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ECOTOXICOLOGICAL ASSESSMENT OF METSULFURON-METHYL AND ISOXAFLUTOLE HERBICIDES TO SOIL FAUNA

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Ecotoxicological assessment of Metsulfuron-Methyl and Isoxaflutole herbicides to soil fauna

Por

FERNANDA BENEDET DE SANTO

Dissertação aprovada como requisito parcial para a obtenção do Grau de Mestre em Ciências, área de concentração Ciências Agrárias, no Programa de Pós-Graduação em Ecossistemas Agrícolas e Naturais, Centro de Ciências Rurais da Universidade Federal de Santa Catarina, pela Comissão formada pelos membros:

Dr. César Augusto Marchioro – UFSC
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Dedico este trabalho a todos que contribuíram para que eu chegasse até aqui

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“Quem elegeu a busca,
não pode recusar a travessia...”
(Guimarães Rosa)

RESUMO

Os herbicidas correspondem a mais de 60% de todo volume de vendas de agrotóxicos no Brasil, sendo a classe de produtos mais empregada na agricultura moderna. Dentre os ingredientes ativos comercializados no país, estão o metsulfuron-methyl e o isoxaflutole. Enquanto o primeiro se destaca pela grande ação em baixas doses, o segundo é peça chave em casos de resistência ao glifosato. Todavia, o elevado uso dos herbicidas não corresponde ao número de informações e estudos avaliando seus potenciais efeitos à fauna do solo. A fim de reduzir as incertezas acerca desses efeitos, o objetivo desta dissertação foi avaliar a ecotoxicidade de herbicidas a base de metsulfuron-methyl e de isoxaflutole a partir de ensaios ecotoxicológicos laboratoriais agudos e crônicos com organismos não-alvo, seguindo normas internacionalmente padronizadas, e de avaliações de campo, onde foi avaliada a atividade alimentar da fauna edáfica através do teste bait lamina, riqueza e abundância dos grupos de mesofauna e quantificação residual dos herbicidas. Os resultados laboratoriais mostraram que tanto o isoxaflutole, como o metsulfuron-methyl não apresentam ecotoxicidade aos organismos de solo testados, exibindo resposta apenas muitas doses acima à aplicada em campo. Entretanto, quando o óleo mineral foi adicionado ao metsulfuron-methyl, simulando aplicação em pós-emergência, verificou-se a toxicidade do composto aos organismos de solo. Os resultados de campo mostraram que a atividade alimentar da fauna edáfica não é afetada pela dose de campo aplicada de metsulfuron-methyl ou pela presença do óleo mineral, como visto em laboratório, porém, a riqueza de grupos da mesofauna se mostrou bastante baixa na área, o que pode ser resultante do tipo de manejo. Ressalta-se, porém, que os resultados obtidos em campo se referem a uma aplicação de cada produto estudado, não sendo possível extrapolar para aplicações contínuas e a longo prazo. Sendo assim, este trabalho mostra a importância de se incluir os adjuvantes na avaliação de risco, bem como de extrapolar os resultados obtidos em laboratório para o campo, a fim de que haja maior entendimento da ação dos agrotóxicos na dinâmica ecossistêmica, e assim, se avance na discussão acerca das limitações existentes na avaliação de risco de agrotóxicos no Brasil.

Palavras-chave: Agrotóxicos. Contaminação ambiental. Ecotoxicidade. Invertebrados do solo.

RESUMO EXPANDIDO

INTRODUÇÃO

O Brasil possui um forte cenário agrícola, que fez do país o maior consumidor de agrotóxicos, colocando os herbicidas como a classe mais comercializada no país. Atendendo a demandas, como resistência de plantas daninhas ao glifosato, na década de 90 foi disponibilizado no mercado o isoxaflutole, um ingrediente ativo pertencente ao grupo químico dos isoxazóis, usado no controle pré-emergente de plantas daninhas de folha larga e estreita no milho e na cana-de-açúcar. Outro grupo químico de herbicidas cujo uso vem crescendo é o das sulfoniluréias, especialmente em razão da sua elevada ação mesmo quando aplicada em baixa doses. Dentre os herbicidas dessa família está o metsulfuron-methyl, um dos ingredientes ativos mais amplamente utilizados para o controle de gramíneas anuais e de plantas daninhas de folhas largas, podendo ser aplicado na pré-emergência do trigo ou pós-emergência do trigo, cevada, centeio, triticale, aveia, cana-de-açúcar e outros.

Estudar os efeitos que os agrotóxicos têm sobre os organismos é um dos objetivos da Ecotoxicologia, ciência que incorpora elementos da ecologia, toxicologia e química, estudando o efeito de substâncias químicas sobre os ecossistemas. Dentre os organismos estudados estão os invertebrados de solo, que participam diretamente nos processos ecossistêmicos relacionados à qualidade do solo.

Ingredientes ativos como o isoxaflutole e o metsulfuron-methyl requerem estudos acerca de seus efeitos ecotoxicológicos na fauna edáfica. Apesar da existência de alguma informação acerca de concentrações efetivas, através de ensaios de laboratório, para que a avaliação de risco dos agrotóxicos seja feita de forma apropriada, é necessário que se realizem ensaios de campo, visto que as propriedades do solo influenciam a biodisponibilidade dos contaminantes, bem como a sua persistência e movimento.

No Brasil, todo agrotóxico antes de ser registrado, passa por avaliações de risco ambiental, que estão a cargo do IBAMA e descritas na Portaria Normativa IBAMA Nº 84, de 15 de outubro de 1996. Entretanto, considerando invertebrados de solo, o único ensaio ecotoxicológicos exigido é o ensaio agudo de minhocas (letalidade), retirado do escopo europeu por ser considerado um *endpoint* não-responsivo. Neste sentido, há incertezas sobre a ecotoxicidade em cenários reais de aplicação de agrotóxicos no Brasil, considerando-se a exposição e a consideração de efeitos crônicos.

Esta dissertação está dividida em três capítulos, onde o Capítulo 1 objetivou avaliar a ecotoxicidade do herbicida isoxaflutole através de ensaios de fuga, letalidade e reprodução com minhocas e colêmbolos; o Capítulo 2 apresenta ensaios de varredura (fuga e letalidade) com o herbicida metsulfuron-methyl com e sem a adição do adjuvante (óleo mineral) e suas respostas para colêmbolos e minhocas; e o Capítulo 3 mostra ensaios laboratoriais (crônicos) e de campo avaliando as diferenças e similaridades entre os resultados obtidos nas duas abordagens.

OBJETIVOS

O objetivo do presente estudo foi avaliar a ecotoxicidade dos herbicidas metsulfuron-methyl e isoxaflutole para a fauna do solo, baseando-se em ensaios ecotoxicológicos laboratoriais com organismos não-alvo e avaliações de campo, discutindo suas vantagens e incertezas. Além de melhor compreender os efeitos dos agrotóxicos no ecossistema solo, este trabalho buscou extrapolar os resultados obtidos em laboratório para as condições de campo, visando contribuir para o avanço da avaliação de risco de agrotóxicos no Brasil.

MATERIAIS E MÉTODOS

No Capítulo 1 foram realizados ensaios agudos (fuga e letalidade) e crônicos (reprodução) com minhocas da espécie *Eisenia andrei* e com colêmbolos da espécie *Folsomia candida,* utilizando solo artificial tropical (SAT) contaminado com o produto comercial Provence® 750 WG (750 $g.L^{-1}$ isoxaflutole). Foram seguidas as normas ISO 17512-1:2008 e ISO 17512-2:2011 para fuga, ISO 11268-2:2012 e ISO 11267:2014 para letalidade e reprodução.

No Capítulo 2, ensaios de fuga e letalidade foram realizados com minhocas (*E. andrei*) e colêmbolos (*F. candida*) em SAT contaminado com os produtos comerciais Ally® (600 $g.L^{-1}$ metsulfuron-methyl) e Assist® (756 $g.L^{-1}$ óleo mineral), como adjuvante.

Já no Capítulo 3, os ensaios foram realizados em laboratório e em campo. Os ensaios em laboratório foram de reprodução, utilizando como espécies não-alvo a minhoca *E. andrei*, o enquitreídeo *Enchytraeus crypticus* e os colêmbolos *F. candida* e *Proisotoma minuta*, em SAT contaminado com os produtos comerciais Ally® (600 $g.L^{-1}$ metsulfuron-methyl) e Assist® (756 $g.L^{-1}$ óleo mineral), como adjuvante. Os ensaios seguiram as normas ISO 11268-2:2012 (minhocas), ISO 11267:2014 (colêmbolos) e ISO 16387:2014 (enquitreídeos). Dois experimentos foram realizados em campo, sendo o primeiro em 2017, com o produto comercial Ally® (600 g.L^{-1} metsulfuron-methyl), e o segundo em 2018, utilizando o produto comercial Metsuram® (600 g.L^{-1} metsulfuron-methyl). Ambos avaliaram a atividade alimentar da fauna edáfica através do teste *bait lamina*, padronizado pela norma ISO 18311:2016. O experimento de 2017 também avaliou a abundância dos grupos da mesofauna, o residual dos herbicidas e o comportamento de fuga de minhocas (ISO 17512-1:2008) e colêmbolos (ISO 17512-2:2011) com solo retirado do experimento.

RESULTADOS E DISCUSSÃO

Os resultados do Capítulo 1 mostraram fuga das minhocas apenas em doses >300 vezes do que a dose de isoxaflutole prevista para ser aplicada em campo, bem como a reprodução diminuiu apenas nas doses >150 vezes a dose de campo. Não foram observados efeitos sobre o comportamento de fuga, sobrevivência ou reprodução para a espécie de colêmbolos avaliada.

No Capítulo 2, o ensaio de letalidade com minhocas não mostrou diferenças quando Ally® foi testado sozinho ou na presença do adjuvante. No ensaio de fuga, o herbicida Ally® apenas causou fuga dos organismos em altas concentrações (5.000 e 10.000 vezes e a dose de campo). Entretanto, a adição do óleo mineral Assist® mudou a resposta dos organismos de solo, aumentando a fuga, mesmo nas doses de campo. A toxicidade do adjuvante foi confirmada nos ensaios em que os colêmbolos e as minhocas foram expostos apenas ao Assist®, resultando em fuga. Esses resultados claramente mostram que a adição do óleo mineral resulta em ecotoxicidade da mistura para as populações edáficas..

Os resultados laboratoriais, no Capítulo 3, mostraram que o metsulfuron-methyl sozinho não é uma ameaça à fauna edáfica, mesmo em exposições crônicas, considerando os grupos avaliados. Todavia, a presença do óleo mineral apresentou ecotoxicidade às espécies *E. andrei, E. crypticus* e *P. minuta,* visto que o adjuvante, quando testado sozinho, já apresentou ecotoxicidade a estas espécies. As avaliações de campo, tanto com Ally®, como com Metsuram® 600 WG, indicaram que os herbicidas à base de metsulfuron-methyl não causaram prejuízo na atividade alimentar da fauna edáfica.

CONSIDERAÇÕES FINAIS

A partir dos resultados laboratoriais, é possível supor que o herbicida Provence® 750 WG não apresenta toxicidade às minhocas e colêmbolos, mesmo nas maiores doses aplicadas em campo, garantindo a segurança das comunidades do solo.

Em geral, os resultados indicaram que os adjuvantes devem ser considerados na avaliação de risco de pesticidas, considerando que, nas condições de campo, estes produtos são aplicados em mistura com os agrotóxicos, o que também leva a uma grande redução e possível perda das comunidades autóctone. Validar os resultados obtidos em laboratório em campo se mostra necessário para o melhor entendimento do comportamento da fauna e para avançar nas avaliações, especialmente na de misturas de agrotóxicos.

ABSTRACT

Herbicides are the most used class of products in modern agriculture, corresponding to more than 60% of all pesticides sales in Brazil. Metsulfuron-methyl and isoxaflutole are among the active ingredients commercialized in the country. While the former is notable for its high low-dose action, the latter is usually used in cases of resistance to glyphosate. However, there is a lack of information about their potential effects to soil fauna. In order to reduce uncertainties about these effects, the aim of this dissertation was to evaluate the ecotoxicity of metsulfuron-methyl and isoxaflutole herbicides-based to non-target soil invertebrates. Laboratory and field tests were used to test both active ingredients and adjuvant addition. Acute (avoidance and lethality) and chronic (reproduction) laboratory tests were performed following standardized guidelines and using non-target organisms. On field trials were evaluated soil fauna feeding activity, richness of mesofauna groups and herbicides determination. Results showed that both isoxaflutole and metsulfuron-methyl do not present ecotoxicity to non-target tested organisms, showing response only in doses several times above those applied in the field. However, when mineral oil was added to metsulfuon-methyl simulating a post-emergence field condition, the mixture caused toxicity to soil fauna, in laboratory tests. Field results showed non-impairment of soil fauna feeding activity by metsulfuron-methyl or mineral oil as recorded in laboratory, but the richness of mesofauna groups was quite low in the area, which may be result of previous soil management. The results obtained in field, however, refer to a single application of each formulated product, so it is not possible to extrapolate these results for continuous and long-term applications. This study shows the importance of including adjuvants in risk assessment as well as to extrapolate results obtained in laboratory to field. Therefore, there will be greater understanding on pesticides action in ecosystem dynamics enabling progress in the discussion about limitations on pesticides risk assessment in Brazil.

Keywords: Ecotoxicity. Environmental contamination. Pesticides. Soil Invertebrates.

FIGURE LIST

CHAPTER I

CHAPTER II

CHAPTER III

TABLE LIST

CHAPTER I

CHAPTER II

CHAPTER III

SUMMARY

CHAPTER I

ECOTOXICITY OF ISOXAFLUTOLE (HERBICIDE) TO SOIL INVERTEBRATES

CHAPTER II

SCREENING EFFECTS OF METSULFURON-METHYL TO COLLEMBOLANS AND EARTHWORMS: THE ROLE OF ADJUVANT ADDITION ON ECOTOXICITY

CHAPTER III
LABORATORY AND FIELD TESTS FOR RISK ASSESSMENT OF METSULFURON-METHYL-BASED HERBICIDES FOR SOIL FAUNA

GENERAL INTRODUCTION

After “The Green Revolution” reached underdeveloped countries with its agricultural model based on mechanization use, irrigation techniques, high yield potential cultivars and indiscriminate use of pesticides (SPADOTTO et al., 2010; BONACHELA et al., 2016), Brazilian agriculture has been growing rapidly, expanding its borders (OLIVEIRA JR., 2011), in order to raise production figures (PYHN; SANTOS, 2003; LIMA BOHNER; ARAUJO; NISHIJIMA, 2013) generating prominence in the contribution to the national GDP (RODRIGUES et al., 2017).However, this agricultural model based on monocultures has made the country become the largest consumer of pesticides worldwide since 2008, surpassing countries like China and the United States (RIGOTTO; VASCONCELOS; ROCHA, 2014). According to Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) more than 500,000 ton/year of pesticides were sold in 2009, with herbicides corresponding to more than 55% of all sales, followed by insecticides (> 10%) and fungicides (<10%) (IBAMA, 2016). In 2015, glyphosate alone corresponded to 66% of all sales, followed by 2,4-D (12%) and atrazine (5%) (IBAMA, 2016), demonstrating that herbicide use increased frighteningly.

