LETICIA SCOPEL CAMARGO CARNIEL

NOVEL APPROACHES AND FUTURE DIRECTIONS FOR PESTICIDE ECOLOGICAL RISK ASSESSMENT TO IN-SOIL FAUNA

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LETICIA SCOPEL CAMARGO CARNIEL

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Tese apresentada à Universidade de Coimbra e à Universidade do Estado de Santa Catarina ao abrigo do Acordo para realização de Doutoramento em Regime de Cotutela assinado entre as duas instituições para a obtenção do Grau de Doutora em Ciência do Solo pela Universidade do Estado de Santa Catarina e de Doutora em Biociências (especialidade em Ecologia) pela Universidade de Coimbra.

Orientadores: Dr. Osmar Klauberg-Filho / Dr. Jose Paulo Filipe Afonso de Sousa

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Lages, 09 de Julho de 2019

Essa obra é dedicada àqueles que lutam por uma agricultura sustentável e pela preservação dos recursos naturais.

This work is dedicated to all who strive for sustainable agriculture and for the preservation of natural resources.

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"(…) *Given time — time not in years but in millennia — life adjusts, and a balance has been reached. For time is the essential ingredient; but in the modern world there is no time. The rapidity of change and the speed with which new situations are created follow the impetuous and heedless pace of man rather than the deliberate pace of nature*."

"(...) Com o correr do tempo - do tempo não em anos, e sim em milênios - a vida ajustou-se, e um equilíbrio foi conseguido. Porquanto o tempo é ingrediente essencial; mas, no mundo moderno, não há tempo. A rapidez da mudança e a velocidade com que novas situações se criam acompanham o ritmo impetuoso e insensato do Homem, ao invés de acompanhar o passo deliberado da Natureza."

Rachel Carson, Silent Spring, 1962.

RESUMO

Os agrotóxicos são moléculas utilizadas para proteger os cultivos agrícolas de pragas e doenças. Contudo, esses produtos podem apresentar efeitos adversos indesejados em organismos não-alvo. O objetivo deste trabalho foi contribuir para aprimorar a análise de risco ecológica (ARE) de agrotóxicos para a fauna do solo na União Europeia (EU) com base na nova Opinião Científica da *European Food Safety Authority* (EFSA) (2017) e executar a mesma abordagem de ARE para cenários brasileiros, verificando a possibilidade de utilização do modelo Europeu para o Brasil. Foi utilizada a abordagem de *tiers*, etapas que avançam de testes mais protetivos para ensaios de maior complexidade, com dois produtos comerciais: o fungicida Bravonil 500 (500 $g.L^{-1}$ clorotalonil) e o inseticida Lorsban 450 (450 $g.L^{-1}$ clorpirifós). No *lower tier* foram elaborados ensaios laboratoriais de reprodução com onze espécies de invertebrados de solo (cinco colêmbolos e seis oligoquetas) utilizando protocolos ISO e OECD com adaptações quando necessário. Os ensaios resultaram em concentrações de efeito (CE) para 10% e 50% das populações que foram utilizadas em um primeiro *tier* intermediário, na elaboração de *species sensitivity distribution* (SSD) *curves*. Em seguida, também foram calculados valores de *hazard concentration* (HC) para proteção de 95% (HC_5) ou de 50% (HC_{50}) das espécies de colêmbolos e oligoquetas. Para um segundo *tier* intermediário foram conduzidos ensaios de comunidade de microartrópodes com solo natural de clima temperado (Portugal) e fauna nativa em condições de laboratório. Além dos dados de toxicidade, a exposição dos organismos também foi estimada por meio das PECs *(predicted environmental concentrations*) dos agrotóxicos em solos para cenários Europeus e Brasileiros. Os dados de toxicidade e de exposição permitiram estimar as TERs (*toxicity-exposure ratio*). Quando esses valores são comparados a um nível de proteção (*trigger value*) o risco pode ser, enfim, estimado. Finalmente, foram executados ensaios em mesocosmos, utilizando *terrestrial model ecosystem* (TMEs) com solo subtropical (Brasil) como proposta de *higher tier*. Para esses experimentos, concentrações de contaminação foram estimadas por meio de diferentes cenários: 1) aplicação continuada (2x) estimado conforme aplicação dos produtos em campo na cultura da soja; 2) aplicação única, estimada por modelagem matemática conforme o executado na análise de risco da União Europeia para o *higher tier*. Resultados do clorotalonil em *lower tier* com colêmbolos indicaram que a espécie padrão mais utilizada, *Folsomia candida*, apresentou o maior CE_{10} (2.44 mg i.a. kg^{-1}), no entanto, os intervalos de confiança de todas as espécies se sobrepuseram, indicando uma sensibilidade similar. Quanto aos oligochaetas, *Eisenia andrei*, não apresentou valores de CE que fossem suficientemente protetivos aos outros organismos testados (CE_{10}: 22.69 mg i.a. kg^{-1}). O clorpirifós apresentou uma alta toxicidade a todos os colêmbolos (CE_{10} < 0.004 mg i.a. kg^{-1}) e entre as oligoquetas, *E. andrei* foi o organismo com menor sensibilidade quanto aos dados de CE_{10} (5.2 mg i.a. kg^{-1}). A falha da espécie de oligoqueta utilizada atualmente na ARE em proteger o grupo taxonômico ao qual pertence é evidenciada pelas SSDs (clorotalonil: HC_5: 2.98 e clorpirifós: HC_5: 0.084 mg i.a. kg^{-1}), levando em consideração que uma etapa intermediária não deveria ser mais sensível que a etapa preliminar (*lower tier*). Os ensaios de comunidades tiveram resultados semelhantes aos das SSDs para os colêmbolos (clorotalonil: CE_{20}: 1.90 – 9.36 e clorpirifós: CE_{20}: 0.0020 - 0.024 mg i.a.kg^{-1}) confirmando a previsão de risco dos ensaios laboratoriais para esse grupo. Os resultados indicam que essa metodologia pode ser utilizada como um tier intermediário, mas uma padronização ainda é necessária devido à alta variabilidade verificada, especialmente para os ácaros. Os resultados de *higher tier* apontam reduções das populações nativas na presença dos dois agrotóxicos, mesmo na concentração mais baixa testada em diferentes cenários de exposição, indicando riscos e ausência de recuperação, especialmente para os colêmbolos, mesmo oito semanas após a contaminação (dissimilaridade de Bray-Curtis > 35%). Possíveis riscos para as minhocas em

TMEs não puderam ser verificados devido ao baixo número de indivíduos presentes. Não houve efeitos dos produtos para os enquitreideos de acordo com a metodologia utilizada. Verificou-se ainda que os *tiers* anteriores foram capazes de prever o risco, que não foi reduzido avançando nas etapas da ARE. As sugestões da opinião científica da EFSA (2017) para mensurar o risco dos pesticidas à fauna do solo são pertinentes, e a adoção de etapas intermediárias pode auxiliar legisladores e reguladores de risco na Europa. Para o Brasil, sugere-se a adoção legislativa de ensaios crônicos de reprodução ao invés de ensaios agudos de letalidade com outras espécies além de *E. andrei*. Além dessa estimativa ser apontada como ineficiente, a espécie não foi o melhor indicador de toxicidade dos dois produtos aos oligoquetas em *lower tier* e no *tier* intermediário com SSDs. Também se sugere o estudo de tempo de dissipação (DT) de agrotóxicos em solos brasileiros para melhores estimativas do risco dos produtos. Sem esses dados torna-se inviável a análise de risco por meio da abordagem em *tiers* utilizada na Europa, uma vez que as estimativas de exposição para os cenários brasileiros com os dados oficiais existentes são irreais.

Palavras chave: Ecotoxicologia terrestre. Análise de Risco Ecológica. Colêmbolos. Enquitreídeos. Minhocas.

ABSTRACT

The aim of the present work was to improve the ecological risk assessment (ERA) of plant protection products (PPPs) to in-soil fauna in European Union based on the new Scientific Opinion of *European Food Safety Authority* (EFSA) (2017) and to execute the same approach in Brazilian scenarios. The tiered approach, which modes from more protective to higher complexity tests was used. Bravonil 500® (500 $g.L^{-1}$ chlorothalonil) and Lorsban 480® (480 $g.L^{-1}$ chlorpyrifos) were used as model PPPs. In the lower tier, reproduction laboratory tests with eleven in-soil organisms (five collembolans and six oligochaete species) were performed using ISO and OECD standard protocols with adaptations when necessary. Effect concentrations to 10% (EC_{10}) and 50% (EC_{50}) of the tested species were estimated and has been used on the first intermediate tier to elaborate the species sensitivity distribution (SSD) curves. These allowed to estimate the hazard concentrations (HC) assumed to protect 95% (HC_5) or 50% (HC_{50}) of the Collembola and Oligochaeta species. In a second intermediate tier, microarthropod community tests in Mediterranean soil using native organisms under laboratory conditions were performed. Besides of toxicity data, exposure was also estimated though PPPs predicted environmental concentrations (PECs) in soils considering European and Brazilian scenarios. Toxicity-exposure ratios (TER) were determined and compared with the trigger values to estimate the potential risk for these organisms. Finally, mesocosms tests in terrestrial model ecosystem (TME) using subtropical soil from Brazil were executed as a surrogate higher tier. For these experiments two different application scenarios were considered: 1) continued application (2x) based on the general agricultural procedures for soybean crop application; 2) single application, estimated based on Europe's ERA instructions for higher tier testing. Lower tier chlorothalonil results to collembolans indicates the higher EC_{10} to the standard *Folsomia candida* (2.44 mg a.i. kg^{-1}). However, there were overlaps on confidence interval to all tested species, indicating similar sensitivity to this fungicide. Among oligochaetes EC_{10} data, *Eisenia andrei* did not present a protective value for all tested species (22.69 mg a.i. kg^{-1}). Chlorpyrifos was highly toxic to all collembolans tested (EC_{10} < 0.004 mg a.i. kg^{-1}). Among oligochaetes, *E. andrei* was the least sensitive species in EC_{10} data (5.2 mg a.i. kg^{-1}). The non-protectiveness of *E. andrei* when estimating risk to all oligochaetes species is clear in the SSDs approach (chlorothalonil: HC_5: 2.98 and chlorpyrifos: HC_5: 0.084 mg a.i.kg^{-1}). The lower tier must be the most protective step in ERA, which was not observed to oligochaetes in the present work. Microarthropod community tests pointed to similar results to those found in the SSDs approach for collembolans (chlorothalonil: EC_{20}: 1.90 – 9.36 and chlorpyrifos: EC_{20}: 0.0020 - 0.024 mg a.i. kg^{-1}), corroborating risk prediction in laboratory tests to Collembola species. Results indicates that the used approach to test for microarthropod communities could be useful as an intermediate tier. However, standardization is still necessary, due the high variability in dataset, mainly due to mites. Higher tier though TMEs tests showed a population reduction of microarthropods due to both products, even at the lowest concentrations tested, regardless the exposure scenario. Results also indicated an absence of recovery eight weeks after application (Bray-Curtis dissimilarity > 35%). Effects on earthworms in TMEs were not observed due the low number of organisms. No effects has been observed in enchytraeids either. The previous tiers were capable of predicting risks, which were still detected at higher tier, mostly for collembolans. EFSA (2017) suggestions to estimate PPPs risks to in-soil fauna are suitable on ERA and the proposed approaches for intermediate tiers could help risk assessors and

management. At Brazil, it is suggested to change the current regulation shifting from acute testing towards the adoption of chronic reproduction tests and the addition of more species than just *E. andrei*. Not only the acute test have been highlighted as inefficient in predicting risks, this earthworm species was not the most sensitive species to both products in lower and intermediate tiers with SSDs. Furthermore, research in dissipation time (DT) of PPPs in Brazilian soils are immediately necessary to estimate accurate PEC values under different scenarios to better predict PPPs risks.

Key words: Soil ecotoxicology. Pesticides. PPPs. Ecological risk assessment. Collembolans. Enchytraeids. Earthworms.

LIST OF ILLUSTRATIONS

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SUMMARY

1. INTRODUCTION

The use of Plant Protection Products – PPPs, which include pesticides, may provide immediate advantages to crop production, although, the adverse effects provoked by these products to the environment may affect ecosystem health, compromising crop production at medium and long-term scales (AKTAR et al., 2009; LARSEN et al., 2017). In order to estimate the probability of negative impacts towards non-target ecological receptors or ecosystems, the Ecological Risk Assessment (ERA) based on a tiered approach has been used in many countries (EC, 2009). It should address the risk with a higher degree of realism and complexity, going from more restrictive *lower tier* to a more realistic *higher tier*. In addition, ERA must to be appropriately protective, internally consistent and cost-effective (BOESTEN et al. 2007; POSTHUMA et al. 2008; DIEPENS et al., 2015).

