FELIPE OGLIARI BANDEIRA

INFLUÊNCIAS DO TIPO DE SOLO, UMIDADE E TEMPERATURA NA TOXICIDADE DO IMIDACLOPRID PARA INVERTEBRADOS EDÁFICOS

Dissertação apresentada ao Curso de Pós-graduação em Ciência do Solo, do Centro de Ciências Agroveterinárias da Universidade do Estado de Santa Catarina, como requisito parcial para obtenção do grau de Mestre em Ciência do Solo.

Orientador: Dr. Dilmar Baretta
Co-orientador: Dr. Paulo Roger Lopes Alves

LAGES, SC
2019

Bandeira, Felipe Ogliari
Influências do tipo de solo, umidade e temperature na toxicidade do imidacloprid para invertebrados edáficos / Felipe Ogliari Bandeira. – Lages, 2019.
114 p.

Orientador: Dilmar Baretta
Co-orientador: Paulo Roger Lopes Alves
Dissertação (Mestrado) – Universidade do Estado de Santa Catarina, Centro de Ciências Agroveterinárias, Programa de Pós-Graduação em Ciência do Solo, Lages, 2019.

1. Mudanças climáticas. 2. Solos tropicais. 3. Fauna edáfica. 4. Avaliação ecotoxicológica
I. Bandeira, Felipe Ogliari. II. Baretta, Dilmar. III. Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência do Solo. IV. Título

Ficha catalográfica elaborada pelo aluno.

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Dissertação apresentada como requisito parcial para obtenção do título de mestre no Curso de Pós-Graduação em Ciência do Solo da Universidade do Estado de Santa Catarina – UDESC.

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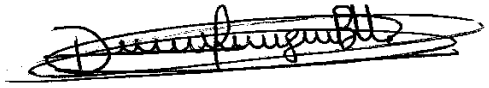

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Dedico,

Aos meus pais, Marlene e Adalberto, à minha irmã, Fabiele, e à minha namorada, Rutinéia, pelo apoio incondicional ao longo desta jornada.

AGRADECIMENTOS

A Deus, pela vida.

Ao professor Dilmar Baretta, pela confiança depositada em mim e pelas contribuições neste trabalho.

Ao professor Paulo Roger Lopes Alves, por me conceder a oportunidade de desenvolver este trabalho. Pelo amparo em todas as dificuldades deste percurso, sempre com muito otimismo e paciência, e pela amizade construída.

Aos professores Osmar Klauberg Filho, Luís Carlos Iuñes de Oliveira Filho e à professora Elke Jurandy Bran Nogueira Cardoso pelos ensinamentos repassados e pelas contribuições para a melhoria deste trabalho.

Aos meus pais, Marlene Ines Ogliari Bandeira e Adalberto Bandeira, e à minha irmã, Fabiele Ogliari Bandeira, pelo apoio incondicional em busca dos meus sonhos, sempre com muito amor e compreensão.

À minha namorada, Rutinéia Jéssica Schuster, pelo companheirismo, carinho e apoio ao longo desta caminhada, e pela compreensão pelos momentos da minha ausência.

Ao Edson e à Ieda Schuster, por todo o carinho, respeito, apoio e confiança durante este período.

Aos colegas Thuanne, Aline, Tânia, Adriano, Marcos, William, Elizeu, Adriano, Jéssica, Eduarda e Ana, do Laboratório de Ecotoxicologia Terrestre da UFFS, por toda a (indispensável) ajuda na realização deste trabalho, pelas amizades e bons momentos vividos no laboratório. Nada disso seria possível sem vocês.

A todos os colegas do Laboratório de Ecologia do Solo da UDESC, pelas amizades e ensinamentos repassados, além de todas as contribuições diretas ou indiretas para que esse trabalho se consolidasse.

Aos colegas do Mestrado e Doutorado do PPGCS, em especial à Vanessa, Diou, Cleiton, Douglas, Gregory, Gustavo, Elston, Dani, Rafa, pelas amizades construídas e pelas diversas contribuições ao longo do projeto.

À CAPES, pela concessão da bolsa de mestrado, ao CNPq, pelo apoio financeiro, e à UDESC e à UFFS, pela oportunidade e estrutura concedida para o desenvolvimento do projeto.

A todos os familiares e amigos que, direta ou indiretamente, sempre me apoiaram na jornada acadêmica.

“Só há duas maneiras de viver a vida: a primeira é vivê-la como se os milagres não existissem. A segunda é vivê-la como se tudo fosse um milagre.”

Albert Einstein

RESUMO

BANDEIRA, Felipe Ogliari. **Influências do tipo de solo, umidade e temperatura na toxicidade do imidacloprid para invertebrados edáficos**. 2019. 114 p. Dissertação (Mestrado em Ciência do Solo) – Universidade do Estado de Santa Catarina. Programa de Pós-Graduação em Ciência do solo, Lages, 2019.

A ecotoxicologia terrestre é um campo de pesquisa relativamente novo no Brasil. Embora algumas avaliações ecotoxicológicas de agrotóxicos já tenham sido realizadas no país, ainda se conhece pouco sobre a influência dos solos tropicais na toxicidade de pesticidas para os invertebrados edáficos. Além disso, apesar da iminente necessidade de se entender as consequências das interações entre os pesticidas e as mudanças climáticas globais na biologia da fauna invertebrada do solo, até onde vai o nosso conhecimento, nenhum estudo com esta abordagem foi realizado em solos tropicais do Brasil. Neste sentido, o objetivo deste estudo foi identificar se o tipo de solo tropical e as mudanças climáticas, nomeadamente, a redução da umidade do solo e o aumento da temperatura atmosférica, influenciam no potencial de toxicidade do imidacloprid para espécies da fauna invertebrada do solo. Para tal foram relizados três experimentos, para avaliar separadamente as influências: a) do tipo de solo, b) da umidade do solo, e c) da temperatura atmosférica. No primeiro experimento, a toxicidade do imidacloprid para minhocas *Eisenia andrei* e colêmbolos *Folsomia candida* foi avaliada em dois solos naturais brasileiros com características distintas (Neossolo e Latossolo) e em um solo artificial tropical (SAT). Neste experimento, verificou-se que a toxicidade do imidacloprid para ambas as espécies foi maior em Neossolo, em comparação ao Latossolo e ao SAT, indicando que o tipo de solo contribui significativamente na regulação da toxicidade deste inseticida para as espécies edáficas. No segundo experimento, os efeitos do imidacloprid sobre *E. andrei* e *F. candida* foram investigados nos mesmos solos, mas sob um regime mais baixo (30% ou 45% da capacidade de retenção de água – CRA) e em um regime mais alto (60% da CRA) de umidade do solo. Para o SAT e Latossolo, verificou-se aumento da toxicidade do imidacloprid em regimes de umidade do solo mais baixos, enquanto que, em Neossolo, a umidade do solo não exerceu clara influência na toxicidade do contaminante para as espécies testadas. No terceiro experimento, a toxicidade de imidacloprid para *E. andrei* e *F. candida* foi avaliada em três solos tropicais (SAT, Neossolo e Latossolo), contudo, os ensaios foram realizados sob três temperaturas crescentes (20, 25 e 28 °C) em uma condição de umidade única (60% da CRA). Em geral, houve aumento da toxicidade do imidacloprid com o aumento da temperatura para ambas as espécies, independentemente do solo utilizado. Os resultados obtidos neste estudo permitem inferir que o imidacloprid poderá causar efeitos mais intensos sobre invertebrados edáficos quando a exposição ocorrer em solos tropicais com baixos teores de silte e argila. Além disso, considerando que a redução da umidade do solo e o aumento da temperatura atmosférica devem ocorrer simultaneamente em várias regiões do globo terrestre, o estresse hídrico ou térmico (ou em combinação) deverão potencializar ainda mais os efeitos do imidacloprid sobre a fauna edáfica, em relação à influência isolada de cada um destes estressores naturais na toxicidade deste inseticida.

Palavras–chave: Mudanças climáticas. Solos tropicais. Fauna edáfica. Avaliação ecotoxicológica.

ABSTRACT

BANDEIRA, Felipe Ogliari. **Influences of soil type, soil moisture and temperature on the toxicity of imidacloprid to edaphic invertebrates**. 2019. 114 p. Dissertation (Master's Degree in Soil Science) –Santa Catarina State University. Postgraduate Program in Soil Science, Lages, 2019.

Soil ecotoxicology is a relatively new research field in Brazil. Although some ecotoxicological assessments of agrotoxics have been carried out in the country, little is known about the influence of the tropical soil type on the toxicity of pesticides to edaphic invertebrates. Moreover, despite the imminent need to understand the consequences of interactions between pesticides and global climate change on the biology of invertebrate soil fauna, to the best of our knowledge, no study with this approach was conducted on tropical soils of Brazil. Hence, the objective of this study was to identify if the type of tropical soil and the climate changes, namely the reduction of soil moisture and the increase in atmospheric temperature, influence the potential toxicity of imidacloprid to invertebrate species of soil fauna. For this, three experiments were carried out, to evaluate separately the influences of: a) soil type, b) soil moisture and c) atmospheric temperature. In the first experiment, the toxicity of imidacloprid to earthworms *Eisenia andrei* and collembolans *Folsomia candida* was assessed in two brazilian natural soils with distinct characteristics (Entisol and Oxisol) and in a tropical artificial soil (TAS). In this experiment, it was verified that the imidacloprid toxicity for both species was higher in Entisol when compared to Oxisol and TAS, indicating that the soil type contributes significantly to the regulation of the toxicity of this insecticide to edaphic species. In the second experiment, the effects of imidacloprid on *E. andrei* and *F. candida* were investigated in the same soils, but under a lower (30% or 45% of the water holding capacity – WHC) and a higher soil moisture regime (60% WHC). For TAS and Oxisol, it was verified an increase in the imidacloprid toxicity in lower soil moisture regimes, whereas in Entisol, the soil moisture did not exert a clear influence on the toxicity of the contaminant for the tested species. In the third experiment, the imidacloprid toxicity to *E. andrei* and *F. candida* was assessed in three tropical soils (TAS, Entisol and Oxisol), however, the assays were performed under three increasing temperatures (20, 25 and 28 °C) in a single soil moisture condition (60% WHC). In general, an increase on imidacloprid toxicity were observed with increasing temperature for both species, regardless the soil used. The results obtained in this study allow us to infer that imidacloprid may cause effects more intenses on soil invertebrates when the exposure occurs in tropical soils with low contents of silt and clay. Furthermore, considering that the decrease in soil moisture and the increase in atmospheric temperature should occur simultaneously in several regions of the world, drought or heat stress (or a combination of both) should further enhance the effects of imidacloprid on edaphic fauna, in comparison to the isolated influence of each one of these natural stressors on the toxicity of this insecticide.

Keywords: Climate changes. Tropical soils. Edaphic fauna. Ecotoxicological assessment.

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SUMMARY

1 INTRODUCTION

The use of pesticides in conventional agricultural systems has been growing in Brazil. During 2010 and 2014, this country was the fourth largest consumer of pesticides in the world (ZHANG, 2018), and approximately 500 thousand tons of active ingredients were sold in 2017 (IBAMA, 2019). The high consumption of pesticides in Brazilian farms, mainly in large-scale food production systems, is generally linked to the need to protect crops against several pests that can damage plants and cause economic losses to farmers.

Among pesticide use in agricultural systems, the seed treatment with pesticides stands out for the high efficiency in relation to their cost, and because of its lower environmental impact, compared to other application ways (JESCHKE et al., 2011; ATWOOD et al., 2018). This technique aims to minimize the occurrence of injuries on seeds through the application of pesticides on its external surface. In addition, after the germination of the treated seeds, part of the pesticide applied to the seeds is absorbed by the roots of the plant and transported along the tissues, protecting the crop from pests and diseases for months after sowing (SIMON-DELSO et al., 2015).

Imidacloprid is a neonicotinoid insecticide widely used in seed treatment in Brazil. This active ingredient (a.i.) acts on the blockage of the insect nervous system receptors, inhibiting the action of acetylcholinesterase and, consequently, leading to an accumulation of the neurotransmitter acetylcholine (BUFFIN, 2003), which may cause paralysis and eventual death of the exposed individuals. Although its use in seed treatment is important to ensure high levels of food production, in recent years this a.i. has been banned in some European countries, due to its impact on wild species, especially on bees (DIVELY et al., 2015). Furthermore, due to its slow degradation in soil, residues of imidacloprid have been found in terrestrial ecosystems (BONMATIN et al., 2014) and may cause negative effects on non-target organisms, especially on soil invertebrates which play important roles in supporting ecosystem services.

The effects of pesticides on non-target soil fauna populations may be assessed through ecotoxicological assays. In order to predict the impact of these substances on terrestrial ecosystems, representative edaphic fauna species are exposed to contaminants in a prospective approach, which allows to identify the effects of contaminants (including pesticides) on soil organisms and to establish their safe levels of exposure.

Some ecotoxicologial studies have shown that imidacloprid can negatively affect edaphic invertebrates. Deleterious effects on growth (CAPOWIEZ et al., 2005; ALVES et al., 2013), reproduction (DE LIMA E SILVA et al., 2017) and survival of earthworms and

collembolans (ALVES et al., 2014; VAN GESTEL et al., 2017) have been identified in laboratory assessments with this insecticide. However, the great majority of these studies were performed in soils from temperate regions or in artificial substrates, which have different characteristics of Brazilian natural soils. Considering that the bioavailability of the contaminants is also regulated by the intrinsic soil properties, such as the type and content of clay, silt and soil organic matter (SOM) (OGUNGBEMI and VAN GESTEL, 2018), and consequently the cation-exchange capacity (CEC), the toxicity of imidacloprid in tropical soils may be even greater than that found in temperate soils, since they normally present higher levels of SOM and CEC if compared to Brazilian natural soils.

In addition to the influence of the soil type on the toxicity of imidacloprid, it must be considered the possible effects of climate change on its toxic potential for edaphic fauna. According to the Intergovernmental Panel on Climate Change (IPCC), the Earth's climate is changing substantially due to the anthropogenic increase in greenhouse gas emissions. Significant changes in global environmental conditions are projected for the next years, such as higher atmospheric temperature and changes in precipitation patterns (IPCC, 2013). Although the consequences of changes are quite variable across regions of the globe, and the predictions have uncertainties about the space and time of occurrence, it has been noted an increase in the overall frequency of extreme weather events, such as heat waves, droughts, storms, among others. A number of studies confirm, for example, that the mean global temperature increased by about 0.7 °C during the 20th century, and an increase of about 2 to 5 times larger is still projected by the end of the 21st century (IPCC, 2013; NOYES et al., 2009).

The expected changes in rainfall patterns and environmental temperature may affect several biological factors of soil organisms. Some invertebrates, such as earthworms, require large amounts of water because of their respiratory system. In the absence of moisture, oligochaetes may induce an estivation process, when they reduce their metabolism and stay protected in small chambers to avoid dehydration (LEE, 1985). Even in the case of insects and other arthropods, which have an exoskeleton that gives them greater protection against dehydration, the water restriction can cause metabolic stress (LIMA et al., 2011) and partially or totally compromise the biological development stages of these organisms (GULLAN and CRANSTON, 2005). Moreover, changes in atmospheric temperatures can lead to changes in the life cycle of species (FAYOLLE et al., 1997). It is known, for example, that temperature exerts a direct influence on the metabolism and feeding activity of the species inside the soil (HACKENBERGER et al., 2018; LIMA et al., 2015). In the presence of very high temperatures,

some species may face up to physiological stresses and have their growth and reproduction impaired (RÖMBKE and MOSER, 2002; ALVES; CARDOSO, 2016).

Besides the direct effects on the physiology of organisms, climate change must also affect the pesticides dynamics in the soil. In some regions, increases in the frequency and intensity of rainfall are expected over the next few years (IPCC, 2013), which may lead to increased dilution of the recently applied pesticides in the soil solution (LIMA et al., 2011) and promote their flow and percolation to aquatic ecosystems (NOYES et al., 2009). On the other hand, in some regions are expected the occurrence of droughts, which may concentrate contaminants in the soil porewater due the reduction of soil moisture (BANDOW et al., 2014b), therefore intensifying the effect of pesticides on soil-dwelling organisms.

Changes in the atmospheric temperature should also affect the dynamics of the contaminants in the terrestrial compartment. Increases in global temperature may favor volatilization and accelerate the degradation of pesticides in soil (RÖMBKE et al., 2007), while at the same time can contribute to the transformation of pesticides into metabolites more toxic than the original compound (NOYES et al., 2009). Moreover, there may be an increase in the uptake and elimination of pesticides by soil invertebrates when exposed to higher temperatures (BEDNARSKA et al., 2017). In this way, it becomes evident that the toxicity of pesticides to edaphic species can be influenced in several ways by the climate changes, especially with regard to changes in environmental temperature and soil moisture regimes.

Ecotoxicological studies have shown that the insecticide imidacloprid can cause negative impacts on earthworms (ALVES et al., 2013; DE LIMA E SILVA et al., 2017; CANG et al., 2017; DITTBRENNER et al., 2010) and collembolans (ALVES et al., 2014; VAN GESTEL et al., 2017; DE LIMA E SILVA et al., 2017; OGUNGBEMI and VAN GESTEL, 2018). However, these experiments were performed only using artificial or temperate soils, and in climatic conditions (temperature and soil moisture) ideal for the species, disregard the influences of natural tropical soils, as well as the global climate changes in the dynamics of exposure to the contaminant. Despite the imminent need to understand the consequences of interactions between pesticides and global climate change on the biology of invertebrate soil fauna, to the best of our knowledge, no study with this approach was carried out on Brazilian tropical soils.

1.1 HYPOTHESES

1. The toxicity of imidacloprid for earthworms *Eisenia andrei* and collembolans *Folsomia candida* is influenced by the type of subtropical soil;
2. The decrease in soil moisture increases the toxicity of imidacloprid for earthworms *E. andrei* and collembolans *F. candida* in subtropical soils;
3. The increase in atmospheric temperature increases the toxicity of imidacloprid for earthworms *E. andrei* and collembolans *F. candida* in subtropical soils.

1.2 GENERAL OBJECTIVE

To identify whether subtropical soil type and climate changes influence the toxicity of imidacloprid to invertebrate soil fauna.

1.3 SPECIFIC OBJECTIVES

1. To verify the influence of two brazilian natural soils (Entisol and Oxisol) and a tropical artificial soil (TAS) on the toxicity of imidacloprid for earthworms *Eisenia andrei* and collembolans *Folsomia candida*;
2. To verify the influence of soil moisture content on the toxicity of imidacloprid for earthworms *Eisenia andrei* and collembolans *Folsomia candida* in TAS, Entisol and Oxisol;
3. To verify the influence of atmospheric temperature on the toxicity of imidacloprid for earthworms *Eisenia andrei* and collembolans *Folsomia candida* in TAS, Entisol and Oxisol.

2 LITERATURE REVIEW

2.1 EDAPHIC FAUNA

Only in the last decades, the environmental services provided by soil fauna, especially by invertebrates, have been globally recognized and the interest in environmental preservation has grown. In terrestrial compartments, these animals are mainly responsible for the fragmentation and distribution of the organic material deposited on the surface of the soils,

although they also play the role of catalysts in the recycling processes of the carbon, nitrogen, phosphorus and sulfur cycles, due to the various relationships (control, distribution, regulation, etc.) that they have with decomposing microorganisms (CORTET et al., 1999).

As a result of their intense activity along the soil profile, these organisms promote significant structural changes in the edaphic system, namely the creation of channels, galleries, among other structures, that interfere in the porosity, oxygenation and percolation of water in the soil. Meso- and macrofauna also contributes by several ways with the distribution and development of the microorganisms into the soil, therefore acting as catalysts of the biogeochemical cycles of important nutrients. As consequences of the transformations carried out by soil invertebrates, essential environmental services are provided for the sustainability of agricultural and forestry ecosystems, such as the regulation of water quality (via filtration processes) and the mineralization of chemical elements from SOM, which has the role of supplying a good part of the nutritional demand of the plants (CORTET et al., 1999; CULIK; ZEPPELINI, 2003).

2.1.1 Assessing pesticide effects on soil fauna bioindicator species

In order to protect the services provided by fauna in the soil, for at least half century, some key-groups of invertebrates have been selected worldwide as bioindicators of the impacts of toxic substances introduced in soils, such as the pesticides. These animals have been employed in a series of toxicity assays in order to predict the ecological risk and, consequently, to establish safe exposure limits of these substances in the soil. In Brazil, safe exposure limits for the presence of pesticides are regulated by CONAMA 420/09 resolution (CONAMA, 2009), which have introduced studies on the ecological risk of pesticides for edaphic fauna in tropical soils into their scope of analysis the terrestrial ecotoxicological assays standardized by the agencies International Organization for Standardization (ISO) and Organisation for Economic Co-operation and Development (OECD) (GARCIA et al., 2008; RÖMBKE et al., 2008; CHELINHO et al., 2012; NUNES; ESPÍNDOLA, 2012; ALVES et al., 2013; 2014; BUCH et al., 2013; DOMÍNGUEZ et al., 2016; NUNES et al., 2016). The methodology of this type of assay is well established in the area of environmental toxicology and has been successfully used worldwide to assess the toxic potential of the presence of pesticides to sensitive soil fauna species.

International organizations have standardized terrestrial ecotoxicological tests (e.g. ISO and OECD) with standard species, such as the collembolan *Folsomia candida* and the

earthworm *Eisenia andrei*. The choice for these species is due, among other factors, to their good suitability for breeding in laboratory environments, short life-cycle (fast reproduction) and to their high sensitivity to several substances (ALVES et al., 2013; 2014). By these ecotoxicological evaluations, reseachers can quantify the lethal (ISO, 1993) and sublethal effects of pollutants (ISO, 2012; 2014) in a short period and in a relatively easy and cheap way.

2.2 IMIDACLOPRID

Imidacloprid is a synthetic neonicotinoid insecticide, widely used for the control of sucking and picking insects, considered as agricultural pests. This active ingredient (a.i.) is commercially available in the formulations: powder, concentrated emulsion, soluble concentrate, granulate, liquid, granulate/pellets, dispersible granules in water and wettable powder (EPA, 2008), and can be used in the chemical treatment of seeds, directly in the soil and also via foliar applications on different crops. This insecticide was registered and approved for use for the first time in 1994 (BACEY, 2000; EPA, 2008), however, in the last years their use has been restricted in several countries due to its side effects on wild species (EFSA, 2018). In Brazil, imidacloprid was the 9th best selling active ingredient in 2017, with more than 9 thousand tons of a.i. sold during this year (IBAMA, 2019). Its mode of action occurs through the inhibition of acetylcholinesterase leading to the accumulation of acetylcholine, blocking the transmission of nerve impulses in exposed organisms (IHARA and MATSUDA, 2018). Under European conditions, the half-life of imidacloprid ranged between 40 and 288 days in the field (EFSA, 2008; van Gestel et al., 2017). Due to its high persistence, imidacloprid residuals are being detected in soils, with concentrations ranging between 0.005 mg a.i. kg^{-1} dry soil (DANKYI et al., 2014) to values higher than 4 mg kg^{-1} (SHARMA and SINGH, 2014).