In addition to protecting agricultural crops, pesticides can pose risks to human health and the environment and their misuse can lead to contamination not only of food, but also of surface water, groundwater and soil, which may present risks to both aquatic and terrestrial organisms (SPADOTTO et al., 2010).

Source of a wide range of essential ecosystem services and ecosystem functions that generate benefits for human populations (MILLENIUM ECOSYSTEM ASSESSMENT, 2005), soils are able to withstand much of the agroforestry systems (production services) through services such as weathering, nutrient cycling and primary production (MAKOVNIKOVA; KANIANSKA; KIZEKOVA, 2017). Additionally, soil participates of the regulation of climate change, through carbon sequestration resulted from its influence on organic matter dynamic and several physical properties (COSTANZA et al., 2014; DI LONARDO et al., 2017). These services are carried out by a large number of organisms, known as soil invertebrates, whose actions are little explored, especially considering the size of their taxonomic groups (LAVELLE et al., 2006).

As macrofauna representatives, earthworms are responsible for a large fraction of the biomass in the soil where they play an important role in the functioning of soil ecosystem as “ecosystems engineers”

(LAVELLE, 2014), organic matter dynamics (DIGNAC et al., 2017), soil structure and microbial community (PELOSI et al., 2014).

Smaller organisms like enchytraeids can be found in all soils with sufficiency of oxygen, moisture and nutrient supplies and are commonly found in areas of intense agriculture (PELOSI; RÖMBKE, 2016). They belong to soil mesofauna and occur in great quantity, having a fundamental role as decomposers of the organic matter and in the nutrient cycling (LAVELLE, 2014).

Collembolans also belong to mesofauna and can be found in all types of soils, especially in tropical soils (FOUNTAIN; HOPKIN, 2001), being considered a key group in the soil ecosystem (DOMENE et al., 2010) because of their place at the base of the food chain. Collembolans are very important in the nutrient cycling, and in organic matter decomposition (ZEPELLINI FILHO; BELLINI, 2004). They are exposed to substances by their epidermis, ventral tube (by water) or intestine (food), and the route of exposure to pesticides is not very clear (DOMENE et al., 2010).

Studying the effects of pesticides on these organisms is one of the aims of the multidisciplinary field of ecotoxicology – a science that combines ecology, toxicology and chemistry, and the connections between these fields (CARDOSO; ALVES, 2012). Through ecotoxicological tests, it is possible to estimate potential effects that a given contaminant may cause on organisms under field conditions, allowing the simulation of a more accurate answer on contaminants toxicity (SISINNO; OLIVEIRA-FILHO, 2013). However, one of the biggest challenges of ecotoxicology is to increase the ecological relevance of laboratory data (SISINNO; OLIVEIRA-FILHO, 2013).

An uncertainty on these evaluations concerns on soil properties and contaminants bioavailability, as well as its persistence and movement (NIEMEYER; CHELINHO; SOUSA, 2017). Another issue that needs attention is pesticides' effects on non-target organisms (BUCH et al., 2013), including soil invertebrates – mediators to ecosystem services occurrence (LAVELLE et al., 2006). Both acute as chronic disturbances can reduce soil invertebrates' biodiversity bringing impacts on ecosystem functions (FILSER et al., 2008). Pesticide use can affect non-target organisms either directly - impacting gene expression, behaviour, reproduction, life cycle - or indirectly - by modifying interactions between individuals and populations (e.g. affecting predation but not predators) (RÖMBKE et al., 2017).

Although herbicides lead the global consumption of pesticides, there are fewer records of ecotoxicological studies for this class of products compared to insecticides and fungicides (NIEMEYER; CHELINHO; SOUSA, 2017). Literature data on LC_{50} and EC_{50} indicate that atrazine, phenmedipham and terbuthylazine are the most toxic herbicides, but herbicide toxicity depends on its chemical group, commercial formulation, application rates, environmental conditions and ecological receptors involved (BUCH; DE SANTO; NIEMEYER, *in press*).

According to Niemeyer, Chelinho and Sousa (2017) all herbicide ecotoxicity tests with soil organisms recorded for Latin America until 2016 were with glyphosate because of its wide use on agriculture. The knowledge on pesticides' use and their impact, specially herbicides, in tropical climate is still incipient when compared to what is already known for temperate climate (NIVA et al., 2016).

When weeds gain resistance against glyphosate, the common procedure is to use another active ingredient to control the range of broadleaves and narrow leaves that appears on the field. One of these active ingredients is isoxaflutole, the common name for (5-cyclopropyl-1,2-oxazol-4-trifluoro-2-mesyl-p-tolyl) methanone, belonging to the isoxazoles chemical class, and used for pre-emergence control of a wide range of broadleaf and grass weeds in maize and sugarcane (PALLET et al. 2001). When in soil, water or plant, isoxaflutole is rapidly converted into diketonitrile (DKT), a stable molecule responsible for the degradation of the (4-hydroxyphenyl) pyruvate dioxygenase (HPPD) enzyme leading to disruption of carotenoid synthesis, with the development of a characteristic bleaching of foliar tissue (RICE; KOSKINEN; CARRIZOSA, 2004; CAVALIERI et al., 2008). Isoxaflutole is effective specially against weeds resistant to other herbicide classes such as glyphosate and atrazine (ISAAA, 2018).

The use of herbicides belonging to the sulfonylureas chemical group increased since their introduction by Dupont Corporation in 1982 (HE at al., 2006; GHOBADI et al., 2015), mainly because of its high levels of activity even when used in low doses (OLIVEIRA JR., 2011). Among these herbicides, metsulfuron-methyl, commom name of methyl 2-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoylsulfamoyl]benzoate, one of the most widely used herbicides from sulfonylurea family, is a common active ingredient used to control a large variety of annual grasses and broad-leaved weeds as pre-emergence application on wheat or as post-emergence application on wheat, barley, flax, triticale, oat and sugarcane (CASTRO et al., 2002; NELEMANS et al., 2017).

Both isoxaflutole and metsulfuron-methyl are active ingredients that require studies on their ecotoxicological effects on soil fauna. Despite the appropriated and useful information about effective concentration (VERSTEEG et al., 1999) provided through laboratory tests, field evaluations should be used to complement risk assessment of pesticides. Laboratory tests cannot outline the effects of organisms' exposure in the field, especially their ecosystem interactions since they focus only on pre-established patterns (RÖMBKE et al., 2017). An assessment under local conditions is useful to reduce uncertainties (CASABÉ et al., 2007), corroborate with laboratory data and to contextualize it ecologically. *In situ* approaches such as TSBF methodology proposed by Anderson and Ingram (1993) and the bait lamina test (VON TÖRNE, 1990; ISO, 2016) allow the observation of effects in a real scenario, where interaction occurs between the environmental parameters and the compound to be evaluated.

This scenario combining laboratory tests and ecological field trials is part of the Ecological Risk Assessment demanded by the European Union (EFSA, 2017), which has one of the most rigid and active committees regarding the release and renewal of pesticide registrations. Since 2002, there are regulations requiring the carrying out of sublethal test, such as reproduction tests, for earthworms, collembolans and mites because of the known difference in sensitivity between organisms and their great functional diversity in soil (EFSA, 2002).

On the other hand, in Brazil the difference is huge. Despite the existence of regulatory laws to assure pesticides registration control (BRASIL, 1989, 2002; NIVA et al., 2016), according to IBAMA Ordinance Nº 84, from October 15, 1996, considering soil invertebrates, the only required test is earthworms' acute toxicity (ABNT, 2014) withdrawn from EU scope for being considered an unresponsive endpoint (EFSA, 2013).

Therefore, the aim of the present study was to evaluate the ecotoxicity of the herbicides metsulfuron-methyl and isoxaflutole to soil fauna based on laboratory ecotoxicological tests with non-target organisms and field evaluations discussing their advantages and uncertainties. In addition to better understanding on the effects of pesticides on soil ecosystem, an effort of this work was to extrapolate the results obtained in laboratory to field conditions contributing to pesticides risk assessment in Brazil.

This dissertation is divided into three chapters. Chapter 1 aimed to evaluate the ecotoxicity of the herbicide isoxaflutole using avoidance, lethality and reproduction tests with earthworms and collembolans.

Chapter 2 presents screening tests (avoidance and lethality) with the herbicide metsulfuron-methyl with and without adjuvant (mineral oil) addition and their responses for collembolans and earthworms. Chapter 3 shows laboratory (chronic tests) and field tests aiming to assess the similarities and differences between results obtained for both approaches.

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CHAPTER I

ECOTOXICITY OF ISOXAFLUTOLE (HERBICIDE) TO SOIL INVERTEBRATES

Based on:
DE SANTO, F.B.; RAMOS, G. A.; RICARDO FILHO, A. M.; MARCHIORO, C.A.; NIEMEYER, J. C. Ecotoxicity of isoxaflutole (herbicide) to soil invertebrates in field predicted doses.

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ABSTRACT

Isoxaflutole (IFT) is an herbicide used for pre-emergence control of a wide range of broadleaf and grass weeds specially the resistant to other herbicide classes such as glyphosate and atrazine. Although its herbicidal potential was identified in the early 90s, IFT is still a new active ingredient in Brazil and little is known about its effects, mainly regarding to ecotoxicity of formulated products to soil macro and mesofaunal groups. This study aimed to assess acute and chronical effects (avoidance, lethality and reproduction) of Provence® 750 WG (750 $g.L^{-1}$ isoxaflutole) on the test organisms *Eisenia andrei* (earthworms) and *Folsomia candida* (collembolans) using standardized ISO guidelines. Results showed avoidance of the earthworm species only at >300 times the field predicted doses (FPD) as well as reproduction decrease over >150 times FPD. Neither avoidance, nor lethality or reproduction response were found to the collembolan species. From the laboratory results, it is possible to assume that Provence® shows no toxicity to earthworms and collembolans even at the highest field applied dose, ensuring the safety of soil communities.

KEYWORDS**:** avoidance behaviour tests; earthworms; pesticides; soil ecotoxicology

1 INTRODUCTION

Isoxaflutole (IFT) is the common name for (5-cyclopropyl-1,2-oxazol-4-trifluoro-2-mesyl-p-tolyl) methanone, belonging to the isoxazoles chemical class, and used for pre-emergence control of a wide range of broadleaf and grass weeds in maize and sugarcane (Pallet et al. 2001). When in soil, water or plant, IFT is rapidly converted to diketonitrile (DKT), a stable molecule responsible for the degradation of the (4-hydroxyphenyl) pyruvate dioxygenase (HPPD) enzyme leading to disruption of carotenoid synthesis, with the development of a characteristic bleaching of foliar tissue (RICE et al., 2004; CAVALIERI et al., 2008).

The use of IFT is effective specially against weeds resistant to other herbicide classes such as glyphosate and atrazine. In 2013, Bayer CropScience developed a genetically modified (GM) event soybean (*Glycine max* L.) with tolerance to glyphosate and IFT herbicides and regulatory approval for use in food, feed and cultivation in Brazil since 2015 (ISAAA, 2018). This event joint seventeen another GM soybean already regulated in Brazil with IFT becoming more expressive in the country sales.

Uncertainties concerning to ecotoxicity of herbicide formulation are specially related to inert substances known as surfactants which, in some cases, can have higher toxicity when compared to the active ingredient (a.i.) itself (GIESY et al., 2000; AGUIAR et al., 2016). Some authors describe the toxicity of inert substances as often more toxic to non-target living organisms than the a.i (TOMINACK et al., 2000; COX; SURGAN, 2006). In this context, rapid sublethal toxicity assessments are of great importance given its ecologically relevant outcome concerning the potential damages on non-target organisms (MARQUES et al., 2009). In contrast, reproduction test is a common laboratory requirement for assessing the effects of chemicals, despite being time-consuming, it has relevance on population parameters because indicate long-term toxicity effects on non-target organisms and, as a consequence, on the ecosystem functions *and the* services that they underpin (HANDY et al., 2012; SALVIO et al., 2016).

A report published in 2003 (EC, 2003) showed endocrine effects caused by IFT classifying it to human health as “toxic to reproduction”. In 2016, EFSA peer review (EFSA, 2016) could not identify endocrine disrupting potential but showed a high risk to mammals (for all representative uses), keeping the classification of “toxic to reproduction category 2” and reclassifying it as “carcinogenic category 2”. On the other hand, according to this report, low toxicity was shown to soil arthropods, macro and microorganisms. Besides the EFSA peer review, there is no information on the toxicity effects of IFT to earthworms and collembolans on the available databases.

Representative of the soil macrofauna, earthworms regulate major soil processes and functions such as soil structure, organic matter decomposition, and microbial and invertebrate population so as plant growth (LAVELLE, 2014). Their representativeness on soil biomass and sensitivity to soil pollutants make them suitable test organisms to using on risk assessment of pesticides (SALVIO et al., 2016). Considered another soil ecosystem key-group (DOMENE et al., 2010), collembolans can accelerate by 20% organic matter decomposition rates, besides interacting in the biological, biochemical and physical processes (LINS et al., 2007; KORBOULEWSKY et al., 2016).

Although its potential as herbicide was identified in 1991, IFT is still a new active ingredient in the Brazilian market and little is known about its effects on soil fauna, especially regarding to ecotoxicity. The aim of this work was evaluating the ecotoxicity of a formulated product of IFT to soil macro and mesofaunal groups, represented by the earthworm species *Eisenia andrei* Bouché, 1972 (Annelida: Lumbricidae) and the collembola species *Folsomia candida* Willem, 1902 (Collembola: Isotomidae) using standardized ISO guidelines for acute and chronical effects assessment.

2 MATERIAL AND METHODS

2.1 TEST CHEMICAL

Provence® 750 WG (750 g a.i. L^{-1} isoxaflutole) marketed in Brazil by BASF S.A. is a water dispersible granule herbicide with recommendations to application in pre-emergence in crops like cassava, corn, cotton, potato, sugarcane and isoxaflutole tolerant soybean. An aqueous solution of 1,5 $g.L^{-1}$ of Provence® 750 WG was prepared for soil contamination. A range of concentrations plus a negative control (soil with distilled water) was set up for each test (Table 1).