Pesticide risk assessment at the European Union started in 1991, under the Directive 91/414/EEC. However, only in 2002 the specific methods to access potential risks of PPPs to non-target in-soil fauna were established (EC, 2002). To consider in-soil fauna as a group of organisms in pesticide risk assessment is crucial, since their activity are deeply related to many biological/ecological processes underlying the provision of key ecosystems services (ES). Soil fauna is involved in several ES, like i) food production; ii) nutrient cycling; iii) organic matter decomposition; iv) water availability in soil; v) erosion control; vi) soil formation and stability and; vii) seed dispersal (Lavelle et al., 2006; Brown et al., 2015). For instance, microarthropod activity has been demonstrated to influence the decomposition of leaf litter, while larger bodied invertebrates, such as earthworms, can have profound impacts on ecosystems through their feeding, burrowing, and nest building (SYNDER; CALLAHAM Jr, 2019). Other important group, smaller than earthworms but tolerant to a wider range of conditions, the enchytraeids, rarely has been studied or often are underestimated. They are abundant in many ecosystems and strongly involved in soil food webs, decomposition and nutrient cycling (DIDDEN, 1993; PELOSI; RÖMBKE, 2018). Using the ES approach as an overarching concept, EFSA (2010) proposed an approach to define specific protection goals (SPGs) for pesticide risk assessment for each key group of organisms (key drivers) using combinations “key drivers – key ES where these are involved”. For example, for earthworms, EFSA proposed that SPGs should be defined according to their role on soil formation, nutrient cycling, and food for other organisms (EFSA, 2010). This approach was further extended by EFSA to other in-soil organisms, by proposing a wide range of SPGs aiming to protect in-soil biodiversity and consequently the provision of ES (EFSA, 2017).

Based on new scientific information and on the necessity of a legislation update, the European Directive 91/414/EEC was replaced in 2009 by the European Regulation EC 1107/2009. Furthermore, new data requirements to in-soil fauna started to be requested for active substances and plant protection products (PPPs) (EU 283 and 284, 2013). Due to this, the number research activities focused on risk of PPPs to soil fauna increased in recent years (CHELINHO et al., 2011; PELOSI et al., 2014; STANLEY; PREETHA, 2016; EIJSACKERS et al., 2017). However, despite these recent updates and new literature data, the current risk assessment scheme in Europe is still carried out according to the 2002 guidance (EC, 2002), and consequently, the mismatch existing between the new data requirements needs to be tackled and a more sound scheme must be developed. For example, on the effects side, there is a need to understand the representativity of the current required tests (and of test species) to fill the desired level of protection, since there are important groups, such as enchytraeids and isopods, which are not included in risk assessment scheme (RÖMBKE et al., 2017), and the level of sensitivity of the tested species in comparison to the plethora of species from the same groups is largely unknown. Moreover, there is a need to develop approaches to proper assess risks to microorganisms, and of approaches for intermediate tiers to include data from more species. Furthermore, is necessary to improve higher tier tests, currently focused mostly on earthworm communities, to embrace other organism groups.

Thus, European Food Safety Authority (EFSA) brought together a Panel of specialists who published a guidance to suggest new approaches (see below, subtopics 1.1 - 1.4) to in-soil fauna ERA tackling some of these issues (see below, subtopic 1.5), and to improve the ERA scheme (EFSA, 2017). This scientific opinion also highlights a demand for more relevant data to establish a new pesticide risk assessment scheme in order to keep ES through the SPGs.

1.1 INCREASING THE NUMBER TEST SPECIES IN LOWER TIER

Although still relatively scarce, the development of research with non-standard Collembola and Oligochaeta species have increased (BUCH et al., 2016, BANDOW et al., 2014). The use of alternative species in soil ecotoxicology beyond the ones required nowadays (*Folsomia candida*, *Hypoaspis aculeifer* and *Eisenia andrei*) is important to estimate the toxicity of PPPs and calibrate trigger values (EFSA, 2017). Despite of that, currently, most tests from the literature are still performed with *F. candida*, *E. fetida/andrei* or *Enchytraeus albidus/crypticus* (PELOSI et al., 2013; FILSER et al., 2014). Increasing the number of test

species will help to understand if the standard species commonly used are good and protective surrogates of the sensitivity to PPPs of their corresponding groups, and it will allow to advance for different steps in ERA, as the intermediate tiers.

1.2 SPECIES SENSITIVITY DISTRIBUTION (SSD) APPROACH AS AN INTERMEDIATE TIER

The SSD approach assumes that the risk posed by a chemical cannot be completely eliminated but should be reduced to an acceptable low level. Based on a distribution function, the SSD approach allows to establish threshold limits (the so called hazardous concentrations; HC) (ALDENBERG; JAWORSKA, 2000; POSTHUMA et al., 2002; BROCK et al., 2006) and the derivation of environmental quality criteria making the bridge between policy makers and single-species toxicity test data for chemicals. This approach has been already used to assess ERA of contaminants for aquatic organisms (SALA et al., 2012; ALDENBERG; RORIJE, 2013; XU et al., 2015). One of the major issues in using this approach to in-soil organisms is the absence of a dataset which an acceptable number of test species to estimate the hazardous concentrations (HC) (FRAMPTON et al., 2006). Since testing alternative species in soil ecotoxicology started recently and is still unclear how to develop the SSDs until more data is available, EFSA (2017) suggested also another possible intermediate tier.

1.3 MICROARTHROPOD COMMUNITY TESTS AS AN INTERMEDIATE TIER.

Despite the many studies involving in-soil fauna and PPPs (e.g., NATAL-DA-LUZ et al., 2012; JEGEDE et al., 2017; MENEZES-OLIVEIRA et al., 2018), there are limited experiences assessing the effects of chemicals through microarthropods community tests. CHELINHO et al. (2014) highlighted that this methodology allowed the introduction of several species into the test-soil and minimizing direct handling of animals. Moreover, its less demanding in terms of space, time and costs, when compared to field and semi-field studies, and with presumably lower associated variability. However, since it is still not standardized and few information are available in literature until now, more information is urgent to improve the method and verify if it could be useful in ERA.

1.4 SEMI-FIELD TESTS THOUGH TERRESTRIAL MODEL ECOSYSTEMS (TME) AS A SURROGATE HIGHER TIER.

The semi-field studies are defined as controlled and reproducible systems which simulates processes and interactions between components of the terrestrial environment, either in the laboratory (small scale), in the field, or somewhere in between (SCHÄFFER et al., 2010). Using the TMEs as a semi-field method is possible to address community composition, population dynamics, indirect effects (predation or competition effects), chronic exposure (eventually repeated exposure), interactions between and within species and exposure mimicking the actual field situation (EFSA, 2017). This approach was already standardized (ASTM, 1993; UBA, 1994; USEPA, 1996; KNACKER et al., 2004) and for so, could be a suitable approach for ecological risk assessment of PPPs.

1.5 ESTABLISHING SUITABLE TRIGGER VALUES OR ASSESSMENT FACTORS (AFs).

One of the threshold values that can be used in classical ERA is provided by the ratio between the toxicity for organisms (ECs or NOEC values) and the predicted environmental concentrations (PECs) - the so-called toxicity-exposure ratio (TER). Currently, when this ratio is smaller than the trigger value (5), some remediating/corrective actions must be implemented, as improving PEC estimation or performing extra toxicity tests. Another threshold could be defined by comparing the ratio between TER and an assessment factor (AF) with the predicted exposure, e.g., PEC values, as used in aquatic assessment (EFSA PPR Panel, 2013). The trigger value 5 was suggested by CHRISTL et al. (2015), who studied the relationship between data from laboratory and field studies for earthworm reproduction testing. However, there are several issues in using this approach, since such value is not established to protect all in-soil species at the desired level to comply with the specific protection goals (SPG) proposed by EFSA (EFSA, 2017). Thus, to derivate an appropriate trigger value or an AF which protect the in-soil organisms complying with the proposed SPGs, a calibration between lower and higher tiers needs to be performed. With lower, intermediate and higher tier data could be possible to verify if the current value is protective and if necessary, it will be possible to estimate new trigger values or assessment factors.

While the ecological risk assessment of PPPs is being discussed and updated in Europe, in Brazil, on the other hand, the required test to ‘approve’ PPPs concerning in-soil fauna is an acute lethality test (LC_{50}) with *Eisenia* sp. This only have the objective to label commercial

products from slight (LC_{50} > 1000 mg a.i. kg^{-1}) to extremely (LC_{50} < 10 mg a.i. kg^{-1}) toxic (IBAMA, 1996). Being Brazil one of the largest producers of agricultural foods and the fifth pesticide consuming country in the world (www.worldatlas.com), this is not conceivable. In addition, this endpoint was considered low sensitive to indicate pesticide risk in tropical conditions (ALVES et al., 2013) (as well as in European climatic scenarios, reason why it was already removed from the data requirement in EU legislation (EU, 2013)). Based on new literature evidence and in European legislative experience, improve the pesticide risk assessment in Brazil is of paramount importance to preserve biodiversity and ecosystem functioning and so, a sustainable food production in the long-term (CAMARGO et al., 2017). Since an ERA scheme to in-soil fauna has not been used in Brazil, international models, as the European, should be tested and adapted in this environment to assess the PPPs potential risks for this important group.

Fungicides are an emerging chemical class of concern, which have not received so much attention as herbicides or insecticides (ELSKUS, 2012). Chlorothalonil (tetrachloroisophthalonitrile; CAS 1897-45-6) is an active ingredient of non-systemic fungicides which is very effective agricultural usage around the world, due to its reported multi-site contact-activity mode of action (SIMÕES et al., 2019). The fungicides which use chlorothalonil as active ingredient (a.i.) are highly efficient and therefore, widely commercialized (ZHANG et al., 2016). On the other hand, more investigation had focused on insecticides, as the organophosphates. These products have been used in agriculture as substitutes for organochlorine insecticides because its lower cost, easy synthesis and lower persistence in the environment (SOLOMON et al., 2014). It can enter the animal body mainly via contact with ingestion of contaminated matrices, and its toxic mode of action involves acting in the nervous system by inhibiting the synthesis of cholinesterase, causing muscular paralysis by excess of acetylcholine (SAVOLAINEN, 2001). A widely commercialized insecticide from this group is chlorpyrifos [O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate]. It is allowed in EU (EC, 2019) but it was banned in several European countries (DE, DK, SI, FI, SE, IE, LV, LT) mostly due to its known risk for human health (RAUH et al., 2012). In Brazil, about eight thousand tons of chlorpyrifos are sold per year (IBAMA, 2018). Due this high use of both chlorothalonil and chlorpyrifos, commercial formulations with these two active substances were used as models PPPs in this thesis.

1.6 MAIN OBJECTIVE

The main objective of this project was to develop a full pesticide Ecological Risk Assessment to in-soil fauna, based on new approaches proposed by EFSA (2017) under European and Brazilian scenarios using one fungicide (Bravonil 500®; 500g of chlorothalonil L^{-1}) and one insecticide (Lorsban 480®; 480g of chlorpyrifos L^{-1}). In order to achieve the main objective, the following specific objectives were established.

1.7 SPECIFIC OBJETICVES

In order to achieve the main objective, the specific objectives were:

1. To determinate effective concentrations (EC) to 10% (EC_{10}) and 50% (EC_{50}) of chlorothalonil and chlorpyrifos to Collembola species (*Folsomia candida, Folsomia fimetaria, Protaphorura fimata, Sinella curviseta, Proisotoma minuta*) and to Oligochaeta species (*Eisenia andrei, Perionyx excavatus, Dendrobaena veneta, Enchytraeus crypticus, Enchytraeus bigeminus, Enchytraeus dudichi*) in tropical artificial soil (TAS) (lower tier);

2. To estimate several predicted environmental concentrations (PEC), i.e., initial ($PEC_{initial}$), for one year of applications (PEC_{year}) and the maximum accumulated in ten years ($PEC_{accumax}$), for European (South, Central and North zones) and Brazilian (*Latossolo* and *Neossolo* or *Argiloso* at 20, 24 and 28ºC) scenarios for both pesticides and use these estimations in ERA;

3. To elaborate SSDs and to estimated HC_5 and HC_{50} values, based on EC_{10} and EC_{50} data for collembolans, oligochaetes (individually and both groups together), to verify if a better assessment could be obtained using this method and if combining both taxonomic groups in the same analysis is a valuable approach (intermediate tier);

4.To perform microarthropod community tests with natural communities in natural soil as an alternative intermediate tier and to establish EC_{20}, EC_{50} and NOEC values for the overall in-soil fauna, to collembolans and to mites for both pesticides (intermediate tier);

5.To conduct TMEs tests with both pesticides to estimate effects and potential community recovery using two different application scenarios, based on the Good Agricultural Practices (GAP) or on the $PEC_{accumax}$ in a Brazilian subtropical soil (surrogate higher tier).