2.2.1 Effects of imidacloprid on non-target soil fauna organisms

Imidacloprid is known to cause toxicity in honey bees (DIVELY et al., 2015), fishes (VIEIRA et al., 2018), birds, mammals and amphibians (VAN DER SLUIJS et al., 2015), as well as in other organisms including several soil fauna individuals (PISA et al., 2015). However, with regard to soil invertebrates, most of the imidacloprid toxicity data comes from tests performed with temperate or artificial soils, and using the pure compound instead of commercial formulations. In studies carried out with pure imidacloprid spiked in the natural LUFA 2.2 soil (about 4% soil organic matter - SOM) under 20 °C, De Lima e Silva et al. (2017)

and van Gestel et al. (2017) found similar toxicity for *F. candida*, with EC_{50} values ranging between 0.10–0.30 mg a.i. kg^{-1} dry soil. In addition, Van Gestel et al. (2017) noted that imidacloprid toxicity persisted over three consecutive generations of collembolans, suggesting that *F. candida* population were not able to recover from the effects of imidacloprid even 90 days after the contamination. Ogungbemi and van Gestel (2018) also reported high toxicity of imidacloprid (using the pure compound) for *F. candida* in two natural and two artificial soils with SOM contents varying between 4% and 12.6% (EC50 ranged between 0.14 and 2.07 mg kg^{-1}). In general, toxicity decreased with increasing SOM content, but clay minerals apparently also influenced the bioavailability of imidacloprid in this study.

Earthworms also seems to be sensitive to this insecticide. Using the pure compound, De Lima e Silva et al. (2017) found an EC_{50} of 0.39 mg kg^{-1} for *E. andrei* in the LUFA 2.2 soil. Similar toxicity values for hatchability (WANG et al., 2015; GE et al., 2018) and number of juveniles (WANG et al., 2019) were observed when *E. fetida* were exposed to pure imidacloprid in an artificial soil composed by 10% of sphagnum peat, 20% kaolinite clay and 70% fine sand (EC_{50} values were 0.92, 0.70 and 0.87 mg kg^{-1}, respectively). On the other hand, a relatively lower toxicity was observed for *F. candida* (ALVES et al., 2014) and *E. andrei* (ALVES et al., 2013) when a commercial formulation of imidacloprid (Gaucho® 600FS – 600 g a.i. L^{-1}) was spiked in a tropical artificial soil (TAS) containing 10% of coconut husk under 23 °C. For collembolans, these authors found an EC_{50} > 1.0 mg kg^{-1}, while an EC_{50} of 4.07 mg kg^{-1} was found for earthworms.

Despite the differences between substrates and compound used in the mentioned studies, imidacloprid proved to be very harmful to *F. candida* and *E. andrei* in all of them. Deleterious effects of this a.i. were also observed in another species of soil invertebrates such as *Aporrectodea nocturna* and *Allolobophora ictérica* (CAPOWIEZ et al., 2005), *Lumbricus terrestris* and *Aporrectodea caliginosa* (DITTBRENNER et al., 2010), *Dendrobaena octaedra* (KREUTZWEISER et al., 2009) and *Enchytraeus crypticus* and *Hypoaspis aculeifer* (AKEJU, 2014).

2.3 INFLUENCE OF THE SOIL TYPE ON PESTICIDE TOXICITY TO SOIL FAUNA

The toxicity responses of edaphic species to the contaminants may vary according to the type of soil used in the ecotoxicological assays. For example, the reproductive performance of soil invertebrates may be influenced by some natural soil properties such as the size of the predominant particles and SOM content. In very clayey textured soils, the reproduction of soft-

body organisms such as earthworms and enchytraeids may be hampered by the lack of porous space required for gas exchange (CHELINHO et al., 2011), which probably does not occur in soils with well-distributed particle-size. In addition, soils with greater amounts of organic material probably provide greater availability of essential resources such as food and water to soil invertebrates. Otherwise, edaphic species may be more susceptible to abiotic stresses in very weathered soils with low levels of SOM (SANCHEZ-BAYO and HYNE, 2011). Soil pH can also influence the performance of soil invertebrates. Edaphic organisms generally fit well in soils with pH close to neutrality, but their reproduction may be impaired in acidic soils (RÖMBKE et al., 2007).

In addition to the direct influence of soil type on the species performance, soil properties can also regulate the bioavailability of the pesticide in terrestrial ecosystems. In recent years, a series of studies have shown that the main route of exposure of soil organisms to pesticides occurs through soil solution (PEIJNENBURG et al., 2012). In this way, the toxicity for some contaminants may be lower in soils with high capacity to adsorb the molecules in the solid matrix, due to the lower fraction of the pollutants available for uptake by the edaphic species in soil pore water (OGUNGBEMI and VAN GESTEL, 2018).

It is known that SOM play a key role in pesticides sorption processes (FELIX et al., 2007). The SOM usually presents a high specific surface area and a great quantity of electric charges capable to retain the polar molecules, like pesticides. Thus, lower bioavailability of organic molecules to the edaphic receptors its expected in soils with higher amounts of SOM (VAN GESTEL and MA, 1998). This influence was verified by Styrishave et al. (2010) when testing the effects of α-cypermethrin in collembolans *F. candida* in two soils with different amounts of SOM. The authors observed a significant decrease on *F. candida* reproduction in the soil with 4% SOM, while in soil with higher SOM content (11.5%), there were no negative effects of this insecticide on the collembolan reproduction. According to Martikainen and Krogh (1999), increasing SOM content also reduced the toxicity of dimethoate to *F. fimetaria*.

The sorption processes of pesticides can also be influenced by soil mineral fraction. For example, pesticides can bind to negative charges of clay minerals and therefore become less available for uptaking by invertebrates (RUTHERFORD et al., 1992). Ogungbemi and van Gestel (2018) identified a key role of clay minerals in the sorption of imidacloprid and therefore, in the toxicity of this a.i. to *F. candida*. The authors reported a relatively higher imidacliprid toxicity in natural soils containing lower clay amounts (4.8 – 7.7%) when compared to that found in artificial soils containing 20% clay. This indicates that the clay fraction may have significative influence on regulating the concentration of the a.i. available for uptake by soil-

dwelling species. According to Owojori et al. (2010), there is also a crucial role of clay content on the bioavailability of metals to *E. fetida*. When earthworms were exposed to copper in OECD artificial soils with the same amount of SOM (10%), but increasing amounts of kaolin clay (5%, 20% and 40%), the authors observed a significant decrease in toxicity and internal earthworm copper concentration with increased clay content.

The type of clay and organic material that compose the soils can also influence its capacity to adsorb pollutants. Soils from hot and humid regions usually have highly weathered clay minerals and organic material in advanced stages of decomposition, when compared to the soils of cold and dry regions (SANCHEZ-BAYO and HYNE, 2011). These differences between the natural characteristics of temperate and tropical soils may lead to different toxicities of the contaminants (DE SILVA et al., 2009). Different compositions between artificial and natural soils can also led to different toxicity responses (MARTIKAINEN, 1996; AMORIM et al., 2005), since the materials generally used in the artificial substrates (i.e. coconut fiber as an organic material source and pure kaolinite and sand setting the mineral fraction) may interact with chemicals differently than natural soil compounds.

Few ecotoxicological studies performed in Brazil have considered the influence of tropical soil types on the dynamics of the pesticide toxicity to edaphic fauna (NUNES and ESPÍNDOLA, 2012; ZORTÉA et al., 2017; ZORTÉA et al., 2018; CARNIEL et al., 2019), although it is evident that soil can strongly alter the maximum exposure limits supported by soil fauna. Hence, there is a lack of studies with widely used pesticides in Brazil in conditions closer to the natural ones.

2.4 CLIMATE CHANGES

The greenhouse effect is a natural phenomenon essential for the regulation of the earth's temperature and, consequently, for the maintenance of life on the planet. A great fraction of greenhouse gas (GHG) emissions are from natural sources, including those emitted by the oceans, volcanoes, forest, among others (YUE and GAO, 2018). However, a number of studies have shown that the Earth's climate has been changing in recent years (IPCC, 2014), and the main cause of these changes have been attributed to the increase in the anthropogenic emissions of GHG. Several human activities have been contributing to these emissions, including the intensive burning of fossil fuels, deforestation, and agricultural and livestock activities (TONG and EBI, 2019). The shifts in GHG atmospheric concentration have been affecting the natural Earth’s warming. As a consequence, substantial climate changes are expected to ocurr until the

end of this century, including the increase in global air, land and ocean temperature, snow melt and rising sea level, changes in average precipitation, and increases in the frequency and duration of extreme climatic events such as storms and heat waves (IPCC, 2013).

Changes in the precipitation pattern are globally expected for the next years. An increase in rainfall frequency and intensity will probably occur in some areas of northern Europe and Asia (NOYES et al., 2009). On the other hand, an intensification of drought conditions is predicted for the end of 21st century in the southern Africa, Asia (NOYES et al., 2009) and also in Brazil (PEREIRA et al., 2018). In some states of the central region of Brazil, annual precipitation is expected to reduce in approximately 50% between 2071-2100 (LYRA et al., 2018). These changes will impact the soil moisture and, consequently, may affect the water availability for soil organisms. If the predicted scenarios of climate change are confirmed, several Brazilian soils may face reductions higher than 10% in moisture content (DAI, 2013). Even in areas where precipitation may increase (REYER et al., 2017), the soil moisture will probably decrease due to the increased temperature and water evaporation (LU et al., 2019).

In addition to changes in precipitation patterns, the IPCC has confirmed a significant increase in global average temperature during last century. An increase in the global mean surface temperature of 0.72 °C over the period 1951–2012 was found (IPCC, 2013), and further warming are still expected for the coming years. Despite the uncertainties associated with projections of future temperature scenarios, an increase of about 1.0 °C until the end of the 21st century are likely to ocurr even considering the low emission of GHG scenario (RCP2.6), but increases above 4 °C may be reached if no efforts to control GHG emissions were made (IPCC, 2013). Considering the worst GHG emission scenario (RCP8.5), Lyra et al. (2018) projected increases of approximately 4 °C above normal in some Southeast Brazil regions until 2040, and the temperature rise can reach up to 8 °C until 2100. In accordance with this warming trend, Marengo and Camargo (2008) reported increases in the minimum (0.5 to 0.8 °C per decade) and maximum air temperature (about 0.4 °C per decade) in Southern Brazil during 1960–2002, and until the end of the century, increases between 2 °C and 5 °C are still projected in the South America (MARENGO et al., 2010).

2.4.1 Influence of soil moisture on pesticide toxicity to soil fauna

Water availability is a substantial environmental factor for the terrestrial ecosystem functioning and is strictly related to rainfall patterns. In drought periods, where in-soil organisms are exposed to situations of water restriction, their metabolism and feeding activity

may be altered, and the internal water balance may be impaired (GONZÁLEZ-ALCARAZ et al., 2019). In addition, drought stress may reduce the normal reproductive performance of edaphic species by impairing several stages of their life cycle. The growth rate and survival time of some species may also be reduced in water stress situations (GUNADI et al., 2003), and thus, faunal community structure and abundance may be negatively affected in low soil moisture contidions, which may result in losses of key ecosystem services.

Shifts in rainfall patterns may also affect the behavior and dynamics of pesticides in the environment. According to Delcour et al. (2015), in areas where rainy events are intensified, it is expected a greater dissipation of pesticides in the environment, which may reach the surface water easily. Furthermore, high soil moisture levels may induce a rapid pesticide degradation (DELCOUR et al., 2015), whilst the opposite may occur in low moisture scenarios. Drought situations may also affect the bioavailability of contaminants to soil invertebrates (GONZÁLEZ-ALCARAZ and VAN GESTEL, 2016), as well as their ability to deal with those substances.

In some ecotoxicological evaluations, significant effects of soil moisture content on the toxicity of different contaminants were identified. Long et al. (2009) observed a lower cocoon production rate of earthworms *Lumbricus rubellus* exposed to fluoretane in soil with lower moisture (15% below the optimum). Similarly, Lima et al. (2011) verified that drought scenarios (10, 20 and 40% of the water holding capacity – WHC) increased the lethal effects of carbaryl to the earthworm *E. andrei*, when compared to the control (60% WHC) or to flood scenarios (80, 100 and 120% WHC). These authors suggested that dehydration caused by drought stress may have led to an increasing concentration of the chemical within the earthworm's body, resulting in a greater toxicity at lower soil moisture scenarios. Moreover, in drought scenarios, the earthworms may lose their ability to regulate internal levels of the pollutants (Friis et al., 2004). Lima et al. (2011) also proposed that the relatively low toxicity observed at higher soil moisture levels may be due to a higher dilution of the chemical in the soil porewater, thereby reducing the concentration of the contaminant effectively available to the organisms.

The toxicity of some contaminants to collembolans can also be affected by soil moisture. Hojer et al. (2001) verified that the acute toxicity of 4-nonylphenol to *F. candida* in realistic soil air humidities that occur during summer drought was at least twice higher than that found under optimal air humidities conditions. Likewise, low relative humidity led to an increased toxicity of pyrene on the survival of the collembolans *Protaphorura armata* (SJURSEN and HOLMSTRUP, 2004). This same effect was observed by Bandow et al. (2014b) when

measuring the toxicity of the fungicide pyrimethanil to collembolans *F. candida* and *Sinella curviseta* under three levels of soil moisture (30, 50 and 70% WHC). These authors observed an increase in the chronic toxicity with the reduction of soil moisture for both species, which was attributed to the higher pyrimethanil concentration in the soil porewater in dry soil, and to the additional stress caused by drought in combination with the toxic effects of the pesticides. Higher toxicity of metal-polluted soils to *Enchytraeus crypticus* was also found at drought conditions (GONZÁLEZ-ALCARAZ et al., 2015; GONZÁLEZ-ALCARAZ and VAN GESTEL, 2016). On the other hand, Bandow et al. (2014a) reported that higher soil moisture led to an increased toxicity of lambda-cyhalothrin to *F. candida* and *S. curviseta*. This discrepancy between the literature data suggests that the influence of soil moisture may vary according to the pesticide classes.

2.4.2 Influence of atmospheric temperature on pesticide toxicity to soil fauna

Temperature is a key factor for the development of soil fauna populations. Changes in temperature may affect several fisiological processes in soil-dwelling organisms (JÄNSCH et al., 2005). When soil organisms are exposed to temperatures higher than those ecologically appropriate, they are subject to deregulation in homeostasis and metabolic stresses (JEGEDE et al., 2017; HACKENBERGER et al., 2018), which can impair their normal growth (JÄNSCH et al., 2005). The reproduction and life cycle of invertebrate species are also sensitive to temperature flutuations (FAYOLLE et al., 1997; WILES and KROGH, 1998; FOUNTAIN and HOPKINS, 2005) and above-optimum temperatures may prevent species to maintaining stable populations.

In addition of acting as an abiotic stress factor to faunal species (when high), temperature changes the dynamics of the contaminants in soils (NOYES et al., 2009). It is known that adsorption of pesticides to the clay and/or SOM may decrease with increasing temperature (MARTIKAINEN, 1996; BROZNIC and MILIN, 2012), leading to its greater bioavailability. On the other hand, the degradation and volatilization of less stable compounds may be accelerated at high temperatures (RÖMBKE et al., 2007).

The exposure level of edaphic invertebrates to pesticides can also be altered in scenarios of climate change, because increments in temperature may promote increases in the species activity pattern and feeding behaviour, leading to shifts in the uptake and elimination rates of pesticides by the exposed individuals (BEDNARSKA et al., 2017).

Some studies have demonstrated the influence of temperature on pesticide toxicity to earthworms. Garcia (2004) assessed the toxicity of the insecticide lambda-cyhalthrin and the fungicides benomyl and carbendazim for *E. fetida* in two different temperatures (20 °C and 28 °C). Lambda-cyhalthrin was more toxic at 28 °C, while a higher toxicity of benomyl and carbendazim was found at 20 °C (RÖMBKE et al., 2007). De Silva et al. (2009) also verified higher impact of the insecticides chlorpyrifos and carbofuran on the survival of *E. andrei* at 26 °C, when compared to 20 °C, however, carbendazim was more toxic at 20 °C when compared to 26 °C. On the other hand, the toxic effects of the pesticides on earthworms reproduction and growth varied inconsistently with temperature and soil used. Lima et al. (2015) reported an increase in the deleterious effects of carbaryl to *E. andrei* survival with increasing temperature. Velki and Ečimović (2015) assessed the influence of temperature (15, 20 and 25 °C) on the toxicity of several insecticides, fungicides and herbicides to *E. fetida* through the filter paper contact method. These authors found that, with the exception of glyphosate, higher temperatures caused increase in the toxicity of the insecticides imidacloprid, chlorpyrifos and lambda-cyhalothrin, the fungicides difenoconazole and azoxystrobin and the herbicides tembotrione and fluazifop-p-butyl. Based on these literature data, it is possible to note that the toxicity of insecticides to earthworms apparently increases with increasing temperature, whilst fungicides toxicity may be increased or decreased as the temperature rises.

The toxicity of some pesticides to collembolans were also affected by temperature. Jegede et al. (2017) observed that the insecticides dimethoate and chlorpyrifos were about 2-3 times more toxic to the *F. candida* reproduction at 26 °C, when compared to 20 °C. Other studies also demonstrated that the chronic toxicity of lambda-cyhalothrin (BANDOW et al., 2014a) and pyrimethanil (BANDOW et al., 2014b) for *F. candida* were higher at 26 °C (than at 20 °C). For carbaryl, Lima et al. (2015) observed a lower chronic toxicity to *F. candida* at higher temperatures (22 – 26 °C) when compared to the results obtained from 8 - 20 °C. On the other hand, the toxicity of lambda-cyhalothrin for *Sinella curviseta* seems to decrease with increasing temperature from 20 °C to 26 °C (BANDOW et al., 2014a). The optimum temperature for this alternative species of collembolans is 30 °C, which may explain the lower toxicity at 26 °C. The above-mentioned works shows that, in general, high temperatures may led to an increase in the toxicity of pesticides for non-thermotolerant species such as *F. candida*, although some exceptions may occur (such as the effect observed by Lima et al. (2015) for carbaryl). On the other hand, species that prefer higher temperatures (such as *S. curviseta*), or those that have greater capacity of adaptation to higher temperature levels, may be less affected by pesticides in future scenarios of climate change.

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3 CHAPTER 1: Toxicity of imidacloprid to edaphic fauna as affected by subtropical soils

3.1 ABSTRACT

Imidacloprid is a widely used seed dressing insecticide in Brazil. However, the effects of this pesticide on non-target organisms of soil fauna are still poorly understood in tropical conditions. This study aimed to assess the toxicity of a commercial formulation of imidacloprid for earthworms *Eisenia andrei* and collembolans *Folsomia candida* under Brazilian subtropical soils. Acute and chronic toxicity assays were performed with both species in a tropical artificial soil (TAS) and in two natural soils (Oxisol and Entisol), at laboratorial temperature of 25 ºC. The ecological risk was calculated for each species and soil by using the Toxicity Exposure Ratio (TER) and Hazard Quotient (HQ) approaches. For collembolans, the acute toxicity was higher in Entisol (LC_{50} = 4.68 mg kg^{-1}) when compared to TAS (LC_{50} = 10.82 mg kg^{-1}), while a lower lethality was observed in Oxisol (LC_{50} = 25.08 mg kg^{-1}). The earthworms survival was also more affected in Entisol (LC_{50} = 0.55 mg kg^{-1}) compared to TAS (LC_{50} = 9.18 mg kg^{-1}). Chronic toxicity for collembolans was similar in TAS and Oxisol ($EC_{50\ TAS}$ = 0.80 mg kg^{-1}; $EC_{50\ Oxisol}$ = 0.83 mg kg^{-1}), whereas higher toxicity was observed in Entisol (EC_{50} = 0.09 mg kg^{-1}). In the chronic assays with earthworms, imidacloprid was also more toxic in Entisol (EC_{50} = 0.21 mg kg^{-1}) when compared to TAS (EC_{50} = 1.89 mg kg^{-1}). TER and HQ values indicate a significant risk of exposure of soil invertebrates to imidacloprid in all soils tested, and the risk in Entisol was at least six times higher than in Oxisol or TAS.

Keywords: Pesticides; Ecotoxicity; Oxisol; Entisol; Earthworms; Collembolans.

3.2 INTRODUCTION

The use of plant protection products (PPPs) in agricultural areas of intensive production has been increasing in Brazil, as a result of human population growth and, consequently, due to the increase in food production. In conventional agriculture the use of pesticides in seed dressing is a common practice, because it allows to prevent or to reduce the incidence of pests and diseases in the early stages of the crops (DOUGLAS and TOOKER, 2015), helping to reach high productivity levels.

Neonicotinoids are a class of insecticides widely used in chemical seed treatment. The choice for this group in agricultural crop areas is mainly due to its high efficiency in the control of insect pests (JESCHKE et al., 2011; GOULSON, 2013). Although imidacloprid (IUPAC: (E)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) has been banned in some countries (EFSA, 2018), this is one of the world's top-selling insecticides for seed dressing and it is also used for foliar applications or via direct application in the soil (VAN DER SLUIJS et al., 2015). This active ingredient (a.i.) blocks the transmission of nerve impulses in insects, preventing the degradation of acetylcholine through the inhibition of acetylcholinesterase (IHARA and MATSUDA, 2018). In this way, this systemic insecticide can cause paralysis and, eventually, the death of exposed individuals.

The seed treatment with insecticides is nowadays often considered an indispensable practice for large-scale food production, but it also is responsible for introducing pollutants into the soil (WOOD and GOULSON, 2017). Due to the persistence and low absorption of imidacloprid by plants (GOULSON, 2013), residues of this a.i. have been found in the soil (DONNARUMMA et al., 2011; GE et al., 2018) and may pose a risk to terrestrial ecosystems, especially for ecological receptors such as soil invertebrates (BONMATIN et al., 2014; PISA et al., 2015).

Considering that the direct effects of xenobiotics on terrestrial ecosystems are difficult to measure, soil invertebrates are used commonly as indicators of soil quality in ecotoxicological assays (DE SILVA et al., 2009; ALVES et al., 2017). This type of study has increased in tropical regions in the last decade, although most of them have been carried out with Tropical Artificial Soil (TAS) (NIEMEYER et al., 2017). Although artificial soils are internationally recommended for terrestrial ecotoxicological assays, their ecological relevance are limited, because the results of the assays based on this type of soil may not accurately represent the toxicity of pesticides in natural soils and therefore should not be directly extrapolated to field conditions (CHELINHO et al., 2014).