To estimate field predicted dose (FPD) the equation described by Jansch et al. (2006) was used:

$$MC5 = \frac{\frac{F.D}{\Delta z}}{p}$$

Where:
MC5 = the maximum concentration of pesticide in the top 5 cm of soil (mg.kg^{-1})
F = factor for conversion from kg.ha^{-1} to mg.m$^{2-1}$
D = the nominal treatment (the application concentration in kg.ha^{-1})
Δz = the layer thickness (0.05m)
p = dry bulk density (kg.m$^{3-1}$)

According to EPPO (2003), the top 5 cm of the soil is the relevant exposure depth to soil invertebrates, and a standard soil has a bulk density of 1,500 kg.m$^{3-1}$. The nominal treatment considered in this calculation was the highest label recommended dose of Provence® 750 WG (467 g a.i. ha^{-1}) which is a pre- emergence concentration for sugarcane on clay soil. FPD was calculated in 0.62 mg.kg^{-1}.

Table 1 - Summary of the tests performed with Provence® 750 WG (750 g.L^{-1} isoxaflutole).

Organism	**Endpoint**	**Concentration range (mg a.i. kg^{-1})**
E. andrei	Avoidance	0; 11.7; 23.4; 46.9; 93.8; 187.5; 375
	Lethality	0; 23.4; 46.9; 93.8; 187.5; 375
	Reproduction	0; 23.4; 46.9; 93.8; 187.5; 375
F. candida	Avoidance	0; 11.7; 23.4; 46.9; 93.8; 187.5; 375
	Lethality	0; 23.4; 46.9; 93.8; 187.5; 375
	Reproduction	0; 23.4; 46.9; 93.8; 187.5; 375

2.2 TEST SOIL

Experiments were carried out using tropical artificial soil (TAS) composed by 75% fine sand (washed and dried), 20% kaolin clay and 5% coir dust (dried at 60°C), adapted from García (2004). This reduced percentage of organic matter in artificial soil (from 10% to 5%) has been proposed by OECD (2016) for being more representative of natural soils.

The pH of the prepared soil was adjusted to 6.0 ± 0.5 adding $CaCO_3$. Moisture was adjusted to 50% of the water holding capacity at the beginning of the tests, adding distilled water and considering the volume of contaminant solution to be added.

2.3 TEST ORGANISMS

The ecotoxicity tests were carried out with the species *E. andrei* and *F. candida*. The tested species were maintained in climatic chambers regulated at 20ºC ± 2, photoperiod 12:12 h light: dark.

Earthworms were cultured in plastic boxes (about 10 L volume), in a moistened mixture of cow manure (free of antibiotics) and coconut powder, and fed with cooked oat once a week. Cultures were maintained according to ISO 11268-1 (ISO, 2012a). Clitellate earthworms (2-12 months old) were used in avoidance, lethality and reproduction tests.

Collembolans were cultured in vessels containing a mixture of plaster of Paris and activated charcoal (10:1), according to ISO 11267 (ISO, 2011a). Biological dry yeast (*Saccharomyces cerevisiae*) was provided as food supply twice a week. Reproduction test was carried out with juveniles (10-12 d old) while three months old adults were used in avoidance and lethality tests.

2.4 AVOIDANCE BEHAVIOUR TESTS

Avoidance tests were carried out with five replicates following ISO 17512-1 (ISO, 2008) for earthworms and ISO 17512-2 (ISO, 2011b) for collembolans. Test vessels were divided into two sections with a removable plastic divider, where one side received 300 g of control soil and the other one received 300 g of contaminated soil. The divider was removed, and 10 earthworms were placed in the center of the vessel. After 48 h of incubation, the divider was reinserted and the number of earthworms in each compartment was recorded.

The same was followed for collembolans, except for the amount of soil (30 g in each side) and the number of organisms used (20 instead of 10). At the end of the test, the number of organisms in each side of the test vessel was recorded using water and stamp ink.

The random distribution of organisms in the absence of contamination was confirmed by dual control tests, performed with the same methodology described above, but receiving control soil in both sides of the test recipients.

2.5 LETHALITY TESTS

Lethality tests with earthworms and collembolans lasted 14 days following ISO guidelines 11268-1 (ISO, 2012a) and 11267 (ISO, 2011a).

The tests were performed with a control moistened with distilled water. Ten clitellate earthworms were placed in each test vessels containing 350 g of contaminated or control TAS. Test vessels were covered with perforated plastic lids to allow aeration, and moisture was adjusted at day 7 by weighting the replicates and replacing the water loss by adding drops of distilled water. Survival was recorded at day 14 by removing and counting the living earthworms. For collembolans, ten organisms from synchronized cultures (about three months old) were put in each test vessels with 30 g of contaminated or control soil. No food was added. At day 14, water and drops of stamp ink were added to allow the count of the floating organisms on water surface. The number of survivals was recorded.

2.6 REPRODUCTION TESTS

Reproduction tests followed ISO guidelines 11268-2 (ISO, 2012b) for earthworms and 11267 (ISO, 2011a) for collembolans.

Test with earthworms were carried out with ten clitellate earthworms put into test vessels containing 350 g of contaminated or control soil. Plastic perforated lids were used to allow aeration. Cow dung free of antibiotics (5 g, dry and ground) was added weekly as food supply. Drops of distilled water were added to replace water loss weekly. At day 28, adults were removed, leaving cocoons to hatch by additional four weeks. At day 56, juveniles were counted using hot extraction by immersing the test vessels in water bath at 60°C, forcing the juveniles to come to soil surface.

Test with collembolans were carried out with ten juveniles put into test vessels containing 30 g of contaminated or control soil. Organisms were fed with approximately 2 mg of dry yeast at days 1 and 14. Twice a week the test vessels were opened allowing aeration. Drops of distilled water were added to replace water loss weekly. At day 28, test vessels were filled up with water and some drops of stamp ink, carefully stirred, and photographed after counting of floating juveniles on the water surface. Counting was carried out using the software ImageJ (SCHNEIDER et al., 2012).

2.7 DATA ANALYSIS

Results obtained in avoidance tests were analysed by Fisher exact test ($p<0.05$) when the highest number of organisms was found in the control section. The null hypothesis assumes that 50% of test organisms stay in the test soil and that no organisms leave that section (non-avoidance) (NATAL-DA-LUZ et al., 2004).

Mean number of juveniles obtained in reproduction tests was analysed by one-way Analysis of Variance (ANOVA), followed by Dunnett test ($p < 0.05$), comparing reproduction in contaminated soils versus control soil. Normal distribution of data and homogeneity of variance were verified by Shapiro Wilk's test and Bartlett test, respectively.

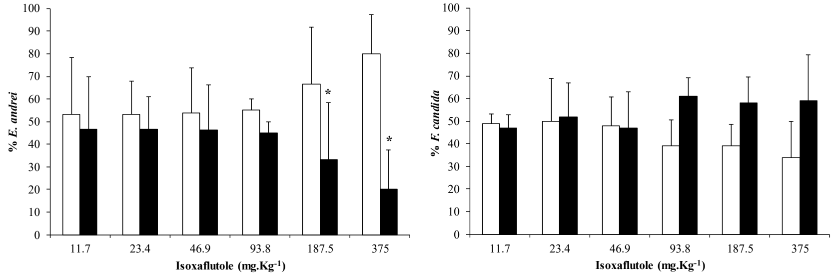
3 RESULTS AND DISCUSSION

3.1 AVOIDANCE BEHAVIOUR TESTS

After 48 h of exposure, lethality observed for earthworms and collembolans in avoidance tests remained <10%, and a random distribution was found in dual control tests (control *versus* control), following the ISO criteria of validation.

Earthworms avoided IFT only at concentrations of 187.5 and 375 mg a.i. kg^{-1}, equivalent to > 300 and >600 times the recommended doses of the commercial product. Collembolans showed non-avoidance behaviour when exposed to IFT, even at the highest tested concentration (375 $mg.kg^{-1}$) equivalent to >600 times the maximum recommended dose (Fig. 1). In general, non-avoidance of earthworms and collembolans can be expect when this product is applied in soil, corroborating with the data showed by EFSA (2016).

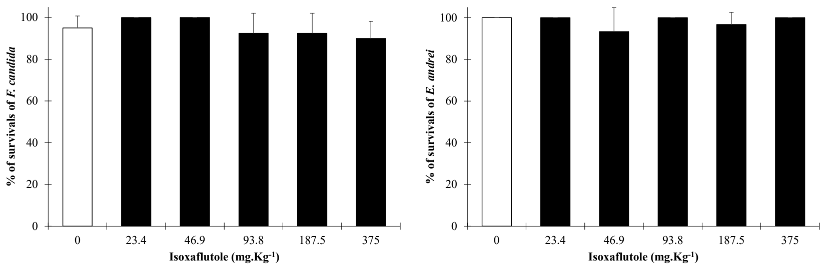
Fig. 1 - Avoidance tests with *E. andrei* and *F. candida*: Percentage of organisms on isoxaflutole contaminated soil (black bars) against the control (white bars). *Asterisks indicate statistical differences ($p<0.05$).



3.2 LETHALITY AND REPRODUCTION TESTS

No lethality was observed to collembolans and earthworms (Fig. 2) even at concentrations > 600 times the maximum recommended dose (375 mg a.i. Kg^{-1}). In general, the tested product cause non-acute effects on these groups of organisms.

Fig. 2 - Lethality tests with *E. andrei* and *F. candida*: Number of survival organisms on isoxaflutole contaminated soil (black bars) and the control (white bars). No statistical difference was recorded.



Reproduction tests showed no impairment on number of juveniles of collembolans (Table 2). The toxicity effects on earthworm reproduction can be expected over 150 times the FPD, but these values are not used under field conditions (Table 2).

Although showing carryover effects to some crops like beans and sugar beets (NELSON; PENNER, 2005) no toxicity effects could be seen to soil fauna in a real scenario. Few studies testing other herbicides like atrazine, pendimethalin, and glyphosate with *E. andrei* (CHELINHO et al., 2010; BUCH et al. 2013) and *F. candida* (BELDEN et al., 2005; AMORIM et al., 2012) found the CE_{50} or CL_{50} values for test organisms, showing the low toxicity of herbicides in general.

Table 2 - Reproduction of the collembolans *F. candida* and the earthworms *E. andrei* exposed to isoxaflutole and its field predicted dose (FPD) according to the nominal dose (isoxaflutole $mg.kg^{-1}$). *Asterisks indicate significant differences in Dunnet test ($p<0.05$)

Treatment	Isoxaflutole ($mg.kg^{-1}$)	FPD	*F. candida*	*E. andrei*
			Number of juveniles	Number of juveniles
Control	0		510.8 ± (69.05)	42.00 ± (9.38)
Provence® 750 WG	23.4	>35	541.00 ± (86.35)	47.25 ± (19.87)
	46.9	>70	580.40 ± (112.16)	19.50 ± (4.12)
	93.8	>150	597.25 ± (68.15)	16.75 ± (11.02)*
	187.5	>300	631.50 ± (81.42)	24.50 ± (7.23)
	375	>600	627.50 ± (85.41)	30.00 ± (11.46)

4 CONCLUSION

Results showed avoidance of the earthworm species only at >300 times the FPD as well as reproduction response over >150 times FPD. Non-avoidance, lethality or reproduction response was found to the collembolan species. From the laboratory results, it is possible to conclude that Provence® 750 WG has no toxicity effects on earthworms and collembolans even at the highest field applied dose, ensuring the safety of macro and mesofaunal soil communities.

The ecotoxicity of herbicides depends on their chemical group, commercial formulation, application rates, environmental conditions and ecological receptors involved. Therefore, it is not possible to extrapolate its toxicity to aquatic ecosystems or humans.

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CHAPTER II

SCREENING EFFECTS OF METSULFURON-METHYL TO COLLEMBOLANS AND EARTHWORMS: THE ROLE OF ADJUVANT ADDITION ON ECOTOXICITY

Based on:
DE SANTO, F.B.; RAMOS, G. A.; RICARDO FILHO, A. M.; MARCHIORO, C.A.; NIEMEYER, J. C. Screening effects of metsulfuron-methyl to collembolans and earthworms: the role of adjuvant addition on ecotoxicity. **Environ Sci Pollut Res**, v. 25, n. 24, p. 24143-24149, 2018. https://doi.org/10.1007/s11356-018-2481-5

ABSTRACT

Metsulfuron-methyl is a common active ingredient recommended for use in pre- and post-emergence control of annual grasses and broadleaf weeds in crops, usually applied with mineral oil as adjuvant to enhance its efficiency. Despite the increasing use of this herbicide, there are no information on its ecotoxicity effects to soil fauna. Avoidance and lethality tests were performed with earthworms and collembolans using tropical artificial soil contaminated with formulated products Ally® (600 $g.L^{-1}$ metsulfuron-methyl) and Assist® (756 $g.L^{-1}$ mineral oil) as adjuvant. Lethality test with earthworms showed no difference when tested with or without adjuvant. When Ally® was tested alone, it caused avoidance behavior only at high concentrations (5,000 and 10,000 times field predicted dose). However, Assist® addition changed the response of soil invertebrates increasing the avoidance even at field predicted doses. The toxicity of the adjuvant was confirmed in tests exposing collembolans and earthworms to Assist® alone resulting in avoidance behavior. The results clearly show that the addition of mineral oil enhanced the ecotoxicity of metsulfuron-methyl. This study provides an important contribution to the knowledge on the toxicity of metsulfuron-methyl, and indicates that adjuvants should be considered in risk assessment of pesticides, considering that under field conditions these products are applied together.

KEYWORDS: avoidance tests; acute toxicity test; herbicides; soil ecotoxicity; soil invertebrates

1 INTRODUCTION

Pesticides are widely used worldwide in agricultural applications to control insect pests, weeds and plant diseases (WANG et al., 2016). Brazil leads the ranking of pesticide consumption since 2008 (REBELO, 2010) and herbicides comprise more than 50% of all sales in the country (IBAMA, 2016). There are serious concerns about contamination of soil environments by the indiscriminate use of pesticides, which are known to affect soil flora and fauna (SINGH; SINGH, 2015; WANG et al., 2016). In this context, obtain information about the potential effects of pesticides in soil, specially to soil fauna, to complement conventional chemical analysis became an important focus in studies involving soil contamination (REINECKE; REINECKE, 2007, RASTETTER; GERHARDT, 2018)

Ecotoxicity tests have been applied to assess the effects of pesticides and mixtures in soil ecosystem (CHELINHO et al., 2014; RÖMBKE et al., 2017). Earthworms and collembolans are the most used test species as model organisms of non-target macro and mesofauna organisms, respectively (AMORIM et al., 2012; PELOSI et al., 2014). Earthworms are responsible for a large fraction of the biomass in the soil, playing an important role in the functioning of soil ecosystem. Such organisms are considered ecosystem engineers because their action on soil structure, consequently promoting microorganisms activity and influencing nutrient cycling (LAVELLE, 2014; PELOSI et al., 2014; DIGNAC et al., 2017). Collembola are considered a key group in soil ecosystem, inhabiting various organic substrates, using a wide range of food sources and being the food chain basis for other species, besides act as nutrient cycling catalyzers as well as by changing the soil structure through litter comminution, casting and other mechanisms (DOMENE et al., 2010; POTAPOV et al., 2016; D'ANNIBALE et al., 2017). Collembolans can be found in all soil types with high abundance in tropical soils (FOUNTAIN; HOPKIN, 2001).