1.8 HYPOTHESES

The mainly hypothesis of this thesis were based on:

1 - The model pesticides used (chlorothalonil and chlorpyrifos) pose differential risks to in-soil fauna species tested according to their sensitivity considering the toxicity and the estimated PECs;

2 - When the number of used species is increased from lower to intermediate tier, it is possible to develop SSDs and the better approach is to perform curves by distinguishing taxonomic groups;

3 - Microarthropods community tests with natural communities and natural soil is a suitable intermediate tier. It is able to predict risks to soil communities and it is an important step to calibrate trigger values in ERA;

4 - TMEs are able to predict risks to in-soil fauna communities as a surrogate higher tier. The GAP scenario has strongly effects on organisms than $PEC_{accumax}$ scenario, and both pesticides pose risks to in-soil fauna;

5 – ERA scheme in Europe could be improved using additional steps (intermediate tier) and increasing the number of tested species in lower tier or a better trigger value calibration;

6 – An ERA scheme in Brazil based on European scenarios is possible but exposure data regarding environmental scenarios must be produced and considered in risk calculations;

The thesis is presented based on papers already elaborate and submitted, or in process of elaboration/submission. In Chapter I, the lower and intermediate tier through SSDs approach for chlorothalonil in European and Brazilian scenarios were assessed. The Chapter II uses the same methodology of chapter I with the insecticide chlorpyrifos. In Chapter III, the microarthropods community tests with chlorothalonil and chlorpyrifos were developed. Lastly, Chapter IV access chlorothalonil and chlorpyrifos risks in TMEs tests as a surrogate higher tier. In addition, a general discussion highlighted the main conclusions.

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2. CHAPTER I: NOVEL APPROACHES TO ECOLOGICAL RISK ASSESSMENT: SPECIES SENSITIVITY DISTRIBUTIONS TO IN-SOIL ORGANISMS USING THE FUNGICIDE CHLOROTHALONIL AS MODEL PPP

2.1 ABSTRACT:

The main objective of this research was to estimate the ecological risk to non-target in-soil organisms using the Species Sensibility Distribution (SSDs) approach as a proposed intermediate tier in a possible future Ecological Risk Assessment scheme. The fungicide chlorothalonil (Bravonil 500®; 500 g a.i. L^{-1}) was used as model PPP. Chronic ecotoxicity tests were performed with five Collembola and six Oligochaeta species. EC_{10}, EC_{50} and protection values to 95% (HC_5) and 50% of the species (HC_{50}) were estimated. In addition, the predicted environmental concentrations (PEC) in soil were calculated for different Brazilian and European scenarios. The ratio between toxicity (EC_{10}, $HC5_{EC10}$ or HC_{50EC10}) and exposure (PEC) (TER) were used to estimated risks by comparison with a trigger value (5). Collembola species had similar dose-responses to chlorothalonil (EC_{10}: 0.92 – 2.44 mg a.i.kg^{-1}). Risks for this group were pointed to all Brazilian and European scenarios (TER < 5). To Oligochaeta group, *Enchytraeus crypticus* was the most sensitivity species (EC_{10}: 3.77 mg kg^{-1}). Using *Eisenia andrei* EC_{10} (22.69 mg a.i. kg^{-1}) data in lower tier, there was no risk (TER > 5) regardless the scenario. However, when HC_{5EC10} Oligochaeta values were used (HC_5: 2.99 mg a.i. kg^{-1}) there was risk in all scenarios. The results indicated that i) by increasing the number of test species the lower tier in ERA was improved; ii) SSDs were a feasible tool as an intermediate tier in ERA for protection of non-target in-soil organisms, and closer taxonomic groups improved the results; iii) its crucial to assess consistent DT_{50} values under Brazilian scenarios to estimate appropriate PECs and so improve the PPPs ecological risk assessment.

Key Words: Soil Ecotoxicology. Pesticides. Collembolans. Earthworms. Enchytraeids.

2.2 INTRODUCTION

In recent years, researchers and legislators have become increasingly interested in plant protection products (PPPs) Ecological Risk Assessment (ERA) to in-soil fauna (EFSA, 2010; RENAUD et al., 2018). It happened mostly since 2009, when the Directive 91/414/EEC (SANCO 2002) was replaced by the Regulation 1107/2009 (EC, 2009) and data requirements for active substances and PPPs (EU 283 and 284/2013), were updated in Europe. However, despite of the recent improvements, some lacks on PPPs ERA process have been pointed out by the European Food Safety Authority (EFSA). For instance, the need of using more species to improve the lower tier and establishing intermediate tiers using Species Sensitivity Distribution (SSDs) approaches or community tests have been proposed (EFSA, 2017).

The SSD approach assumes the principle that the risk posed by a chemical cannot be completely eliminated but should be reduced to an acceptable low level. From SSDs threshold limits may be established, the so-called hazardous concentrations (HC) based on a distribution function (ALDENBERG; JAWORSKA, 2000; POSTHUMA et al., 2002; BROCK et al., 2004). It allows the derivation of environmental quality criteria making the bridge between policy makers and single-species toxicity test data for chemicals. This approach is largely used to assess ERA of contaminants for aquatic organisms community (SALA et al., 2012; ALDENBERG; RORIJE, 2013; XU et al., 2015) but to in-soil fauna, there are still some issues to be assessed. It is unclear how the toxicity could be combined, since there is not enough information to perform this evaluation (EFSA, 2017) and species from distinct taxonomic groups have been used in the same SSD for soil organisms, what might not be the most appropriate approach. Silva et al. (2014) used plants, arthropods, oligochaetes and microorganism sensitivity to tributyltin in the same SSD, as Kwak et al. (2018), who included plants, oligochaetes, arthropods and algae sensibility to Bisphenol. However, Daam et al. (2011) argue that a taxonomic distance between organisms could to imply directly in sensitivity, which could underestimate risk doses for some group (MALTBY et al., 2009).

PPPs have different toxic modes of action as organisms have different exposure routes. A general example is the study of Frampton et al. (2006), which showed that insecticides are more toxic for arthropods than for oligochaetes while fungicides seams to behave in the opposite way. Researches highlighted that to incorporate both oligochaetes and arthropods already was described as not directly applicable to the risk assessment scheme. Moreover, they argued that the number of available studies to find out how the toxicity data could be combined

are normally scarce: 96% of pesticides do not have toxicity data from more than five in-soil fauna species. Due to these data limitations, a minimum of five species was used in this study to perform SSD and estimates HC_5, even that Maltby et al. (2005), argued that at least six data points should be the minimum number to support an SSD. Diepens et al. (2016) defines that at least eight species, from the same taxonomic group, are required to apply the SSD approach for pelagic organisms.

Consequently, to perform SSDs with in-soil fauna is hardly possible using more than five organisms from the same taxonomic group and using the new required endpoints (EC_{10} or EC_{20}) (EFSA, 2017), especially when the current European legislation requires only three species (EC_{10}, EC_{20} and NOEC of Eisenia sp., *Folsomia candida* and *Hypoaspis aculeifer*) which not belong to the same group (EC, 2009; EU 284, 2013). So, more ecotoxicological data is necessary using either species for which standard ISO or OECD protocols do exist, or species whose protocols are not yet standardized (although are based on those that are), but the scientific literature data has enough quality allowing its use in SSD determinations.

Although European Union (EU) countries has issues to clarify to in-soil fauna PPPs ERA, some countries still have not even developed a risk assessment scheme. In Brazil, one of the biggest food producers, which consequently applies a large amount of pesticides (CAMARGO et al., 2017), the required test to 'approve' pesticides concerning in-soil fauna is an acute lethality (LC_{50}) with Eisenia sp. This only has the objective to label commercial products from slight (LC_{50} > 1000 mg a.i.kg^{-1}) to extremely (LC_{50} < 10 mg a.i.kg^{-1}) toxic (IBAMA, 1996). This endpoint was considered low sensitive to indicate pesticide risk in tropical conditions (Alves et al., 2013) and was already removed from the data requirement in EU legislation (EU 284/2013).

Currently, research including laboratory tests with non-standard Collembola and Oligochaeta species have increased (BUCH et al., 2016, BANDOW et al., 2014). However, most tests are still performed only with *Folsomia candida*, *Eisenia fetida/andrei*, *Enchytraeus albidus* and *Enchytraeus crypticus* (PELOSI et al., 2013; FILSER et al., 2014). For these common species, even that some are required in the EU legislation, there is a lack of information for many PPPs, mainly for chronic endpoints. Moreover, even that the absence of enchytraeids in legislation already has been criticized (EFSA, 2007) no changes have been performed to include this group until the present moment.

The risk in the current ERA scheme is evaluated through the ratio between the toxicity (ECs or NOEC values) and the exposure (Predicted Environmental Concentration - PEC), so called toxicity-exposure ratio (TER). This value is compared with a trigger value of 5, where

values <5 indicate risk. This value was defined by Christl et al. (2015), which studied the relationship between laboratory and field study for earthworm reproduction testing. However, there are several issues in using this approach, since it was not established in order to protect all in-soil species at the desired levels to comply with the specific protection goals (SPG) proposed by EFSA (EFSA, 2017). Thus, to derivate an appropriate trigger value which protects the in-soil SPG, a calibration between lower and higher tiers needs to be performed. The major issue to perform this calibration and assess additional uncertainties is to decide how the trigger value could change as more information becomes available (EFSA, PPR Panel, 2017). Intermediate tiers could be a powerful tool to contribute and help to fulfill this gap. If laboratory tests are available for additional species and SSDs could be performed, the uncertainty about the lower tier is expected to be reduced and this may lead to a change to the overall trigger value.

Fungicides are emerging chemical class of concern, which have not received so much attention as herbicides or insecticides (ELSKUS, 2012). Chlorothalonil (tetrachloroisophthalonitrile) is an active ingredient of non-systemic fungicides which is very effective agricultural usage around the world, due to its reported multi-site contact-activity mode of action (SIMÕES et al., 2019a). The fungicides which use chlorothalonil as active ingredient (a.i.) are highly efficient and therefore, widely commercialized (ZHANG et al., 2016). However, as other PPPs, still few information about soil fauna toxicity does exist. Tu et al., (2011) observed that chlorothalonil applied through Daconil Ultrex use, may reduce feeding activity and abundance of earthworms in the field. Leitão et al. (2014) estimated toxic values (EC_{20} 18.2; 39,4; 20.8 mg a.i.kg^{-1}) of Bravo® (40% a.i. w:w) to *F. candida, E. crypticus* and *E. andrei* in a natural soil in standard laboratory tests and more recently, Simões et al (2019a) also using Bravo® evaluated genomic alterations caused by chlorothalonil in *F. candida*.

Aiming to improve ERA process, namely contributing to produce data tackling some of the research gaps identified by EFSA (EFSA PPR Panel, 2017), this project has been developed with key experiments comprising acquisition of both lower and higher tier data. The present chapter presents data obtained for Chlorothalonil integrated in SSD approaches, as an intermediate tier of ERA. Five collembolan species (*Folsomia candida*, *Folsomia fimetaria*, *Sinella cuviseta*, *Protaphorura fimata*; *Proisotoma minuta*) and six Oligochaeta species (*Eisenia andrei*, *Perionyx excavatus*, *Dendrobaena veneta*, *Enchytraeus crypticus, Enchytraeus bigeminus, Enchytraeus dudichi*) were used in laboratory tests with tropical artificial soil (TAS). The main objectives of this manuscript were i) to improve the toxicity data available for

chlorothalonil, by testing a wide range of species, in order to support ERA of this PPP; ii) to understand the importance/relevance of using in-soil species of the same taxonomic group in SSD approaches and; iii) to evaluate the adequacy of using SSDs as an intermediate tier in ERA. To attain these purposes, hazard concentrations with 95% and 50% protection levels (HC_5 and HC_{50}), based on EC_{10} data, were estimated for Collembola and Oligochaeta species. In addition, all available data for both groups were combined in a single SSD. These values were compared to PEC values estimated for six Brazilian and three European scenarios (South, Center and North), establishing the low and intermediate tiers of chlorothalonil ERA. Based on these comparisons, the pros and cons of SSD approaches were discussed.

2.3 MATERIAL AND METHODS

2.3.1 Test substance

A commercial formulation of the fungicide isophthalonitrile chlorothalonil (Bravonil 500®, 500 g a.i.L^{-1}) was used for soil contamination. Physical and chemical characterization of this active ingredient are shown in Table 1.

Table 1 - Physicochemical characteristics of Chlorothalonil. Data collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and IUPAC (https://sitem.herts.ac.uk/aeru/iupac/Reports/).

Characteristic	**Chlorothalonil**
CAS	1897-45-6
IUPAC name	tetrachloroisophthalonitrile
Empirical formula	$C_8Cl_4N_2$
Molecular mass (g mol^{-1})	265.91
Relative density (g cm^{-1})	1.8
Solubility (pH = 7) (mg L^{-1} 20ºC)	0.81
Log K_{ow} (at 20ºC)	2.94
Henry's Law constant (25ºC Pa m^3 mol^{-1})	2.50 x 10^{-02}
Degradation/Dissipation (days)	
Soil (20°C/aerobic)	DT50: 386
Field	DT50: 27.6

2.3.2 Test Soil

Tropical Artificial Soil (TAS) was used in the laboratory experiments. This soil is a modified version of the artificial soil proposed by Garcia (2004) and is composed by 75% of

fine sand (washed and dried), 20% of kaolin clay and 5% of coconut coir dust. The pH was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$.