In addition, the bioavailability of contaminants to soil organisms is directly influenced by intrinsic soil characteristics, such as the amount and type of clay minerals and soil organic matter, among others (AMORIM et al., 2005b; OGUNGBEMI and VAN GESTEL, 2018). Although these soil attributes have influence on the toxicity of pesticides to soil fauna species (DE SILVA et al., 2009; CHELINHO et al., 2014), they are frequently neglected in ecotoxicological studies, especially in those using only artificial soils. Particularly in Brazil, where a wide variety of soil textural classes occur (EMBRAPA, 2006), the tolerance of species to pesticides may vary according to soil type, especially between soils with distinct textural

characteristics (e.g., clayey and sandy soils). Thus, to reduce the uncertainty of the toxicity values and risk os pesticides for the regulation of pesticides used in tropical regions, some authors recommend to use natural tropical soils as a test substrate in terrestrial ecotoxicological assays (NIVA et al., 2016; NIEMEYER et al., 2017).

Therefore the aim of this study was to assess the influence of soil type on the potential toxicity and risk of imidacloprid for earthworms *E. andrei* and collembolans *F. candida*. Laboratory ecotoxicological assays were set up using an artificial soil and two natural soils from southern Brazil (ISO, 2012).

3.3 MATERIAL AND METHODS

3.3.1 Test species

The species *E. andrei* (Oligochaeta) and *F. candida* (Collembola) were reared in the laboratory, in a climate room with atmospheric temperature of 20 ± 2 °C, according to the recommendations from ISO guidelines (ISO, 2012; 2014). Earthworms were maintained in plastic boxes containing a mixture of horse manure, coconut fiber, distilled water and fine sand in a proportion of 100:50:330:15 (weight-based), respectively. Weekly, the earthworms were fed with cooked oat flakes and the moisture of the breeding medium was adjusted with distilled water.

F. candida were maintained in substrate containing activated charcoal (powdered), plaster of Paris (powdered) and distilled water, in a proportion of 1:10:6 (weight-based), respectively. Twice a week, the collembolans were fed with granulated dry yeast (*Saccharomyces cerevisiae*), and the moisture of the substrate was adjusted with few drops of distilled water.

3.3.2 Test soils and substance

A Tropical Artificial Soil (TAS) and two natural tropical soils were used as test substrate in this study. The TAS is a mixture of fine sand (more than 50% of the particles sized between 0.05 and 0.2 mm), kaolinite clay and powdered coconut husk, in a proportion of 75:20:5 of dry weight (dw), respectively (GARCIA, 2004; DE SILVA and VAN GESTEL, 2009). The pH of the TAS was adjusted to 6.0 ± 0.5 with $CaCO_3$, according to ISO 11268-2 (ISO, 2012). Two soils with different characteristics were also selected for the study: an Entisol with a higher sand

content (above 90%) and an Oxisol with a higher clay content (approximately 59%). The soil samples were taken from the surface layer (0-20 cm) of the soil profile in areas with no history of pesticide contamination in the municipalities of Araranguá (29º00′S; 49°31′W) and Chapecó (27°06'S; 52°42'W), Brazil, respectively. The natural soils were sieved (# 2.0 mm opening) and all existing macrofaunal individuals were eliminated by a freeze-thaw defaunation process, as described in Alves et al. (2013).

The following physical and chemical properties of the soils (Table 3.1) were determined according to Tedesco et al. (1995): available contents of Al, Ca, Mg and Mn (KCl); P and K (Mehlich 1); Cu, Zn and Fe (Mehlich); H+Al (Shoemaker–McLean–Pratt – SMP buffer method); Soil Organic Matter (SOM) by espectroscopy; clay (densitometry), sand (gravimetry) and silt (total sample mass discounted the amounts of clay and sand). Cation-exchange capacity (CEC) was calculated by the sum of available K, Ca, Mg and Al contents. The pH (1 M KCl) and the maximum water holding capacity (WHC) were determined following ISO (2012).

The commercial formulation MUCH 600 FS® containing imidaloprid (600 g of a.i. L^{-1}) was choose for the study because of its frequent use in seed treatments in Brazilian agricultural areas.

Ecotoxicological assays were carried out individually in each soil. Immediately before the beginning of the tests, the three different soils were spiked with five solutions, containing increasing concentrations of imidacloprid. For all treatments, the soil moisture was adjusted with distilled water to approximately 60% of their WHC. A control treatment containing only distilled water (also 60% of the WHC) was run for each test soil. The range of nominal imidacloprid concentrations (in mg of a.i. per kg of dry soil – mg kg^{-1}) used in acute and chronic toxicity assays (Table 3.2) were based on a range-finding test and literature data (ALVES et al., 2013; ALVES et al., 2014; DE LIMA E SILVA et al., 2017).

Table 3.1 - Physical and chemical characteristics of Tropical Artificial Soil (TAS), Entisol and Oxisol, used in the ecotoxicological tests with the species *E. andrei* and *F. candida*.

Parameter	TAS	Entisol	Oxisol
pH (1 M KCl)	5.9 ± 0.1	4.2 ± 0.1	3.9 ± 0.1
SOM % (m/v)	1.4 ± 0.0	2.2 ± 0.1	3.7 ± 0.1
P (mg dm^{-3})	23.4 ± 4.8	4.8 ± 1.0	3.4 ± 0.4
K (mg dm^{-3})	422.0 ± 48.1	42.0 ± 2.8	136.0 ± 11.3
Ca ($cmol_c$ dm^{-3})	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Mg ($cmol_c$ dm^{-3})	1.1 ± 0.3	0.5 ± 0.1	0.5 ± 0.0
Al ($cmol_c$ dm^{-3})	< 0.1	0.3 + 0.0	4.4 ± 0.3
H + Al ($cmol_c$ dm^{-3})	1.8 ± 0.4	4.0 ± 1.3	16.6 ± 4.5
Cu (mg dm^{-3})	0.9 ± 0.1	0.7 ± 0.1	1.9 ± 0.1
Fe (g dm^{-3})	> 5.0	> 5.0	> 5.0
Mn (mg dm^{-3})	< 2.5	< 2.5	21.6 ± 0.1
Zn (mg dm^{-3})	0.6 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
CEC ($cmol_c$ dm^{-3})	3.3 ± 0.2	1.4 ± 0.2	5.9 ± 0.2
Clay (g kg^{-1})	143.0 ± 0.0	41.5 ± 2.2	593.0 ± 7.1
Silt (g kg^{-1})	185 ± 0.0	20.5 ± 1.4	352.5 ± 57
Sand (g kg^{-1})	672.0 ± 0.0	938.0 ± 4.2	5.4 ± 1.4
WHC (%)	46.3 ± 1.7	31.6 ± 1.1	45.1 ± 0.8

Values are expressed in mean ± standard deviation; n = 2.
SOM – Soil Organic Matter.
CEC – Cation Exchange Capacity.
WHC – Water Holding Capacity.
Source: prepared by the author, 2019.

Table 3.2 - Predicted imidacloprid environmental concentration (PEC) for each soil. Nominal concentrations of imidacloprid used in the acute and chronic toxicity tests with *E. andrei* and *F. candida* in Tropical Artificial Soil (TAS), Entisol and Oxisol. Concentrations are expressed as mg of imidacloprid per kg^{-1} of dry soil (mg kg^{-1}).

Test soil	PEC (mg kg^{-1})	Test species	Imidacloprid concentrations (mg kg^{-1})	
			Acute toxicity assays	Chronic toxicity assays
TAS	0.24	*E. andrei*	0; 10; 16; 24; 36; 54	0; 0.25; 0.5; 1; 2; 4
		F. candida	0; 4; 8; 16; 32; 64	0; 0.25; 0.5; 1; 2; 4
Entisol	0.16	*E. andrei*	0; 0.1; 0.2; 0.4; 0.8; 1.6	0; 0.12; 0.18; 0.27; 0.4; 0.6
		F. candida	0; 4; 8; 16; 32; 64	0; 0.25; 0.5; 1; 2; 4
Oxisol	0.24	*F. candida*	0; 4; 8; 16; 32; 64	0; 0.25; 0.5; 1; 2; 4

Source: prepared by the author, 2019.

3.3.3 Acute toxicity assays

The ecotoxicological tests with both species were conducted in a climate room with controlled temperature of 25 ± 2 ºC and photoperiod of 12 h. At least twenty-four hours before the beginning of the tests, the earthworms were acclimated into the respective test soil in a climate room with a temperature of 25 ± 2 °C.

Impacts of imidacloprid on the survival of *E. andrei* were assessed through acute toxicity tests, according to ISO 11268-1 (ISO, 1993). Ten adult earthworms (with apparent clitellum) weighing between 250 and 600 mg were randomly inserted into cylindrical plastic containers (14.8 cm diameter and 9.8 cm height) containing approximately 600 g of wet soil, contaminated with imidacloprid or only distilled water (control soil). Individuals were fed at the start of the test and after 7 days with 10 g of uncontaminated equine manure and distilled water (5 mL), per experimental unit. The soil moisture was adjusted weekly (weight-based) with distilled water. Four replicates were performed for each treatment. After 14 days, the surviving earthworms were manually removed, gently rinsed with distilled water, weighed, and the survival and biomass loss of earthworms were calculated.

Acute toxicity assays with *F. candida* were based on guideline ISO 11267 (ISO, 2014). 30 g of wet soil (contaminated or control) were added to glass containers (7.5 cm diameter and 6.0 cm height). Then, ten collembolans with synchronized ages between 10 and 12 days were inserted in each experimental unit. Five miligrams of dried granulated yeast was supplied as food for the organisms at the beggining of the test. The containers were hermetically sealed during the test period except when they were opened for air renewal and soil moisture adjustment (addition of distilled water based on weight loss), twice a week. There were five replicates for each treatment. After 14 days, the adult survival collembolans were counted. The content of each container was carefully transferred to a larger container, which was filled with water and a few drops of black ink, to facilitate the counting of floating individuals which survived.

3.3.4 Chronic toxicity assays

Chronic toxicity assays with *E. andrei* were performed according to ISO 11268-2 (ISO, 2012). Reproduction assays were carried out in a similar way to acute toxicity assays, except for a longer exposure time (56 days), feeding frequency (worms were fed weekly during the test period) and test concentrations (Table 3.2). Four replicates were performed for each

treatment, and after 28 days of exposure, surviving adult earthworms were removed, rinsed with water, counted and weighed. Only the soil, the eventual juveniles and the generated cocoons remained in the container during the last 28 days of the test. On day 56, the plastic containers were inserted in a water bath (60 ± 5 ºC) for 1 hour, where juveniles were counted as describe by Alves et al. (2013).

The effect of imidacloprid on the reproduction of *F. candida* was assessed according to ISO 11267 (ISO, 2014). The procedures for the assays were identical to those of the acute toxicity tests with *F. candida*, differing only in the concentrations (Table 3.2), exposure time and the counting method. Five replicates were prepared for each treatment. After 28 days, the contents of each experimental unit were submerged in distilled water similarly to the procedure described for the acute toxicity test however, in this case, the experimental units containing the living floating individuals were photographed in high resolution. The images were analyzed using the computational software ImageJ® (SCHNEIDER et al., 2012) to account for the number of juveniles generated during the assay.

3.3.5 Estimation of PEC and PNEC

The Predicted Environmental Concentrations (PEC) were calculated for each soil based on the procedure described by Alves et al. (2013), simulating a worst-case scenario. Our calculated PEC of imidacloprid were based on the assumption of a soybean field with a sowing density of 100 kg of seeds ha^{-1} in a depth of 0-5 cm, with soil densities of 1.0 g cm^{-3} for TAS and Oxisol and 1.5 g cm^{-3} for Entisol. According to the commercial recommendation, its necessary 200 mL of the insecticide Much 600 FS (600 g a.i. L^{-1}) for treating 100 kg of soybean seeds, resulting in an amount of 120 g of imidacloprid ha^{-1}. The calculated PEC values (Table 3.2) were checked by methodology proposed by the European and Mediterranean Plant Protection Organization (EPPO, 2003), using the software ESCAPE®, assuming a single application of the pesticide, without interception of the crops, during one planting cycle. The Predicted No-Effect Concentrations (PNEC) were calculated by dividing the lowest EC_{10} of each soil by an assessment factor of 100 (EC, 2003), as described in Renaud et al. (2018).

3.3.6 Data analysis

The results of the ecotoxicological tests were analyzed using Statistica 7.0® software (STATSOFT, 2004), with the exception of the lethal concentration values of 50% (LC_{50}) of the

populations for the acute toxicity tests that were estimated through the probitic model, using PriProbit® software (SAKUMA, 1998). The normality and homoscedasticity of the reproduction data were ascertained through the Kolmogorov-Smirnov and Bartlett's tests, respectively; when necessary, logarithmic transformations were applied in order to comply with the analysis of variance (ANOVA) assumptions. When significant differences ($p < 0.05$) were detected by ANOVA, the means of the treatments were compared with the control through Dunnet's post-hoc test, in order to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC). In addition, concentrations that reduced reproduction by 10% and 50%, in relation to the control treatment (EC_{10} and EC_{50}, respectively), were estimated using the non-linear regression models (Exponential, Gompertz, Hormesis or Logistic model) recommended by Environmental Canada (2007). .

To calculate the ecological risk of imidacloprid for both soil fauna species in the tropical soils, the PEC was compared with the EC_{10} and PNEC, respectively, through two different approaches: 1) Toxicity-Exposure Ratios (TER), obtained by dividing the PEC by EC_{10} (TER = PEC/EC_{10}), as described in EC (2002); 2) Hazard Quotients (HQ), obtained by dividing the PEC by PNEC (HQ = PEC/PNEC), in accordance with the guidelines for the risk assessment of new and existing substances, proposed by the European Commission (EC, 2003). Significant risk was considered for the tested species when HQ > 1 or TER < 5.

3.4 RESULTS

3.4.1 Suitability of soils for ecotoxicological assays

In the controls of the acute and chronic assays with TAS and Entisol, no mortality of *E. andrei* was observed. On the other hand, in a preliminary test, the number of *E. andrei* juveniles generated in Oxisol was lower than 30 in all control replicates (data not shown). Therefore, the results for earthworms in Oxisol were not presented in this study. The mean mortality for adult collembolans in the controls of the assays with TAS, Entisol and Oxisol was respectively of 7.5%, 4% and 12% in the acute toxicity tests, and 12%, 6% and 30% in the chronic tests. The mean number (± standard deviation) of *E. andrei* juveniles generated in the controls of TAS and Entisol was 163 ± 36 and 208 ± 15, respectively. In the controls of TAS, Entisol and Oxisol, the mean number of juvenile collembolans (± sd) was, respectively, 298 ± 48, 210 ± 46 and 100 ± 28. For all bioassays, the coefficients of variation in the controls were lower than 30%. The number of adult (7 individuals = 30% mortality) and of juvenile collembolans found in two

replicates of the Oxisol control (R4 = 88 and R5 = 72 juveniles) did not meet the validation criteria established by ISO 11267 (ISO, 2014) for the chronic toxicity tests. Nevertheless, the results of this assay were considered in this study because an interesting dose-response relationship could be observed in this natural soil. In general, the validation criteria for the toxicity tests with the three soil types were met for both species (ISO, 1993; 2012; 2014), except for the assays with *F. candida* (chronic) and *E. andrei* (acute and chronic) in Oxisol.

3.4.2 Acute toxicity assays

The survival of *E. andrei* and *F. candida* was reduced in all spiked soils (Figure 3.1), with the intensity of the effect varying according to the soil type. The LOEC values for mortality in the natural sandy soil (Entisol) were, in general, lower than in the other soils (Table 3.3). Lowest LC_{50} values for both species were found in Entisol, confirming that the lethal effect of imidacloprid on this species was higher in this soil (Table 3.3). Significant mortality of collembolans started at 8.0 mg kg^{-1} for TAS and at 16.0 mg kg^{-1} for Oxisol. In TAS, acute toxicity was similar for earthworms (LC_{50} = 9.18 mg kg^{-1}) and collembolans (LC_{50} = 10.82 mg kg^{-1}), whereas, higher toxicity was found for earthworms (LC_{50} = 0.55 mg kg^{-1}) when compared to collembolans (LC_{50} = 4.68 mg kg^{-1}) in Entisol.

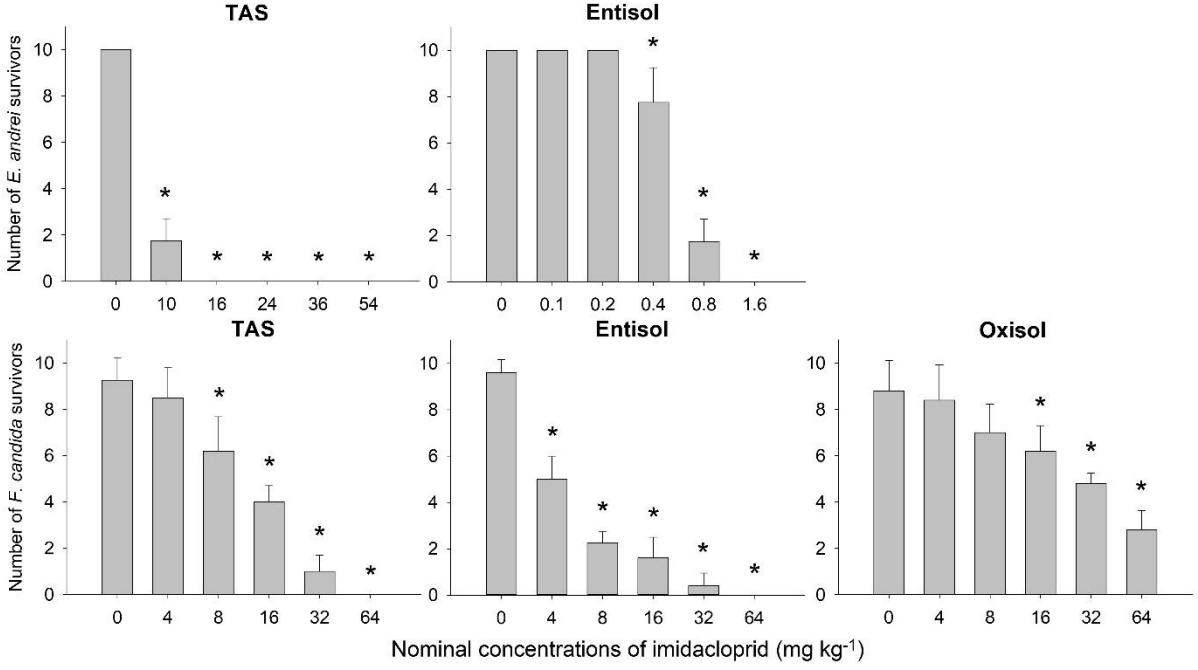
Table 3.3 - Ecotoxicological parameters (NOEC, LOEC, LC_{50}, EC_{10} and EC_{50}) obtained in acute and chronic toxicity tests with the species *E. andrei* and *F. candida*, in artificial soil (TAS) and in natural tropical soils (Entisol and Oxisol). The concentrations are expressed in mg of imidacloprid per kg^{-1} of dry soil (mg kg^{-1}).

Test species	**Endpoint**	**Parameter**	**Concentration (mg kg^{-1})**		
			TAS	**Entisol**	**Oxisol**
E. andrei	14-d mortality	NOEC	< 10.0	0.20	n.a.
		LOEC	10.0	0.40	n.a.
		LC_{50}	9.18 (8.81 - 9.57)	0.55 (0.48 - 0.62)	n.a.
	56-d reproduction	NOEC	1.0	< 0.12	n.a.
		LOEC	2.0	0.12	n.a.
		EC_{10}	0.66 (0.20 - 1.13)	0.03 ([a])	n.a.
		EC_{50}	1.89 (1.36 - 2.42)	0.21 (0.10 - 0.32)	n.a.

F. candida	14-d mortality	NOEC	4.0	< 4.0	8.0
		LOEC	8.0	4.0	16.0
		LC_{50}	10.82 (8.99 - 12.82)	4.68 (3.17 - 6.07)	25.08 (18.43 - 37.24)
	28-d reproduction	NOEC	0.5	< 0.25	0.5
		LOEC	1.0	0.25	1.0
		EC_{10}	0.20 (0.01 - 0.39)	0.02 (0.01 - 0.03)	0.20 (0.01 - 0.39)
		EC_{50}	0.80 (0.53 - 1.07)	0.09 (0.07 - 0.11)	0.83 (0.42 - 1.24)

The 95% confidence interval is in parenthesis.
[a] Data did not allow the estimation of the 95% confidence interval.
n.a. – Not available because the earthworms did not survive in the soil.
Source: prepared by the author, 2019.

Figure 3.1 - Mean number of adult *Eisenia andrei* (upper) and *Folsomia candida* (lower) survivors found in soils treated with increasing imidacloprid concentration, after 14 days of exposure. Asterisk (*) indicates significant differences ($p < 0.05$) between the treatment and control (Dunnet's test). (┬) Standard deviation (n = 4 for earthworms; n = 5 for collembolans).

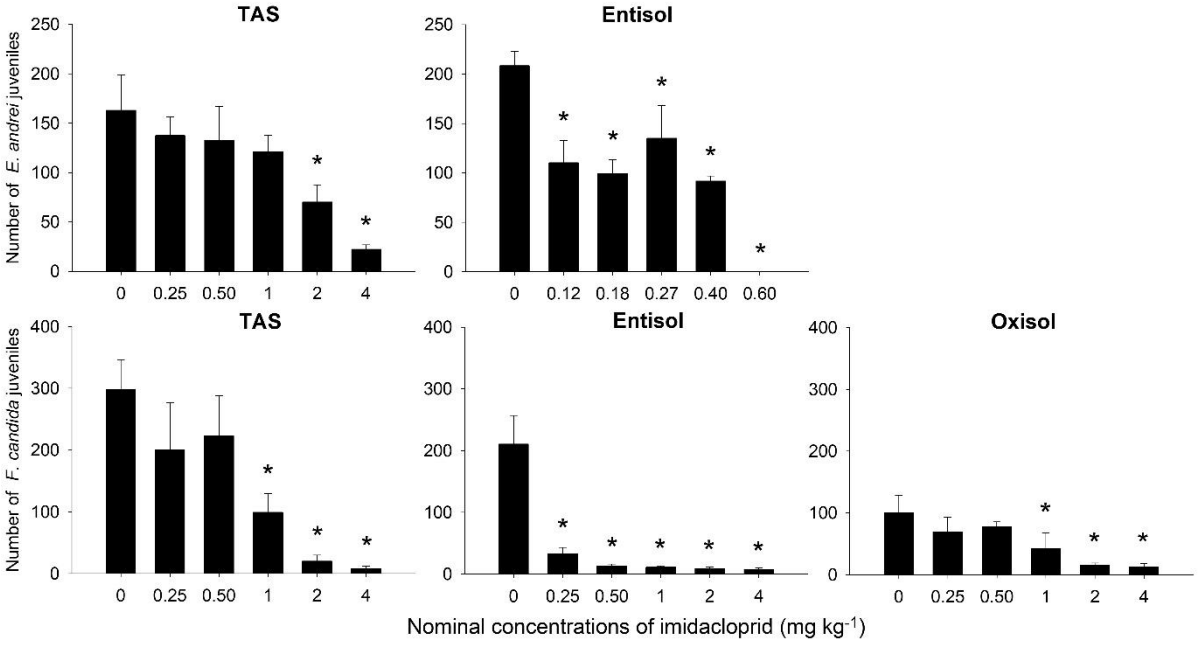


Source: prepared by the author, 2019.