Considered an ecologically relevant endpoint, avoidance behavior of earthworms and collembolans have been used as screening tests to evaluate soil quality (NATAL-DA-LUZ et al., 2004; ISO, 2008, 2011; BRAMI et al., 2017) demonstrating sensitive responses to pesticides addition in soil (MARQUES et al., 2009). Decline of populations occasioned by avoidance behavior can have effects on ecosystem functioning in concentrations lower than that causing lethality or impaired reproduction (BUCH et al., 2013; LOWE et al., 2016). These tests have the advantage of short duration and reduced effort if compared with chronic tests, not replacing them but being an early warning of pesticides effects to soil fauna populations.

The use of herbicides belonging to the sulfonylureas chemical group increased dramatically since their introduction by Dupont Corporation in 1982 (HE at al., 2006; GHOBADI et al., 2015), mainly because of its high levels of activity even when used in low doses (OLIVEIRA JR., 2011). Among these herbicides, metsulfuron-methyl, one of the most widely used herbicides from sulfonylurea family, is a common active ingredient used to control a large variety of annual grasses and broad-leaved weeds as pre-emergence application on wheat or as post-emergence application on wheat, barley, flax, triticale, oat and sugarcane (CASTRO et al., 2002; NELEMANS et al., 2017). This herbicide is commonly applied combined with adjuvants to increase its efficiency, especially mineral oil. Despite the studies demonstrating that the combination of some herbicides with adjuvants may result in higher toxicity to environment when compared to active ingredients or commercial products applied alone (CAQUET et al., 2005; SURGAN,et al., 2010), these mixtures are not considered in regulatory risk assessment schemes of pesticides (EFSA *Draft* 2017).

Despite the increasingly use of metsulfuron-methyl to control weeds in agricultural crops, there is a lack of laboratory data on the effects of this herbicide to soil fauna. In this context, standardized lethality tests with earthworms and avoidance tests with collembolans and earthworms were carried out to screen the effects of the metsulfuron-methyl to soil fauna. Considering that under field conditions this product is applied combined with adjuvants, the response of collembolans and earthworms to metsulfuron-methyl combined with mineral oil was also tested, as well as the mineral oil alone.

2 MATERIAL AND METHODS

2.1 TEST ORGANISMS

The ecotoxicity tests were carried out with the species *Eisenia andrei* Bouché, 1972 (Annelida: Lumbricidae) and *Folsomia candida* Willem, 1902 (Collembola: Isotomidae). The tested species were maintained in climatic chambers regulated at 20ºC ± 2, photoperiod 12:12 h light: dark.

Earthworms were cultured in plastic boxes (about 10 L volume), in a moistened mixture of cow manure (free of antibiotics) and coconut powder, and fed with cooked oat once a week. Cultures were maintained according to ISO 11268-1 (ISO, 2012). Clitellate earthworms (2-12 months old) were used in lethality and avoidance tests.

Collembolans were cultured in culture vessels containing a mixture of plaster of Paris and activated charcoal (10:1), according to ISO 17512-2 (ISO, 2011). Biological dry yeast (*Saccharomyces cerevisiae*) was provided as food supply twice a week. Avoidance tests were carried out with adults from synchronized cultures of three months old.

2.2 TEST SOIL

Experiments were carried out using tropical artificial soil (TAS) composed by 75% fine sand (washed and dried), 20% kaolin clay and 5% coir dust (dried at 60°C), adapted from García (2004). This reduced percentage of organic matter in artificial soil (from 10% to 5%) has been proposed by OECD (2016) for being more representative of natural soils. The pH of the prepared soil was adjusted to 6.0 ± 0.5 adding $CaCO_3$. Moisture was adjusted to 50% of the water holding capacity at the beginning of the tests, adding distilled water and taking into account the volume of contaminant solution to be added.

2.3 CHEMICALS

Ally® (600 $g.kg^{-1}$ metsulfuron-methyl), marketed in Brazil by Du Pont do Brasil S.A, is a water dispersible granule herbicide with recommendations to application in pre-emergence in sugarcane and post-emergence in rice, oat, wheat, coffee and barley to broadleaf weed control. An aqueous solution of 3 $g.L^{-1}$ of Ally® was prepared for soil contamination. For each test, a range of concentrations plus a negative control (soil with distilled water) was set up (Table 1).

Assist® (756 $g.L^{-1}$ mineral oil) from BASF S.A. was used as adjuvant in a proportion of 0.5% v/v (5mL of adjuvant for 1L of water) following label recommendation.

The test chemicals were added into the premoistened soil (until 50% of the water holding capacity), homogeneous mixed, and introduced into the test vessels. TAS moistened with distilled water was used in all tests as control soil.

In order to estimate field concentration of Ally®, the equation described by Jansch et al (2006) was used:

$$MC5 = \frac{\frac{F.D}{\Delta z}}{p}$$

Where:

MC5 = the maximum concentration of pesticide in the top 5 cm of soil ($mg.kg^{-1}$)

F = factor for conversion from $kg.ha^{-1}$ to $mg.m^{2-1}$

D = the nominal treatment (the application concentration in $kg.ha^{-1}$)

Δz = the layer thickness (0.05m)

p = dry bulk density ($kg.m^{3-1}$)

According to EPPO (2003), the top 5 cm of the soil is the relevant exposure depth to soil invertebrates and a standard soil has a bulk density of 1,500 $kg.m^{3-1}$. The nominal treatment considered in this calculation was the highest label recommended dose of Ally® (18 g a.i. ha^{-1}) which is a pre- emergence concentration for sugarcane. The FPD was calculated in 0.03 $mg.kg^{-1}$.

Table 1. Summary of the tests performed with Ally® (metsulfuron-methyl) with and without the adjuvant Assist® (mineral oil in a mixture of 0.5% v/v)

Organism	Endpoint	Contaminants	Concentration range (mg a.i. kg^{-1})
E. andrei	Avoidance	Ally® + Assist®	0; 0.59; 1.17; 2.34; 4.69; 9.38; 18.75; 37.5; 75; 150; 300
	Avoidance	Ally®	0; 9.38; 18.75; 37.5; 75; 150; 300
	Lethality	Ally® + Assist®	0; 18.75; 37.5; 75; 150; 300
	Lethality	Ally®	0; 18.75; 37.5; 75; 125; 150; 300
F. candida	Avoidance	Ally® + Assist®	0; 0.07; 0.15; 0.29; 0.59; 1.17; 2.34; 4.69; 9.38; 18.75; 37.5; 75; 150; 300
	Avoidance	Ally®	0; 9.38; 18.75; 37.5; 75; 150; 300

2.4 LETHALITY TESTS

Lethality tests with earthworms were carried out with four replicates and lasted 14 days following the guidelines of ISO 11268-1 (ISO, 2012). Ten clitellate earthworms were placed in each test vessels (200 cm^3) containing 350 g (wet weight; ww) of contaminated or control TAS. The adjuvant was also tested alone. Test vessels were covered with perforated plastic lids to allow aeration, and moisture was adjusted at day 7 by weighting the replicates and replacing the water loss by adding drops of distilled water. Survival was recorded at day 14 by removing and counting the living earthworms.

2.5 AVOIDANCE TESTS

Avoidance tests were carried out with five replicates following ISO 17512-1 (ISO, 2008) for earthworms. Test vessels (lenght, 22 cm; width, 14 cm; height, 7 cm) were divided into two sections with a removable plastic divider, one side received 300 g (ww) of control soil, and the other one received 300 g (ww) of contaminated soil. The divider was removed, and 10 earthworms were placed in the center of the vessel. After 48 h of incubation, the divider was reinserted and the number of earthworms in each compartment was recorded.

The avoidance test with collembolans followed ISO guideline 17512-2 (ISO, 2011) with similar procedures as for earthworms' test, but using test vessels of 125 mL capacity. The amount of soil was 30 g (ww) in each side and the number of organisms per replicate was 20. At the end of the test, the number of organisms in each side of the test vessel was recorded using water and stamp ink.

The random distribution of organisms in the absence of contamination was confirmed by dual control tests, performed with the same methodology described above, but receiving control soil in both sides of the test recipients. Additionally, a test was also conducted with the adjuvant Assist®, applied in one side of the recipient.

2.6 DATA ANALYSIS

Results obtained in the avoidance tests were analyzed by Fisher exact test ($p<0.05$). The null hypothesis assumes that 50% of test organisms stay in the test soil and that no organisms leave that section (non-avoidance) (NATAL-DA-LUZ et al., 2004).

3 RESULTS AND DISCUSSION

3.1 LETHALITY TESTS

After 14 days of exposure, lethality was <10% in all treatments. Lethality ranged between 0 and 3.3% ± 0.58% when Ally® was applied alone and between 0 and 17% ± 2.08% when Ally® was applied combined with Assist®. Results showed no effects of metsulfuron-methyl on earthworm's lethality even at high concentrations, equivalent to 10,000 times the recommended doses for oat desiccation.

Acute lethality tests with earthworms are considered less sensitive than reproduction or avoidance tests. For this reason, this test is no longer required for pesticide's risk assessment in European Union (EFSA, 2007). However, Brazilian legislation considers only earthworms' lethality as soil invertebrates' protection endpoint to risk assessment of new pesticides (IBAMA, 1996; NIVA et al., 2016).

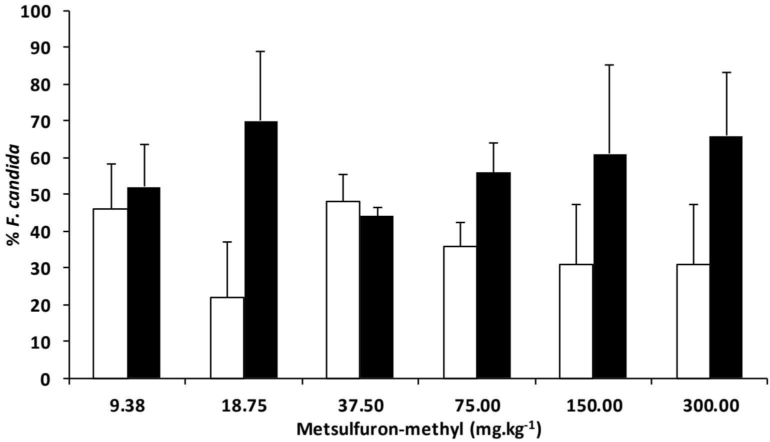
3.2 AVOIDANCE TESTS

After 48h of exposure, all the validity criteria were fulfilled for collembolans and earthworms.

3.2.1 Collembolans

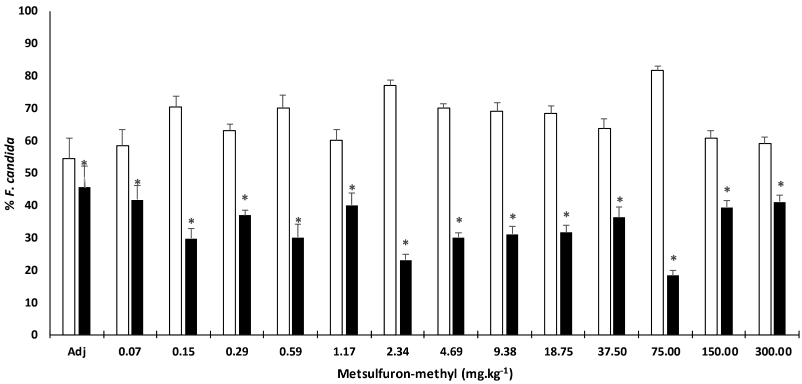
Collembolans showed non-avoidance behavior when exposed to metsulfuron-methyl, even at the highest tested concentration (300 $mg.kg^{-1}$) (Fig. 1). However, when adjuvant was added, avoidance behavior was observed in all tested concentration, reaching field predicted concentration (0.03 $mg.kg^{-1}$), calculated according to Jänsch et al. (2006). The observed avoidance behavior can be related to the mixture of Ally® and Assist®, given its negative effects on *F. candida* when applied alone (Fig.2). Unsurprisingly, avoidance behavior was similar in all tested concentration when Assist® was added, possibly because its addition is based on water volume instead of product volume; therefore, the concentration of the adjuvant was the same in the tested solutions.

Fig. 1 - Avoidance tests with *F. candida*: Percentage of organisms on metsulfuron-methyl contaminated soil (black bars) against the control (white bars). No statistical difference was recorded.



Also used as biocides, based-oil adjuvants can be the cause of the recorded avoidance behaviour. Assist® label recommends its use as insecticide and acaricide in addition to its adjuvant properties. Martins et al. (2013) reported that when collembolans are exposed to oil, an impermeable film is formed enveloping its body and inhibiting breathing. Also, in the presence of amphipathic substances their physiology may be affected. Furthermore, an avoidance response can be related to a complex of sensory "hairs" and receptors that collembola have in their antenna which contain receptive cells to chemical and mechanical stimuli (HOPKIN, 1997).