2.3.3 Test Organisms

Collembola species used in the laboratory tests were *Folsomia candida* Willem, 1902; *Folsomia fimetaria* Linnaeus, 1758 and *Proisotoma minuta* Tullberg, 1971 (Isotomidae); *Sinella curviseta* Brook, 1882 (Entomobryidae) and *Protaphorura fimata* Gisin, 1952 (Onychiuridae). In addition, six Oligochaeta species were used: the earthworms species *Eisenia andrei* Bouché, 1972; *Dendrobaena veneta* Rosa, 1886 (Lumbricidae)*; Perionyx excavatus* Perrier, 1872 (Megascolecidae) and the enchytraeids species *Enchytraeus crypticus* Westheide and Graefe, 1992; *Enchytraeus bigeminus* Nielsen & Christensen, 1963 and *Enchytraeus dudichi* Dózsa-Farkas, 1995 (Enchytraeidae). All species were obtained from laboratory cultures except *D. veneta* that was obtained from a vermiculturist.

F. candida is a parthenogenetic and euedaphic species widely distributed and recommended as test species by ISO guidelines (ISO, 2007; ISO, 2011). *F. fimetaria* has sexual reproduction and have a smaller body size than *F. candida*. This species is euedaphic, have common occurrence in European agricultural soils and was already pointed as a suitable species for ecotoxicity tests (FOUNTAIN; HOPKIN, 2005) being included in an OECD guideline for reproduction tests since 2016 (OECD, 2016). An alternative species recommended by OECD (OECD, 2016), is the epigeic Collembola *S. curviseta*, which colonizes similar habitats to those where *F. candida* is usually found, have sexual reproduction and a bigger size than *F. candida* and *F. fimetaria* (BANDOW et al., 2014). The OECD guideline (OECD, 2016) also suggests the use of the hemiedaphic Collembola *P. minuta*, which also reproduces sexually and is a cosmopolitan species. Despite to be present in large number of habitats (LARSEN et al., 2009), it is often found in tropical regions, mostly within agriculture, forestry and pasture areas (BUCH et al., 2016). Lastly, the *P. fimata* is an euedaphic species that has sexual reproduction, a wide distribution and a high abundance in soil, leaf litter and composts (FJELLBERG, 1998; MITSCHUNAS et al., 2006).

Belonging to the oligochaete species, *E. andrei* is one of the most commonly studied species for standard ecotoxicological testing, being included in ISO and OECD standards (e.g. ISO 11268-2, 2012; OECD 222, 2016). This epigeic species has a relatively small size when compared to *D. veneta*, also an epigeic species. Both species have active role on decaying

organic matter process (RORAT et al., 2013). Due to the large body mass of *D. veneta*, researches with this species have mostly investigated its use in vermicomposting (MUYIMA et al., 1994; FAYOLLE et al., 1997; EDWARDS et al., 2010) and its use in laboratory ecotoxicological tests is scarce (VERDU et al., 2018). *P. excavatus* is an epigeic non-standard species that have been used in ecotoxicological studies to evaluate ecotoxicity of substances like gasoline (AN; LEE, 2008), metals (REINECKE et al., 2001) and pesticides (REINECKE et al., 2002). Biology and life cycle of this species has been extensively studied (JOSHI; DABRAL, 2008). The enchytraeid *E. crypticus* is a test species commonly used in standardized ecotoxicity tests (ISO, 2004; KUPERMAN et al., 2004; ZHANG et al., 2019). With sexual reproduction by self-fertilization and possibly also by cross-fertilization (SCHMELZ; COLLADO, 2012; GONÇALVES et al., 2016), this species has been largely used in laboratory studies. Notwithstanding, the ecology of this species is still not entirely known, as it has so far been found only in a compost plant (WESTHEIDE; GRAEFE, 1992; CHELINHO et al., 2011). *E. bigeminus* reproduces mostly by fragmentation (CHRISTENSEN, 1973) but, at low densities, may reproduces sexually. This species usually colonizes soils rich in organic matter (DÓZSA-FARKAS, 1995; SCHMELZ et al. 2000; BANDOW et al., 2013). *E. dudichi* also reproduces by fragmentation and physiologically has some similarities with *E. bigeminus* (NIVA et al., 2012).

All collembolan and oligochaete species were maintained in the laboratory under a photoperiod of 16:8h light:dark and a temperature of 20 ± 2ºC. The exception was for *P. excavatus* which was kept at 25ºC due to its biological requirements (EDWARDS et al., 1998; de SILVA et al., 2010). Earthworms were kept in boxes (12 L) with perforated lids, containing a moistened mixture of horse manure (previously defaunated through two freeze–thawing cycles of 48 h at -20ºC followed by 48 h at 25ºC) and coconut coir dust with sand (7:2:1, w:w:w) as substrate. The organisms were fed twice a month with oat porridge, except for *D. veneta* which were kept in *Sphagnum* peat (with pH corrected to 6.5 through the addition of $CaCO_3$) and fed weekly with defauned cow manure. The enchytraeids were cultured in plastic vessels with perforated lids, containing moistened TAS as substrate and fed weekly with oat. Collembolan cultures were kept in a mixture of plaster of Paris and activated charcoal (11:1, w:w) as substrate and fed weekly with dried baker's yeast. Synchronized cultures were established before the beginning of the tests to ensure that the organisms were synchronized with the adequate age for the tests. All substrates were moistened with deionized water once a week.

2.3.4 Experimental procedure

For each test, a gradient of laboratory spiked soils with increasing concentrations of chlorothalonil was achieved. Each gradient of soils was prepared by a stock solution diluting Bravo 500© in distilled water. The concentrations of each gradient were selected based on literature data and preliminary laboratory tests to assess the full dose-response relationships and to allow the estimation of EC_{10} and EC_{50} values for each species. The range of concentrations used in reproduction tests is presented in table 2. Over the experiments, test containers were opened weekly to allow aeration and weight loss of the replicates was reestablished by the addition of distilled water to compensate water losses. All tests were performed under a photoperiod of 16:8h light:dark and at 20ºC, except tests with *P. excavatus* that were performed at 25ºC.

The procedures adopted in the reproduction tests with collembolans were based on the methods described in the international standards available for *F. candida* and *F. fimetaria* (ISO, 2011; OECD, 2016), and in papers from literature for other collembolan species (BANDOW et al., 2014; BUCH et al., 2016; NAKAMORI et al., 2008). At the end of the experiments, test containers were emptied to larger vessels and flooded with water. After the addition of few drops of blue ink (to increase contrast between collembolans and water surface), and after carefully stirring, the water surface was photographed to allow juveniles counting using the software ImageJ (SCHNEIDER et al., 2012).

Laboratory reproduction tests with Oligochaetes were carried out based on the procedures described in ISO standards 11268-2 (ISO, 2012) for earthworms and on the methods described in the ISO standard 16387 (ISO, 2014) and Bandow et al. (2013) for enchytraeids. At the end of the tests with earthworms (*D. veneta*, *E. andrei* and *P. excavatus*), 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.

After the test period of tests with enchytraeids (*E. crypticus*, *E. bigeminus* and *E. dudichi*) 15 mL of alcohol (95%), few drops of bengal rose (1% in ethanol) and 15 mL of water were added in the test containers to preserve and color the organisms. After a minimum of 48 h, the organisms were counted using a stereomicroscopic microscope (60× of magnification). For further experimental details on laboratory tests see Table 2.

Table 2 - Procedures adopted in laboratory reproduction tests with the collembolans *Folsomia candida, Proisotoma minuta, Sinella curviseta, Protaphorura fimata, Folsomia fimetaria,* and the oligochaetes *Eisenia andrei, Dendrobaena veneta, Perionix excavatus, Enchytraeus crypticus, Enchytraeus bigeminus and Enchytraeus dudichi* using chlorothalonil as the model PPP in tropical artificial soil (TAS).

Collembola	*F. candida*	*P. minuta*	*S. curviseta*	*P. fimata*	*F. fimetaria*
Range of concentrations used (mg a.i. kg^{-1})	1.5 - 200	1.0 -300	1.5 - 200	1.5 - 200	1.5 – 200
Test period (days)			28		21
Number of organisms per replicate	10	10	20	20	20
Age of starting organisms (days)	10 - 12	10 - 12	20 - 23	20 - 23	23 – 26
Days of food supply			Weekly		
Number of replicates per treatment			5+1[a]		
Test container (ml)			~150		
Food source			Dry yeast		
Food per test container (mg of FW)			2		
Soil per test container (g of DW)			~30		

Oligochaeta	*D. veneta*	*E. andrei*	*P. excavatus*	*E. crypticus*	*E. bigeminus*	*E. dudichi*
Range of concentrations used (mg a.i. kg^{-1})	1.5 - 300	5 -200	5 - 750	5 - 500	5 - 300	5 - 150
Test period for adults (days)	56	28	28		21	
Test period for juveniles (days)	84	28	28		21	
Number of organisms per replicate			10			
Days of food supply			Weekly			
Number of replicates per treatment		4			5+1[a]	
Test container (ml)	~2000	~1000	~1000		~150	
Weight of starting organisms (g)	1 - 2	0.3 – 0.6	0.3 – 0.6		n.d.	
Length of starting organisms (mm)		n.d.		n.d.	8 - 12	8 - 12
Food source	Cow manure	Horse manure	Horse manure		Rolled oats	
Food per test container (g of FW)	20	5	5		0.001	
Soil per test container (g of DW)	1000	500	500	30	25	25

n.d. – not determined. [a] - Additional replicate without organisms to control soil pH and moisture content at the end of the test.

2.3.5 Chemical analysis

At the beginning of each laboratory test, a soil sample of 10g was taken from each test treatment. These samples were used in chemical analyses to determine the actual concentrations of chlorothalonil in each treatment. Chemical measurements were performed using a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155) and Gilson Unipoint System software. The quality of chemical analyses was checked using chlorothalonil standards (purity >99%, Sigma Aldrich, Steinheim, Germany). The recovery of the standard was always in average >70%. For detailed information on nominal and measured concentrations, see the table 6 in annex I.

Soil samples from tests with *P. minuta* were accidentally lost and thus, could not be used for chlorothalonil quantification. In this test, actual concentrations were estimated by assuming the average % of nominal concentrations obtained in the chemical analyses performed in all soil samples collected in the other Collembola tests (81.5%).

2.3.6 Predicted Environmental Concentrations

An initial predicted environmental concentration ($PEC_{initial}$) was estimated considering that all the fungicide applied reached the soil (0% of interception by the crop was assumed - worst case scenario) and was distributed homogeneously in the 5 cm top soil layer in a soil with bulk density of 1.5 (FOCUS, 1997). Additionally, the time-weighted average concentration for one year (PEC_{year}) and ten years (as the maximum accumulated in ten years; $PEC_{accumax}$) were estimated considering percentage of interception by crops, DT_{50} values, product characteristics and environmental data, according to data from EFSA (2015) using the software ESCAPE (KLEIN, 2015).

Three European (South, Center and North) and six Brazilian scenarios were considered for each predicted value. EU scenarios were stablished for carrying out tier-1 soil exposure assessments (EFSA, 2015). For each region, air temperature (12, 10, 7 ºC for South, Center and North zones, respectively), soil texture (medium fine to South; coarse to Center and North) and the respective DT_{50} values for chlorothalonil (according to local properties) were taken into consideration for PEC values estimation. These data were obtained from data available in the Rapporteur Assessment Report (RAR) of chlorothalonil

(https://www.efsa.europa.eu/en/consultations/call/161024). Good application practices (GAP) of chlorothalonil were assumed according to specific recommendations available for each region (see annex I, table 7).

The crops considered to estimate PEC values were based on those allowing the highest application doses and presented the lowest crop interception at each scenario. The crop interception was measured by the Biologische Bundesanstalt, Bundesortenamt und Chemische Industrie (BBCH) code, which is a decimal code ranging from 0 to 99 to characterize the crop development stage (MEIER, 2001). Through the BBCH code, its possible to estimate the fraction of the pesticide dose that was not covered by the crops and consequently, reaches the soil (f_{soil}) (EFSA, 2015). In annex I (table 7), more information on the variables used to estimated PEC_{year} and $PEC_{accumax}$ are presented.