3.4.3 Chronic toxicity assays

Reduction of juveniles number was observed in all tested soils for both species (Figure 3.2), being the effect higher with increasing concentrations of imidacloprid. The highest chronic toxicity was observed in Entisol ($EC_{50earthworms}$ = 0.21 mg kg^{-1}; $EC_{50collembolans}$ = 0.09 mg kg^{-1}), where the number of *E. andrei* and *F. candida* and juveniles were reduced at the lowest tested concentration (Table 3.3). For earthworms, the chronic toxicity (EC_{50}-based) in the Entisol was about 9 times higher compared to the TAS (Table 3.3). Similar EC_{50} values were found in the collembolans assays with Oxisol (0.83 mg kg^{-1}) and TAS (0.80 mg kg^{-1}).

Figure 3.2 - Mean number of *Eisenia andrei* (upper) and *Folsomia candida* (lower) juveniles found in soils treated with increasing imidacloprid concentration, after 56 and 28 days of exposure, respectively. Asterisk (*) indicates significant differences (p < 0.05) between the treatment and control (Dunnet's test). (┬) Standard deviation (n = 4 for earthworms; n = 5 for collembolans).



Source: prepared by the author, 2019.

3.4.4 Ecological risk estimation

Both approaches used to calculate the potential risk of imidacloprid for the tested species (TER and HQ) indicated a significant risk of exposure for soil fauna to the expected levels of the a.i. in the environment (PEC), in all tested soils (Table 3.4). Considering the values of TER, the risk for collembolans is similar when they were exposed to a.i. in TAS or Oxisol. The lowest TER values for *F. candida* and *E. andrei* were found in Entisol, revealing a highest risk in this soil. HQ values confirm these results, indicating that the risk is at least six times higher in Entisol than in the other soils (Table 3.4).

Table 3.4 - Ecological risk for the exposure to imidacloprid in Tropical Artificial Soil (TAS), Entisol and Oxisol, estimated via Toxicity Exposure Ratio (TER) and Hazard Quotient (HQ) approaches.

Soil	Tested species	PNEC [a] (mg kg^{-1})	TER [b]	HQ [c]
TAS	*E. andrei*	0.002	2.75 [e]	120 [d]
	F. candida		0.83 [e]	
Entisol	*E. andrei*	0.0002	0.19 [e]	800 [d]
	F. candida		0.12 [e]	
Oxisol	*F. candida*	0.002	0.83 [e]	120 [d]

[a] PNEC = lowest EC10 / 100;
[b] TER = EC10 / PEC;
[c] HQ = PEC / PNEC;
[d] HQ > 1, indicating significant risk and the need for future investigations;
[e] TER < 5, indicating a significant risk for the tested species.
Source: prepared by the author, 2019.

3.5 DISCUSSION

The species had different reproductive performance in each tested soil. The lower survival and reproduction of *E. andrei* in Oxisol indicate that this soil was not able to maintain their population during the test period (data not shown). In the same way, the mean number of *F. candida* juveniles was lower than the minimum for validation of the test (ISO, 2014), indicating that both species presented lower reproductive performance in Oxisol, when compared to the other soils. This may be due to the pedogenetic characteristics of this soil, such as its higher clay content (Table 3.1). Similar effects were observed for *F. candida* (DOMENE et al., 2011) and oligochaetes *E. andrei* and *E. crypticus* (CHELINHO et al., 2011) in fine

textured soils, possibly due to the greater difficulty of colonization and occupation of porous space by these organisms in clayey soils. In addition, according to Amorim et al. (2005b), there may be a positive influence of the porous structure in sandy soils (e.g., Entisol and TAS) on the reproduction of this species that allows for better mobility and reproductive performance of the soil invertebrates.

In our study, negative effects of imidacloprid on the biological parameters of both species were detected in all assays. Adverse effects of this a.i. on *F. candida* and *E. andrei* were also seen in other studies (ALVES et al., 2013; ALVES et al., 2014; CHEVILLOT et al., 2017), and they have been attributed to the mode of action of this molecule on soil organisms. This molecule acts on the blockage of the acetylcholine receptors in the nervous system of the organisms, through irreversible agonist connections, leading to the accumulation of the neurotransmitter acetylcholine (CATAE et al., 2018). The systemic action of this a.i. may trigger a number of negative effects on exposed individuals, such as reduced fecundity (LAYCOCK et al., 2012; GE et al., 2018), disorientation, paralysis (SIMON-DELSO et al., 2015), changes in DNA and in detoxifying enzymes (WANG et al., 2016; SILLAPAWATTANA and SCHÄFFER, 2017), and even death (DE LIMA E SILVA et al., 2017).

Although the mode of action of this molecule seems to be similar for the two tested species, the toxicity values were different (Table 3.3). In general, collembolans were more sensitive to imidacloprid than earthworms in the chronic toxicity assays, due to the lower EC_{50} values found for *F. candida* in all soils. The higher sensitivity of *F. candida* to imidacloprid compared to *E. andrei* has been also identified in other studies (ALVES et al., 2013; ALVES et al., 2014; DE LIMA E SILVA et al., 2017) and may be explained by their different intrinsic sensitivity to this class of pesticide, as well as different exposure routes of the species. When acting on the nervous system of the exposed species, the neonicotinoids are generally more selective for the nicotinic acetylcholine receptors (nAChRs) of arthropods when compared to oligochaetes (AKEJU, 2014). A higher selectivity of the imidacloprid by the collembolan's receptor sites can also be expected due to the phylogenetic proximity of the arthrophods to the insects (DE LIMA E SILVA et al., 2018), which are the main target of action of imidacloprid.

On the other hand, its known that earthworms absorb the contaminants mainly by the passive diffusion of porewater through the dermis, in addition of the intestinal absorption due to the ingestion of soil particles (BELFROID et al., 1994), while collembolans are exposed mainly through the absorption of porewater by specific organs, such as the ventral tube

(FOUNTAIN and HOPKIN, 2005). When earthworms are exposed to high concentrations of imidacloprid in the soil, it is possible that the absorption of the a.i. from the soil solution through the skin may have been favored. This could be an explanation for the higher mortality of *E. andrei* compared to *F. candida* in the acute toxicity assays in Entisol (Table 3.3), especially because earthworms were in a compulsory exposure to high concentrations of the contaminant (dermal contact + feeding). For collembolans, the short-time exposure at high concentrations (as in the case of acute toxicity assays) seems to be less harmful, because they can avoid the consumption of pore water and also stay protected from the direct contact with contaminated soil by an exoskeleton.

Some studies have evaluated the chronic toxicity of imidacloprid to soil fauna species using representative soils from temperate regions. Wang et al. (2019) and Ge et al. (2018) found EC_{50} values of 0.70 and 0.87 mg kg^{-1}, respectively, for hatchability and number of juveniles when exposed *E. fetida* to pure imidacloprid in an OECD artificial soil. Ogungbemi and van Gestel (2018) found EC_{50} of 0.63 and 2.07 mg kg^{-1} when *F. candida* was exposed to the pure a.i. in OECD artificial soils containing 5% and 10% of peat, respectively. Similarly, Mabubu et al. (2017) reported an EC_{50} of 0.82 mg kg^{-1} for *F. candida* reproduction when the pure compound was tested in OECD artificial soil (5% peat). On the other hand, a relatively lower toxicity was found by Alves et al. (2013; 2014) when soil invertebrates were exposed to the commercial formulation Gaucho (600 mg a.i. L^{-1}) in a tropical artificial soil with 10% coconut husk. They found EC_{50} values of 4.07 mg kg^{-1} for *E. andrei* (ALVES et al., 2013) and > 1 mg kg^{-1} for *F. candida* (ALVES et al., 2014). The toxicity of imidacloprid (pure a.i.) were also investigated in a natural LUFA 2.2 soil, and it was found an EC_{50} of 0.39 mg kg^{-1} for *E. andrei* (DE LIMA E SILVA et al., 2017), while the EC_{50} values ranged from 0.1 – 0.3 mg a.i. kg^{-1} in studies with *F. candida* (DE LIMA E SILVA et al., 2017; VAN GESTEL et al., 2017; OGUNGBEMI and VAN GESTEL, 2018).

Despite the methodological differences between the reported studies, a relatively similar imidacloprid toxicity for collembolans may be observed between artificial substrates with resembled compositions, especially with regard to the organic material content. The EC_{50} values reported in the assays with OECD soils containing 5% peat (MABUBU et al., 2017; OGUNGBEMI and VAN GESTEL, 2018) are similar from our EC_{50} values in TAS, which contains 5% of coconut fiber. A relatively lower imidacloprid toxicity could be identified in assays where artificial substrates containing 10% of organic material were used (ALVES et al., 2013; 2014; OGUNGBEMI and VAN GESTEL, 2018), suggesting that, when the soil texture

is quite similar, the SOM has an important influence on imidacloprid bioavailability. On the other hand, different toxicities can be identified between natural soils which have similar SOM contents but with distinct textures. The chronic toxicity for *F. candida* found in natural LUFA 2.2 soil with about 4% of SOM and less than 20% of clay + silt content (DE LIMA E SILVA et al., 2017; VAN GESTEL et al., 2017) were at least twice higher than those found for Oxisol in this study (Table 3.3), which has 3.7% SOM but high levels of silt + clay (> 90 %). These results suggest that the toxicity of imidacloprid is not exclusively driven by the organic material but is also regulated by the mineral soil fraction (such as the amount and type of clay), which probably had a stronger influence on the sorption of the imidacloprid molecules than SOM in our assays.

Some studies have identified a key role of the fine-textured soil mineral fraction in the pesticide toxicity (RUTHERFORD et al., 1992; OGUNGBEMI and VAN GESTEL, 2018). Mineral particles with high specific surface area are able to adsorb the imidacloprid molecules and therefore decrease their bioavailability to soil invertebrates (OGUNGBEMI and VAN GESTEL, 2018). Thus, the higher amounts of clay and silt in Oxisol used in this study could partially explain the relatively lower toxicity of imidacloprid, when compared to that reported by De Lima e Silva et al. (2017) and van Gestel et al. (2017) in the natural LUFA 2.2 soil, as well as the lower acute toxicity for collembolans in this soil when compared to TAS (Table 3.3). Likewise, the highest toxicity found in Entisol in this study could be due to its low contents of clay and silt, which probably led to a greater fraction of the contaminant available for uptake by the organisms in the soil solution (VAN GESTEL, 2012; PEIJNENBURG et al., 2012). In line with our results, Zortéa et al. (2018) observed that fipronil was more toxic to *F. candida* reproduction in Entisol than in TAS and Oxisol. For animal wastes such as swine manure (SEGAT et al., 2015) and pig manure (MACCARI et al., 2016), deleterious effects on soil invertebrates were also higher in Entisol, when compared to Oxisol. Anyway, the differences between the toxicity values reported in the literature and ours should be interpreted carefully and can not be exclusively attributed to the differences in soil properties, because the most of the above-mentioned studies were performed at 20 °C instead of 25 °C and were based on the effects of the pure substance instead of a commercial formulation of imidacloprid. However, we consider that the use of commercial formulations on ecotoxicologial assessments are more ecologically relevant than the pure active ingredient, since pesticides are used via formulations in agricultural areas (RENAUD et al., 2018).

Although our PEC values (Table 3.2) has been estimated based on a conservative approach by considering the worst-case scenario, similar imidacloprid concentrations have been found in agricultural soils. In soils from cocoa plantations, residues of imidacloprid ranged between 0.005 – 0.25 mg kg^{-1} (DANKYI et al., 2014). DONNARUMMA et al. (2011) identified residues of imidacloprid of 0.65 mg kg^{-1}, 30 days after the sowing of seeds treated with the commercial formulation Gaucho 350 FS. When considering direct applications of imidacloprid in the crops, residues in soil may reach concentrations > 4 mg kg^{-1} (SHARMA and SINGH, 2014), and even higher levels of this a.i. may ocurrs in the environment after sucessive applications (GE et al., 2018). The detected imidacloprid concentrations in the reported studies are closer or are even higher than our LOEC vaules for chronic assays with earthworms (0.12 – 2 mg kg^{-1}) and collembolans (0.25 – 1.0 mg kg^{-1}), indicating that species reproduction may be affected by the expected levels of imidacloprid in tropical regions.

Despite differences between soils in the toxicity of imidacloprid (Table 3.3), a significant ecological risk was detected for all soils using TER and HQ approaches (Table 3.4). TER and HQ values for collembolans in TAS and Oxisol were the same, while in Entisol the estimated risk values for *F. candida* and *E. andrei* were at least 6 times higher than in the other soils. These results indicate that data produced from ecotoxicological tests with TAS should not be directly extrapolated to the field, since they may not accurately represent the real exposure in tropical conditions. Niemeyer et al. (2017) have also highlighted the importance of considering physical and chemical properties of tropical soils demonstrated here. This must be considered when conducting ecological risk assessments and for the registration and application of pesticides in Brazil.

In addition, this study found lethal effects of imidacloprid in Entisol at concentrations 2.5 and 25 times higher than the PECs for earthworms and collembolans (Tables 3.2 and 3.3), respectively. In TAS and Oxisol, the species survival was affected only at concentrations higher than those causing mortality in Entisol. Although in the European Union the acute toxicity tests with earthworms are no longer required for the registration of Plant Protect Products (EU, 2013), in Brazil, this is the unique assay with soil organisms currently required for pesticide registration (BRAZIL, 1996). Because of this, our results also highligh for the need to include more sensitive and ecologically relevant endpoints (such as reproduction) as requirement for pesticides registration into the national regulations (NIVA et al., 2016).

3.6 CONCLUSIONS

Imidacloprid caused mortality and reduced the reproduction of *E. andrei* and *F. candida* in all performed assays, but the intensity of the toxic effects was influenced by the soil type. The highest toxicity for both species was observed in Entisol ($EC_{50earthworms}$ = 0.21 mg kg^{-1}; $EC_{50collembolans}$ = 0.09 mg kg^{-1}), probably due to the lower CEC and lower clay and silt contents of this soil. Similar dose-response patterns were found in reproduction tests with collembolans in TAS (EC_{50} = 0.80 mg kg^{-1}) and Oxisol (EC_{50} = 0.83 mg kg^{-1}), soils that have higher CEC and higher sum of clay and silt contents compared to Entisol. For earthworms, the lowest chronic toxicity was observed in TAS (EC_{50} = 1.89 mg kg^{-1}). These results indicate that imidacloprid toxicity to soil fauna is highly driven by soil type (and their properties), which is not represented properly by the single use of artificial substrates in soil ecotoxicological assays. The species survival was affected at concentrations higher than the PEC, while *E. andrei* reproduction was reduced at concentrations lower than those predicted in the environment, when the molecule is used for seed dressing in Entisol. Significant ecological risk was found for all tested soils, being at least six times greater in Entisol than in Oxisol and TAS.

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4 CHAPTER 2: Influence of soil moisture on imidacloprid toxicity to earthworms and collembolans in tropical soils

4.1 ABSTRACT

The chemical treatment of seeds with imidacloprid contributes to increased food production although it can impact terrestrial ecosystems. Shifts in precipitation patterns, due to the predicted climate changes, can reduce the water content in tropical soils and, consequently, alter the toxicity of pesticides to edaphic fauna. The objective of this work was to assess the influence of soil moisture on the toxicity of the active ingredient (a.i.) imidacloprid to invertebrates in tropical soils. Acute and chronic toxicity tests with earthworms *Eisenia andrei* and collembolans *Folsomia candida* were performed using a Tropical Artificial Soil (TAS) and two tropical natural soils (Entisol and Oxisol), which were spiked with increasing imidacloprid concentrations. The soils were submitted to situations of normal water availability (60% of the water holding capacity - WHC) and water restriction (30% or 45% WHC). In Oxisol, the toxicity (EC_{50}-based) to collembolans was more than seven times higher at 45% WHC (0.32 mg kg^{-1}), when compared to the exposure at 60% WHC (2.49 mg kg^{-1}). Increasing toxicity at lower soil moisture was also identified for earthworms in TAS ($EC_{50\ 45\%}$ = 1.96 mg kg^{-1}; $EC_{50\ 60\%}$ = 2.77 mg kg^{-1}). No clear influence of soil moisture on imidacloprid toxicity was observed for the Entisol. Imidacloprid toxicity varied with the sensitivity of edaphic organisms and soil type. In addition, in two soils (TAS and Oxisol) with lower water content the a.i. toxicity was intensified, indicating that the hazard of pesticide contamination to soil invertebrates increased due to climate changes. This study also reinforced the importance of considering the alterations in environmental factors caused by climate changes in the assessment of pesticide risk to edaphic fauna, especially in tropical regions.

Keywords: Soil Ecotoxicology; *Eisenia andrei*; *Folsomia candida*; Climate Changes; Seed dressing; Neonicotinoids.

4.2 INTRODUCTION

The main model of agricultural production in Brazil is based on the intensive use of pesticides to increase crop productivity (EMBRAPA, 2014), which placed the country among the world's top pesticide consumers. From 2000 to 2017, the commercialization and use of pesticides in Brazil increased by 240% (IBAMA, 2017).

Seed treatment with pesticides is a practice that allows the control of pests that can harm crop development in the early stage (DOUGLAS and TOOKER, 2015). Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-N-nitro-1H-imid azol-2-amine) is a neonicotinoid insecticide widely used for seed dressing (VAIKKINEN et al., 2015; WU et al., 2016) due to its efficient mechanism of action on a large variety of insects that may damage plants. This active ingredient (a.i.) acts on the nervous system of these organisms, causing blockage of receptors and accumulation of the neurotransmitter acetylcholine, which leads to paralysis and death (BUFFIN, 2003).

Despite the benefits for agricultural productivity, the use of imidacloprid can lead to negative consequences for the environment, such as impacts on soil fauna (ALVES et al., 2013; ALVES et al., 2014; WANG et al., 2016; VAN GESTEL et al., 2017). In soils, the invertebrates play crucial functions in nutrient cycling, organic matter breakdown, soil structure maintenance, among others (EDWARD et al., 2009; OGUNGBEMI and VAN GESTEL, 2018). Thus, exposure of soil invertebrates to imidacloprid may result in losses of key ecosystem services.

The current anthropogenic increase in the emission of greenhouse gases will result in climate changes, which include shifts in the precipitation patterns over the next years (IPCC, 2013). Soil moisture is considered another important factor for the maintenance of terrestrial ecosystems (BRADY and WEIL, 2013). The ability of faunal populations to develop in their natural habitat will not depend only on their reaction to changes in conditions such as temperature and precipitation, through physiological or behavioral adaptations (Chown and Gaston, 2008), but also on their ability to deal with the modified pesticide dynamics due to interactions with climate change (TRIPATHI et al., 2015).

In addition, climate may also affect the dynamics of pesticides in the soil (DELCOUR et al., 2015; NADAL et al., 2015). Several studies have shown that the increase of temperature and/or the reduction of soil moisture may affect pesticide availability in soils, as well as their toxic potential for ecological receptors (DELCOUR et al., 2015; GONZÁLEZ-ALCARAZ & VAN GESTEL, 2016; BARMENTLO et al., 2017; OGUNGBEMI and VAN GESTEL, 2018).

Some ecotoxicological studies indicate that reductions of soil moisture associated with contamination can cause decreases in the reproduction of soil meso- and macrofauna and other negative biological effects (LONG et al., 2009; BANDOW et al., 2014a; BANDOW et al., 2014b; BARMENTLO et al., 2017; HACKENBERGER et al., 2018). However, the influence of soil moisture on pesticide toxicity to edaphic organisms in tropical soils is not clear.

Therefore, the aim of this study was to assess the influence of soil moisture on the toxicity of imidacloprid to edaphic fauna in tropical soils. Chronic and acute toxicity assays with earthworms and collembolans were performed in two natural tropical soils and in an artificial tropical soil, with moisture regimes that simulate situations of normal water availability and water restriction.

4.3 MATERIAL AND METHODS

4.3.1 Test organisms

For the ecotoxicological assays, two species of soil invertebrates were used, *Folsomia candida* and *Eisenia andrei*. The breeding of these organisms and the ecotoxicological assays were performed under controlled temperature and luminosity (20 ± 2 ºC and photoperiod of 12 h), following the guidelines ISO 11267 (ISO, 2014) and ISO 11268-2 (ISO, 2012). Although the average tropical climate temperature is around 25 ° C, the assays were performed at 20 ± 2 ºC in order to promote a better development of the species and, therefore, to isolate the influence of the soil moisture on the toxicity of imidacloprid to edaphic species.

Collembolans were bred in plastic containers with a mixture of plaster of Paris, water and activated charcoal in the proportion of 10:7:1, respectively. Twice a week, the organisms were fed dry granulated yeast (*Saccharomyces cerevisiae*) and the moisture of the breeding medium was adjusted with a few drops of distilled water. Synchronized cultures of collembolans (10 to 12 days old) were used in the assays (ISO, 2014).

The substrate to rear the earthworms was a mixture of dry and sieved horse manure (free of veterinary drugs), powdered coconut husk, and fine sand in the proportion of 20:10:3, respectively. The moistened substrate was transferred to plastic boxes and small holes were opened on the lids to allow gas exchange. Once a week, the moisture was calibrated and 10 g of cooked oat flakes were offered as food for the oligochaetes. Adult earthworms with a clitellum and individual weight between 250 and 600 mg were used in the assays. The

individuals were acclimatized in the test soils for at least 24 hours before the start of the assays (ISO, 2012).

4.3.2 Test soils

All the bioassays were conducted in an artificial soil and in two natural tropical soils. The artificial soil consisted of a modified version of the soil of OECD (1984), called Tropical Artificial Soil (TAS), proposed by Garcia (2004) as an adaptation to tropical climate conditions. This soil is a mixture of fine sand, kaolinitic clay and powdered coconut husk in the proportion of 75:20:5, respectively. When necessary, the pH of the TAS was adjusted to 6.0 ± 0.5 with $CaCO_3$.

Two natural soils with distinct characteristics were used in the assays: an Entisol (sandy soil) sampled in the municipality of Araranguá (SC) (29º 00'S; 49º 31'W) and an Oxisol (clay soil), sampled in the municipality of Chapecó (SC) (27º 5'S; 52º 37'W). Soil samples collected in areas with no history of contamination by pesticides, in the superficial layer of 0 to 20 cm of the soil profile.