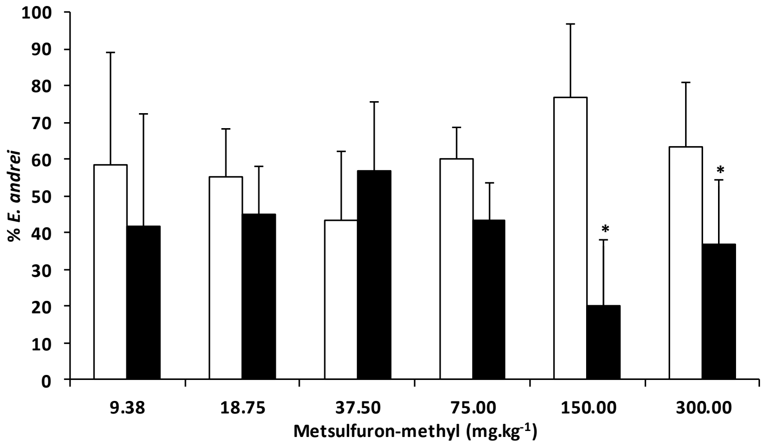
Fig. 2 - Avoidance tests with *F. candida*. Percentage of organisms on soil contaminated with metsulfuron-methyl + adjuvant (black bars) against the control (white bars). Asterisks indicate statistical differences ($p<0.05$). Adj = control where Assist® was applied



3.2.2 Earthworms

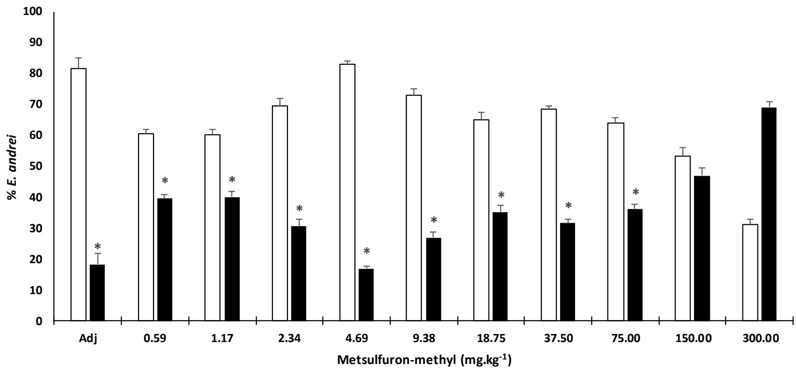
Earthworms showed an avoidance behavior in the two highest concentrations of metsulfuron-methyl (150 and 300 $mg.kg^{-1}$) when Ally® was tested alone (Fig. 3). On the other hand, when adjuvant was added, avoidance behavior was recorded from 0.59 $mg.kg^{-1}$ to 75 $mg.kg^{-1}$ (Fig. 4). The exposure of earthworms to Assist® alone confirmed that the avoidance response was caused by the adjuvant. The addition of Assist® to Ally® generated a bell-shaped avoidance response curve as concentration increased, reaching a peak at the concentration of 4.69 $mg.kg^{-1}$. Gradually, avoidance decreased until there is non-avoidance behavior in the concentrations of 150 and 300 $mg.kg^{-1}$, probably due to neurotoxic effects (Fig. 4).

Fig. 3 - Avoidance tests with *E. andrei*. Percentage of organisms on metsulfuron-methyl contaminated soil (black bars) against the control (white bars). *Asterisks indicate statistical differences (p<0.05).



Considering that adjuvant addition is proportional to water volume, it was expected that all the concentration range tested presents avoidance behavior. Loss of avoidance behavior with the increase of the active ingredient concentration can occurs when organisms are exposed to acetylcholinesterase (AChE) inhibitors. The function of this enzyme is extremely important for several physiological processes, such as predator evasion and orientation toward food (MIRON et al., 2005). Organophosphates and carbamates are able to specifically inhibit the enzymatic activity of AChE but various environmental chemicals are found to impair AChE function (XIE et al., 2016). Its inhibition in brain or muscle tissues can lead to adverse effects in movement (FERNÁNDEZ-VEGA et al., 2002). Miron et al. (2005) studying the effects of metsulfuron-methyl in silver catfish (*Rhamdia quelen*) showed that AChE activity, in concentrations of 400 to 1200 mg.L^{-1}, in muscle tissue, was inhibited from 47 to 56% when compared to control. Neurotoxic effects of sulfonylurea herbicides in fishes were also observed with nicosulfuron in goldfish (*Carassius auratus*), where AChE had its activity inhibited 9.8% in fishes' brain when submitted to 500 µg.L^{-1} (BRETAUD et al., 2000). Caselli et al. (2006) characterized AChE in *E. andrei*, confirming the usefulness and sensitivity of this species to soil contamination, indicating that it can act as sentinel to pesticide contamination.

Fig. 4 - Avoidance tests with *E. andrei*. Percentage of organisms on soil contaminated with metsulfuron-methyl + adjuvant (black bars) against the control (white bars). *Asterisks indicate statistical differences ($p<0.05$). Adj = control where Assist® was applied



Active ingredients may have their toxicity increased when adjuvants are added to formulated products (Marques et al. 2009). Adjuvant-dependent toxicities have been reported for bacteria (NOBELS et al., 2011), cyanobacteria (CACQUET et al., 2005; MA et al., 2005), algae (MA et al., 2004; CACQUET et al., 2005), snails (COUTELLEC et al., 2008) and earthworms (MARQUES et al., 2009). The information available in the literature and the results obtained in this study can help policy-makers in elaborating guidelines for environmental risk assessment for pesticides.

4 CONCLUSIONS

Results indicate that the use of earthworms' acute lethality as the only test to assess soil contamination may underestimate the effects of contaminants on soil macrofauna.

Metsulfuron-methyl based herbicide applied alone caused avoidance behavior only at high concentrations. However, adjuvant addition changed the effects to soil invertebrates, which avoided the herbicide even at predicted field doses. When collembolans were exposed to adjuvant alone, an avoidance behavior was observed, which can be explained by the insecticide effects of the mineral oil.

Adjuvant addition should be considered in risk assessment of pesticides, once this product changes the expected effects of herbicide applied alone. Currently, effects of addition are not considered in products regulation. Furthermore, comparison among adjuvant toxicity is important to choose the less toxic products for soil ecosystem.

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CHAPTER III

LABORATORY AND FIELD TESTS FOR RISK ASSESSMENT OF METSULFURON-METHYL-BASED HERBICIDES FOR SOIL FAUNA

Based on:
DE SANTO, F.B.; GUERRA, N.; VIANNA, M.S.; TORRES, J.P.M.; MARCHIORO, C.A.; NIEMEYER, J.C. Laboratory and field tests for risk assessment of metsulfuron-methyl-based herbicides for soil fauna.

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ABSTRACT

Metsulfuron-methyl is one of the sulfonylurea herbicides most used, being applied alone in pre-emergence and with an adjuvant in post-emergence. Ecotoxicity tests were applied to assess the effects of pesticides and mixtures under laboratory conditions, but they could not outline the effects of organisms' exposure in field, being necessary an assessment under local conditions to contextualize it ecologically. Considering the differences between field and laboratory, and the lack of data on the effects of metsulfuron-methyl in natural soils, this study performed a set of tests under laboratory and field conditions to assess the ecotoxicity of this herbicide applied alone, combined with mineral oil and the mineral oil applied alone, providing information on the effects and differences between tests performed under different conditions. Reproduction tests with four non-target organisms were performed in laboratory while two experiments were performed in field evaluating avoidance behaviour, feeding activity, mesofauna abundance and pesticide residual. Laboratory results showed that metsulfuron-methyl alone is not a threat to soil fauna. However, the presence of mineral oil showed ecotoxicity to Eisenia andrei, Enchytraeus crypticus and Proisotoma minuta on laboratory tests, because the adjuvant itself presented ecotoxicity. Field evaluations indicated that metsulfuron-methyl do not cause impairment on soil fauna feeding activity. Considering that under field conditions where the mixture was applied, reduction and possible loss of native communities, especially due to soil management system was noticed Results showed that extrapolations between laboratory and field are necessary to better understanding of soil fauna behaviour, and move forward on pesticides mixture assessment.

KEYWORDS: avoidance behaviour tests, herbicide, pesticides mixture, reproduction tests.

5 INTRODUCTION

The increase in global demand for food (TILMAN et al., 2011) leads greater use of pesticides to control insect pests, weeds and plant diseases (WANG et al., 2016). Since 2008, Brazil has been leading the ranking of pesticide consumption (REBELO et al., 2010) with herbicides comprising more than 50% of all sales (IBAMA, 2016). Sulfonylureas chemical group was introduced by Dupont Corporation in 1982 and its use increased dramatically (HE et al., 2006; GHOBADI et al., 2015) probably because of its high levels of activity even when applied in low doses (OLIVEIRA Jr., 2011). One of the most used herbicides belonging to the sulfonylurea family is metsulfuron-methyl, a common active ingredient used to control a large variety of annual grasses and broad-leaved weeds in pre and post-emergence application (CASTRO et al., 2002; NELEMANS et al., 2017), where it is commonly applied combined with mineral oil (as adjuvant) to increase its efficiency (DE SANTO et al., 2018). Despite the studies demonstrating that pesticides associations may result in higher toxicity to environment when compared to active ingredients or commercial products applied alone (CAQUET et al., 2005; SURGAN,et al., 2010; DE SANTO et al., 2018), these associations are not considered in regulatory risk assessment schemes of pesticides (EFSA *Draft*, 2017). As their use increased rapidly (GIANESSI, 2013), the concerns about soil contamination by indiscriminate use of pesticides, which are known to affect soil flora and fauna (SINGH; SINGH, 2015; WANG et al., 2016) increase as well. Therefore, it is necessary to obtain information about the potential effects of pesticides in soil, specially to soil fauna, to complement conventional chemical analysis (REINECKE; REINECKE, 2007; RASTETTER; GERHARDT, 2018).

Earthworms, collembolans and enchytraeids are commonly found in a wide range of soils across the world (CICCONARDI et al., 2013; AMOSSÉ et al., 2018) which make them the most used non-target macro and mesofauna representatives under laboratory conditions (AMORIM et al., 2012; PELOSI et al., 2014). Earthworms (Annelida: Oligochaeta) are responsible for a large fraction of the soil biomass, playing an important role in the soil ecosystem functioning, and considered ecosystem engineers because of their action on soil structure, consequently promoting microorganisms’ activity and influencing nutrient cycling (LAVELLE, 2014; PELOSI et al., 2014; DIGNAC et al., 2017). Collembolans (Arthropoda: Hexapoda) are considered a key group in soil ecosystem, inhabiting various organic substrates, using a wide range of food sources and being the food chain basis for other species. They act as nutrient cycling catalysers and change the soil structure through litter comminution, casting and other mechanisms (DOMENE et al., 2010; POTAPOV et al., 2016; D'ANNIBALE et al., 2017). Enchytraeids (Annelida: Oligochaeta) can occur in very high densities, up to several hundred thousand individuals per square meter and are classified as "biological regulators" (TURBÉ et al., 2010; RÖMBKE et al., 2017) because of their role on soil quality maintenance, including nutrient cycling, decomposition of organic matter, stress indicators, and maintaining soil structure and porosity (LAAKSO; SETÄLÄ, 1999; JÄNSCH et al., 2005; RÖMBKE et al., 2017).

Ecotoxicity tests have been applied to assess the effects of pesticides and mixtures in soil ecosystem (CHELINHO et al., 2014; RÖMBKE et al., 2017). Under laboratory conditions, single-species tests (Cairns 1983) can provide appropriate and useful information about effective concentration (VERSTEEG et al., 1999) from acute (avoidance and lethality) and chronic endpoints (reproduction) (ISO, 2008, 2011, 2012a, 2012b, 2014a, 2014b). Furthermore, acute endpoints can be used as screening tests to evaluate soil quality (NATAL-DA-LUZ et al., 2004; ISO, 2008, 2011, 2012a, 2014a, 2014b; BRAMI et al., 2017), but long-term assessments like chronic endpoint or field evaluations should be used to complement risk assessment of pesticides application to soil fauna. However, laboratory tests cannot outline the effects of organisms' exposure in-field, especially regarding their ecosystem interactions, since they focus only on pre-established patterns for those species (RÖMBKE et al., 2017). So, it is necessary to consider that soils are sources of a wide diversity of essential ecosystem services and ecosystem functions (MILLENNIUM ECOSYSTEM ASSESSMENT, 2005) and have components that can influence pesticides effects (KÖRDEL; RÖMBKE, 2001). An assessment under local conditions is useful to reduce uncertainties, complementing laboratory data and to contextualize it ecologically (CASABÉ et al., 2007).

Considering the differences between field and laboratory tests, as well as the lack of data on the effects of the herbicide metsulfuron-methyl in natural soils, this study conducted a set of tests under laboratory and field conditions to assess the effects of the herbicide applied alone and combined with mineral oil, and the mineral oil applied alone on soil fauna in order to provide information about the effects and differences between tests performed under different conditions and using different species.

6 MATERIAL AND METHODS

6.1 TEST ORGANISMS

Earthworms of the species *Eisenia andrei* Bouché, 1972*,* collembolans of the species *Folsomia candida* Willem 1902 and *Proisotoma minuta* (Tullberg, 1871), and enchytraeids of the species *Enchytraeus crypticus* Westheide & Graefe, 1992 were obtained from laboratory cultures, maintained under photoperiod of 12 h light: 12 h dark and temperature of 20 ± 1°C.

Earthworms were cultured according to ISO guideline 11268-2 (ISO, 2012b) in plastic boxes of 11 L of capacity, in a mixture of cattle dung free of antibiotics and coconut dust (1:1, v:v), moistened with distilled water, and fed weekly with cooked oat. Both species of collembolans were cultured according to ISO 11267 (ISO, 2014a) in culture vessels lined with a mixture of plaster of Paris and activated charcoal in a ratio of 10:1, fed twice a week with granulated dry yeast *Saccharomyces cerevisiae*. Enchytraeids were cultured according to ISO 16387 (ISO, 2014b) in culture vessels with moistened Tropical Artificial Soil (TAS), fed twice a week with oat flour.

Synchronized organisms were used in the tests: *E. andrei* (2 – 12 months old; 250-600 mg weight and clitellated), *F. candida* and *P. minuta* (10-12 d old for reproduction; 60 d old adults for avoidance and lethality); and *E. crypticus* (clitellated adults with similar size).

6.2 LABORATORY SPIKING AND CHEMICALS

Laboratory experiments were carried out using tropical artificial soil (TAS) composed by 75% fine sand (washed and dried), 20% kaolin clay and 5% coir dust (dried at 60°C), adapted from García (2004). This reduced percentage of organic matter in artificial soil (from 10% to 5%) has been proposed by OECD (2016) for being more representative of natural soils. The pH of the prepared soil was adjusted to 6.0 ± 0.5 adding CaCO3. Moisture was adjusted to 50% of the water holding capacity at the beginning of the tests, adding distilled water and considering the volume of contaminant solution to be added.

Ally® (600 $g.kg^{-1}$ metsulfuron-methyl) was used as a commercial formulation. This product is recommended for application in pre-emergence of sugarcane and post-emergence of rice, oat, wheat, coffee and barley to broadleaf weed control. An aqueous solution of 3 $g.L^{-1}$ of Ally® was prepared for soil contamination. For each test, a range of concentrations plus a negative control (soil with distilled water) was set up (Table 1). To simulate post-emergence scenario, Assist® (756 $g.L^{-1}$ mineral oil) marketed in Brazil by BASF S.A. was used as adjuvant in a proportion of 0.5% v/v (5mL of adjuvant for 1L of water) following label recommendation.