For Brazil, GAPs for chlorothalonil were taken from MAPA database (MAPA, 2019). As performed for EU regions, the worst-case scenario (WCS) for chlorothalonil persistence in soil was established considering the total number of applications for the crop allowing the highest application doses and higher value of f_{soil}. Information are available in annex I (table 8). Since official DT_{50soil} data for Brazilian soils were not found in open databases, this values were provided by a Brazilian Institute of Environment (IBAMA) through an online government public communication channel (https://esic.cgu.gov.br/sistema/site/index.aspx). Brazilian legislation accepted DT values measured or DT values converted from the percentage (%) of radiolabeled carbon dioxide ($^{14}CO_2$) detached (IBAMA, 1996) (IBAMA method of conversion available in annex I, table 9). Since the information provided by IBAMA was $^{14}CO_2$ detached of chlorothalonil, these data were converted to DT_{50} values. Brazilian law from 1996 requires information on three soils (*Latossolo Vermelho Escuro*, *Latossolo Roxo* and *Glei Húmico*). However, considering the current Brazilian soil classification (Embrapa, 2018), *Latossolo Vermelho Escuro* and *Latossolo Roxo* belong now to the same group (*Latossolo Vermelho)*, for so, the evaluation of ERA in this paper was conducted to *Latossolo Vermelho* soil, which have a clay texture. In addition, the *Glei Húmico* soil has a low representativeness for agriculture (EMBRAPA, 2019) and, because of that a *Neossolo quartzarênico* soil (loamy fine sand texture) was used in alternative, since is representative (EMBRAPA, 2019) and there are IBAMA official data on this soil.

Thus, data taken from IBAMA ($^{14}CO_2$ converted to DT_{50}, texture and OM) for two soils (*Latossolo Vermelho* and *Neossolo quartzarênico*) were used in three different temperatures (20º, 24º and 28º C representing the annual average temperatures of South, Centre and North of Brazil, respectively; INMET, 2019), making a total of six scenarios.

2.3.7 Data analysis

In each reproduction test, the number of juveniles was compared between treatments and control through one-way ANOVA, followed by Dunnett's post hoc test. Normality and homogeneity of variances of data were verified by Kolmogorov-Smirnov test and Levene's tests, respectively. Statistical differences found in this analysis allowed to establish NOEC concentrations.

Effective concentrations for 10% and 50% of effect (EC_{10}, and EC_{50}) were estimated considering measured concentrations and using nonlinear models (ENVIRONMENTAL CANADA, 2007) in Statistica 7.0 software (STATSOFT, Inc., 2004). The estimation method was Levenberg-Marquardt and the fit of the model was evaluated by the analysis of the normality of the residuals via Q-Q plots.

EC_{10} and EC_{50} values were used to generate SSD curves through ETX 2.2 software (VAN VLAARDINGEN et al., 2004) and to calculate hazardous concentration for a protection level of 95% and 50% based on EC_{10} ($HC5_{EC10}$ and $HC50_{EC10}$, respectively) and EC_{50} values ($HC5_{EC50}$ and $HC50_{EC50}$, respectively). As proposed by Maltby et al. (2009) the interspecific variation in sensitivity for each SSD curve may be measured though the HC_{50}:HC_5 ratio. The greater the ratio, the shallower the slope of the SSD and hence the greater the interspecific variation.

The toxicity to exposure ratio (TER) is a value estimated by the ratio of the sensitivity (EC_x) to exposure (PEC) and its used in ERA to establish if there is risk. The result should be compared with a trigger value, which is currently 5: if TER is below the trigger value, this indicates risk for the evaluated organisms. According with the current legislation in Europe (EC, 2009), the TER values were estimated, firstly, using the standard species *(F. candida, E. andrei*) and $PEC_{initial}$ for the lower tier. Since other species are not required, estimations between other EC_{10} data with $PEC_{initial}$ were not performed. For the intermediate tier proposed in this project, $HC5_{EC10}$ data to Oligochaeta and $HC5_{EC10}$ data to Collembola were compared with PEC_{year} and $PEC_{accumax}$.

2.4 RESULTS

Reproduction tests with Collembola species fulfilled the validity criteria proposed by ISO 11267 (ISO, 2011). Even the non-standard species had an adult mortality < 20%, a

reproduction rate of >100 instars per vessel and a coefficient of variation for reproduction < 30% in control vessels. In reproduction tests with oligochaetes, while the earthworms *E. andrei* and *P. excavatus* species fulfilled the validity criteria defined in the ISO 11268-2 (ISO, 2011), *D. veneta* had a number of juveniles lower than 30 in some control replicates. Thus, the earthworms *E. andrei, D. veneta* and *P excavates* had an adult mortality ≤10%, and a coefficient of variation of reproduction <30% in control vessels. The number of juveniles ≥ 30 per vessel was accomplished only by *E. andrei* and *P. excavatus*. In tests with enchytraeids, the validity criteria defined in ISO 16387 (ISO, 2013) could not always be confirmed. The number of juveniles >25 per vessel and a coefficient of variation of reproduction < 50% was always attained in control vessels. However, adult mortality < 20% could be confirmed only in test with *E. crypticus*. For tests with *E. bigeminus* and *E. dudichi* the number of adults at the end of the test could not be determined as these species may reproduce through fragmentation. Toxic values estimated to each species for chlorothalonil exposure are presented in Table 3.

Table 3 - Reproduction EC_{10}, EC_{50} and NOEC values estimated for Collembola and Oligochaeta species when exposed to artificial soil spiked with increasing concentrations of Bravonil 500® (with chlorothalonil as active ingredient). Values are expressed in mg a.i. kg^{-1}.

Collembola	EC_{10}	EC_{50}	NOEC
Folsomia candida	2.44 (0.90 - 3.97)	16.80 (13.38 - 20.23)	1.33
Folsomia fimetaria	1.70 (0.83 - 2.57)	9.81 (6.96 - 12.69)	< 1.35
Sinella curviseta	1.84 (0.55 - 3.13)	9.11 (6.81 - 11.41)	1.26
Protaphorura fimata	1.42 (0.42 - 2.42)	11.07 (8.52 - 13.63)	0.89
Proisotoma minuta	0.92 (0 - 1.85)	14.05 (7.64 - 20.45)	0.82
Oligochaeta			
Eisenia andrei	22.69 (19.21 ± 26.18)	113.49 (96.08 ± 130.91)	13.51
Dendrobaena veneta	62.80 (40.74 ± 84.86)	119.75 (63.28 ± 176.22)	150
Perionyx excavatus	8.22 (3.55 ± 12.89)	32.41 (26.12 ± 38.70)	4.55
Enchytraeus crypticus	3.77 (1.19 ± 6.35)	31.51 (22.26 ± 40.75)	< 4.60
Enchytraeus bigeminus	44.39 (34.59 ± 54.19)	79.91 (68.14 ± 91.67)	62.62
Enchytraeus dudichi	22.92 (12.31 ± 33.54)	41.32 (35.05 ± 47.59)	19.30

Within Collembola species, the highest EC_{50} value was found for *F. candida* (16.80 mg a.i. kg^{-1}) and the lowest for *S. curviseta* (9.11 mg a.i. kg^{-1}). For EC_{10} data, the highest value was also to *F. candida* (2.44 mg a.i. kg^{-1}) but the lowest was found to *P. minuta* (0.92 mg a.i. kg^{-1}) Ordering Collembola species according to their sensitivity against chlorothalonil for EC_{10} data, from the least to the most sensitive we obtain: *F. candida* < *S. curviseta* < *F. fimetaria* < *P. fimata* < *P. minuta*. Among Oligochaeta group, *E. crypticus* was the most sensitive species with an EC_{50} value of 31.51 mg a.i. kg^{-1}, while *D. veneta* presented the highest toxic values (EC_{50}: 119.75 mg a.i. kg^{-1}). The same order of sensitivity was observed to EC_{10} values (*E. crypticus*: 3.77 and *D. veneta*: 62.80). Ordering Oligochaete species from the least to the most sensitive to chlorothalonil based on the EC_{10} values of each species we obtain: *D. veneta* < *E. bigeminus* < *E. dudichi* < *E. andrei* < *P. excavatus* < *E. crypticus*. Using EC_{10} and EC_{50} data for each taxonomic group or to both as a single group, SSDs were performed (figures 1-3). In addition, HC_5 and HC_{50} were also estimated (table 4).

Figure 1 - SSD curves using EC_{10} and EC_{50} values of Collembola species to a commercial formulation with chlorothalonil as active ingredient. For information about the species used in this SSD see Table 3.

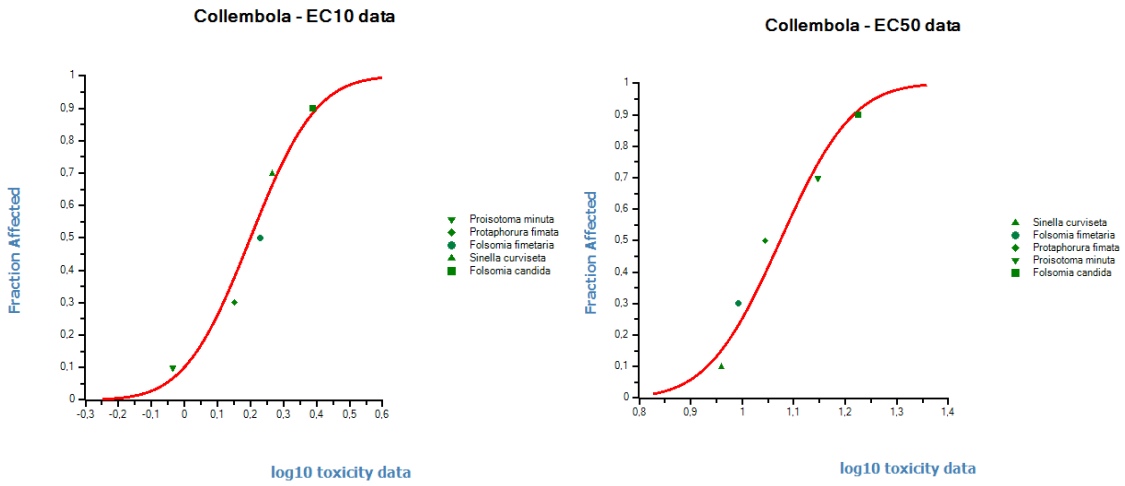

Figure 2 - SSD curves using EC_{10} and EC_{50} values of Oligochaeta species to a commercial formulation with chlorothalonil as active ingredient. For information about the species used in this SSD see Table 3.

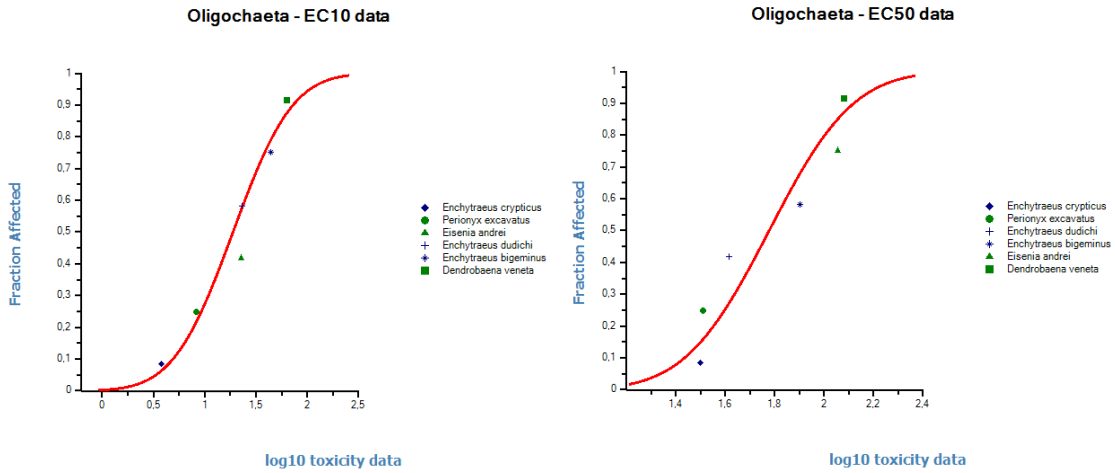

Figure 3 - SSD curves using EC_{10} and EC_{50} values of Collembola and Oligochaeta species to a commercial formulation with chlorothalonil as active ingredient. For information about the species used in this SSD see Table 3.

