Therefore, studies of terrestrial ecotoxicology to short and long-term are excellent alternatives for understanding the toxic effects of veterinary medicines in edaphic organisms (Kuperman et al., 2009). Invertebrate edaphic as earthworms, springtails and enchytraeids are important for the organic matter decomposition, release and cycling nutrient (Botinelli et al., 2015). Moreover, these organisms are commonly employed as quality bioindicators of soils since are easily adapted in laboratory conditions and pollutant-sensitive (Greenslade and Vaughan, 2003; Havlicek, 2012) in addition to have short life

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cycles (Fountain and Hopkin, 2005; Jänsch et al., 2006).

Fipronil is widely employed as synthetic medicine for avoiding external parasites on bovines due to its physiochemical properties and slow action inside the animal (Gunasekara and Troung, 2007). Despite fipronil is widely employed in many countries, it is not allowed in Europe due to its toxicity to insects (Stevens et al., 1998). In view of this, the toxic effects of fipronil for edaphic organisms have been recently studied (Alves et al., 2014b; Qu et al., 2014; Rehbein et al., 2013; Zortéa et al., 2017, 2018). Veterinary medicines products containing plant chemicals may be used safely because of the possibility of having low toxicity compared to synthetic compounds (Broglio-Micheletti et al., 2010). Therefore, many studies have been conducted to make feasible veterinary medicines containing natural compounds to decrease the environmental impacts after application in animals as well as after the use of manure in fertilization of soils. A natural product as Indian neem (*Azadirachta indica*) is an excellent alternative to be used as natural veterinary medicine since it has acaricide effects for ticks (Landau et al. (2009); Denardi et al. (2010); Giglioti et al. (2011); Remedio et al. (2016) and low toxicity for mammals (Silva et al., 2008; Xu et al., 2018; Šefčíková et al., 2018). However, some studies should be conducted to know its toxic effects for the environment.

The hypothesis of the work is based on the toxicity of fipronil being greater to the edaphic organisms, whereas the natural phytotherapeutic compounds, the base of neem, have smaller effects. The objective of the present study was to evaluate the effects on survival and reproduction of three soil quality bioindicators (*Eisenia andrei*, *Enchytraeus crypticus* and *Folsomia candida*) when exposed to natural soil contaminated with doses of a chemical compound based on fipronil, and two phytotherapeutic compounds, neem cake and neem extract, all used to control parasites in cattle.

## 2. Materials and methods

### 2.1. Soils

Oxisol samples were collected at depths ranging from 0 to 20 cm in the City of Chapecó – SC, Brazil (27°05′274″S; 052°38′085″W). The soil samples were dried in an oven at 65 °C, milled and sieved to 2 mm using a granulometric sieve. TAS sample containing 75% of sand, 20% of caulim and 5% of cocoon fiber was prepared mixing each component and adjusting the pH for values ranging from 5.5 to 6.5 using $CaCO_3$. The humidity of both soils was corrected to 65% of the maximum retention capacity. The physiochemical properties were measured according Embrapa (2006); the Oxisol had 3.9% organic matter, pH 4.3 and clay 55%. The SAT had 5% organic matter, pH 5.8% and 20% clay.

### 2.2. Veterinary medicines

Natural neem cake, purchased from Dogneem® and extract neem from Sigma-Aldrich®, were employed as phytotherapic medicines and fipronil purchased from Top Line-Merial® as synthetic medicine. Doses of neem cake and extract were calculated based on the Azadirachtin concentration in each product. Neem cake containing 2.5% of Azadirachtin was applied in soil in the following doses: 0; 5.0; 10; 20; 40; 80; 200; 500 and 1000 mg extract per kg soil. Neem extract containing 38% of Azadirachtin was applied in soil in the following doses: 0; 1.5; 3.0; 7.0; 15; 35; 70 and 140 mg extract per kg dry soil. Commercial fipronil containing 5% of active ingredient was applied in soil containing earthworms and enchytraeid in the following doses: 0; 0.25; 0.50; 1.0; 2.0 and 5.0 mg fipronil per kg soil. In soil containing springtails were applied doses of 0; 0.01; 0.02; 0.04; 0.08; 0.15; 0.30; 0.60 and 1.0 mg fipronil per kg soil. The doses, for all products, were calculated taking into consideration the recommended dose for tick control and the rate of excretion of each medicine, for the calculations was considered a bovine with 300 kg. These soil samples were prepared mixing 25.0 mL of fipronil in acetone for each 100 g of soil. After homogenization, the fipronil-containing soil was introduced in an exhaustion chamber for 24 h up to complete evaporation of the organic solvent. Finally, water was added in the prepared soil to keep 65% humidity. A control experiment containing pure acetone was carried out to evaluate the solvent effects.

### 2.3. Ecotoxicological tests – *Eisenia andrei*

Reproduction and survival of earthworms were evaluated according to ISO 11268-2 (1998). Plastic vessels of 200 $cm^2$ containing 500 g of soil with doses of veterinary medicines were employed. Ten earthworms, obtained from laboratory creation, with apparent clitellum and masses ranging from 250 to 600 mg were added to each flask. These edaphic organisms were feed each 14 days with 5 g of equine non-contaminated manure, according to Segat et al. (2015). After 28 days of start test, adult earthworms were removed for counting the organisms that survived and rate survival was measured. The soil containing earthworm cocoons was incubated for 28 days. Finally, the plastic vessels were introduced in water baths at 60 °C for 1 h to force the migration of juvenile earthworms to the soil surface and these juveniles were counted, thus establishing the reproduction of earthworms

### 2.4. Ecotoxicological test – *Enchytraeus crypticus*

Reproduction and survival of enchytraeid were evaluated according to ISO 16387 (2004). Ten organisms with apparent clitellum were placed in the same plastic vessels with 3 cm diameter and 5 cm height, containing 30 g of soil. At the beginning and at 14 days of testing the organisms received 0.5 g of oat flakes. The number of juveniles of enchytraeid, after 28 days, was determined fixing these organisms in ethylic alcohol and rose bengal 1%, thereby determining the reproduction of enchytraeid. To determine the number of survivors in this test, the organisms fixed in rose bengal are separated by body size, enchytraeid adults added at the beginning of the test and survived are noticeably larger than the organisms generated while conducting the test.

### 2.5. Ecotoxicological test – *Folsomia candida*

Reproduction and survival of springtails were evaluated according to ISO 11267 (1999). Each experimental sample was prepared in plastic vessels 3.5 cm diameter, 11.5 cm height and 30 g of previously contaminated soil. Ten springtails with ages from 10 to 12 days were added to each vessel. These organisms were fed in the first day and after 14 days of experiment with 2 mg of *Saccharomyces cerevisiae*. After 28 days of experiment water and black ink were added in these vessels to contrast with the white coloration of springtails. After shaking, the organisms that were alive floated and were counted. Adult accounting is done based on the difference in body size between the adults and the juveniles generated. Thus, accounting is done at the same time, separating adults by size from juveniles and obtaining survival and reproduction.

### 2.6. Validation tests

Criteria for validation of the tests are determined by the ISO protocols of each organism. For earthworms the criteria are, survival index above 90%, number of juveniles greater than 30 and less than 30% of coefficient of variation. For enchytraeids the survival need be above 80%, number of juveniles greater than 25 and the coefficient of variation lower than 50%. For springtails in all the control vessels, the adult survival rate need be higher than 80%. The number of juveniles higher than 100 and coefficient of variation lower than 30%.