The predicted environmental concentration (PEC) was calculated assuming the highest label recommended dose of Ally® (18 g a.i. ha^{-1}), a soil density of 1.5 g cm^{-3} and a depth of incorporation in the soil profile of 0–5 cm. The software ESCAPE Version 2.0 (Estimation of Soil-Concentrations After Pesticide Applications) was used (KLEIN, 2008).

Table 1. Summary of the tests performed with Ally® (metsulfuron-methyl) with and without the adjuvant Assist® (mineral oil in a mixture of 0.5% v/v)

Organism	Contaminant	Concentration (mg a.i. kg^{-1})
Eisenia andrei	Ally®	0; 0.03; 0.3; 3
	Ally® + Assist®	0; 0.03; 0.3; 3; 6; 12
	Ally® + Assist®	0; 18.75; 37.5; 75; 150; 300
Folsomia candida	Ally®	0; 300
	Ally® + Assist®	0; 18.75; 37.5; 75; 150; 300
Enchytraeus crypticus	Ally®	0; 0.01; 0.1; 1; 10; 33.33
	Ally® + Assist®	0; 0.01; 0.1; 1; 10; 33.33
Proisotoma minuta	Ally®	0; 10
	Ally® + Assist®	0; 0.01; 0.1; 1; 10

6.3 REPRODUCTION TESTS

Reproduction tests with collembolans were performed with *F. candida* and *P. minuta*. The tests for each species were carried out following the ISO guideline 11267 (ISO, 2014a). Ten juveniles were kept in recipients containing 30 g of contaminated or control soil, with five replicates per treatment. Organisms were fed with approximately 2 mg of dry yeast at days 1 and 14. Twice a week the recipients were opened allowing aeration. Drops of distilled water were added to replace water loss weekly, according to weight loss of the replicates. At day 28, recipients were filled up with water and some drops of stamp ink, carefully stirring, and photographed to after counting of floating juveniles at the water surface. Counting was carried out using the software ImageJ (SCHNEIDER et al., 2012).

Reproduction tests with *E. andrei* were carried out following the ISO guideline 11268-2 (ISO, 2012b). Ten clitellated earthworms were put into test vessels containing 300 g of contaminated or control soil, with four replicates per treatment. Plastic perforated lids were used to allow aeration. Cow dung free of antibiotics (5 g, dry and ground) was added weekly as food supply as well as drops of distilled water to replace water loss, according to weight loss of each replicate. At day 28, adults were removed, leaving cocoons to hatch by additional four weeks. At day 56, juveniles were counted using hot extraction by immersing the test vessels in water bath at 60°C, forcing the juveniles to come up to soil surface.

Reproduction tests with *E. crypticus* were carried out following ISO 16387 (ISO, 2014b). Ten clitellated organisms were put into test vessels containing 30 g of contaminated or control soil, using five replicates per treatment. Organisms were fed with oat flour once a week when test vessels were opened allowing aeration. Drops of distilled water were added to replace water loss. At day 28, alcohol (70%) was added until the soil was fully covered by the solvent, adding some drops of bengal rose to preserve and colour the organisms. After a period of 48 h, the organisms were counted using stereomicroscope (60x).

6.4 FIELD TRIALS DESIGN

To evaluate the ecotoxicity of the herbicide metsulfuron-methyl under field conditions, two experiments were performed on the Experimental Farm of the Federal University of Santa Catarina (UFSC), in Curitibanos, Santa Catarina State, Southern Brazil. (-27.274049º Lat and -50.502828º Long). The soil is classified as haplic cambisol (4.4% organic matter, clay texture) and climate type is Cfb, with mean temperatures reaching 4 ºC in the colder month and less than 22 °C in the warmer month and annual precipitation around 1.400 mm. Temperature and rainfall were provided by the Meteorological Station located in the Experimental Farm, and managed by the Information Center for Environmental Resources and Hydrometeorology of Santa Catarina.

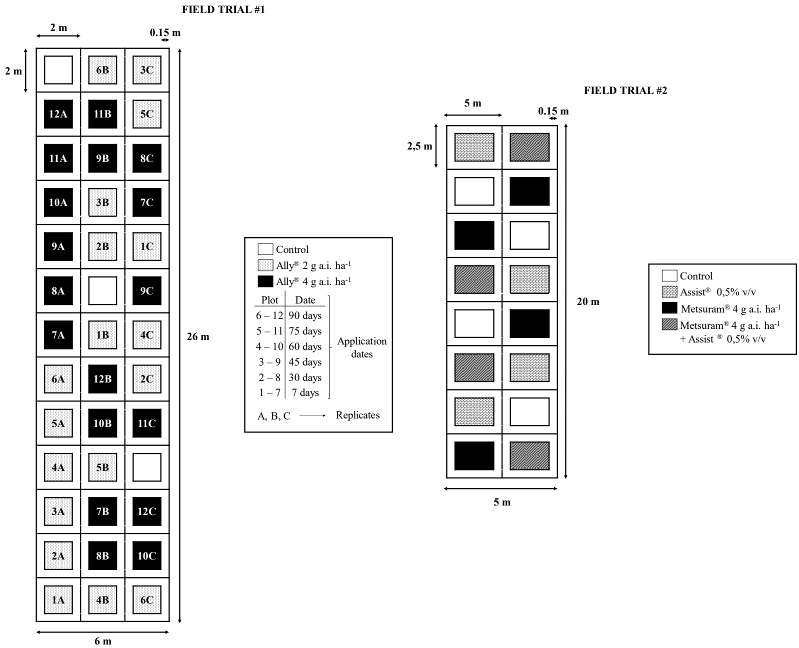
The first experiment, named Field Trial #1 (Fig. 1), applied the commercial product Ally® (600 $g.L^{-1}$ metsulfuron-methyl) commercialized in Brazil by DuPont do Brasil S.A. as a water-dispersible granular herbicide alone on black oat (*Avena strigosa*), simulating a pre-emergence condition. The trial was carried out from July to October 2017 with six dates of applications (27/07, 11/08, 27/08, 11/09, 25/09 and 17/10/2017) named 90 days, 75 days, 60 days, 45 days, 30 days and 7 days, representing the time after the herbicide application. The applied doses were the minimum (2 g a.i. ha^{-1}) and the maximum (4 g a.i. ha^{-1}) recommended for black oat. This experiment was performed in a randomized block design with thirteen treatments (six for the maximum dose, six for the minimum and one for control) with three replicates for each one (Fig. 1). The plots were 4m² in size.

The second experiment, named Field Trial #2 (Fig. 1) was performed in august 2018 to complement the data found on Field Trial #1. Metsuram® 600 WG (600 $g.L^{-1}$ metsulfuron-methyl) was used as commercial product from Rotam do Brasil Agroquímica Produtos Agrícolas Ltda. as a water-dispersible granular herbicide, and Assist® (756 $g.L^{-1}$ mineral oil) from BASF S.A was used as adjuvant. Applied doses were 4 $g.ha^{-1}$ for metsulfuron-methyl and 0.5% v/v for mineral oil. The experiment was performed in plots sized 12.5 m² in randomized blocks with four treatments and three replicates:

- Met = Metsuram® 600 WG (metsulfuron-methyl);
- Ass = Assist® (mineral oil);
- Met + Ass = Metsuram® 600 WG (metsulfuron-methyl) + Assist® (mineral oil);
- Control = no application

A pressurized sprayer with CO_2 was used for pesticides application. Such equipment contained a 2.0 m long bar with four sprays tips MAGNO 110015-BD interspaced 50 cm from each other at a distance of 50 cm of the cultivation, kept at a work pressure of 37.5 kgf.cm² and speed of 3.6 km h^{-1}, providing an application volume of 200 $L.ha^{-1}$ on Field Trial #1 and 150 $L.ha^{-1}$ on Field Trial #2.

Fig. 1. Experimental design of Field Trial #1 performed in 2017 and Field Trial #2 performed in 2018



6.5 BAIT LAMINA TEST

This test standardized by ISO 18311 (ISO, 2016) consists on inserting 16-hole-bearing plastic strips vertically, filled with 70% cellulose, 25% wheat flour and 5% activated charcoal, aiming to determine site-specific soil fauna feeding activity (RÖMBKE et al., 2006).

On Field Trial #1, the baits were exposed in field eight days after the last herbicide application (10/25/2017). On Field Trial #2, baits were exposed seven days after the last herbicide application (08/13/2018). Both tests design consisted on three groups of five baits per plot with three plots as replicates on Field Trial #1 and four replicates on Field Trial #2 (shown in 2.4). The baits remained on field during 40 d according to recommendations to this mesoregion (NIEMEYER et al., 2018). The percentage of consumption was determined by recording the number of empty holes (>30% of bait consumed).

6.6 MESOFAUNA EXTRACTION

On Field Trial #1, a composite sample for each treatment was collected by three withdrawals with stainless steel rings of 94 cm³ (5.2 cm height x 4.8 cm width) at soil surface. The composed sample was placed in plastic bags and transported to the laboratory. The bags were not completely closed to allow gas exchange.

The mesofauna extraction started at the same day of sampling using an alternative Berlese-Tüllgren funnels that consists on creating a gradient of heat/dryness at soil surface (ANDERSON; INGRAM, 1993). Plastic funnels with a mesh to retain the soil were put on a collector tube containing alcohol 70% (v/v) to collect the organisms. The funnels were exposed to lamps of 40W during 7 d, forcing the edaphic organisms to go down into the collector tube. The extracted organisms were identified at the Order level using a stereomicroscope (40x). .

6.7 AVOIDANCE BEHAVIOUR TESTS WITH FIELD SOIL

Avoidance tests were carried out with field soil following ISO 17512-1 (ISO, 2008) for earthworms of the species *E. andrei*, and ISO 17512-2 (ISO, 2011) for collembolans of the species *F. candida*.

Tests were performed with soil samples obtained from Field Trial #1 using paired combinations between control soil *versus* soils obtained from treatments Control, 7 days, and 90 days comprising both minimum and maximum recommended doses applied in the field (see topic 2.4) All tests were performed with five replicates.

Tests with earthworms were carried out using test vessels (200 cm³) divided into two equal sections with a removable plastic card, where one side received soil from control (300 g wet weight) and the other side the contaminated soil (300 g ww). A dual control test using control soil in both sides was carried out to check the random distribution of organisms when no contamination is present (ISO, 2008). After putting the soil, the card was removed, and 10 clitelated earthworms were placed in the centre of the recipient. The tests were incubated for 48 h at 20 ºC ± 1 with a photoperiod of 12h:12h (light:dark), after that, the plastic card was reinserted and the number of earthworms in each compartment was counted.

Avoidance tests with collembolans were performed similarly as the earthworms, differentiating in the recipient size (125 cm³), soil content (30 g in each side) and in number of organisms (20 instead of 10). At the end of the test, the number of organisms in each side of the test recipient was recorded using water and stamp ink, following instructions described in the ISO guideline (ISO, 2011).

To calculate and express Avoidance (A) as percentage, the formula $A = ((C - T)/N) \times 100$ was used, where C is the number of organisms in control soil, T is the number of organisms in the contaminated soil, and N is the total number of organisms at the end of the test. If more than one earthworm per test vessel (10%) were dead or missing, the test was invalidated. The number of dead or missing collembolans per test vessel to invalidate the test was four (20%).

6.8 CHEMICAL ANALYSIS OF METSULFURON-METHYL IN SOIL

Ten days after the last herbicide application on Field Trial #1 (October 28) the soil was collected from each treatment at 0-20 cm of depth, frozen and sent to the Laboratory of Toxicology at the Federal University of Rio de Janeiro (UFRJ) to determinate the chemical residual of metsulfuron-methyl.

Using 125mL Erlenmeyers, 15g of mass from each sample were added to 40mL of phosphate buffered saline (PBS) and centrifuged. The soil extracts were taken out by shaking with dichloromethane for 1 hour after centrifugation and separation of the supernatant and stored in a glass container at -20°C. The extraction was developed with light protection. Before chromatographic analysis, the extracts were subjected to total solvent evaporation and resuspension with acetonitrile. The soil samples from the control plot followed the same extraction procedure.

To determine metsulfuron-methyl, the extracts from the soil samples were analyzed by high performance liquid chromatography. The analytical curve was constructed from duplicate injections of standard metsulfuron solution at the following concentrations: 0.5 $\mu g.mL^{-1}$; 1.0 $\mu g.mL^{-1}$; 2.5 $\mu g.mL^{-1}$; 5.0 $\mu g.mL^{-1}$; 10 $\mu g.mL^{-1}$; 20 $\mu g.mL^{-1}$.

For the chromatographic measurements was used Agilent 110 Series HPLC with quaternary pump and diode array detector (HPLC-DAD). For the separation of metsulfuron-methyl, Zorbax® SB-C18 column 4.6 mm i.d. x 150 mm (3.5 µm particle size) was used under the following elution conditions: mobile phase composed of acetonitrile (acidified water under flow 1 $mL.min^{-1}$). A volume of 20 µL was injected (automatic injection) and the herbicide detection performed at 230 nm. All samples were filtered prior to injection into 0.22 µm mesh membrane.

6.9 STATISTICAL ANALYSIS

Statistical analysis, except Fisher's Exact test, were carried out using the software Statistica 13.0 (DELL INC., 2015) and R Studio (R CORE TEAM, 2017).

Results obtained in avoidance tests were analyzed with Fisher's Exact test ($p<0.05$) comparing the expected distribution (random) with the observed distribution in each test (NATAL-DA-LUZ et al., 2004).

Analysis of Variance (ANOVA) was used to assess differences among treatments followed by Dunnett test ($p < 0.05$), comparing each concentration versus control soil. Normal distribution of data and homogeneity of variance were verified by Shapiro Wilk's test and Bartlett test, respectively.

Median effective concentrations (EC_{50}) reducing 50% of reproduction were estimated using non-linear regressions, according to Environmental Canada (2007). The best fitting model was applied according to Chelinho et al. (2014).

Feeding activity in bait-lamina was analysed using ANOVA. Normal distribution was checked by Shapiro Wilk's test and Kurtosis, while homogeneity of variance was checked by the Levene's test. Dunnett's post-hoc test was used for multiple-comparisons among treatments ($p<0.05$). Two-way ANOVA, interaction, contrast, Dunnet and Tukey post-hoc tests were used in order to evaluate possible differences among treatments in soil depth.

Abundance and richness values for mesofauna groups were presented for all treatments and control in a qualitative form.

7 RESULTS AND DISCUSSION

7.1 REPRODUCTION TESTS

Results showed differences in the sensitivity of non-target organisms when exposed to Ally® alone and Ally+Assist® (Table 2). While the collembolan species *F. candida* presented no response to both treatments, another collembola species, *P. minuta* appear to be most sensitive to mineral oil presence, showing an $EC_{50} = 0.003$ mg.kg^{-1} (0 – 0.0010). However, Buch et al. (2016) evaluated the ecotoxicity of mercury and found lower values of EC_{50} to *F. candida* than to *P. minuta,* showing that the sensitivity of each species is dependent of the substance or mixture to which they are exposed (Baas and Kooijman, 2015).