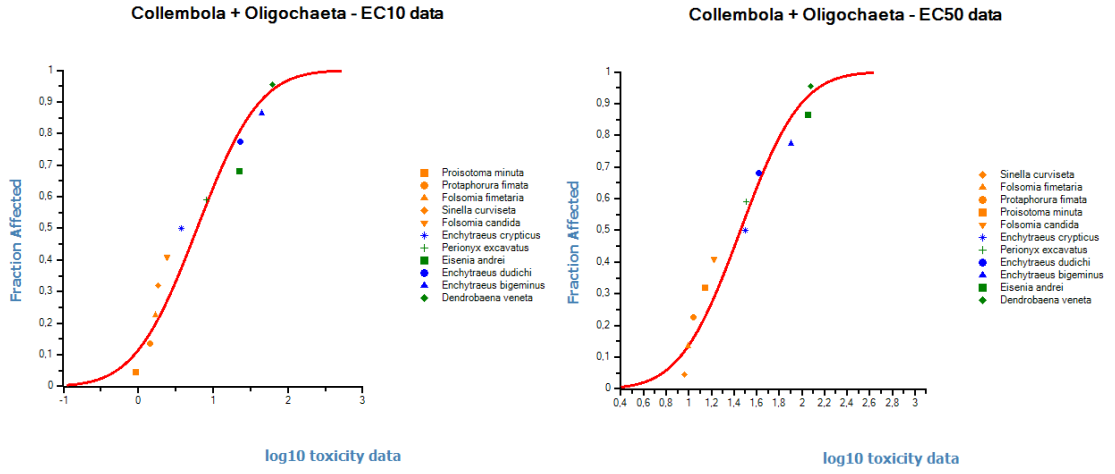

Table 4 - Hazard concentrations (with respective 95% confidence intervals) expressed in mg a.i. kg^{-1} for a protection level of 95% and 50% based on EC_{10} (HC_{5EC10} and HC_{50EC10}, respectively) and EC_{50} values (HC_{5EC50} and HC_{50EC50}, respectively) and estimated through Species Sensitivity Distribution (SSD) curves generated from Collembola species, Oligochaeta species and both Collembola and Oligochaeta species. Chlorothalonil was the model PPP. For information about the species used in each SSD see in the text.

	$HC5_{EC10}$	$HC50_{EC10}$	$HC5_{EC50}$	$HC50_{EC50}$
Collembola	0.83	1.58	7.52	11.84
	(0.34 - 1.17)	(1.12 - 1.99)	(4.05 - 9.61)	(9.29 - 15.11)
Oligochaeta	2.99	18.85	20.34	59.82
	(0.38 - 7.51)	(7.93 - 44.79)	(6.08 - 34.89)	(36.03 - 99.31)
Collembola +	0.47	6.11	5.58	28.65
Oligochaeta	(0.08 - 1.27)	(2.68 - 13.95)	(1.89 - 10.49)	(16.91 - 48.55)

Considering the HC values based on EC_{10} data, the larger interspecific variation in sensitivity was associated to SSDs with Collembola and Oligochaeta plotted together (HC_{50}/HC_5: 13), followed by Oligochaeta (6.32) and Collembola (1.90). The result obtained in

the combined SSD was expected and is justified by the large sensitivity differences between the two taxonomic groups.

About ERA, the estimation for the lower tier was assessed though TER values to standard species (EC_{10}) and $PEC_{initial}$ (table 5). In addition, TER values for the intermediate tier using the ratio between $HC5_{EC10}$ or $HC50_{EC10}$ and PEC_{year} or $PEC_{accumax}$ to each taxonomic group (table 5).

Table 5 - Ecological risk assessment estimated in lower tier and intermediate tier using the predicted environmental concentration estimated immediately after pesticide Bravonil 500® (chlorothalonil a.i.) application (PEC_{intial}), one (PEC_{year}) and ten years ($PEC_{accumax}$) after a normal scenario of pesticide application. Brazilian (BR 20, BR 24, BR 28) and European scenarios (North, Center and South) with different temperatures and distinguish to soil type (LS: loamy sand; L/SL: loamy/sand loamy; Clay: clay; LFS: loamy fine sand). Toxicity exposure ratios (TER) using HC_5 or HC_{50} with EC_{10} data for Oligochaeta, Collembola or both in SSDs. If TER value is lower than 5, there is risk (*).

Lower tier								
Scenario	Soil	$PEC_{initial}$			TER *F. candida* EC_{10}		TER *E. andrei* EC_{10}	
BR all	-	1.70			1.44*		13.35	
EU North	LS	1.33			1.83*		17.06	
EU Center	LS	1.70			1.44*		13.35	
EU South	L/SL	1.33			1.83*		17.06	
Intermediate tier								
PEC_{year}			Collembola		Oligochaeta		Collembola + Oligochaeta	
Scenario	Soil	PEC	TER $HC5_{EC10}$	TER $HC50_{EC10}$	TER $HC5_{EC10}$	TER $HC50_{EC10}$	TER $HC5_{EC10}$	TER $HC50_{EC10}$
BR 20	Clay	16.73	0.050*	0.094*	0.18*	1.13*	0.028*	0.37*
	LFS	12.56	0.066*	0.13*	0.24*	1.50*	0.037*	0.49*
BR 24	Clay	15.93	0.052*	0.10*	0.19*	1.18*	0.030*	0.38*
	LFS	11.96	0.069*	0.13*	0.25*	1.58*	0.039*	0.51*
BR 28	Clay	14.91	0.056*	0.11*	0.20*	1.26*	0.032*	0.41*
	LFS	11.19	0.074*	0.14*	0.27*	1.68*	0.042*	0.55*
EU North	LS	1.14	0.78*	1.49*	2.81*	17.78	0.44*	5.76*
EU Center	LS	3.49	0.24*	0.45*	0.85*	5.40	0.13*	1.75*
EU South	L/SL	1.50	0.55*	1.05*	1.99*	12.57	0.31*	4.07*
$PEC_{accumax}$								
BR 20	Clay	22.16	0.037*	0.071*	0.13*	0.85*	0.021*	0.28*
	LSF	16.64	0.050*	0.095*	0.18*	1.13*	0.028*	0.37*
BR 24	Clay	18.69	0.044*	0.085*	0.16*	1.01*	0.025*	0.33*
	LSF	14.03	0.059*	0.11*	0.21*	1.34*	0.033*	0.44*
BR 28	Clay	16.09	0.052*	0.10*	0.19*	1.17*	0.029*	0.38*
	LSF	12.08	0.069*	0.13*	0.25*	1.56*	0.039*	0.51*
EU North	LS	1.14	0.73*	1.39*	2.61*	16.54	0.41*	5.36*
EU Center	LS	3.59	0.23*	0.44*	0.83*	5.25	0.13*	1.70*
EU South	L/SL	1.50	0.55*	1.05*	1.99*	12.57	0.31*	4.07*

TER values in the lower tier based on EC_{10} data indicates risk to *F. candida* (TER < 5) but not to *E. andrei* (TER > 5) in all scenarios. In intermediate tier, regardless the PEC used, TER $HC5_{EC10}$ values indicates risk to Collembola and Oligochaeta in all exposure scenarios. However, if TER $HC50_{EC10}$ (protecting just 50% of the species) to Oligochaeta was considered, Europe scenarios do not posed risks, regardless the PEC used. For Brazilian scenarios, PEC_{year} and $PEC_{accumax}$ values were higher than those from the European scenarios. In addition, PEC values were inversely proportional to temperature, and higher in clay than in loamy fine sandy soil. In European scenarios, the values of PEC from the highest to lowest were: Center > South > North. This ranking was inversely proportional to TER values, since the higher the PEC, the lower the TER, increasing the risk.

2.5 DISCUSSION

2.5.1 Ecotoxicological data

Despite of the validation criteria has been fulfilled for all Collembola species, there was difference between the reproductive outcome of species, with a variability from 484 to 189 juveniles per control replicate (see annex I, table 10). For earthworms, *D. veneta* did not fulfil the minimum of 30 juveniles per control replicate and this agrees to the mean cocoon production reported in the literature that is 0.28 day^{-1} with a low hatching viability (20%). Moreover, the mean number of earthworms hatching from each viable cocoon was reported as about 1.1 (VILJOEN et al. 1991, 1992; MUYIMA et al. 1994). To enchytraeids, despite of *E. crypticus* had fulfilled validation criteria in the number of adults, this could not be verified in fragmentation species. The methodology available in literature and followed in this work, uses enchytraeids with size between 8 and 12 mm (BANDOW et al., 2013). However, at the end of the test period (21 days) much more than 10 organisms with this size were found in control vessels. This information highlights the need of establish validity criteria adequate for the new species integrated in laboratory reproduction tests.

Regarding sensitivity, Collembola species had similar dose-response to chlorothalonil and the higher ECx value was observed to *F. candida* (EC_{50}: 16.80 mg a.i. kg^{-1}). There were overlaps of 95% confidence interval of all toxic values which reflect the low difference between each other. This is also noted by similar NOEC values. The results for *F. candida* contrasts with data reported by Leitão, et al. (2014), which used a sandy clay loam natural soil (pH = 5, OM

= 5.7%) contaminated with Bravo 500® (40% chlorothalonil, w:w). These researchers found an EC_{50} of 31.1 mg a.i. kg^{-1}, which is c.a. two times higher than the EC_{50} estimated in the present paper for the same species. Simões et al. (2019b), with the same species and also using Bravo 500® in a sandy loam natural soil (pH = 7), reported an EC_{50} of 127.3 mg a.i. kg^{-1}, which was seven times higher than the EC_{50} estimated in the present work. These authors also linked effects of chlorothalonil, at different levels of biological organization for *F. candida,* and verified that this pesticide affects functional proteins of vital processes, like embryonic development and cellular energy homeostasis. A possible reason for this discrepancy might be that differences in soils affects the sensitivity of organisms, as already argued by Chelinho et al. (2011) and Carniel et al. (2019). In addition, the difference in sensitivity could be associated with the commercial product, since we had used Bravonil 500® and not Bravo 500®. de Santo et al. (2019) already pointed that inert ingredients might have adverse effects in soil fauna, which could to increase the product toxicity.

Concerning the other Collembola species, up to our knowledge, few studies have been conducted with these species in laboratory reproduction tests, mainly with PPPs. Notwithstanding, to our knowledge, the sensitivity of the Collembola species (other than *F. candida*) used in this work against fungicides were never assessed. Despite that, some tests have been conducted with other contaminants, as copper sulfate (PEDERSEN; VAN GESTEL, 2001), detergent and hydrocarbons (JENSEN et al., 2002) and some veterinary pharmaceutical products (JENSEN et al., 2003) namely with the standard species *F. fimetaria* (KROGH et al., 2009). Other alternative species with some scientific literature data are *S. curviseta*, investigated by Bandow et al. (2014) about pyrethroid effects and *P. minuta*, used in tests with the herbicide metsulfuron-methyl (DE SANTO et al., 2019) and mercury (BUCH et al., 2016).

Regarding the representativeness of sensitivity of the standard species *F. candida* to the other Collembola species with chlorothalonil, our data show that this standard species generated toxic values in the same order of magnitude of values from the other species. This fact suggests that this *F. candida* has high representativeness for chlorothalonil. In fact, despite the low 95% confidence interval of *F. candida* toxic values, this interval overlaps almost with all confidence intervals generated from the other species (the exception was for the EC_{50} of *S. curviseta* that had a 95% confidence interval lower than that of *F. candida*).

Among earthworms, the lower EC_{50} was estimated to *P. excavatus* (32.41 mg kg^{-1}). This species was less sensitive than *E. andrei* in avoidance tests with chlorpyrifos and carbofuran in OECD artificial soil (10% of sphagnum peat) under tropical conditions (25º C) in a study conducted by De Silva et al. (2009). Authors argued that this difference in sensitivity might

lead to a wrong estimate of potential effects in the tropics using only *E. andrei*. However, García-Santos and Keller-Forrer (2011) argued that there are uncertainties when using avoidance tests with pesticides, mostly to organophosphorus insecticides. Their research highlighted that such tests may provide false negatives, since this type of substances might interfere in the capacity of earthworms to choose the least toxic soil. Thus, with the aim of investigating *P. excavatus* sensitivity in reproduction tests, de Silva et al. (2010) performed laboratory tests with this species and using OECD artificial soil (10% sphagnum peat) and reported EC_{10}, EC_{50} and NOEC values to chlorpyrifos, carbofuran and mancozeb. Finally, researchers concluded that all these pesticides were more toxic to *P. excavatus* than to the standard test species *E. andrei* at temperatures representative of tropical conditions.

D. veneta was the least sensitive species in the present work (EC_{50}: 119.75 mg kg^{-1}). Despite of the absence of reproduction studies with pesticides to this species, it was already indicated as less sensitive to Bisphenol A in artificial soil than *Eisenia fetida* (VERDÚ et al., 2018). In addition, Kostecka and Garczyńska (2008) tested the efficiency of *D. veneta* in vermicomposting when insecticides (teflubenzuron, diflubenzuron and chlorfenvinfos) were applied to control the occurrence of dipterans *Sciaridae*. These researchers concluded that, under recommended doses, the addition of all insecticides into the ecological box did not differentiate a daily rate of vermicomposting of organic wastes.

Nevertheless, the results obtained in the present study show that *E. andrei* sensitivity was not representative for all earthworm species used against chlorothalonil. This agrees to a recent research which highlighted that *E. andrei* is not representative of in-soil fauna for some pesticides, namely insecticides (ALVES et al., 2013; PELOSI et al., 2014).