Physical and chemical properties of the soils (Table 4.1), such as pH, organic matter, contents of P, K, Ca, Mg, Al, H + Al, Cu, Fe, Mn and Zn, effective cation exchange capacity (CEC), base saturation, as well as sand, clay and silt contents were determined according to Tedesco et al. (1995). The water holding capacity (WHC) of the soils was determined according to the Annex C of ISO 11268: 2 (ISO, 2012), as described in Alves et al. (2019). For the ecotoxicological assays, the soil samples were sieved (# 2 mm) and defaunated by undergoing three freeze-thaw cycles to eliminate the presence of natural soil organisms.

Table 4.1 - Physical and chemical characterization of Tropical Artificial Soil (TAS), Entisol and Oxisol, before spiking with imidacloprid.

Parameter	TAS	Entisol	Oxisol
pH (1 M KCl)	5.7 ± 0.1	4.5 ± 0.1	3.8 ± 0.1
SOM (%)	1.4 ± 0.0	2.2 ± 0.1	3.7 ± 0.1
P (mg dm^{-3})	23.4 ± 4.8	4.8 ± 1.0	3.4 ± 0.4
K (mg dm^{-3})	422.0 ± 48.1	42.0 ± 2.8	136.0 ± 11.3
Ca ($cmol_c$ dm^{-3})	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Mg ($cmol_c$ dm^{-3})	1.1 ± 0.3	0.5 ± 0.1	0.5 ± 0.0
Al ($cmol_c$ dm^{-3})	<0.1	0.3 ± 0.0	4.4 ± 0.3
H + Al ($cmol_c$ dm^{-3})	1.8 ± 0.4	4.0 ± 1.3	16.6 ± 4.5
Cu (mg dm^{-3})	0.9 ± 0.1	0.7 ± 0.1	1.9 ± 0.1
Fe (g dm^{-3})	>5.0	>5.0	>5.0
Mn (mg dm^{-3})	<2.5	<2.5	21.8 ± 0.1
Zn (mg dm^{-3})	0.6 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
CEC ($cmol_c$ dm^{-3})	3.33 ± 0.2	1.4 ± 0.4	5.9 ± 0.2
Base Saturation (%)	65.0 ± 6.6	22.2 ± 3.3	8.6 ± 2.3
Clay (g kg^{-1})	143.0 ± 0.0	41.5 ± 2.2	593.0 ± 70.7
Silt (g kg^{-1})	185.0 ± 0.0	20.5 ± 1.4	352.5 ± 57.3
Sand (g kg^{-1})	672.0 ± 0.0	938.0 ± 4.2	54.5 ± 13.4
WHC (%)	46.3 ± 1.7	31.6 ± 1.1	45.1 ± 5.6

Values are expressed as mean ± standard deviation; n=2
SOM – Soil Organic Matter
CEC – Cation Exchange Capacity
WHC – Water Holding Capacity

4.3.3 Test substances

The soils were spiked with increasing concentrations of the a.i. imidacloprid, in the commercial formulation Much 600 FS® (600 g a.i. L^{-1}), which is recommended for seed dressing in Brazil.

The concentrations (expressed as mg a.i. per kg^{-1} dry soil - mg kg^{-1}) used in the assays (Table 4.2) were based on preliminary laboratory assays (data not shown) and on literature data (ALVES et al., 2013; ALVES et al., 2014; VAN GESTEL et al., 2017). Based on the calculations described by Alves et al. (2013), the predicted environmental concentrations (PEC) were estimated assuming a soil density of 1.0 g cm^{-3} for TAS and Oxisol, and 1.5 g cm^{-3} for Entisol. For the calculation of the PEC (Table 4.2), the commercial recommendation of Much 600 FS® for soybean seeds was considered, consisting of a single application of 120 g a.i. ha^{-1} and incorporation of the insecticide in the soil to a depth of 0-5 cm (ALVES et al., 2013).

The spiking of the imidacloprid concentrations in the soil samples was through an aqueous solution, prepared with a pre-calculated volume of distilled water sufficient to achieve the intended soil moisture. In general, the assays were conducted under moisture regimes of

30% and 60% WHC, except for those carried out with Oxisol, and the chronic toxicity assays with earthworms in TAS, which were performed with 45% and 60% WHC due to the lower adaptation capacity of most species to lower soil moisture contents.

The acute and chronic toxicity assays were performed with earthworms and collembolans in the three soil types, using two of the above-mentioned soil moisture regimes. Assays with collembolans had five replicates, while the assays with earthworms were performed with four replicates, whose experimental units were opened once a week to allow gas exchange and soil moisture correction by difference of weight.

Table 4.2 - Concentrations of imidacloprid (mg of a.i. kg^{-1} dry soil) expected in the environment (PEC) and concentrations used in the acute and chronic toxicity assays carried out in Tropical Artificial Soil (TAS), Oxisol and Entisol with *F. candida* and E. *andrei*.

Soil	PEC values (mg kg^{-1})	Species	Imidacloprid concentration (mg kg^{-1})	
			Acute toxicity	Chronic toxicity
TAS	0.24	*F. candida*	0; 4; 8; 16; 32; 64	0.25; 0.50; 1.00; 2.00; 4.00
		E. andrei	0; 10; 16; 24; 36; 54	0.25; 0.50; 1.00; 2.00; 4.00
Entisol	0.16	*F. candida*	0; 4; 8; 16; 32; 64	0.25; 0.50; 1.00; 2.00; 4.00
		E. andrei	0; 0.10; 0.20; 0.40; 0.80; 1.60	0; 0.12; 0.18; 0.27; 0.40; 0.60
Oxisol	0.24	*F. candida*	0; 4; 8; 16; 32; 64	0.25; 0.50; 1.00; 2.00; 4.00

Source: prepared by the author, 2019.

4.3.4 Acute toxicity assays

The acute toxicity assays with collembolans were based on ISO 11267 guidelines (ISO, 2014). 30 g of contaminated soil (wet weight) or control soil were added in cylindrical glass containers with hermetic closures (4 cm diameter and 9 cm height). Ten individuals were inserted in each experimental unit. The organisms were fed approximately 2 mg of yeast on the first day of the assay. After 14 days, the contents of the experimental unit (soil + collembolans) were submerged in water with a few drops of black ink, and the survivors (those who floated) were counted.

Acute toxicity assays with *E. andrei* were carried out according to ISO 11268-1 (ISO, 1993). 600 g of moist soil (control or contaminated) were added in cylindrical plastic containers (15 cm diameter and 10 cm height) with perforated lids. Then, 10 adult earthworms were inserted in each experimental unit. 10 g of wet horse manure (1:2 manure / water) was offered

as food. After 14 days of the start of the assay, the surviving earthworms were manually counted.

4.3.5 Chronic toxicity assays

Reproduction assays with *F. candida* were performed according to ISO 11267 (ISO, 2014). The procedures adopted in the setup of this assay are similar to those performed in the acute toxicity assays, differing only in the concentrations used (Table 4.2) and in the exposure time (28 d). The collembolans were fed at the beginning of the assay and after 14 days. Moisture maintenance and gas exchange were also performed weekly. At 28 days after the start of the test, the collembolans were counted in a similar way to that of the acute toxicity assay. However, after the flotation, the replicates were photographed from a superior angle, allowing the counting of juveniles through ImageJ® software (ALVES et al., 2014).

The earthworm reproduction assays were performed according to ISO 11268-2 (ISO, 2012). The spiking and the insertion of 10 adult worms into each experimental unit was performed in the same way as for the acute toxicity assay. The oligochaetes were fed weekly with moist equine manure. After 28 days from the beginning, surviving adult worms were removed, counted and weighed. During the next 28 days, only the soil, cocoons and generated juveniles remained in the containers. On the day 56, the experimental units were immersed in a water bath (60 ± 5 ºC) for one hour and the *E. andrei* juveniles generated were counted.

4.3.6 Data analysis

The homoscedasticity and normality of the chronic toxicity assays data were verified by the Bartlett and Kolmogorov-Smirnov tests, respectively, and, when necessary, logarithmic transformations were applied to meet the assumptions. Then, the results of the assays were submitted to analysis of variance (ANOVA) and, when significant differences ($p < 0.05$) were detected, treatments with different concentrations of imidacloprid were compared to the control treatment using Dunnett's test through Statistica 7.0® software. Thus, NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) values were determined. Effect concentrations causing reductions of 20% and 50% in the species reproduction (EC_{20} and EC_{50}, respectively) were estimated by using non-linear regression models predefined by Environment Canada (2007), in Statistica 7.0® software. In addition, the

LC_{50} values (lethal concentration of 50%) of the acute toxicity assays were estimated through PriProbit® software.

4.4 RESULTS

4.4.1 Assays validation

The mean number of surviving adults in the controls of the acute toxicity assay was nine (± 1) for *F. candida* and, for the *E. andrei* assays, 10 living individuals were found in all control units. Therefore, the validation criteria for all mortality assays were met (ISO, 2012; 1993).

For the chronic toxicity assays with *F. candida*, the validation criteria were also met (ISO, 2014), since the mean number of *F. candida* juveniles in the controls of Oxisol, Entisol and TAS were 185 (± 65), 247 (± 56) and 280 (± 91), respectively. The earthworms *E. andrei* did not survive in the assays carried out with Oxisol, so these results were not considered in this study. On the other hand, in controls of Entisol (159 ± 56) and TAS (140 ± 28), the mean number of juveniles generated met the the validation criteria for ISO (2012). The adult survival in the chronic toxicity assays was greater than 80% and 90% for *F. candida* and *E. andrei*, respectively. In addition, the coefficients of variation obtained for the assays were lower than 30%.

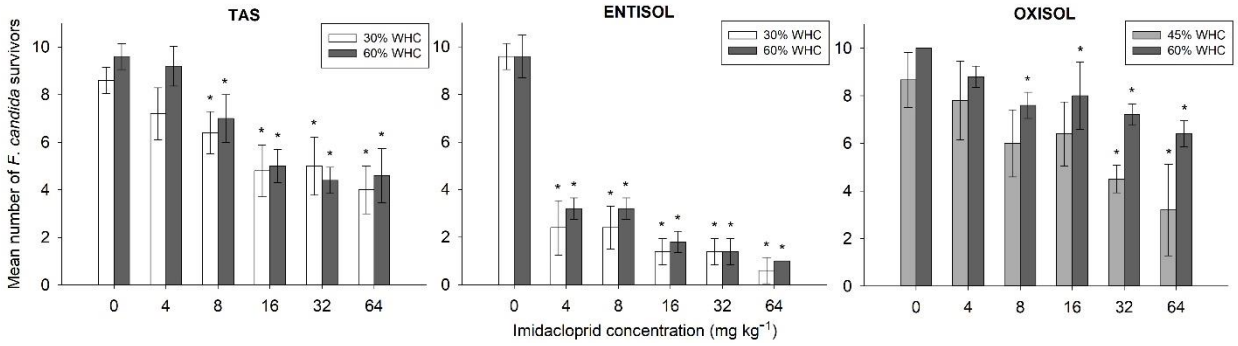
4.4.2 Acute toxicity assays

There was a significant mortality of *F. candida* in all soils for both moisture regimes tested (Figure 4.1). The LOEC values found for the different moisture regimes were the same for TAS and Entisol ($LOEC_{\text{TAS 30\% and 60\%WHC}}$ = 4 mg kg^{-1}; $LOEC_{\text{ENTISOL 30\% and 60\% WHC}}$ = 8 mg kg^{-1}). However, in Oxisol, adult survival at 45% WHC was only affected at a concentration (LOEC = 32 mg kg^{-1}) four times higher than that under the 60% WHC condition (LOEC = 8 mg kg^{-1}). In all soils, the LC_{50} values were lower under soil drought conditions ($LC_{50\ \text{TAS 30\%WHC}}$ = 23.58 mg kg^{-1}; $LC_{50\ \text{ENTISOL 30\%WHC}}$ = 0.44 mg kg^{-1}; $LC_{50\ \text{OXISOL 45\%WHC}}$ = 23.84 mg kg^{-1}), when compared to the values obtained for 60% WHC ($LC_{50\ \text{TAS 60\%WHC}}$ = 29.88 mg kg^{-1}; $LC_{50\ \text{ENTISOL 60\%WHC}}$ = 1.18 mg kg^{-1}; $LC_{50\ \text{OXISOL 60\%WHC}}$ > 64 mg kg^{-1}).

There was also a significant mortality of *E. andrei* in TAS and Entisol for both moisture regimes (Figure 4.2). In the acute toxicity assays with earthworms at 30% WHC, the effect of a.i. was at least six times higher in Entisol (LOEC = 1.6 mg kg^{-1}) than in TAS (LOEC = 10 mg

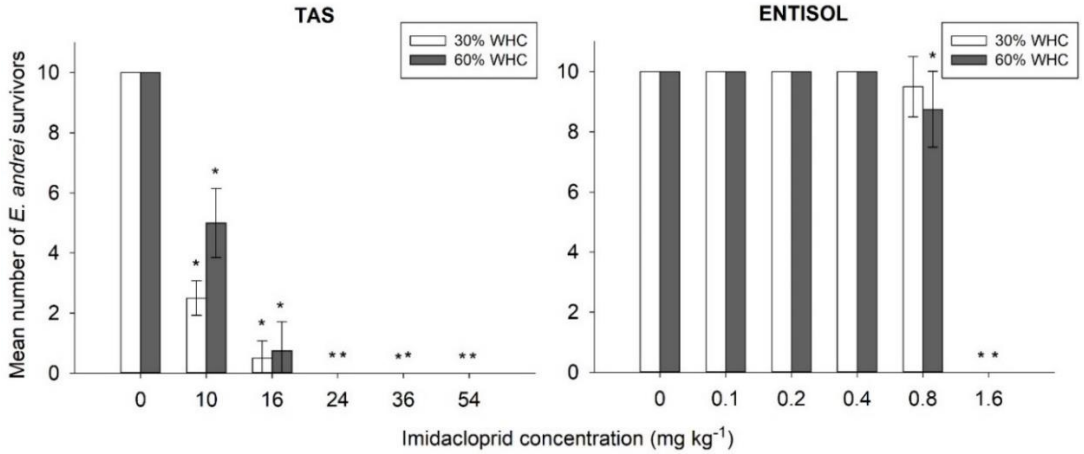
kg^{-1}; Table 4.3). The influence of soil moisture content on the toxicity of imidacloprid to earthworms was higher in TAS, where the LC_{50} was lower for the dry condition ($LC_{50\ TAS\ 30\%WHC}$ = 7.56 mg kg^{-1}) when compared to 60% WHC ($LC_{50\ TAS\ 60\%WHC}$ = 10.04 mg kg^{-1}). For Entisol, the estimated LC_{50} values were similar for the two soil moisture contents tested (Table 4.3).

Figure 4.1 - Mean number (n = 5, ± standard deviation) of adult *F. candida* survivors after 14 days of exposure to different concentrations of imidacloprid in Tropical Artificial Soil (TAS), Entisol and Oxisol, under regimes of 30% (white bars) or 45% (light gray bars), and 60% (dark gray bars) of soil WHC. (*) indicates a significant mortality in comparison to the respective control (Dunnett test - $p \leq 0.05$).



Source: prepared by the author, 2019.

Figure 4.2 - Mean number (n = 4, ± standard deviation) of adult *E. andrei* survivors after 14 days of exposure to different concentrations of imidacloprid in Tropical Artificial Soil (TAS) and Entisol, under regimes of 30% (white bars) and 60% (dark gray bars) of soil WHC. (*) indicates a significant mortality in comparison to the respective control (Dunnett test - $p \leq 0.05$).



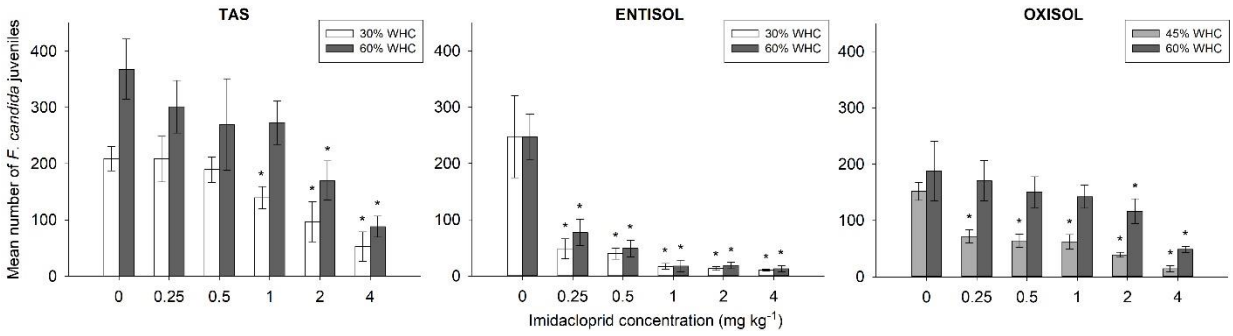
Source: prepared by the author, 2019.

4.4.3 Chronic toxicity assays

The LOEC values of the chronic toxicity assays with *F. candida* in Entisol did not differ when comparing the soil moisture regimes ($LOEC_{ENTISOL\ 30\%\ and\ 60\%\ WHC}$ = 0.25 mg kg^{-1}). In TAS, there was higher toxicity under lower moisture content ($LOEC_{TAS\ 30\%\ WHC}$ = 1 mg kg^{-1}; $LOEC_{TAS\ 60\%\ WHC}$ = 2 mg kg^{-1}). In the same way, in Oxisol there was a significant reduction in the number of juveniles at a concentration eight times lower under 45% WHC (LOEC = 0.25 mg kg^{-1}), when compared to 60% WHC (LOEC = 2 mg kg^{-1}; Figure 4.3). In general, the EC_{20} and EC_{50} values were similar for the different moisture contents in TAS and Entisol (Table 4.3). In Oxisol, the EC values under 45% WHC (EC_{50} = 0.32 mg kg^{-1}; EC_{20} = 0.18 mg kg^{-1}) were approximately eight (EC_{50}) and five (EC_{20}) times lower than those under 60% WHC (EC_{50} = 2.49 mg kg^{-1} and EC_{20} = 0.99 mg kg^{-1}).

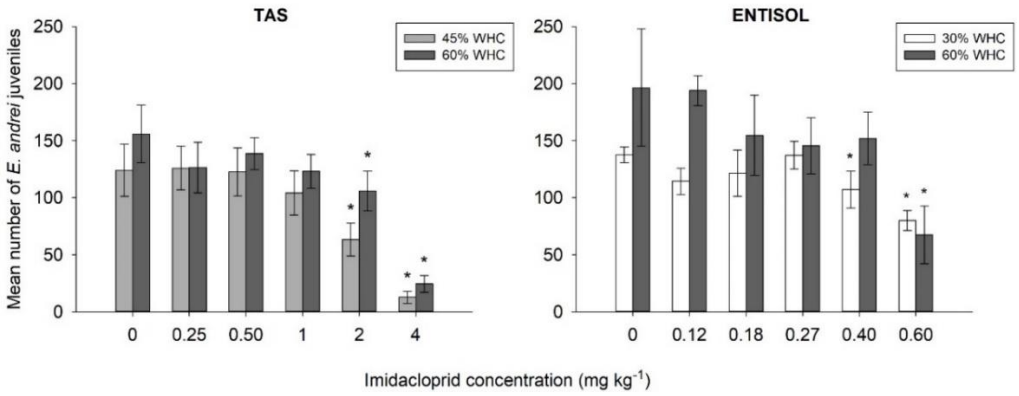
The LOEC values for earthworm reproduction assays in TAS were the same for both soil moisture contents (Table 4.3). In Entisol, the effect of the a.i. on *E. andrei* reproduction was higher under 30% WHC (LOEC = 0.4 mg kg^{-1}), in comparison to 60% WHC (LOEC = 0.6 mg kg^{-1}) (Figure 4.4). The EC values for the TAS were higher in the condition of higher soil moisture and were similar in the Entisol for the two moisture contents (Table 4.3). In general, the mean number of juveniles of *F. candida* and *E. andrei* generated under lower soil moisture (30% or 45% WHC) was lower, when compared to 60% WHC for the same treatments (same tested concentration or control) in each soil.

Figure 4.3 - Mean number (n = 5, ± standard deviation) of *F. candida* juveniles generated after 28 days of exposure to different concentrations of imidacloprid in Tropical Artificial Soil (TAS), Entisol and Oxisol, under regimes of 30% (white bars) or 45% (light gray bars), and 60% (dark gray bars) of soil WHC. (*) indicates a significant reduction of juveniles in comparison to the respective control (Dunnett test - $p \leq 0.05$).



Source: prepared by the author, 2019.

Figure 4.4 - Mean number (n=4, ± standard deviation) of *E. andrei* juveniles generated after 56 days of exposure to different concentrations of imidacloprid in Tropical Artificial Soil (TAS) and Entisol, under regimes of 30% (white bars) or 45% (light gray bars), and 60% (dark gray bars) of soil WHC. (*) indicates a significant reduction of juveniles in comparison to the respective control (Dunnett test - $p \leq 0.05$).



Source: prepared by the author, 2019.

Table 4.3 - Ecotoxicological parameters (NOEC, LOEC, LC50, EC50 and EC20) of the acute and chronic toxicity assays with *Folsomia candida* and *Eisenia andrei* exposed to increasing concentrations of imidacloprid in Tropical Artificial Soil (TAS), Entisol and Oxisol under different soil moisture regimes (30% or 45%, and 60% of WHC).

Species	Endpoint	Ecotoxicological parameter	Imidacloprid concentration (mg kg^{-1})						
			TAS			Entisol		Oxisol	
			30% WHC	45% WHC	60% WHC	30% WHC	60% WHC	45% WHC	60% WHC
F. candida	14-d mortality	NOEC	4.00	(-)[b]	4.00	<0.25	<0.25	16.00	4.00
		LOEC	8.00	(-)[b]	8.00	4.00	4.00	32.00	8.00
		LC_{50}	23.58	(-)[b]	29.88	0.44	1.18	23.84	>64.00
		Limits (95%)	(-)[a]	(-)[b]	(-)[a]	(-)[a]	(-)[a]	(-)[a]	(-)[a]
	28-d reproduction	NOEC	1.00	(-)[b]	1.00	<0.25	<0.25	<0.25	1.00
		LOEC	1.00	(-)[b]	2.00	0.25	0.25	0.25	2.00
		EC_{50}	1.74	(-)[b]	1.94	0.11	0.16	0.32	2.49
		Limits (95%)	(1.23-2.25)	(-)[b]	(1.20-2.68)	(0.06-0.15)	(0.12-0.19)	(0.20-0.45)	(1.60-3.37)
		EC_{20}	0.67	(-)[b]	0.53	0.04	0.06	0.18	0.99
		Limits (95%)	(0.29-1.04)	(-)[b]	(0.07-0.98)	(0.02-0.06)	(0.05-0.07)	(0.11-0.26)	(0.30-1.68)
E. andrei	14-d mortality	NOEC	<10.00	(-)[b]	<10.00	0.80	0.40	(-)[b]	(-)[b]
		LOEC	10.00	(-)[b]	10.00	1.60	0.80	(-)[b]	(-)[b]
		LC_{50}	7.56	(-)[b]	10.04	0.87	0.85	(-)[b]	(-)[b]
		Limits (95%)	(3.94-9.27)	(-)[b]	(8.50-11.12)	(0.85-0.91)	(0.83-0.87)	(-)[b]	(-)[b]
	56-d reproduction	NOEC	(-)[b]	1.00	1.00	0.27	0.40	(-)[b]	(-)[b]
		LOEC	(-)[b]	2.00	2.00	0.40	0.60	(-)[b]	(-)[b]
		EC_{50}	(-)[b]	1.96	2.77	0.68	0.53	(-)[b]	(-)[b]
		Limits (95%)	(-)[b]	(1.57-2.34)	(2.27-3.27)	(0.54-0.82)	(0.38-0.68)	(-)[b]	(-)[b]
		EC_{20}	(-)[b]	1.17	1.85	0.48	0.28	(-)[b]	(-)[b]
		Limits (95%)	(-)[b]	(0.76-1.59)	(1.36-2.33)	(0.36-0.60)	(0.12-0.44)	(-)[b]	(-)[b]

[a] The data did not allow calculating the 95% confidence intervals.
[b] Assay not performed.
Source: prepared by the author, 2019.