Table 2. Median effective concentration of Ally® (metsulfuron-methyl) with and without the adjuvant Assist® (mineral oil in a mixture of 0.5% v/v) to *E. andrei, F. candida, E. crypticus* and *P. minuta* in reproduction tests with Tropical Artificial Soil (5% M.O.)

Organism	**Contaminants**	**EC_X value ($mg.kg^{-1}$)**	**Model fitting**
Eisenia andrei	Ally®	-	-
	Ally® + Assist®	EC_{20} = 0.009 (0 – 0.026)	Exponential
	Ally® + Assist®	EC_{50} = 10.74 (4.84 – 16.64)	Exponential
Folsomia candida	Ally®	EC_{50} > 300	-
	Ally® + Assist®	EC_{50} > 300	-
Enchytraeus crypticus	Ally®	EC_{50} > 33.33	-
	Ally® + Assist®	EC_{50} = 5.73 (1.76 – 13.23)	Gompertz
Proisotoma minuta	Ally®	EC_{50} > 10	-
	Ally® + Assist®	EC_{50} = 0.003 (0 – 0.010)	Exponential

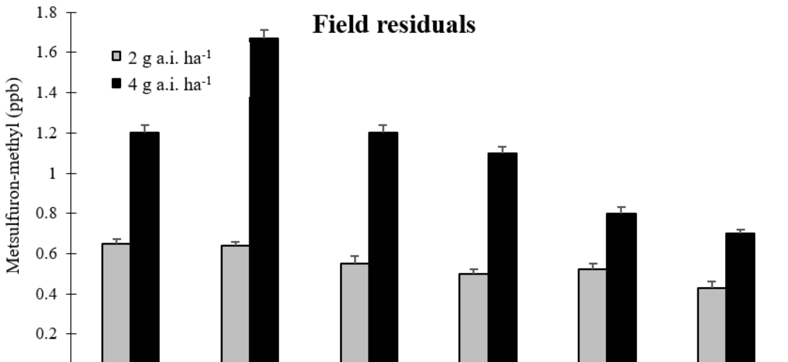
Both oligochaetes species (*E. andrei* and *E. crypticus*) showed similar EC_{50} values when exposed to Ally®+Assist® with *E. andrei* presenting an EC_{50} = 10.74 $mg.kg^{-1}$ (4.84 – 16.64) and *E. crypticus* an EC_{50} = 5.73 (1.76 – 13.23). Considering that ecotoxicological studies mainly focuses on earthworms to assess the ecotoxicity of the compounds (ALVES et al., 2013; PELOSI et al., 2014; LI et al., 2018; GE et al., 2018) this EC_{50} values show the need to widen the scope and understand the ecotoxicological effects (PRINCZ et al., 2018) of pesticides to other species like collembolans (AMORIM et al., 2005; SANTOS et al., 2010; NIEMEYER et al., 2018) and enchytraeids (RÖMBKE, 2003; NOVAIS et al., 2012; RÖMBKE et al., 2017).

Brazilian policy only evaluates earthworms' acute toxicity on pesticides risk assessment scheme (IBAMA, 1996), despite being considered an unresponsive endpoint and withdrawn from EU scope (EFSA, 2007). Studies assessing pesticides ecotoxicity in Brazil (CHELINHO et al., 2012; ALVES et al., 2013; NIVA et al., 2016; NIEMEYER et al., 2018; DE MENEZES-OLIVEIRA et al., 2018) have already shown the need to re-evaluate this policy changing the requested endpoint and adding other soil organisms to pesticide risk assessment.

7.2 CHEMICAL ANALYSIS

Plots treated with 2 $g.ha^{-1}$ of metsulfuron-methyl presented a concentration average of 0.65 ± 0.02 ppb in the 0-20 cm soil layer 7 days after application, while plots treated with 4 $g.ha^{-1}$ of metsulfuron-methyl presented 1.20 ± 0.04 ppb. Thirty days after application, plots sprayed with 2 $g.ha^{-1}$ decayed to 0.64 ± 0.02 ppb, and plots with 4 $g.ha^{-1}$ presented their highest levels of metsulfuron-methyl residues: 1.67 ± 0.04 ppb. After 45 days, both treatments showed a decrease in the residual concentrations, which remained similar until the 90 d plot. For 2 $g.ha^{-1}$: 0.55 ± 0.04 ppb (45d); 0.50 ± 0.02 ppb (60d); 0.52 ± 0.03 ppb (75d); 0.43 ± 0.03 ppb (90d). For 4 $g.ha^{-1}$: 1.20 ± 0.04 ppb (45d); 1.10 ± 0.03 ppb (60d); 0.80 ± 0.03 ppb (75d); 0.70 ± 0.04 ppb (90d) (Fig. 2).

Fig. 2. Measured residuals of metsulfuron-methyl (mean ± standard deviation) in soil samples from Field Trial #1 identified as days after herbicide application on different treatments (2 g a.i. ha-1, minimum recommended dose; 4 g a.i. ha-1, maximum recommended dose)



According to Azcarate et al. (2015) herbicides mobility and leaching through soil profile can be influenced by water availability for downward or lateral movement, chemical nature of the product, chemical persistence and the soil sorption capacity. This may be the reason why the concentration found 7 d after application was lower than 30 d. Metsulfuron-methyl has already demonstrated a similar behaviour to water down from soil profiles (SONDHIA, 2009). Considering that sulfonylureas are weak acids and their sorption is low enough in Brazilian soils to be ranked as leachers (OLIVEIRA JR et al., 2001), the low recovery can be attributed to chemical hydrolysis, mineralization, formation of different metabolites, as well as formation of bound residues (TANDON et al., 2016).

7.3 BAIT LAMINA TEST

After the exposure period of 40 d, bait lamina strips on Field Trial #1 and Field Trial #2 showed similar consumption in all treatments (Table 3 and Fig. 3). Neither Field Trial #1 nor Field Trial #2 presented significant differences ($p<0.05$) between treatments when compared to control. On Field Trial #1, rainfall during the period of bait exposition was 194.4 mm and mean temperature of 17.6ºC (11.3ºC – 22.3ºC). On Field Trial #2, rainfall was higher reaching the accumulate of 325 mm and mean temperature was lower reaching 12.6ºC (7.0ºC – 19.0ºC).

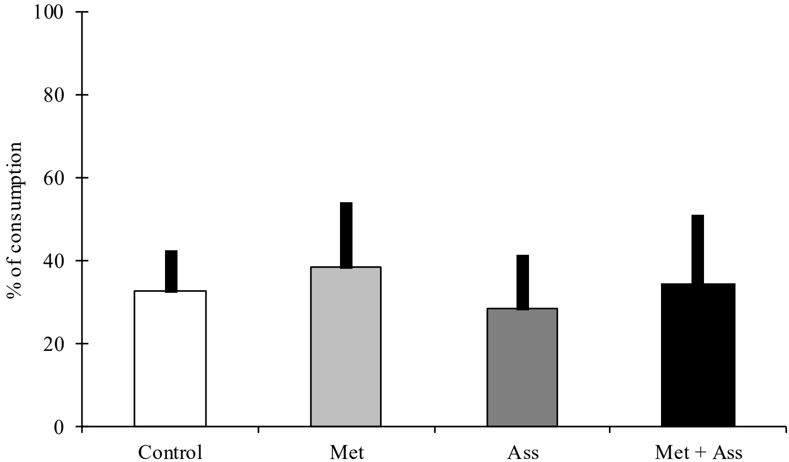
The results found on Field Trial #1 support the screening laboratory tests described by de Santo et al. (2018) where Ally® applied alone did not cause effects to non-target organisms. However, the results found on Field Trial #2 contradict the ones described by this same author (DE SANTO et al., 2018), where the mixture of metsulfuron-methyl herbicide-based and Assist® caused avoidance for collembolans and earthworms likewise Assist® applied alone. A reasonable explanation for these differences is that de Santo et al. (2018) performed the tests under laboratory conditions, considered the worst-case scenario, while in the field, physical structure and chemistry of soils (PIOLA et al., 2009) as well as fate and behaviour of the pesticide (VON MEREY et al., 2016) have a strong influence on pesticide bioavailability. Furthermore, pesticides degradation is directly affected by microbial communities present in soil (PARTE et al., 2017). All these factors can contribute to non-effects observed in the field experiment when compared to laboratory exposure.

Analysing the depth of consumption on Field Trial #2, a higher rate of consumption could be observed on the top 3.0 cm (Fig. 4), corroborating with Römbke et al. (2006), that found higher activity in top surface hypothesizing that oribatid mites could be the mainly responsible and that mesofauna have an important role process. Despite being related to earthworms feeding activity (VAN GESTEL et al., 2003), collembolans and enchytraeids can present high feeding activity when earthworms are absent (HELLING et al., 1998). Amossé et al (2018) analysing two different fungicides and the relationship between their application and oligochaetes feeding activity in French soils, found no differences on feeding rate and on the density of annelid families.

Table 3. Bait lamina consumption (%) on Field Trial #1 according to treatment and application dose

Treatment	Application Date	Days after application	Application dose ($g.ha^{-1}$)	Consumption rate (%)	Standard deviation
1	17/oct	7 days	2	82.78	3.07
2	25/sep	30 days		82.92	7.98
3	11/sep	45 days		79.58	7.95
4	27/aug	60 days		81.94	5.93
5	11/aug	75 days		82.22	9.25
6	27/jul	90 days		81.39	5.21
7	17/oct	7 days	4	81.67	14.53
8	25/sep	30 days		83.61	8.90
9	11/sep	45 days		85.69	3.37
10	27/aug	60 days		80.28	9.90
11	11/aug	75 days		84.03	5.02
12	27/jul	90 days		80.42	5.22
Control	--	--	0	82.78	6.68

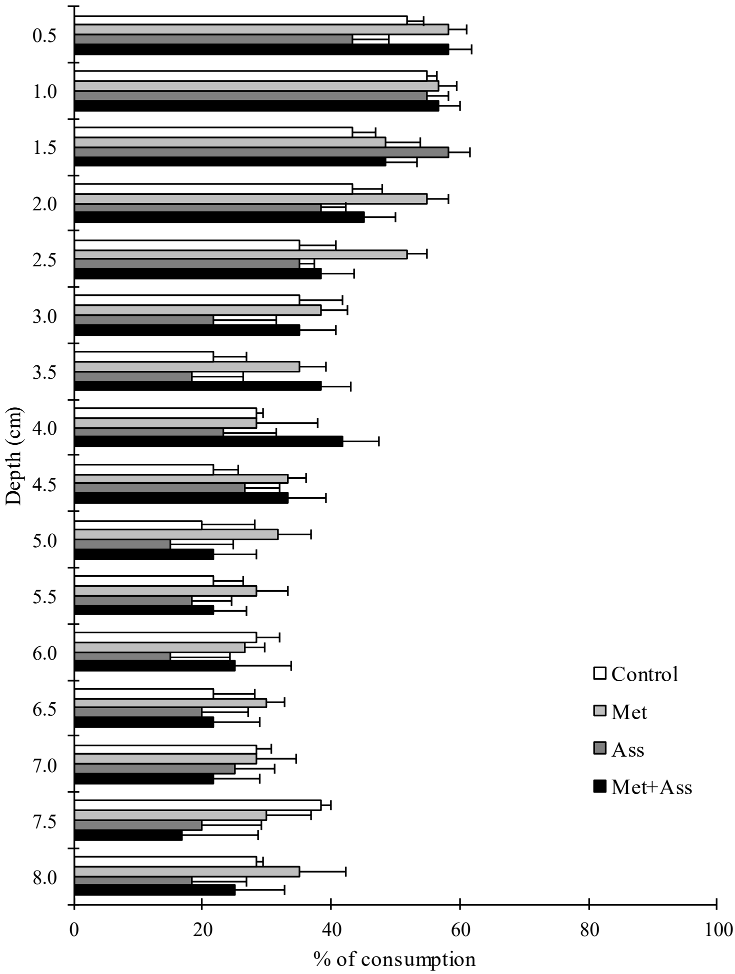
Fig. 3. Bait lamina consumption (%) on Field Trial #2. Control = no pesticide; Met = Metsuram® 600 WG;
Ass = Assist®; Met + Ass = tank mixture of Metsuram® 600 WG + Assist®



The consumption rate was higher on Field Trial #1. This is well represented by two studies placed in Argentina. While Casabé et al. (2007) found a significant decrease on bait lamina consumption on soils treated with chlorpyrifos, Piola et al. (2009) found nonsignificant decrease in the bait consumption exposed to the same pesticide. Biological activity may be affected not only by the pesticide application itself but also by environment (site by year interaction) and tillage intensity (MARWITZ et al., 2014).

Fig. 4. Bait lamina consumption (%) on Field Trial #2 in depth (cm). Control = no pesticide;

Met = Metsuram® 600 WG; Ass = Assist®; Met + Ass = tank mixture of Metsuram® 600 WG + Assist®

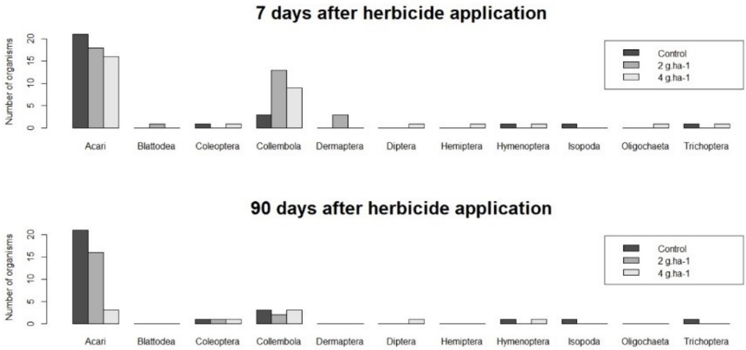


7.4 MESOFAUNA DIVERSITY

Eleven orders were found on Field Trial #1, considering recent (7 days) and oldest (90 days) applications. In general, there was a low abundance of organisms and species richness in all treatments (Fig. 5).

Acari presented the highest number of organisms in control treatment on both dates of applications (21 organisms for both). According to Bedano et al. (2006), soil mites tend to be sensitive to changes in the soil environment, but some family groups respond different to disturbance and increase their number. Collembola showed higher number of organisms on the recent application treatment (13 organisms for minimal dose and 9 for maximum) compared to the oldest one (2 and 3, respectively). This result corroborate that found by Coulibaly et al (2017) in which Collembola were less sensitive to residue management than tillage intensity.