Among the enchytraeids, *E. bigeminus* was the less sensitive species, with EC_{10} and EC_{50} values of 44.39 and 79.91 mg kg^{-1}, respectively, followed by *E. dudichi* that had EC_{10} and EC_{50} values of approximately half of the dose (22.92 and 41.32 mg a.i. kg^{-1}, respectively). Although being species from the same genus and with the same preferential reproduction type (fragmentation), *E. bigeminus* and *E. dudichi* had differences in the kind of coelomocyte vesicles, mean segment and number of chaetae per bundle (COLLADO et al., 2012) and some of these features could have influence in pesticides toxicity. For example, the coelomocytes play a key role in the defense reactions of most invertebrates and they are involved in important immune functions (e.g. phagocytosis, encapsulation, graft rejection, inflammation), as well in the synthesis and secretion of several humoral factors, especially in annelids and echinoderms (TAHSEEN, 2009). *E. crypticus* was the most sensitive species with EC_{10} and EC_{50} values of

3.77 and 31.51 mg a.i. kg^{-1}, respectively. Leitão et al (2014) reported also a lower sensitivity of *E. crypticus* compared to *E. andrei* (EC_{50} = 40.9 mg a.i. kg^{-1}), which does not agree with the data obtained in our tests. Apparently, the toxicity of chlorothalonil observed in the present study was higher than that reported in the literature, mostly to *F. candida* and to *E. crypticus*, which already has been tested with this active ingredient. This discrepancy might be related to the differences in physical and chemical properties of soils used in the different studies. It is known that soil properties like OM content and quality, beyond texture and other characteristics, may interfere in contaminants availability in soil and in sensitivity of organisms, as previously argued by Chelinho et al. (2011) and more recently by Carniel et al. (2019). In addition, the difference in sensitivity could be associated to the commercial product. While we used Bravonil 500®, data from literature was obtained using Bravo 500®.

Earthworm species have been used for years in ERA studies (PELOSI et al., 2013; UWIZEYIMANA et al. 2017) and currently, enchytraeid species are increasingly being used as well. Enchytraeids have been recognized as good soil quality bioindicators due to their important role in soil systems (PELOSI; RÖMBKE, 2016) and high sensitivity against contaminants presence in soil (JANSCH et al., 2005; NIVA et al., 2015). Their high sensitivity to a broad range of pesticides has been attributed to their close contact with the soil pore water, high ingestion rate and the thin cuticle that lines their bodies (RÖMBKE et al., 2017). Although enchytraeids tests are not actually included in current legislation (both from Europe and Brazil), their absence in pesticide regulation has been criticized by EFSA (EFSA, 2007) and PPR Panel (EFSA, 2017) already suggested the inclusion of species from this group in the EU data requirements.

2.5.2 Hazardous Concentrations values and Ecological Risk Assessment

Regarding the SSDs and HCs, Collembola data presented the closest EC_{10} values between organisms (0.92 – 2.44 mg a.i. kg^{-1}) and consequently the smallest CI in HC evaluation (HC_5: 0.34 – 1.17 mg a.i. kg^{-1}). This distribution was not observed in the Oligochaeta data, which was much more variable in EC_{10} data (3.77 – 62.80 mg a.i. kg^{-1}) and therefore, presented a higher CI in HC evaluation (HC_5: 0.38 – 7.51 mg a.i. kg^{-1}) comparing to Collembolan data. Generating SSDs and HCs using data from both taxonomic groups (HC_5: 0.008 – 1.27 mg a.i. kg^{-1}) the CI interval was reduced in comparison to Oligochaeta alone but increased in comparison to Collembola alone. Despite that, the HC_5 was protective for all organisms ($HC5_{EC10}$: 0.47 mg a.i. kg^{-1}) and there was a clear separation among Collembola and

Oligochaeta species. Similar conclusions were reported by Frampton et al. (2006) when plotting together in the same SSD arthropod and Oligochaeta species. In fact, in the present study and comparing sensitivity of Collembola and Oligochaeta species, it is evident that Collembolan species were the most sensitive. The least sensitive Collembola species (*F. candida* EC_{50}: 16.80 mg kg^{-1}) was about two times more sensitive to chlorothalonil than the most sensitive Oligochaeta species (*E. crypticus* EC_{50}: 31.51 mg a.i. kg^{-1}).

Some approaches to perform SSDs with pesticides have been suggested to aquatic organisms. It has been defended that the most sensitive group, generally depending on the mechanism of action of the test substance, should be used. Based on this assumption, for instance, arthropods should be used for insecticides (MALTBY et al., 2005) and plants for herbicides (VAN DEN BRINK et al., 2006). However, for the fungicides, Maltby et al., (2009) suggested to generate SSDs using data from all major taxonomic groups (vertebrates, invertebrates, and primary producers). These researchers highlighted that whereas fungicides are designed to control fungal pathogens, their modes of action are not specific to this group. Therefore, it could be toxic to a bigger range of organisms than insecticides or herbicides, which normally are more specific. Even fungicides, that specifically inhibit the production of the fungal sterol ergosterol, could interact with enzymes that are highly conserved across fungi, plants, and animals (STENERSEN et al., 2004). In order to understand how toxicity data of non-target in-soil organisms could be combined, information for other pesticides are necessary and the dataset, mostly for other collembolans and enchytraeids, needs to be increased. By this point of view, the present work constitutes an important improvement to the existing dataset concerning in-soil organisms.

The SSD approaches present many advantages when compared to methods used in the current ERA of PPPs, which are normally based on few single species tests and field experiments when legally required. Posthuma et al. (2002) highlighted as advantages of the SSDs: i) their conceptual transparency to decision makers and stakeholders; ii) their general acceptability by regulators and practitioners; iii) and their versatility regarding the possibility to choose percentiles and confidence limits based on the risk manager's preferences. On the other hand, these authors also pointed out the need of requiring relatively large data sets as a disadvantage of the SSD approaches. This issue has been also indicated by other authors (FRAMPTON et al., 2006; MALTBY et al., 2009; VAN WIJNGAARDEN et al., 2010). About issues in SSDs approach statistical methods, Posthuma and collaborators (2002) also pointed that i) there are no mechanistic components, purely empirical; ii) fits of standard functions may

be poor; iii) diverse species sets result in polymodal distributions. Wang et al. (2008) argued that, compared to traditional approaches, SSDs have greater statistical significance and ecological meaning. However, the same researches have also highlighted that there is still no uniform standard method to develop SSDs and to estimate HC_5 values. Both parametric and nonparametric methods have been used and to the second, any assumptions about the distribution have been applied. Furthermore, SSD is assumed as a pseudo-continuous distribution: the basic bootstrap method cannot choose values other than original elements in the dataset. The probability of distribution concentrated only in few points may unable the representability of the true distribution.

Wang et al. (2008) also highlighted other uncertainties as the extrapolation from either simple laboratory to complex field environments or from single species to populations and ecosystems. Due to several reasons already presented, SSDs could not be taken as the higher tier of the ERA and should not be taken as the only analysis possible. However, since SSDs represent the probability of effects on a biodiversity of species (HC values) or communities' level (fraction affected), reducing uncertainties from lower tier and predicting effects for the higher tier, could be a feasible alternative to an intermediate tier. Other approaches to be used as intermediate tiers have been suggested. Ernst et al. (2015) argued that a two-generation study with *F. candida* could be used as an intermediate tier to improve ERA to Collembola. However, differences between species sensitivity associated with the specific roles in ecosystem services that are performed depending on the Collembola traits must not be overlooked (EISENHAUER et al., 2011; SILVA et al., 2016). Thus, testing just one Collembola species could imply in non-protect other important species. On the other hand, microarthropods community tests were also highlighted as a possible intermediate tier (CHELINHO et al. 2014), but this methodology has some uncertainties, related to representativeness of soil communities used and sampling effort, that needs to be fulfilled before its implementation/recommendation (EFSA, 2017). Moreover, such tests seem more adequate for region-specific scenarios than SSD approaches.

PEC values estimated are presented in table 4. For the $PEC_{initial}$, as this consider only the applicated dose, Brazilian scenarios had always the same value (1.70 mg.a.i. kg^{-1}) since the recommendation is the same for all country. EU scenarios had two different values calculated: 1.33 mg a.i. kg^{-1} for both south and north scenarios, and 1.70 mg a.i. kg^{-1} for center scenario. Despite of $PEC_{initial}$ be considered in lower tier, EFSA (2017) suggests that in all other tiers, crop interception and subsequent dissipation at the crop canopy should be considered. EFSA (2015) also suggests the use of the $PEC_{accumax}$ concentration until 5 cm depth for risk

assessment, since it considers the crop interception and the rate application in ten years – which is the time of pesticides to reassess PPP's risks in Europe (EC, 2009).

Regardless of the PEC (initial, annual or maximum in ten years), in general terms, Brazilian scenarios had higher values than EU scenarios. This is due to the higher application doses, number of applications, and DT_{50} considered. It is generally accepted that in tropical conditions, higher degradation rates of pesticides happen in comparison to the rate found in temperate climates (MARTINEZ et al., 2008). However, in the official data accepted by IBAMA for chlorothalonil, DT_{50} values ranges from 180 to 360 days and it is considered higher than that occurred in real scenarios. Besides the few studies investigating the DT_{50} values in Brazilian soils, de Souza et al. (2017), evaluated the role of different factors on chlorothalonil degradation and reported that the DT_{50} values was less than 1 day in all tested treatments. Potter at al. (2001) found a DT_{50} < 1−3.5 days for chlorothalonil and between 10 and 22 days for its principal degradation product (4-hydroxychlorothalonil) in a field experiment at Tift County Georgia, EUA. The same work suggested that the 30-day field half-life often used in risk assessment in EUA to chlorothalonil might be too long.

On the other hand, to EU scenarios, DT_{50} values for natural soils are always available, since it is required in the legislation (EC, 2009). However, some issues have been observed. The initial risk assessment provided for a Member State and based on the applicant's dossier (RAR) accepts DT_{50} information for non-European soils, which is potentially dissimilar. For example, Simões et al. (2019a) estimated a DT_{50} of 1.1 days for Bravo 500® (40% chlorothalonil w:w) and 2.9 days for chlorothalonil (a.i.) in a Portuguese natural soil whereas another study using a EUA natural soil found a DT_{50} of 4.2 days and was accepted in chlorothalonil RAR.

About the ERA, for all the lower tier scenarios, there was risk to *F. candida* (TER < 5) but not for *E. andrei* (TER > 5). Since the standard specie *F. candida* was an adequate sensitivity indicator of Collembola species for chlorothalonil, all the Collembola species tested will be at risk. On the other hand, despite the absence of risk to the standard species *E. andrei*, the other oligochaete species might be at risk, especially *E. crypticus* (EC_{10}: 3.77 mg a.i. kg^{-1}) and *P. excavatus* (EC_{10}: 8.22 mg a.i. kg^{-1}) that were the most sensitive species of enchytraeids and earthworm species, respectively. If beyond *E. andrei, F. candida* and *H. aculeifer*, the enchytraeid *E. crypticus* was required in legislation at the lower tier, this species would be able to predict risk to oligochaete species, since it was the most sensitive species in this group. Thus, our data suggest that *E. andrei* should not be the only Oligochaeta species included in the data

requirements. Apparently, the improvement of the number of species in data requirements is important to ensure adequate predictions of the risk in lower tier.

Concerning the use of SSD approaches as intermediate tier, there were risks to Collembola group (TER < 5) independently of the scenario considered, even though using PEC_{year} - normally lower than $PEC_{accumax}$. Besides the trigger value of 5 seems to be protective to Collembola in this study, EFSA (2017) highlighted that this trigger value was not properly calibrated at the time of their inclusion in the regulation. Furthermore, EFSA panel suggested that the current test battery with the use of an appropriate (calibrated) assessment factor might cover the intra- and interspecies variability in toxicological sensitivity of in-soil organisms. Indeed, for Oligochaeta, TER estimated using $HC50_{EC10}$ in European scenarios did not indicate risk (TER > 5). Besides EFSA (2017) has been questioned the use of 5 as a trigger value, the PPR Panel defined that HC5 should be preferably used, as well EC_{10} or EC_{20} and NOEC data must be provided. In general, the risk was higher in the intermediate tier (TER < 5) than in the lower tier of study, especially for Oligochaeta species. This fact suggest that the standard Oligochaeta species used in the current legislation (1107/2009 – *Eisenia andrei/fetida*) is insufficient for lower tier, since lower tiers should be more protective in ERA scheme than higher tiers (MURALIKRISHNA; MANICKAM, 2017).