4.5 DISCUSSION

The data obtained for the acute and chronic toxicity assays performed in TAS (*E. andrei* and *F. candida*) and Oxisol (*F. candida*) indicated that the condition of lower soil moisture may lead to higher mortality and lower reproduction of the tested organisms in these soils. According to the literature, lower soil moisture can be harmful to some edaphic species because it may cause metabolic stresses and, consequently, intensify the effects of pesticides on organisms (KILIÇ, 2011; LIMA et al., 2011; PEIJNENBURG et al., 2012). This effect was seen in this study, since a higher number of juveniles was observed in the controls with 60% WHC in TAS (for *F. candida* and *E. andrei*), in Oxisol (for *F. candida*) and in Entisol (for *E. andrei*), when compared to the lower moisture conditions (30% or 45% WHC; Figures 4.3 and 4.4). Therefore, it is possible to state that when drought stress situations are associated with imidacloprid contamination, the risk to the edaphic fauna may be higher, since a greater inhibition of the reproduction was observed in soils with lower moisture.

Furthermore, it was evidenced in this study that the restriction of soil moisture was more harmful to the survival of collembolans than to earthworms (LC_{50} values - Table 4.3). Since the main route of exposure of the collembolans to pesticides is through the absorption of water from soil pores (PEIJNENBURG et al., 2012), the most severe effect occurs under the conditions of lower soil moisture (30% WHC - TAS, Entisol and Oxisol). This may be due to the higher concentration of the contaminant in the soil pore water, when compared to the assays performed at 60% WHC, in which the contaminant may be more diluted in the soil solution and, therefore, have a lower impact (BANDOW et al., 2014a).

When exposed to 45% WHC in Oxisol, the survival of *F. candida* was affected at a concentration four times higher than that observed for 60% WHC (Table 4.3). However, it is important to note that all collembolans were found alive in the control of Oxisol under 60% WHC, whereas in the bioassay carried out under 45% WHC, some individuals did not survive after 14 days in the control (Figure 4.1). Thus, the mortality attributed exclusively to the lower water content may have increased the variability of the data and, consequently, the LOEC value was determined at a higher concentration (32 mg kg^{-1}). In addition, the lower survival in the control with 45% WHC can also indicate that the reduction of soil moisture, regardless of soil contamination, is harmful to edaphic species.

For the chronic toxicity assays with *F. candida* in TAS and Entisol, although the EC_{50} and EC_{20} values were similar for both moisture contents, it was noted that the number of *F. candida* juveniles at all concentrations tested was lower in the soil drought situation, when

compared to the higher soil moisture condition (Figure 4.3). Thus, it is possible that the interpretation of the toxic potential of imidacloprid (when affected by soil moisture) based only on the values of EC can lead to an underestimation of the effect on the collembolans in some soils, because there is the double impact of the intrinsic toxicity of the pesticide and that of water restriction.

Literature data indicate that the reproductive performance of *F. candida* is higher at soil moisture contents varying between 53% and 74% WHC (VAN GESTEL and VAN DIEPEN, 1997; CROUAU et al., 1999; BANDOW et al., 2014a; BANDOW et al., 2014b), which corresponds to an "optimum" moisture range for this species. Similarly, Reinecke and Venter (1987) observed a better generation of cocoons and juveniles of *Eisenia fetida* in soil moisture regimes varying between 65% and 70% WHC. Thus, the imminent soil moisture restriction, which is predicted due to climate change, may act as an additional stressor to soil organisms, compromising the production of offspring to maintain a population in dry soil (BANDOW et al., 2014b).

For the bioassays carried out with *E. andrei* in TAS, the toxicity was higher under drought conditions. In this case, in addition to the imidacloprid effects on the nervous system (BUFFIN, 2003), lower soil water contents can cause dehydration of tissues and concentrate the a.i. in the earthworm's body, leading to death (LIMA et al., 2011).

In general, the most severe effects of imidacloprid on the biological parameters of both species were observed in Entisol. However, a clear influence of the moisture content could not be verified in this soil, since most of the EC_{50} and EC_{20} values for both species were similar for both soil moisture regimes (Table 4.3). These results indicate that probably there is another factor with greater power to drive imidacloprid toxicity in this soil, which overlaps the influence of soil moisture. Assuming that the bioavailability of pesticides in the soil solution is equally influenced by the physical and chemical properties of the soil (STYRISHAVE et al., 2010), the lower CEC and clay content of the Entisol (Table 4.1), compared to the others, probably leads to lower capacity to adsorb the contaminant in the solid phase. Hence, lower concentrations of the a.i. may have been enough to saturate the binding sites of the soil matrix, increasing the concentration of the contaminant in the soil pore water, even in situations of higher soil moisture (60% WHC).

The EC_{20} values (Table 4.3) for both species in TAS were higher than the PEC estimated for this soil (PEC = 0.24 mg kg^{-1}). However, EC_{20} values derived from Entisol are close to (for *E. andrei*) or even lower than (for *F. candida*) its PEC (0.16 mg kg^{-1}; Table 4.2). In Oxisol, the EC_{20} for *F. candida* was higher than its PEC (0.16 mg kg^{-1}) under 60% WHC (EC_{20}= 0.99 mg

kg^{-1}). However, when collembolans were exposed to imidacloprid concentrations under 45% WHC, EC_{20} decreased to a value (EC_{20} = 0.18 mg kg^{-1}) lower than the PEC (Table 4.2). Therefore, the reduction of soil moisture may increase the ecological risk associated with the exposure of *F. candida* to imidacloprid in natural tropical soils with a clayey texture.

Lakowski et al. (2010) studied interactions between various toxic substances (metals, pesticides, herbicides, fungicides, drugs, etc.) and environmental factors such as temperature, dissolved oxygen, aur humidity and soil moisture, in a wide range of bioindicator species, including enchytraeids, crustaceans and collembolans, and found that water stress increases the toxicity of the substances (mortality and reduction of reproduction) by about 52%. Bandow et al. (2014a) studied the response of collembolans *F. candida* and *Sinella curvisetta* exposed to Lambda-cyhalothrin in an OECD artificial soil (OECD, 2009), under different soil moisture and temperature regimes, and found that the temperature did not influence the toxicity of the tested pesticide, while a decrease in soil moisture significantly inhibited the reproduction of the two species tested. Drought situations, associated with the exposure to the tested pesticide, lead to a greater impact on these populations. Højer et al. (2001) also found that *F. candida* had higher mortality when submitted to different concentrations of 4-nonylphenol in drier soils. González-Alcaraz & Van Gestel (2016) verified that low soil moisture, associated with exposure to metalloids and some soil physical characteristics, negatively affected the survival of *Enchytraeus crypticus*.

Although some of these cited data have been obtained by using different organisms, contaminants and/or soils from those of the present study, the statements corroborate that soil moisture may have a significant influence in modeling the toxicity of pollutants in soils. Although the interactions between contaminants, environmental compartments and climate are complex, it is possible to infer that predicted changes in precipitation patterns and consequent decreases in soil moisture may represent threats to edaphic populations, especially when associated with soil contamination by pesticides (LIMA et al., 2011; BANDOW et al., 2014a; BANDOW et al., 2014b; OGUNGBEMI and VAN GESTEL, 2018).

In countries with a tropical climate, such as Brazil, the problem of soil water availability may be even more serious in future scenarios of climate change, since precipitation regimes are naturally influenced by an annual drought period (SANCHEZ-BAYO and HYNE, 2011). It is also important to highlight that it was chosen to perform the assays at the temperature of 20 ± 2ºC, disregarding the regional temperature conditions (25ºC - 28ºC), as well as those predicted by the changes in the climate by the end of the 21st century (+ 1.1 to 6.4 ºC; IPCC, 2007) with the purpose of ensuring a better development of the species and understanding the influence of

soil moisture on the toxicity of imidacloprid to each edaphic invertebrate in each one of the tropical soils tested. However, additional studies are suggested, including atmospheric temperature as an experimental variable, in order to understand the impacts of pesticides in a more complex climate change scenario.

4.6 CONCLUSIONS

In general, increased imidacloprid toxicity was observed in lower soil moisture regimes, but the influence of moisture content on the toxic effects varied between soil types. A decrease in soil moisture clearly increased the negative effects of imidacloprid on *F. candida* reproduction in Oxisol, and the EC_{50} value under 60% WHC (2.49 mg kg^{-1}) was almost 8-fold higher than that under 45% WHC (0.32 mg kg^{-1}). In addition, the EC_{20} value estimated for the collembolan reproduction assay under 45% WHC in Oxisol (0.18 mg kg^{-1}) was below the estimated concentration in this soil (PEC_{OXISOL} = 0.24 mg kg^{-1}), indicating a potential risk for *F. candida* under a drought scenario in this clayey tropical soil. A higher toxicity under lower soil moisture was also observed for *E. andrei* in TAS ($EC_{50\ TAS\ 45\%WHC}$ = 1.96 mg kg^{-1}, $EC_{50\ TAS\ 60\%WHC}$ = 2.77 mg kg^{-1}). On the other hand, a clear influence of soil moisture could not be identified for the species in Entisol, since the toxicity responses under both moisture regimes were similar. Nevertheless, the exposure of soil invertebrates to imidacloprid in this soil cannot be considered harmless because negative effects were found at concentrations close to its PEC. The data obtained in the present study also demonstrate the importance of considering changes in precipitation regimes, resulting from climate change scenarios for the next years, as well as the use of natural soils, in the assessments of the ecotoxicological potential of pesticides.

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5 CHAPTER 3: The increase in temperature increases the toxicity of imidacloprid to *Eisenia andrei* and *Folsomia candida* in tropical soils

5.1 ABSTRACT

The influence of climate changes on the chronic toxicity of imidacloprid to soil fauna was assessed in tropical soils. Earthworms *Eisenia andrei* and collembolans *Folsomia candida* were exposed to imidacloprid in a tropical artificial soil (TAS) and in two natural tropical soils (Entisol and Oxisol) under increasing atmospheric temperatures of 20, 25 and 28 °C. The ecological risk was calculated through the Hazard Quotient (HQ) approach for each exposure scenario. In general, an increase on imidacloprid toxicity (EC_{50}-based) were observed with increasing temperature for both species, regardless the soil used. The toxicity responses observed in each soil were clearly different at 20 °C but become more similar at 28 °C, indicating that the influence of soil type was not greater than the influence of temperature on imidacloprid toxicity. The HQ values also revealed an increase in ecological risk with increasing temperature in all soils. The toxicity (and risk) were greater in Entisol at high temperatures, while the lowest ones were found in TAS at 20 °C. This preliminary investigation indicates that temperature affects the toxicity of imidacloprid to edaphic species in tropical soils and reinforces the need of considering future scenarios of climate changes in the pesticide risk assessments.

Keywords: Earthworms; Collembolans; Temperature rise; Neonicotinoids; Risk Assessment

5.2 INTRODUCTION

The seed treatment with neonicotinoid insecticides is a common practice in conventional agriculture systems in Brazil. Imidacloprid is the largest selling neonicotinoid in the world (SIMON-DELSO et al., 2015; JESCHKE et al., 2011), and its wide use on seed dressing is mainly due to its good cost-effectiveness in controlling a broad-spectrum of insects (JESCHKE et al., 2011; GOULSON, 2013; ATWOOD et al., 2018). Although this active ingredient (a.i.) can help to achieve high levels of food production, the residues of imidacloprid introduced into the soil through treated seeds may cause deleterious effects on non-target soil populations (EL-NAGGAR and ZIDAN, 2013; SMITH et al., 2016).

In order to protect the environmental services promoted by soil fauna (THIMM et al., 1998), laboratory ecotoxicological tests have been made to identify the effects and to (preliminarly) assess the risk of pesticides to soil invertebrates (DE SILVA et al., 2009; ALVES et al., 2013; NIEMEYER et al., 2018). In these investigations, soil fauna species are exposed to pollutants under standardized environmental conditions originally developed for temperate regions (ISO, 2012; 2014). The assays are generally conducted under controled atmospheric temperature (20 °C is generally used) and artificial substrates are often employed. However, if the predicted changes in the global temperature are considered in the ecotoxicological assessments, the exposure scenario of soil invertebrates to pesticides may change (HOLMSTRUP et al., 2010), and soil organisms may be more frequently exposed to toxicants under stressfull temperatures (NOYES et al., 2009).

Increases in temperature over the 21st century have been projected to values close to 2°C, if greenhouse gas emissions were mitigated, and to values greater than 4°C, if no efforts to control de emissions were made (IPCC, 2014). When species are exposed to above-optimum temperatures, they can face up to alterations in the homeostasis that can promote disturbances in the metabolism (JEGEDE et al., 2017). The life cycle (FAYOLLE et al., 1997; WILES AND KROGH, 1998) and the normal growth and reproduction rates of soil fauna invertebrates may also be affected (JÄNSCH et al., 2005), and thus the health and the size of edaphic communities may be altered.

Besides the direct impacts of global warming on the biological factors of soil organisms, changes in temperature patterns may also alter the environmental fate of pesticides in the terrestrial compartments (NOYES et al., 2009). For example, the degradation and volatilization of the pesticides may be increased under high temperatures (RÖMBKE et al., 2007). The uptake of the chemicals by the organisms may also be enhanced at high temperatures (LIMA et al., 2015). These shifts in the dynamics of soil invertebrates exposure may potentialize the effects of the pesticides to soil fauna species.

Literature data suggest that temperature may affect the toxicity of different pesticides in opposite directions. For example, the increase in temperature apparently increases the toxicity of some insecticides (GARCIA, 2004; DE SILVA et al., 2009; LIMA et al., 2015; VELKI and EČIMOVIĆ, 2015) whilst may decreases the fungicides toxicity to earthworms (RÖMBKE et al., 2007; DE SILVA et al., 2009). In general, collembolans *Folsomia candida* seems to be more sensitive to insecticides (BANDOW et al., 2014a; JEGEDE et al., 2017) and fungicides (BANDOW et al., 2014b) at high temperatures. However, the role of species intrinsic

preferences in the toxicity responses can not be ruled out, and probably termophilic species will be less affected by pesticides in warmer temperatures (BANDOW et al., 2014b).

Although the works cited above indicates that climate change may affect the toxicity of pesticides, the most of these studies were performed with different classes of pesticides other than neonicotinoids, and the influence of temperature on the toxicity of this pesticide class to soil fauna are limited at best (HOLMSTRUP et al., 2010; LASKOWSKI et al., 2010; VELKI and EČIMOVIĆ, 2015). To our knowledge, no chronic toxicity studies assessing the effects of climate changes on the imidacloprid toxicity have been carried out with standard soil invertebrate species in tropical soils. To fill this knowledge gap, this study aimed to assess the influence of increasing atmospheric temperatures on the toxicity of imidacloprid for *Eisenia andrei* (Oligochaeta) and *F. candida* (Collembola) in one artificial tropical soil and two natural tropical soils.

5.3 MATERIAL AND METHODS

5.3.1 Test species

Earthworms *E. andrei* were reared in the laboratory, following the recommendations of ISO 11268-2 (ISO, 2012). This species was maintained in plastic boxes containing a mixture of distilled water, horse manure, coconut fiber, and fine sand in a proportion of 330:100:50:15 (weight-based), respectively. The boxes contained an opening on the lids to allow air ventilation and, once a week, they were opened to adjust the medium moisture and to add approximately 10g of cooked oat flakes, offered as food. At least twenty-four hours before the assay start, the earthworms were acclimatized into the respective test soils (TAS, Entisol or Oxisol), in a climate room with the same temperature at which the test was performed (20, 25 or 28 °C).

The *F. candida* culture was kept in laboratory according to ISO 11267 (ISO, 2014). Collembolans were reared in a substrate composed by plaster of Paris (powdered), distilled water and activated charcoal (powder), in a proportion of 10:6:1 (weight-based), respectively. Twice a week, the substrate moisture was adjusted with distilled water, and granulated dry yeast (*Saccharomyces cerevisiae*) were offered as food.

5.3.2 Test soils and contaminant

A tropical artificial soil (TAS) and two natural tropical soils were used in this study. The TAS, a standard substrate recommended for terrestrial ecotoxicological assays in tropical and subtropical regions, is composed by 75% of fine sand (> 50% of the particles sized between 0.05 and 0.2 mm), 20% of kaolinite clay and 5% of powdered coconut husk (GARCIA, 2004; DE SILVA and VAN GESTEL, 2009). The TAS pH was adjusted to 6.0 ± 0.5 with $CaCO_3$. A natural sandy soil (Entisol) and a natural clayey loam soil (Oxisol) from the south of Brazil were also used in the assays (FAO, 2014). The Entisol were collected in the municipality of Araranguá (29º00′S; 49°31′W) and it is composed by more than 90% of sand and 2.2% of organic matter (O.M.). The Oxisol was collected in Palmitos (27º04'S; 53º 09'W) and it is composed by 35.5% of clay and 3.2% of O.M. Both soils were collected in the surface layer of the profile (0-20 cm) in areas with no history of pesticide application. Soil samples were sieved (#2.0 mm), air-dried and defaunated through three freezing and thawing cycles, as described in Alves et al. (2019). The pH (1 M KCl) and the maximum water holding capacity (WHC) of the soils were determined according to ISO (2012). Methodologies used for the determination of additional physical and chemical soil properties (Table 5.1) were described in Tedesco et al. (1995).

Due to its common use for the treatment of seeds planted in agricultural areas in Brazil, the commercial formulation MUCH 600 FS® (containing 600 g of the active ingredient imidacloprid L^{-1}) was chosen as test substance for this study.

Ecotoxicological assays were set up according to a factorial design where two species were exposed to increasing concentrations of imidacloprid under three increasing temperatures (20, 25 and 28 °C) and soil types (TAS, Entisol and Oxisol), totalizing 9 toxicity assays per species. Due the large number of experimental units, each test was run separately. Immediately before the beginning of each bioassay, the soil samples were spiked with five solutions containing increasing concentrations of imidacloprid. The moisture of each soil sample was maintained at 60% of WHC of the respective soil test. A control treatment containing only distilled water was also prepared for each test soil. Nominal concentrations (Table 5.2) of imidacloprid (expressed as mg a.i. kg^{-1} dry soil - mg kg^{-1}) used in the chronic toxicity assays with *E. andrei* and *F. candida* were based on the results of range-finding tests (data not shown).

We calculated the Predicted Environmental Concentration (PEC) for each soil following EC (2003). Initially, a sowing density of 100 kg of soybean seeds per hectare was assumed. Following the commercial recommendation for seed dressing, we admit an application of 120

g i.a. per 100 kg of seeds, which resulted in an incorporation of 120 g a.i. ha^{-1} in the first 5 cm of the soil profile, simulating a worst-case scenario. Soil densities of 1.5 g cm^{-3} for Entisol and 1.0 g cm^{-3} for TAS and Oxisol were used to calculate the total soil mass in contact with the amount of insecticide applied, allowing to derive the PEC for each soil. To validate the calculated PEC values, we checked it using the software ESCAPE® (EPPO, 2003), assuming a single application of 120 g i.a. ha^{-1} without crop interception. In addition, for each soil and temperature combination, EC_{10} values from the collembolans and earthworms tests were compared and the lowest value was divided by an assessment factor of 100 to obtain a Predicted No-Effect Concentration (PNEC), as described in EC (2003). Both parameters (PEC and PNEC) were used to calculate de ecological risk.

Table 5.1 - Physical and chemical properties of Tropical Artificial Soil (TAS), Entisol and Oxisol, used in the chronic toxicity assays with *E. andrei* and *F. candida* at 20, 25 and 28 °C.

Parameter	TAS	Entisol	Oxisol
pH (1 M KCl)	5.9 ± 0.1	4.2 ± 0.2	4.8 ± 0.1
SOM % (m/v)	1.4 ± 0.0	2.2 ± 0.1	3.2 ± 0.8
CEC ($cmol_c\ dm^{-3}$)	3.3 ± 0.2	1.4 ± 0.1	10.8 ± 0.4
Clay (g kg^{-1})	143.0 ± 0.0	41.5 ± 2.2	355.0 ± 14.1
Sand (g kg^{-1})	672.0 ± 0.0	938.0 ± 4.2	315.0 ± 1.4
WHC (%)	46.3 ± 1.7	31.6 ± 1.1	53.0 ± 1.4

Values are expressed in mean ± standard deviation; n = 2.
SOM – Soil Organic Matter.
CEC – Cation Exchange Capacity.
WHC – Water Holding Capacity.
Source: prepared by the author, 2019.

Table 5.2 - Nominal concentrations of imidacloprid used in the chronic toxicity assays with *E. andrei* and *F. candida* in Tropical Artificial Soil (TAS), Entisol and Oxisol, under 20 °C, 25 °C and 28 °C. Concentrations are expressed as mg of imidacloprid per kg^{-1} of dry soil (mg kg^{-1}).

Test soil	Test species	Nominal concentrations (mg kg^{-1})	
		20 and 25 °C	28 °C
TAS	*E. andrei*	0; 0.25; 0.5; 1; 2; 4	0; 0.25; 0.5; 1; 2; 4
	F. candida	0; 0.25; 0.5; 1; 2; 4	0; 0.06; 0.12; 0.25; 0.50; 1
Entisol	*E. andrei*	0; 0.12; 0.18; 0.27; 0.40; 0.60	0; 0.12; 0.18; 0.27; 0.40; 0.60
	F. candida	0; 0.25; 0.5; 1; 2; 4	0; 0.03; 0.06; 0.12; 0.25; 0.50
Oxisol	*E. andrei*	0; 0.25; 0.5; 1; 2; 4	0; 0.25; 0.5; 1; 2; 4
	F. candida	0; 0.25; 0.5; 1; 2; 4	0; 0.06; 0.12; 0.25; 0.50; 1

Source: prepared by the author, 2019.