Fig. 5. Abundance of mesofauna groups 7 d and 90 d after herbicide application



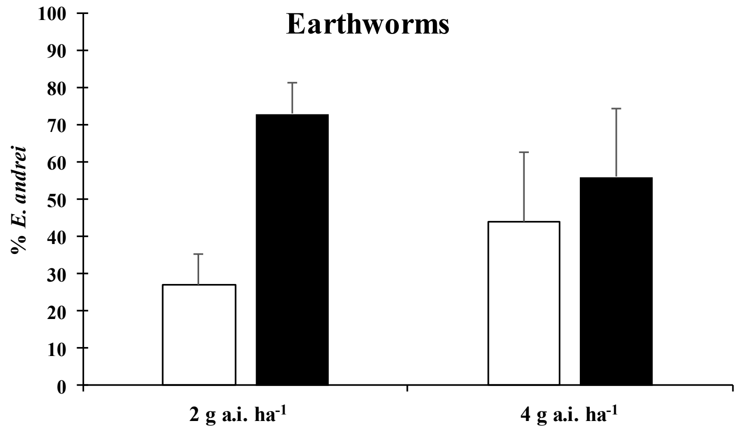
The results may reflect the management of the studied area characterized by no-tillage with crop rotation. Baretta et al. (2003) studying seven types of soil management in the west of Santa Catarina, Southern Brazil, found significant differences in soil fauna diversity, highlighting the importance of conservation practices for the maintenance of soil biodiversity. Similar results were found by Tsiafouli et al. (2015) analysing four agricultural regions across Europe, where land-use intensity decreased soil faunal taxonomic groups diversity, as well as diversity among functional groups and the average trophic level in the soil food web. Changing crop species is another factor that can reduce the density of soil organisms (SCHEUNEMANN et al., 2015).

7.5 AVOIDANCE BEHAVIOUR TESTS WITH FIELD SOIL

After 48h of exposure, all the validity criteria were fulfilled for collembolans and earthworms (>20 and >10% lethality).

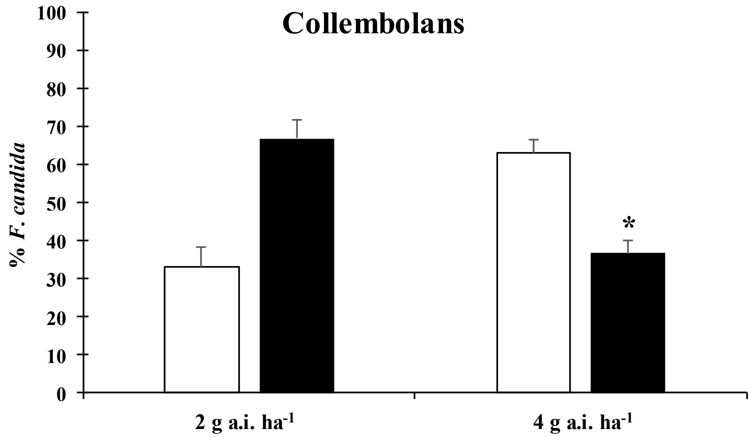
Earthworms showed non-avoidance behaviour for both field doses (Fig. 6). This means that Ally® doses applied on field do not impair earthworms' habitat function. However, collembolans presented avoidance behaviour for the highest dose (4 g.ha^{-1}) (Fig. 7) contradicting laboratory results found by de Santo et al. (2018), where avoidance happened only when the herbicide was applied mixture to mineral oil, simulating a post-emergence condition. Such effects of mineral oil can be expected because it forms an impermeable film enveloping collembolans body and inhibiting their breath (MARTINS et al., 2013).

Fig. 6. Avoidance tests with *Eisenia andrei*. Percentage of organisms on metsulfuron-methyl contaminated soil (black bars) against the control (white bars). No statistical differences ($p<0.05$)



Differences observed to Ally® can be related to the soil used, which can influence the exposure of soil organisms and contaminants bioavailability. While de Santo et al. (2018) used TAS, an artificial substrate, in the present work we used natural soil. The avoidance behaviour could be a result of a complex of sensory "hairs" and receptors that collembolans have in their antenna which contain receptive cells to chemical and mechanical stimuli (HOPKIN 1997). The meaning of this results is that the application of the maximum field dose can cause the avoidance of collembolans, and consequently, impairment on soil quality.

Fig. 7. Avoidance tests with *Folsomia candida*. Percentage of organisms on metsulfuron-methyl contaminated soil (black bars) against the control (white bars). *Asterisks indicate statistical avoidance behavior differences ($p<0.05$)



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Commonly used because of its ease in preparing, artificial soil allows comparisons between laboratories (CHELINHO et al., 2014), but has its limitations, especially with regard to soil properties, one of the most important factors to explain the different toxicity results found in different soils (DOMENE et al., 2010). Despite the assumptions that can be made from single-species tests on TAS, it is necessary to consider that soils are sources of a wide diversity of essential ecosystem services and ecosystem functions (MILLENNIUM ECOSYSTEM ASSESSMENT, 2005) and have components that can influence on pesticides effects (KÖRDEL; RÖMBKE, 2001).

8 CONCLUSIONS

Laboratory results showed that metsulfuron-methyl alone is not a threat to soil fauna. However, the presence of mineral oil showed ecotoxicity to *E. andrei, E. crypticus* and *P. minuta* on laboratory tests, because the adjuvant itself presented ecotoxicity when tested alone.

Field evaluations with Ally® and Metsuram® 600 WG indicated that metsulfuron-methyl do not cause impairment on soil fauna feeding activity. On Field Trial #1 mesofauna abundance was measured and low rates were registered in both recent and oldest treatments, indicating that the management of the area may be influencing the soil diversity. An avoidance behaviour test was realized with collembolans and earthworms using soil from control and from maximum and minimum doses, where collembolans showed avoidance behaviour contradicting laboratory results.

On Field Trial #2 was possible to test all pesticide combinations used in laboratory and no significant differences were found, contradicting the results found in laboratory where species tend to avoid mineral oil.

Field trials contribute to better understanding the effects of pesticides on non-target organisms in the agricultural ecosystem. This study provides an important contribution to the knowledge of the toxicity of metsulfuron-methyl and indicates that adjuvants should be considered in the risk assessment of pesticides. Considering that under field conditions where metsulfuron-methyl is applied mixed with mineral oil, it is remarkable the reduction and possible loss of native communities, especially due to soil management system.

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OUTLOOK AND GENERAL CONCLUSION

For isoxaflutole, results of laboratory tests showed avoidance of the earthworm species only at >300 times the field predicted dose as well as reproduction response over >150 times the field predicted dose. Non-avoidance, lethality or reproduction response was found to the collembolan species. From the laboratory results, it is possible to conclude that Provence® 750 WG has no toxicity effects on earthworms and collembolans even at the highest field applied dose, ensuring the safety of macro and mesofaunal soil communities.

For metsulfuron-methyl, results indicate that the use of earthworms' acute lethality as the only test to assess soil contamination may underestimate the effects of contaminants on soil macrofauna. This herbicide applied alone caused avoidance behaviour only at high concentrations. Chronic laboratory results ensured that this active ingredient is not a threat to soil fauna. However, adjuvant addition changed the effects to soil invertebrates. Earthworms and collembolans avoided the herbicide with adjuvant addition even at predicted field doses. On reproduction tests, *E. andrei, E. crypticus* and *P. minuta* presented ecotoxicity to herbicide with adjuvant addition on laboratory tests, because the adjuvant itself presented ecotoxicity when tested alone.

Adjuvant addition should be considered in risk assessment of pesticides, once this product changes the expected effects of herbicide applied alone. Currently, effects of addition are not considered in products regulation. Furthermore, comparison among adjuvant toxicity is important to choose the less toxic products for soil ecosystem.

Field evaluations with Ally® and Metsuram® 600 WG indicated that metsulfuron-methyl do not cause impairment on soil fauna feeding activity, but collembolans showed avoidance behaviour in field predicted doses.

Field trials contribute to better understanding the effects of pesticides on non-target organisms in the agricultural ecosystem. This study provides an important contribution to the knowledge of the toxicity of metsulfuron-methyl and indicates that adjuvants should be considered in the risk assessment of pesticides. Considering that under field conditions where metsulfuron-methyl is applied mixed with mineral oil, it is remarkable the reduction and possible loss of native communities, especially due to soil management system.

The ecotoxicity of herbicides depends on their chemical group, commercial formulation, application rates, environmental conditions and ecological receptors involved. Therefore, it is not possible to extrapolate its toxicity to aquatic ecosystems or humans.

Further studies are necessary to evaluate other adjuvants and its addition to pesticides considering the real scenario of application. Brazilian environmental legislation should include chronic tests and other non-target organisms in pesticides risk assessment to reduce uncertainties and bring more realistic parameters to the scope. Field evaluations are necessary since pesticides have different formulations which can lead to different behaviour depending on soil physical-chemical and biological characteristics. Besides that, other factors such as temperature and raining can contribute to changes in soil fauna exposure, active ingredient degradation rate, and transport of contaminants into each region.

APPENDIX I

PHOTOGRAPHIC RECORD OF PERFORMED EXPERIMENTS

Fig. 1. Non-target organisms used on laboratory ecotoxicity tests: A) Earthworm species *Eisenia andrei,* B) Enchytraeid species *Enchytraeus crypticus,* C) Collembola species *Folsomia candida,* D) Collembola species *Proisotoma minuta*

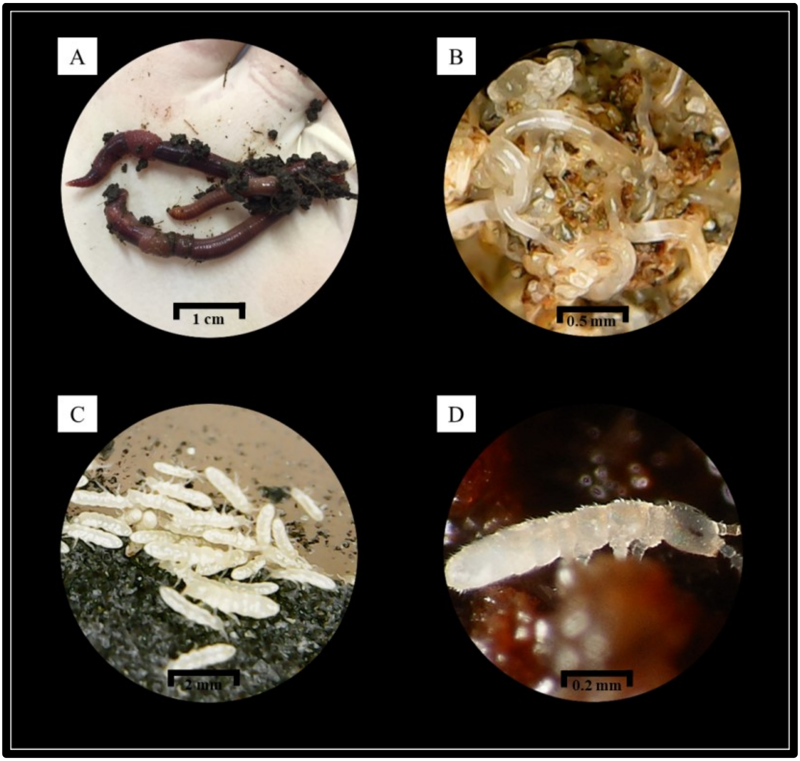

Fig. 2. Summary of laboratory tests: A) Tropical artificial soil at 5% organic matter, composed by 75% fine sand, 20% kaolin clay and 5% coir dust (Picture by J.C. Niemeyer); B) Earthworm avoidance test; C) Collembola avoidance test; D) Lethality test with earthworms; E) Counting juveniles on a reproduction test with enchytraeids on stereoscopic microscope using an adapted methodology by the author; F) Reproduction test with enchytraeids coloured with Bengal rose; G) Reproduction test with earthworms on bain-marie; H) Reproduction test with collembolans (*F. candida*) before counting; I) Reproduction test with collembolans (*P. minuta*) after counting on ImageJ software

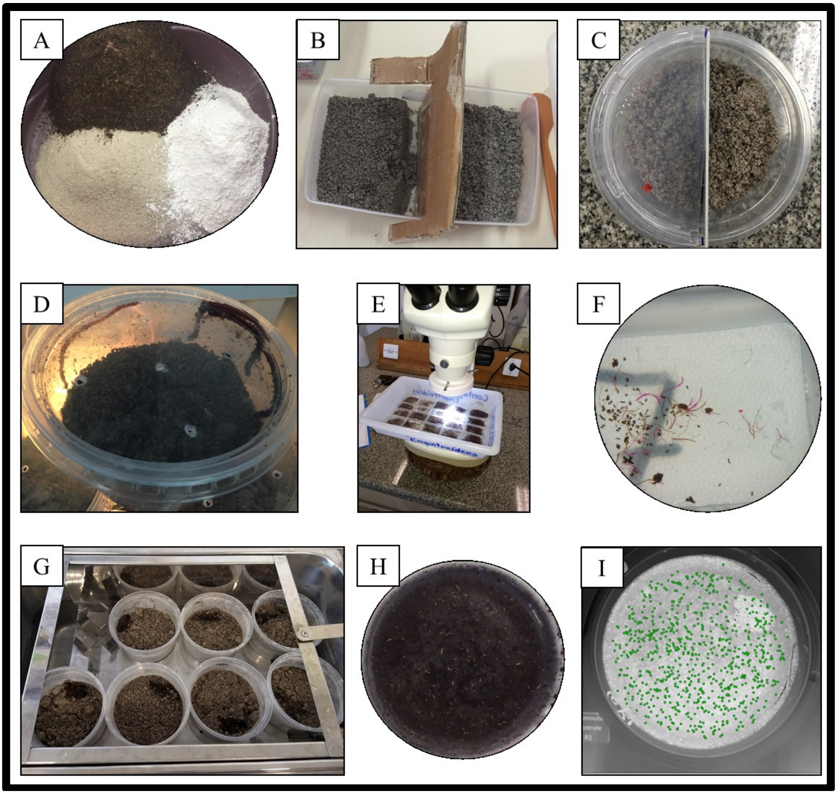

Fig. 3. Field trials: A) Bait lamina test in field; B) Experimental area on when bait laminas were inserted and mesofauna were extracted (Field trial #1); C) Mesofauna ring after a sample extraction; D) When extracted, a bait strip presented an earthworm next to the 12th role; E) Appearance of the experimental area when bait laminas where removed from soil; F) Maize growth after 40 days and the set of bait strips in the growing line; G) Experimental area on Field Trial #2 (black oat); H) Straw cover with bait lamina barely visible; I) Experimental area when bait laminas were removed, 40 days after picture G)