5.3.3 Chronic toxicity assays

The effect of imidacloprid in the reproduction of *E. andrei* and *F. candida* was assessed in each one of the three soils and temperatures, following the general recommendations described in the ISO guidelines (ISO, 2012; 2014).

Earthworm reproduction tests were performed in plastic containers (14.8 cm diameter and 9.8 cm height) with perforated lids to allow air ventilation. Approximately 600 g of wet soil, contaminated with imidacloprid or containig only distilled water (control soil), were added in each container. Ten adult *E. andrei* with well-developed clitellum and individual weight between 250 and 600 mg were inserted in each experimental unit. The initial soil moisture was maintained (weight-based) by the addition of distilled water twice a week. Four replicates were prepared for each treatment. The organisms were fed weekly with moist equine manure free of contaminants. After 28 days, adult earthworms were removed from each replicate, counted, washed and weighed. Only the soil, generated cocoons and juveniles remained in the containers for another 28 days. Then, the plastic vessels were placed in a water bath (60 ± 5 °C) for one hour in order to force the emergence of juveniles to the soil surface, allowing the counting of the individuals generated during the test.

Chronic toxicity tests with collembolans were performed in glass vessels (7.5 cm diameter and 6.0 cm height) containing 30g of wet soil (contaminated or control). Ten *F. candida* individuals aged between 10 and 12 days were inserted in each experimental unit. The organisms were fed with approximately 2 mg of dried granulated yeast at the beginning of the test and after 14 days. Twice a week, the containers were opened to allow air exchange, and the soil moisture of each replicate was adjusted based on its initial weight. Five replicates were performed for each treatment. After 28 days, the content of each vessel were submerged in water to force the collembolans to float. A few drops of black ink were added to water in order to produce a dark background and facilitate the visualization of the surviving juveniles and adults. All replicates were photographed in high resolution and the images were used to account for the number of juveniles generated during the test in the software ImageJ®.

5.3.4 Data analysis

All the results from ecotoxicological assays were analyzed using the software Statistica® (STATSOFT, 2004). Initially, normality and homogeneity of variances of data were checked using Kolmogorov-Smirnov and Bartlett's tests, respectively. When necessary,

logarithmic transformations were used to confirm the analysis of variance (ANOVA) assumptions. An ANOVA was ran separately with the data set of each test. If significant differences ($p < 0.05$) were detected, the means of each treatment were compared to the control using the Dunnet's test, which allows to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC). The concentrations that decreased reproduction by 10% (EC_{10}) and 50% (EC_{50}), when compared to the respective control, were also estimated for each test, through non-linear regression models (ENVIRONMENTAL CANADA, 2007). Due to the large variability of the data obtained under 28 °C, the EC_{10} and EC_{50} confidence limits were estimated with a confidence level of 90%. EC_{50} values were considered different only when confidence intervals did not overlap (JEGEDE et al., 2017).

The ecological risk of the exposure of each species to the expected imidacloprid concentration in the soil (PEC) were estimated through the recommendations for the risk assessment of new and existing substances, established by European Comission (EC, 2003). Hazard Quotients (HQ) were calculated for each scenario by dividing the PEC by PNEC (HQ = PEC/PNEC). A significant ecological risk was identified when HQ values are higher than 1.

5.4 RESULTS

5.4.1 Tests validation

The number of *E. andrei* juveniles produced in the control replicates at 20 and 25 °C ranged between a maximum of 270 (in Entisol at 20 °C) and a minimum of 96 juveniles (in Oxisol at 25 °C). Considering only the controls of the assays performed at 20 and 25 °C, the highest number of *F. candida* juveniles was found in TAS at 20 °C (439 juveniles), whilst the lowest occurred at Entisol at 25 °C (157 juveniles). At these temperatures, the coefficient of variation (CV) of control treatments was < 30% for both species in all tested soils, and the adult survival in the controls averaged above 80% and 90%, respectively, for *F. candida* and *E. andrei*. Thus, the validity criteria were met in these assays (ISO, 2012; 2014). At 28 °C, the mean number of juveniles (± standard deviation) in TAS, Entisol and Oxisol controls was, respectively, 38 ± 14, 34 ± 9 and 23 ± 11 for *E. andrei*, and 55 ± 22, 44 ± 14 and 58 ± 20 for *F. candida*. Thus, the minimum number of juveniles established by the ISO guidelines for the tests validation (at least 30 for earthworms and 100 for collembolans) was not reached in the majority of the control replicates under 28 °C. However, these results were considered in this paper

(including control replicates with less than 30 and 100 juveniles of *E. andrei* and *F. candida*, respectively), since a dose-response relationship could be identified despite the lower number of juveniles in the controls.

5.4.2 Chronic toxicity assays

In general, the chronic toxicity of imidacloprid (based on EC_{50} values) for earthworms increased with increasing temperature (Table 5.3). In TAS, negative effects on *E. andrei* reproduction became significant at 2.0 mg kg^{-1} in all temperatures (Figure 5.1). However, higher temperatures clearly increased the toxicity in the artificial soil, as can be seen by the decrease in EC_{50} values with temperature rise. The toxicity for earthworms in Entisol was similar at 25 °C and 28 °C, with significant reductions in the number of juveniles starting at 0.12 mg kg^{-1} and 0.27 mg kg^{-1}, respectively. At 20 °C, in this soil, the earthworms reproduction was only affected at the highest tested concentration (0.60 mg kg^{-1}), and the EC_{50} was approximately twice higher than the values found for the other temperatures (Table 5.3). In the Oxisol, increases in temperature increased the negative effects of imidacloprid on *E. andrei*: EC_{50} was decreased by about four times with temperature rising from 20 to 25 °C, and it was further lower when organisms were exposed at 28 °C (Table 5.3).

The effects of imidacloprid on *F. candida* reproduction were also influenced by temperature in all soils (Figure 5.2). In TAS and Entisol, the toxicity for collembolans increased with increasing temperature, which led to a clear decrease in EC_{50} values (Table 5.3). In TAS, the LOEC for collembolans decreased with increasing temperature, and the EC_{50} obtained at 20 °C was at least 2 and 13 times higher than those estimated at 25°C and 28°C, respectively (Table 5.3). In Entisol, the negative effects on the reproduction of *F. candida* were seem at 0.25 mg kg^{-1} under temperatures of 20 and 25 °C, whereas at 28 °C, the reproduction was affected at 0.06 mg kg^{-1}. In addition, the toxicity (EC_{50}-based) in this soil was approximately two and three times greater at 28 °C, respectively, when compared to 25 and 20 °C. In assays performed in Oxisol with *F. candida*, a similar toxicity was found at 25 and 28 °C, whilst a toxicity at least 2.5 times lower was observed at 20 °C.

In each soil tested the toxicity was clearly lower at 20 °C when compared to 25 or 28 °C for both species. It was also noted that at 20 °C, EC_{50} values were different in each soil (Figure 5.3). At 28 °C, the EC_{50} values for earthworms were the same for Entisol and Oxisol, whilst for collembolans, similar EC_{50} values were found for TAS and Oxisol (Figure 5.3).

Table 5.3 - NOEC, LOEC, EC_{10} and EC_{50} values derived from chronic toxicity assays with *E. andrei* and *F. candida* performed at temperatures of 20 °C, 25 °C and 28 °C in TAS, Entisol and Oxisol.

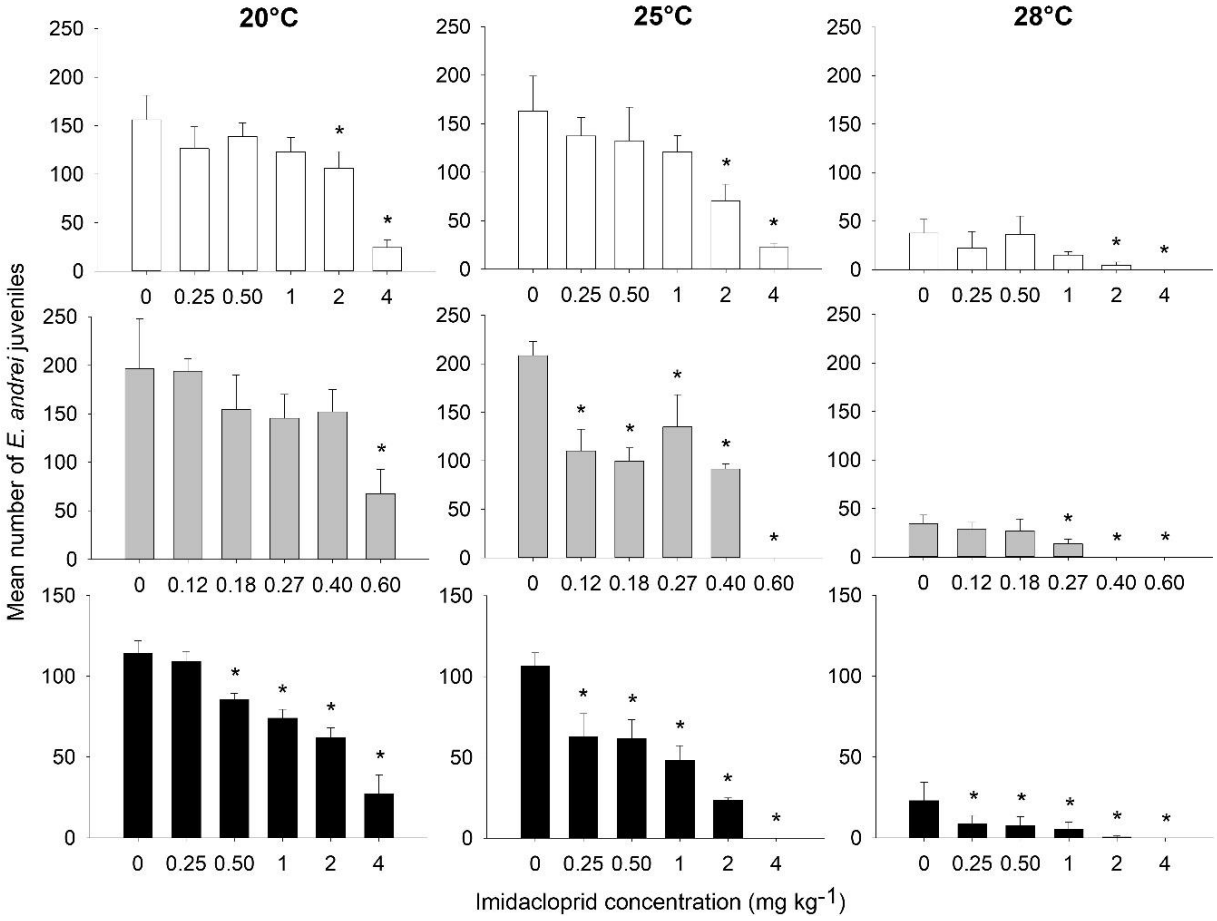
Species	Soil	Parameter	Temperature		
			20 °C	25 °C	28 °C
E. andrei	TAS	NOEC	1.0	1.0	1.0
		LOEC	2.0	2.0	2.0
		EC_{10}	1.48 (1.05 - 1.91)	0.45 (0.11 - 0.79)	0.40 ([a])
		EC_{50}	2.77 (2.36 - 3.19)	1.89 (1.45 - 2.33)	1.06 (0.53 - 1.58)
	Entisol	NOEC	0.4	< 0.12	0.18
		LOEC	0.6	0.12	0.27
		EC_{10}	0.19 (0.06 - 0.33)	0.03 ([a])	0.14 (0.07 - 0.21)
		EC_{50}	0.53 (0.40 - 0.66)	0.22 (0.13 - 0.31)	0.25 (0.21 - 0.29)
	Oxisol	NOEC	0.25	< 0.25	< 0.25
		LOEC	0.5	0.25	0.25
		EC_{10}	0.21 (0.09 - 0.34)	0.07 (0.01 - 0.12)	0.06 (0.01 - 0.11)
		EC_{50}	1.89 (1.55 - 2.23)	0.59 (0.39 - 0.79)	0.25 (0.11 - 0.39)
F. candida	TAS	NOEC	1.00	0.50	0.06
		LOEC	2.00	1.00	0.12
		EC_{10}	0.30 (0.03 a 0.58)	0.20 (0.04 - 0.36)	0.06 (0.02 - 0.10)
		EC_{50}	1.94 (1.33 - 2.55)	0.80 (0.58 - 1.02)	0.14 (0.07 - 0.21)
	Entisol	NOEC	< 0.25	< 0.25	0.03
		LOEC	0.25	0.25	0.06
		EC_{10}	0.04 (0.03 - 0.05)	0.02 (0.01 - 0.03)	0.01 (0.001 - 0.02)
		EC_{50}	0.16 (0.13 - 0.19)	0.09 (0.07 - 0.11)	0.05 (0.03 - 0.07)
	Oxisol	NOEC	0.25	< 0.25	0.25
		LOEC	0.50	0.25	0.50
		EC_{10}	0.16 (0.07 - 0.25)	0.09 (0.05 - 0.13)	0.06 ([a])
		EC_{50}	0.53 (0.40 - 0.66)	0.20 (0.17 - 0.23)	0.20 (0.10 - 0.30)

90% confidence limits of the ECs are presented in the parenthesis.

[a] Data did not allow the estimation of the confidence interval

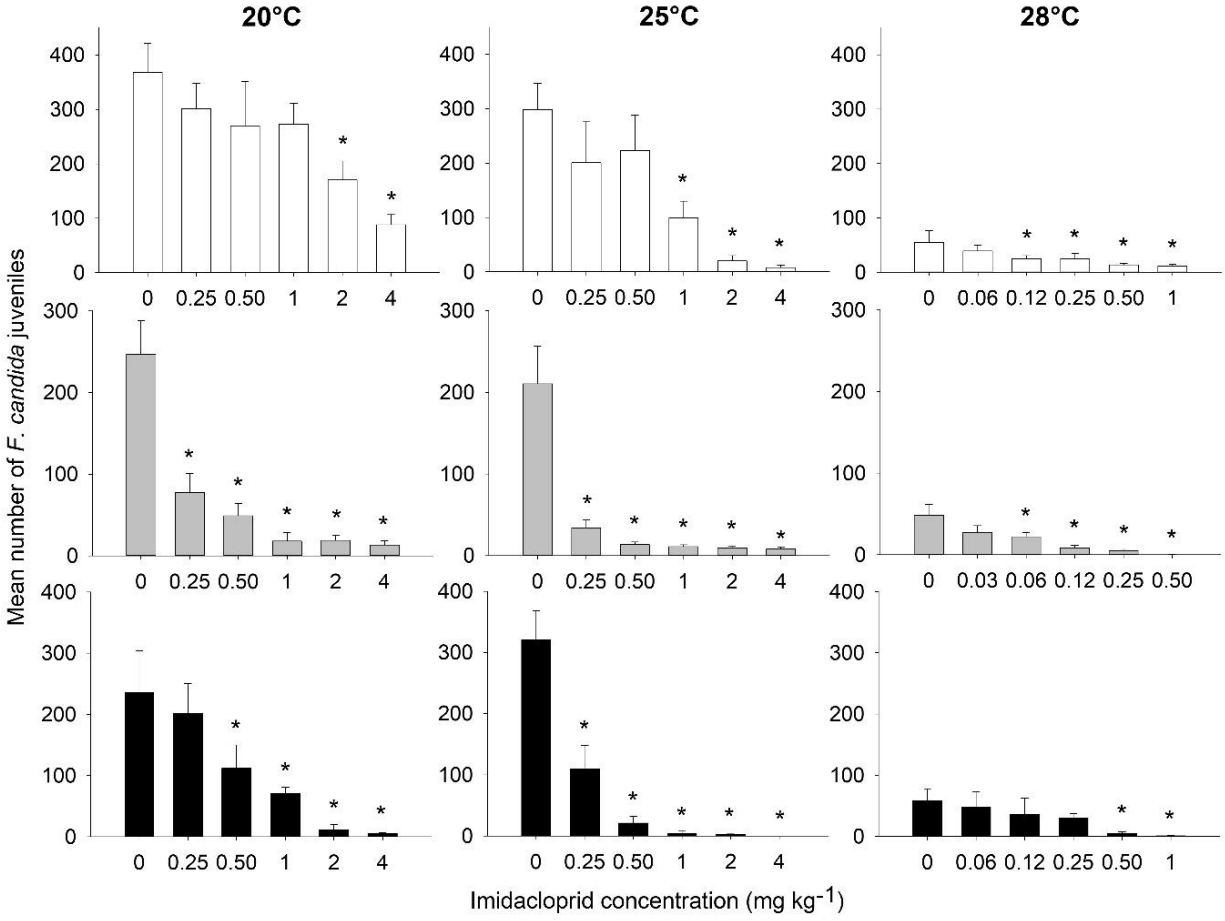
Source: prepared by the author, 2019.

Figure 5.1 - Mean number (+ standard deviation, n = 4) of *E. andrei* juveniles found in TAS (white bars), Entisol (grey bars) and Oxisol (black bars), after 56 days of exposure to increasing imidacloprid concentrations, at temperatures of 20 °C, 25 °C and 28 °C. Asterisk (*) indicates significant difference (p <0.05, Dunnet's test) between the treatment and control.



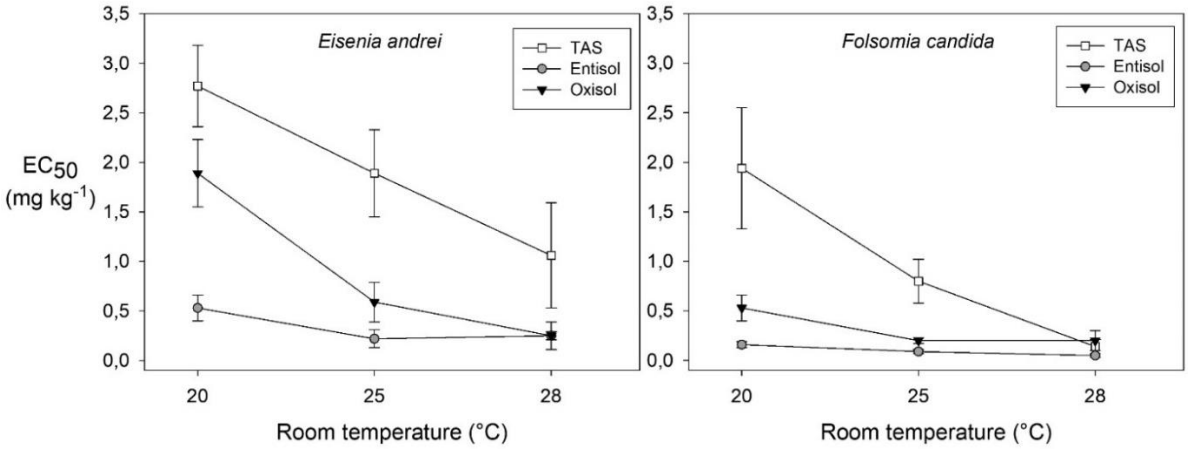
Source: prepared by the author, 2019.

Figure 5.2 - Mean number (+ standard deviation, n = 5) of *F. candida* juveniles found in TAS (white bars), Entisol (grey bars) and Oxisol (black bars), after 28 days of exposure to increasing imidacloprid concentrations, at temperatures of 20 °C, 25 °C and 28 °C. Asterisk (*) indicates significant difference (p <0.05, Dunnet's test) between the treatment and control.



Source: prepared by the author, 2019.

Figure 5.3 - EC_{50} values estimated from chronic toxicity assays with *E. andrei* (left) and *F. candida* (right) performed in TAS, Entisol and Oxisol, at temperatures of 20 °C, 25 °C and 28 °C. (┬) 90% confidence intervals.



Source: prepared by the author, 2019.

5.4.3 Preliminary risk assessment

The ecological risk, calculated through the HQ approach, was significant for the species exposure to the predicted imidacloprid levels in all the combination scenarios of soils and temperatures. However, a clear increase in HQ values with increasing temperature could be identified for all tested soils (Table 5.4). In addition, the calculated risk for natural soils was generally higher than those from artificial soil in the three temperature regimes. Considering all the exposure scenarios admitted in this study, the lowest HQ value was found in TAS at 20 °C, whilst the highest were identified in Entisol at 28 °C.

Table 5.4 - Hazard Quotient (HQ) values for the exposure of *E. andrei* and *F. candida* to the Predicted Environmental Concentrations of imidacloprid in TAS, Entisol and Oxisol, under temperatures of 20 °C, 25 °C and 28 °C.

Test soil	Test species	PEC (mg kg^{-1})	20 °C		25 °C		28 °C	
			PNEC	HQ	PNEC	HQ	PNEC	HQ
TAS	*E. andrei* *F. candida*	0.24	0.0148	16.22	0.002	120	0.0006	400
Entisol	*E. andrei* *F. candida*	0.16	0.0004	400	0.0002	800	0.0001	1600
Oxisol	*E. andrei* *F. candida*	0.24	0.0016	150	0.0009	266.67	0.0006	400

PNEC = lowest EC_{10} / 100; HQ = PEC / PNEC.
HQ values higher than 1 point out significant risk and the necessity of further investigation.
Source: prepared by the author, 2019.

5.5 DISCUSSION

When considering each soil in isolation, the mean number of juveniles in the control replicates at 20 °C is similar to that found at 25 °C (Figures 5.1 and 5.2), indicating that this temperature increase probably did not affect the normal reproductive performance of the species. However, the number of *E. andrei* and *F. candida* juveniles in the controls decreased at 28 °C, regardless the soil. The highest temperature tested in this study is probably inadequate to the development of both invertebrate species, which may be the reason to the lower reproductive performance in the control treatments. *E. andrei* better growth and reproduce at moderate temperatures from 20 °C to 25 °C (JÄNSCH et al., 2005), but temperatures above the optimal range (such as 28 ºC) may lead to weight loss and reductions in the growth rates (PRESLEY et al., 1996) and cocoon hatchability (JÄNSCH et al., 2005). Collembolans *F.*

candida are adapted to lower temperatures, being the optimal for their reproduction about 21 °C (LIMA et al., 2015). Fountain and Hopkins (2005) showed that hatching of the *F. candida* eggs is compromised at 28 °C, and at 30 °C they probably are in the biological survival limit (DE BOER et al., 2010). This may indicate that populations of both species may be affected in future scenarios of climate changes, even disregarding soil pollution with pesticides, due to the lower reproduction under higher temperatures. The results also suggest that the use of native species of temperate regions, such as *F. candida*, may be inadequate to assess the effects of pesticides under scenarios of climate change in tropical regions, and the search for thermotolerant bioindicator species is needed to better understand the toxicity of pesticides under higher temperatures.

In our study, the toxicity of imidacloprid for *E. andrei* increased with temperature rise from 20 °C to 25-28 °C, both in artificial soil than in natural soils. The influence of temperature on the toxicity of different substances has already been evaluated in other studies. In a filter paper toxicity assay with *E. fetida*, Velki and Ečimović (2015) observed that several pesticides (including imidacloprid, chlorpyrifos + cypermethrin and lambda-cyhalothrin) increased their toxicity with increasing temperature (20 vs 25 °C), and only the herbicide glyphosate presented a decrease in the toxicity at high temperature. Garcia (2004) found that when *E. fetida* was exposed to an insecticide (lambda-cyhalothrin) higher toxicity was found at high temperatures, on the other hand, when the earthworms were exposed to fungicides (benomyl and carbendazim) the toxicities were higher at 20 °C, in comparison to 28 °C. De Silva et al (2009) also identified higher lethality caused by insecticides (chlorpyrifos and carbofuran) to *E. andrei* at 26 °C, when compared to 20 °C in OECD artificial soil, and for a fungicide (carbendazim) the toxicity was higher at the lower temperature. The toxicity observed in the above cited studies are not directly comparable with ours due to the differences between the substances, species and/or endpoints used, but the results suggest that the influence of temperature may depend on the pesticide class, i.e., the toxicity of the insecticides to earthworms were generally increased, while for fungicides usually decrease as temperature rises. This may be due to the faster degradation of this fungicides at higher temperatures (RÖMBKE et al. (2007), which probably does not occur with more persistent pesticides such as the insecticides.

Similar patterns of increase toxicity of different insecticides with increasing temperature can be identified in the literature, and in most cases it have been attributed to the increase in the metabolic activity of the earthworms at higher temperatures, which may have led to a higher uptake of the insecticide on this conditions (DE SILVA et al., 2009; LIMA et al., 2015; HOLMSTRUP et al., 2010) leading to an increased toxicity. BEDNARSKA et al. (2017)

corroborates with these statements, by observing that the assimilation and elimination rates of the organic insecticide chlorpyriphos by the earthworm *E. fetida* increased with increasing temperature from 10 to 20°C. While assimilation rate increased almost 4-fold from 10 to 20 °C, elimination rate increased only 2.3-fold, indicating that at high temperatures the pesticide internal accumulation may be higher in earthworms. Thus, it is possible that the absorption of imidacloprid by *E. andrei* has been increased with increasing temperature in our study, which may have contributed to the higher toxicity at 25 and 28 °C, when compared to 20 °C, in all tested soils.

In general, the sensitivity of *F. candida* to imidacloprid also increased with increasing temperature in our study, and this same trend has already been observed for another pesticides. The toxicities of lambda-cyhalothrin (BANDOW et al., 2014a), pyrimethanil (BANDOW et al., 2014b) dimethoate and chlorpyrifos (JEGEDE et al.,2017) on *F. candida* reproduction were higher at 26 °C than at 20 °C. However, when collembolans *Sinella curviseta* were used, the increase in the temperature from 20 to 26 °C led to a decrease in the toxicity of lambda-cyhalothrin (BANDOW et al., 2014a), while temperature apparently did not affect the toxicity of pyrimethanil for *S. curviseta* (BANDOW et al., 2014b). This suggest that the effect of temperature on the pesticide toxicity is also species-dependent, and probably thermophilic species will be less affected if the global temperature increase.

Broznic et al. (2012) verified that the temperature may also affect the sorption and desorption dynamics of imidacloprid in soil. The authors observed a decrease in the values of organic carbon partition coefficient (K_{OC}) with increased temperature in six soils, indicating a higher imidacloprid solubility at higher temperatures. Although the sorption-desorption kinetics of imidacloprid has not been measured in our experiments, the increase in temperature may also have led to a decrease of imidacloprid adsorbed in the solid matrix while increased the bioavailable fraction in the soil solution.

Considering that the pore water represents the main route of exposure of the collembolans to the soil contaminants (PEIJNENBURG et al., 2012; OGUNGBEMI and VAN GESTEL, 2018), the possible increase of imidacloprid concentration in the soil solution, caused by the higher temperatures, may have contributed to the higher toxicity for *F. candida*. This hypothesis may also be valid for earthworms, because the main exposure of soft-body organisms to the chemicals usually occurs by diffusion of the porewater through the skin (BELFROID et al., 1994; RENAUD et al., 2018). Nevertheless, it must be recognized that metabolic disorders may ocurr when the species are exposed to the pesticides under termal stress (HACKENBERGER et al., 2018). Thus, both species may be forced to spend more

energy to maintain basic biological functions and to regulate internal body temperature when exposed to above-optimum temperatures, becoming more susceptible to the effects of the contaminants.

There is a clear difference between EC_{50} values found in TAS, Oxisol and Entisol in the tests performed at 20°C (Table 5.3; Figure 5.3). However, when temperature increases to 28°C, this difference tend desapear and the toxicity response to become more similar for the three soils (Figure 5.3). These results reveal that when organisms are exposed to imidacloprid at higher temperatures, the great influence of soil properties on the toxicity, specially observed at 20 °C, tends to decrease, and the effect of temperature probably overlaps other environmental factors such as soil characteristics.

Our preliminary risk assessment indicates that the increase in temperature may enhance the risk of imidacloprid to *E. andrei* and *F. candida* under the tested tropical soils (Table 5.4). In accordance with our results, Jegede et al. (2017) reported a higher (chronic) risk of chlorpyrifos (for *F. candida* and *Hypoaspis aculeifer*) and deltamethrin (for *H. aculeifer*) at 28 °C when compared to 20 °C, using the Toxicity Exposure Ratio (TER) approach. We also noted that the HQ values were generally higher for natural soils, when compared to the TAS, indicating that the risk estimated only in artificial soil may not be representative of the real conditions which the organisms are exposed in tropical soils. In addition, the risk values were higher in Entisol when compared to Oxisol. The higher toxicity observed in Entisol (Table 5.3) is probably due to its lower CEC and clay content (Table 5.1) which may have favored a lower imidacloprid adsorption in the solid phase, leading to a higher bioavailability of the a.i. through the soil solution (OGUNGBEMI and VAN GESTEL, 2018). Our results suggests that the temperature increase due to climate changes may be especially hazard to soil fauna if the exposure to imidacloprid ocurr in very sandy tropical soils.

Since soil dwelling invertebrates like *E. andrei* and *F. candida* has preference for fresh organic material (FLEUREN et al., 2003; KLIRONOMOS et al., 1992), it is predicted that those organisms will be found in superficial soil layers and thus might be more exposed to the atmospheric temperature alterations due to climate changes (VELKI and EČIMOVIĆ, 2015; DE BOER et al., 2010). Thus, an increased on the potential risk of the exposure of this species to imidacloprid is expected under future scenarios, especially because climate changes has been occurring much faster than the time required for species to adapt to the new temperature conditions. Anyway, further investigation under more realistic scenarios (such as semi-field assays) may confirm the effects of temperature on the toxicity of imidacloprid observed in the laboratory.

5.6 CONCLUSIONS

The results of this study reveal that the toxicity of imidacloprid to *E. andrei* and *F. candida* in tropical soils was clearly influenced by the temperature increase. For the three tested soils, the toxicity (EC_{50}-based) observed in asssays performed at 20 °C for both species were lower than those at 25 °C and 28 °C. In addition, the toxic effects of imidacloprid were generally higher in natural soils, when compared to the artificial soil. For earthworms, the highest toxicities were found in Entisol (at 25 and 28 °C) and in Oxisol (at 28 °C), while for collembolans highest toxicity was seem in Entisol at 28 °C. The lowest toxicity for both species were identified in TAS, at 20 °C. There was a clear increase in ecological risk with increasing temperature in all the soils, and the exposure to imidacloprid in Entisol, especially at high temperatures, constituted the greater risk scenario for soil fauna species. The results of this work also reinforce the importance of considering the influence of climate changes on pesticide toxicity, which allows a more accurate estimation of the ecologial risk.

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6 GENERAL DISCUSSION AND CONCLUSIONS

Although imidacloprid has been considered efficient for the pests control, this study demonstrated that its use may cause deleterious effects on non-target soil fauna organisms. The results of experiment 1 proved that the toxicity of imidacloprid to earthworms and collembolans is influenced by the type of tropical soil. It was demonstrated that, in addition to the known role of SOM on the regulation of the toxic effects, silt and clay contents may also contribute to reduce the toxicity of imidacloprid to soil fauna. Based on the comparison of EC_{50} values, a similar toxicity could be observed in Oxisol when compared to TAS (imidacloprid was about 3% less toxic in Oxisol when compared to TAS). On the other hand, the relative toxicity increased about 800% in Entisol, when compared to that found in TAS, indicating that the exposure of soil invertebrates should be particularly harmful in sandy tropical soils.

The results obtained in experiment 2 revealed that the toxicity of imidacloprid on soil invertebrates can be generally increased by lower soil moisture. The reduction of soil moisture (relative to the standard soil moisture condition – 60% WHC) resulted in increases in toxicity (EC_{50}-based), being (on average) 26% higher in TAS and 678% higher in Oxisol, confirming the hypotheses of increased imidacloprid toxicity with the decrease of soil moisture for these soils. However, when lower moisture was performed in Entisol, the toxicity decreased in about 22% for earthworms and increased about 45% for collembolans. In this case an unclear influence of soil moisture on the imidacloprid toxicity was found in this soil. It was also noted that even when the EC_{50} and EC_{20} values were similar for both moisture contents, the number of juveniles found in the assays of drought scenarios was in general lower than that found at 60% of WHC. This indicates that the ecological risk assessment from climate change scenarios should not be interpreted by only using the "ECx" approach, because it may lead to an underestimation of the actual toxic potential. In addition to the possible impacts of climate change expected for tropical climate regions (i.e., rainfall reduction and, consequently, soil moisture reduction), it should be considered that periods of annual drought naturally occur in Brazil. Thus, the climate changes may intensify the problems of water restriction for soil-dwelling organisms, especially if the exposure to imidacloprid occurs in drought scenarios in Oxisol.

The hypothese of increasing imidacloprid toxicity with the increase of the atmospheric temperature was confirmed by the results of the experiment 3. It was observed a higher toxicity at higher temperatures (25 and 28 °C) for both species in all soils tested, indicating a key influence of temperature on imidacloprid toxic potential. By comparing the EC_{50} values

obtained in the assays at 20 °C with those found at 28 °C, a relative increase in the toxicity of 166%, 410% and 723% could be identified for Entisol, Oxisol and TAS, respectively, at the highest temperature. In addition, at 28 °C, the toxic values (EC_{50}) between the soils were more similar. Thus, we suppose that in future scenarios of climate change, imidacloprid toxicity will be mostly driven by temperature and less affected by the soil type. Furthermore, the contribution of higher temperatures appears to be greater than the contribution of lower soil moistures for the increment in the toxicity of imidacloprid in TAS and Entisol, whilst in Oxisol, both factors seems to contribute similarly for the increase in the toxic response.

When considering how climate changes will affect tropical countries, like Brazil, it is predicted that decreases in soil moisture and increases in the atomspheric temperature will occur simultaneously. In this case, the drought and heat stress will further enhance the effects of pesticides on edaphic fauna (Table 7.1 and Figures 7.1 and 7.2, supplementary data), in comparison to their single influence on the imidacloprid toxicity (Chapters 2 and 3). Considering a worst case-scenario where soil fauna could be simultaneously exposed to imidacloprid at higher temperatures, lower soil moisture contents and in soils with lower capacity to adsorb pesticides (i.e., 28 °C + 30% WHC in Entisol), the toxicity (EC_{50} = 0.16 mg kg^{-1} for earthworms and EC_{50} = 0.03 mg kg^{-1} for collembolans, Table 7.1) may be at least 1600% and 6300% higher (for earthworms and collembolans, respectively) than those found at standard conditions (20 °C + 60% WHC in TAS). These results reveal that the toxicity (and risk) of imidacloprid for soil invertebrates, when considering tropical regions and climate changes, can be strongly underestimated if the ecotoxicological assessments are performed only under standard conditions of temperature, moisture and soil. In this way, in addition to the natural tropical soils (for tropical regions), drought and heat scenarios should be included as abiotic stressors in ecological risk assessments, especially in regions most affected by climate change, since they probably will induce additive effects and, consequently, increase the toxicity in comparison to the standard conditions of exposure.

Although the experiments carried out in this work demonstrated influences of tropical soils, as well as of the climate changes in the toxicity of imidacloprid for edaphic species, the extrapolation of these results to the field conditions should be done with caution, since the influences of several environmental factors occurring in nature were not considered in laboratory tests. The temperature and soil moisture content were kept unchanged during the assays, but in the field, fluctuations in the levels of these parameters occur naturally during the day and night cycles. Thus, in improved climate change scenarios, the exposure time of soil fauna to critical levels of environmental factors (such as drought and heat scenarios) should be

adjusted for more realistic periods. In addition, the sensitivity of the standard species used in this work may not be the same that of native species from tropical regions, which can have different sensitivity to higher temperatures. Therefore, it is also suggested to carry out ecotoxicological tests with native species, not only in the natural soils used in this study but also in other soils representative of tropical agricultural areas, in exposure scenarios that allow to consider the consequences of climate change on the environment, like those of the semi-field assays.

7 SUPPLEMENTARY DATA

Table 7.1 - Ecotoxicological parameters (NOEC, LOEC, EC_{10} and EC_{50}) values derived from chronic toxicity assays with *E. andrei* and *F. candida* performed at temperatures of 20, 25 and 28 °C in TAS, Entisol and Oxisol, under 30 or 45% of WHC.

Species	Soil (%WHC)	Parameter	Temperature		
			20 °C	25 °C	28 °C
E. andrei	TAS (45% WHC)	NOEC	1.00	1.00	1.00
		LOEC	2.00	2.00	2.00
		EC_{10}	0.87 (0.52 – 1.22)	1.08 (0.73 – 1.43)	([a])
		EC_{50}	1.96 (1.64 – 2.28)	2.15 (1.86 – 2.44)	0.78 (0.19 – 1.38)
	Entisol (30% WHC)	NOEC	0.27	0.18	0.18
		LOEC	0.40	0.27	0.27
		EC_{10}	0.39 (0.25 – 0.53)	0.19 (0.14 – 0.24)	0.08 (0.02 – 0.14)
		EC_{50}	0.68 (0.56 – 0.80)	0.28 (0.25 – 0.31)	0.16 (0.11 – 0.21)
	Oxisol (45% WHC)	NOEC	([b])	([b])	([b])
		LOEC	([b])	([b])	([b])
		EC_{10}	([b])	([b])	([b])
		EC_{50}	([b])	([b])	([b])
F. candida	TAS (30% WHC)	NOEC	0.50	0.50	0.50
		LOEC	1.00	1.00	1.00
		EC_{10}	0.38 (0.14 – 0.62)	0.14 (0.02 - 0.26)	([a])
		EC_{50}	1.74 (1.32 – 2.17)	0.52 (0.33 - 0.72)	0.60 (0.39 - 0.82)
	Entisol (30% WHC)	NOEC	< 0.25	< 0.25	< 0.03
		LOEC	0.25	0.25	0.03
		EC_{10}	0.03 (0.02 – 0.04)	0.03 (0.02 – 0.04)	< 0.01
		EC_{50}	0.11 (0.07 – 0.15)	0.13 (0.10 – 0.16)	0.03 (0.02 – 0.04)
	Oxisol (45% WHC)	NOEC	1.00	< 0.25	0.06
		LOEC	2.00	0.25	0.12
		EC_{10}	0.49 (0.18 – 0.79)	0.10 (0.02 – 0.18)	0.02 (0.01 – 0.04)
		EC_{50}	1.08 (0.80 – 1.37)	0.21 (0.16 – 0.25)	0.08 (0.05 – 0.11)

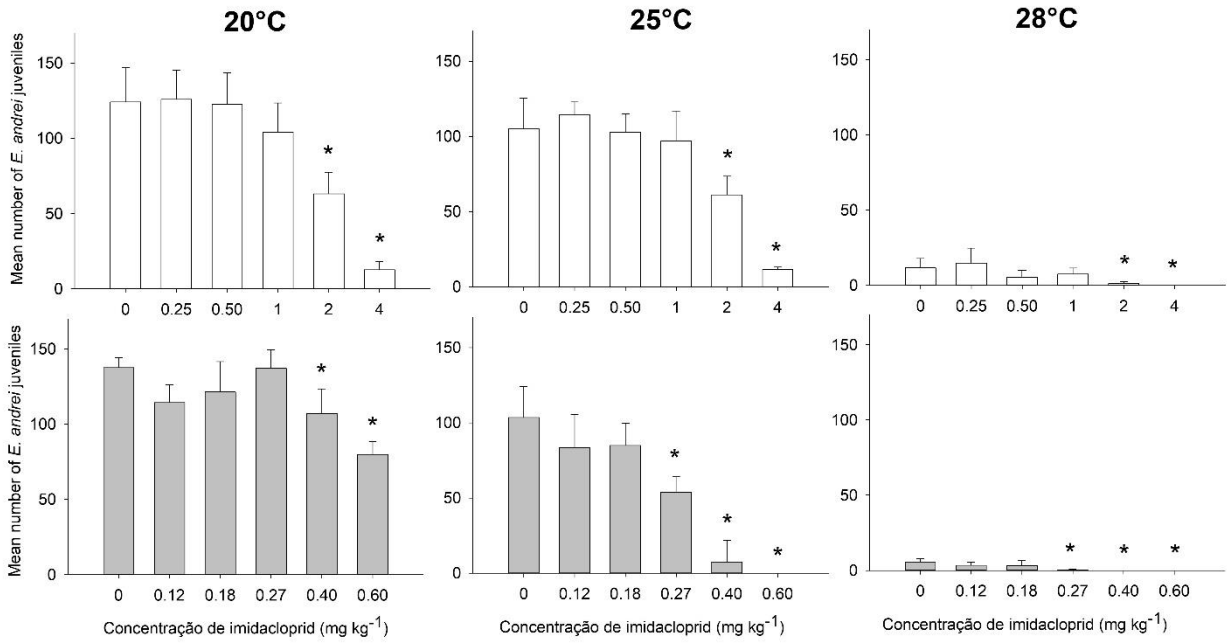
90% confidence limits of the ECs are presented in the parenthesis.

[a] Data did not allow the estimation of the parameter.

[b] Assay not performed. In preliminary assays, the earthworms *E. andrei* were not able to reproduce in Oxisol (45% WHC).

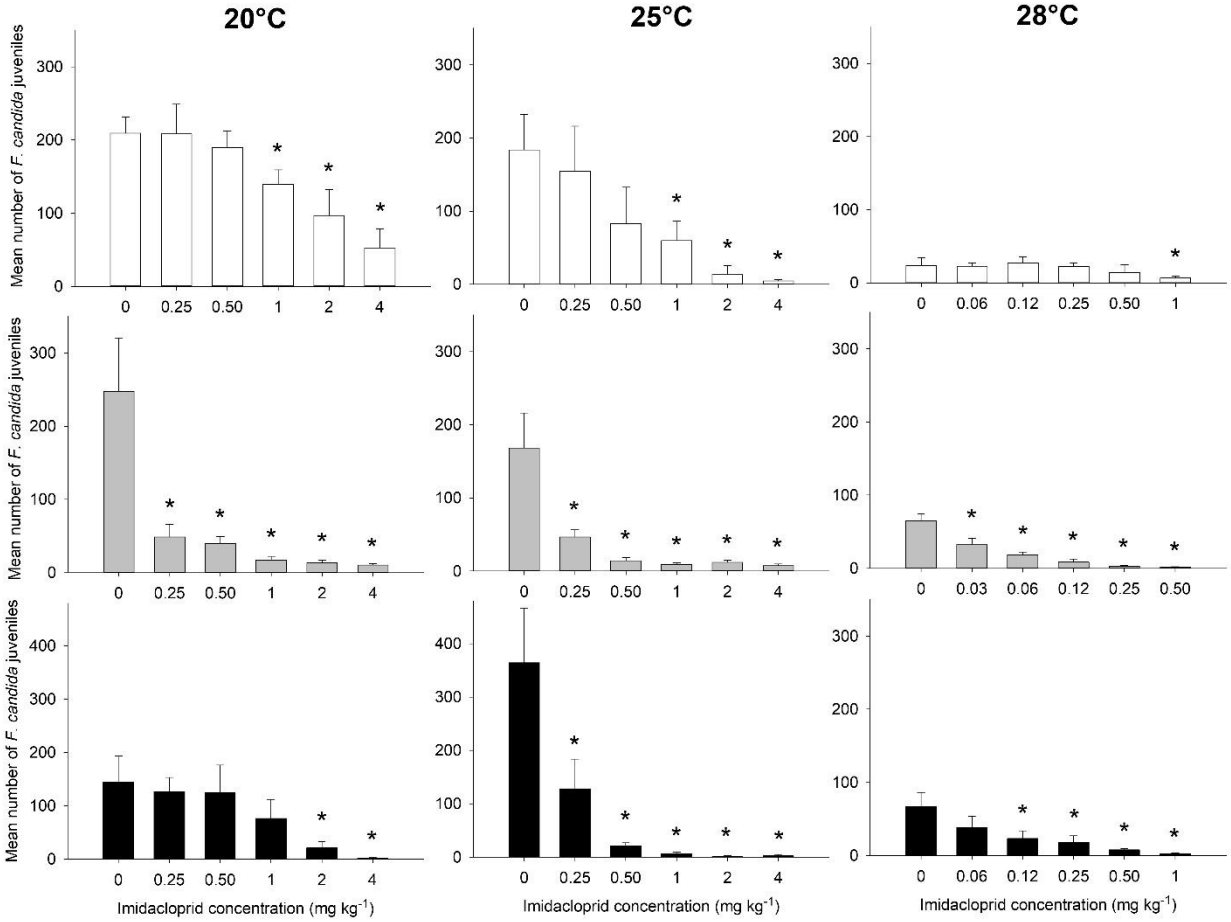
Source: prepared by the author, 2019.

Figure 7.1 - Mean number (+ standard deviation, n=4) of *E. andrei* juveniles found in TAS (white bars, 45% of WHC) and Entisol (grey bars, 30% of WHC), after 56 days of exposure to increasing imidacloprid concentrations, at temperatures of 20, 25 and 28 °C. Asterisk (*) indicates significant difference (p <0.05, Dunnet's test) between the treatment and control.



Source: prepared by the author, 2019.

Figure 7.2 - Mean number (+ standard deviation, n=5) of *F. candida* juveniles found in TAS (white bars, 30% of WHC), Entisol (grey bars, 30% of WHC) and Oxisol (black bars, 45% of WHC), after 28 days of exposure to increasing imidacloprid concentrations, at temperatures of 20, 25 and 28 °C. Asterisk (*) indicates significant difference (p <0.05, Dunnet's test) between the treatment and control.



Source: prepared by the author, 2019.