2.6 CONCLUSION

The Collembola species presented higher sensitivity (lower ECx values) to chlorothalonil than Oligochaeta species. Sensitivity shown by *F. candida* was representative of the sensitivity found to the other Collembola species which suggest that this standard species is an adequate risk indicator in lower tier to Collembola. On the other hand, for Oligochaeta group, sensitivity observed for *E. andrei* was not representative for the rest of Oligochaeta species used in the tests. This suggest that other species of Oligochaeta should be used to adequately evaluate the risk of Chlorothalonil. The SSD curves generated by Collembola sensitivity data had low variation compared to SSDs based on Oligochaeta sensitivity data. Data obtained showed that, in general, using both species groups (Collembola and Oligochaeta) together in the same SSD generate HC values less realistic and, thus, such approach should not be used for an adequate risk estimation. The improvement of sensitivity data of in-soil species allowed to build more robust SSDs, providing more adequate and protective HC values. Improvements on the available data related to DT_{50} and PEC values in soil for PPPs, especially in Brazil, will contribute to improve the estimation of risk, allowing a higher and more reliable definition of

threshold values to protect soil fauna. Higher tier tests in field or semi field conditions are recommended to verify the risk previous tiers.

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2.8 **ANNEX I**

Table 6 - Range of concentrations (mg a.i.kg^{-1}) used in reproduction tests using chlorothalonil as a model PPP (continue)

Test	**Nominal**	**Measured**	**% of nominal**
Folsomia candida	0.0	0.00	
C0	1.5	1.33	88.34
C1	2.5	2.21	88.29
C2	5.0	4.50	89.90
C3	10.0	8.82	88.18
C4	20.0	16.86	84.31
C5	35.0	28.30	80.85
C6	50.0	41.28	82.57
C7	120.0	96.09	80.08
C8	150.0	140.84	93.90
C9	200.0	189.96	94.98
*Proisotoma minuta***			
C0	0	0.00	
C1	1	0.82	
C2	3	2.45	
C3	5	4.08	
C4	15	12.23	
C5	30	24.46	
C6	60	48.92	
C7	90	73.38	
C8	120	97.84	
C9	150	122.30	
C10	300	244.59	
** Estimated based on average 81.53% SD			
Folsomia fimetaria			
C0	0.0	0.00	
C1	1.5	1.35	90.13
C2	3.0	2.44	81.30
C3	6.0	4.10	68.29
C4	20.0	16.02	80.11
C5	30.0	22.73	75.76
C6	50.0	43.47	86.94
C7	80.0	86.36	107.95
C8	150.0	112.11	74.74
C9	200.0	151.97	75.98

Table 6 - Continuation

Protaphorura fimata			
C0	0	0.00	
C1	1.5	0.89	59.17
C2	2.5	1.76	70.22
C3	5	3.68	73.65
C4	10	6.54	65.38
C5	20	14.72	73.58
C6	30	20.59	68.64
C7	50	36.74	73.48
C8	80	61.60	77.00
C9	150	120.40	80.27
C10	200	125.37	62.69
Sinella curviseta			
C0	0	0.00	
C1	1.5	1.26	84.07
C2	2.5	1.97	78.72
C3	5	3.53	70.54
C4	10	7.31	73.11
C5	20	15.32	76.59
C6	30	23.36	77.86
C7	50	39.64	79.28
C8	80	63.39	79.24
C9	150	114.38	76.25
C10	200	167.75	83.88
Eisenia andrei			
C0	0	0.00	
C1	5	4.11	82.16
C2	10	13.51	135.13
C3	20	23.97	119.84
C4	50	58.43	116.85
C5	100	118.86	118.86
C6	200	216.84	108.42
Perionyx excavatus			
C0	0	0.00	
C1	5	4.55	91.00
C2	10	9.25	92.51
C3	25	22.82	91.27
C4	50	46.48	92.95
C5	100	78.49	78.49
C6	250	241.47	96.59
C7	500	453.12	90.62
C8	750	698.61	93.15

Table 6 - Continuation

Dendrobaena veneta			
C0	0	0.00	
C1	1.5	1.11	74.04
C2	3	2.30	76.80
C3	6	4.79	79.82
C4	15	10.99	73.27
C5	25	22.77	91.09
C6	50	47.66	95.31
C7	150	105.92	70.61
C8	300	229.44	76.48
Enchytraeus crypticus			
C0	0	0.00	
C1	5	4.60	92.00
C2	10	8.28	82.81
C3	20	15.66	78.28
C4	30	29.64	98.81
C5	40	32.20	80.51
C6	60	60.72	101.20
C7	90	85.93	95.48
C8	150	155.54	103.69
C9	250	268.52	107.41
C10	500	258.79	51.76
Enchytraeus bigeminus			
C0	0	0.00	
C1	5	5.23	104.50
C2	10	11.93	119.25
C3	20	20.97	104.87
C4	30	31.63	105.44
C5	40	46.22	115.55
C6	60	62.62	104.36
C7	90	110.88	123.20
C8	150	176.36	117.57
C9	200	260.21	130.11
C10	250	306.84	122.74
C11	300	327.83	109.28
Enchytraeus dudichi			
C0	0	0.00	
C1	5	5.23	104.50
C2	10	11.79	117.94
C3	20	19.30	96.48
C4	30	35.06	116.88
C5	40	46.92	117.30
C6	60	64.05	106.75
C7	90	111.70	124.11
C8	150	170.58	113.72

Table 7 - Good application practices (GAP) of chlorothalonil according to specific recommendations available for each European region.

Zone	Commercial product (PC)	g i.a.kg or L PC	Crop	rate application (L ha)	dose (mg a.i. ha^{-1})	nº app	interval	BBCH code	reaching soil (fraction)	Interception (%)
EU South	FONGIL FL ARASTAR	500	Tomato	2.0	1000	3	10 d	40	0.7	30
EU Center	TWIN 480 SC	480	Carrots	2.0 – 2.5	1200	2	10 d	40-89	1	0
EU North	Amistar Opti	400	Spring barley	2.0 – 2.5	1000	1	-	30 - 59	0.7	30

Table 8 - Good application practices (GAP) of chlorothalonil according to specific recommendations available for Brazil.

Zone	Commercial product (PC)	g i.a.kg or L PC	Crop	rate application (L ha)	dose (mg a.i. ha^{-1})	nº app	interval	BBCH code	reaching soil (fraction)	Interception (%)
BR 1 to BR 6	Bravonil 500	500	Potato	2.5 – 3.0	1500	8	10 d	40	0.85	15

Table 9 - Biodegradability of PPPs according with IBAMA legislation (1996).

Biodegradability			Class	Classification	DT_{50} conversion		
0 ≤	% CO_2	< 1	1	Highly Persistent	360 ≤	$T_{1/2}$ (days)	
1 ≤	% CO_2	< 10	2	Very Persistent	180 ≤	$T_{1/2}$ (days)	< 360
10 ≤	% CO_2	< 25	3	Moderately Persistent	30 ≤	$T_{1/2}$ (days)	< 180
25 ≤	% CO_2		4	Low Persistent	0 ≤	$T_{1/2}$ (days)	< 30

Table 10 - Mean number of juveniles (± standard deviation) and coefficient of variation (CV) of control replicates of reproduction tests with Collembola and Oligochaete species. The tested substance was the a.i. chlorothalonil.

Species	**Mean ± SD in control**	**CV (%)**
Folsomia candida	250 ± 20	8.00
Folsomia fimetaria	248 ± 35	14.24
Sinella cuviseta	484 ± 81	16.65
Protaphorura fimata	143 ± 23	15.83
Proisotoma minuta	189 ± 12	6.34
Dendrobaena veneta	27 ± 7	25.92
Perionyx excavatus	33 ± 3	9.09
Eisenia andrei	57 ± 3	6.15
Enchytraeus crypticus	172 ± 22	12.81
Enchytraeus bigeminus	310 ± 37	11.92
Enchytraeus dudichi	247 ± 25	10.22

3. CHAPTER II: FROM LOWER TO INTERMEDIATE TIER: NEW APPROACHES FOR ECOLOGICAL RISK ASSESSMENT OF PLANT PROTECTION PRODUCTS USING THE INSECTICIDE CHLORPYRIFOS AS A MODEL SUBSTANCE.

3.1 ABSTRACT:

Ecological Risk Assessment (ERA) of pesticides to in-soil organisms is required in EU regulation, however, the current guidance does not match with the ongoing legislation. On the other hand, some countries do not even have an ERA to in-soil organisms to date. In Brazil, acute tests with earthworms has been used to label products only. This paper proposed to increase the number of Collembola and Oligochaeta species used in order to perform the Species Sensibility Distribution (SSDs) approach as an intermediate tier in ERA. Chlorpyrifos (Lorsban 450® 480 g a.i. L^{-1}) was used as model pesticide. Effect concentrations to 10 (EC_{10}) and 50% (EC_{50}) to each species were estimated, and based on these values, protection level to 95 (HC_5) or 50% (HC_{50}) were predicted. The risk has been calculated considering the Predicted Environmental Concentration (PEC) in different European and Brazilian scenarios. *Eisenia andrei* was the least sensitive organism if EC_{10} data is observed (5.20 mg kg^{-1}), and when it was used in EU scenarios for lower tier, no risk has been pointed. SSDs were more protective to Oligochaeta group (HC_5: 0.084 mg a.i. kg^{-1}). Chlorpyrifos was also highly toxicity to all collembola tested (EC_{10} < 0.005 mg a.i. kg^{-1}) and *Protaphorura fimata* could be considered the most sensitive species (EC_{10}: 0.00125 mg a.i.kg^{-1}). SSD curves with Collembolas were also more protective than use just *Folsomia candida* data, even that this species has been also sensitive (EC_{10}: 0.0023 mg a.i. kg^{-1}). In general, SSDs could be used in terrestrial ecotoxicology, but more studies are necessary in order to evaluate the best species to use. Also, to indeed improve ERA in Brazil, studies which aim to evaluated fate and behavior of pesticides in soils are necessary.

KEY WORDS: Soil Ecotoxicology. Chlorpyrifos. Pesticides. In-soil fauna. Collembola. Enchytraeid. Earthworm.

3.2 **INTRODUCTION**

Among organisms of in-soil fauna, Collembolans are the most abundant arthropods after mites in most arable soils worldwide (FILSER et al., 2014). They have significantly influence on soil microbial ecology, nutrient cycling and soil fertility, feeding on microorganisms and organic matter. In addition, they respond to a range of environmental and ecological factors, such as changes in soil chemistry, microhabitat configuration, agricultural practices and consequently, play a key role as soil quality bioindicators (HOPKIN, 1997; PONGE et al., 2003; PARISI et al., 2005; SOUSA et al., 2006; BARETTA et al., 2008; BARTZ et al., 2014). *Folsomia candida* is the most used standard species of this group in laboratory ecotoxicological tests (ISO, 2011). Its sensitivity towards several substances, like Plant Protection Products (PPPs), namely insecticides (NATAL-DA-LUZ et al., 2012; OWOJORI et al., 2014) makes them good bioindicators to evaluate soil habitat function. The research using non-standard Collembola species (in alternative to the standard species *F. candida* and *F. fimetaria*) has increased over the last decade (e.g. BUCH et al., 2016) reporting differences in sensitivity between standard and non-standard species (BANDOW et al., 2014). Another important group of in-soil fauna is Oligochaeta, which contributes to the provision of several ecosystem services (e.g. soil formation), sustaining many ecological niches (PANT et al., 2017). In soil ecotoxicology, earthworms (namely the species *Eisenia andrei/fetida*) have been used as risk indicators in Ecological Risk Assessment (ERA) of PPPs for years (PELOSI et al., 2013; UWIZEYIMANA et al. 2017). Within Oligochaeta, enchytraeids have been recognized as organisms important to terrestrial systems and their sensitivity to contaminants presence makes them good soil quality bioindicators (JÄNSCH et al., 2005; NIVA et al., 2015; PELOSI:RÖMBKE, 2016). Research with non-standard Oligochaeta species has also increased over the last decade (DE SILVA et al., 2010; BANDOW et al., 2013; BUCH et al., 2017), although, most of the tests are still performed with the earthworms *Eisenia andrei/fetida*, and the enchytraeids *Enchytraeus albidus* and *Enchytraeus crypticus* (PELOSI et al., 2014).

The increasing use of PPPs in the maintenance of agricultural crops as promoted the use of laboratory single-species tests to characterize its environmental toxicity. In fact, PPPs are potentially harmful to soil organisms, namely to Collembola (MENEZES-OLIVEIRA et al., 2018) and oligochaete species (FRANCO et al., 2016; BART et al., 2017). The organophosphorus insecticides for example, can enter in the animal body mainly via contact with ingestion of contaminated matrices, and its toxic mode of action involves acting in the

nervous system by inhibiting the synthesis of cholinesterase, causing muscular paralysis by excess of acetylcholine (SAVOLAINEN, 2001). It is used in agriculture as substitutes for organochlorine insecticides due several advantages (SOLOMON et al., 2014) but already were reported as highly toxic to soil fauna.

Chlorpyrifos [O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate] is a organophosphate insecticide allowed in EU (EC, 2019) but banned in several European countries (DE, DK, SI, FI, SE, IE, LV, LT) mostly due to its known risk for human health (RAUH et al., 2012). In Brazil, about eight thousand tons of this active ingredient are sold per year (IBAMA, 2018). Their effects to in-soil fauna have been reported in field tests to native collembolan communities (FRAMPTON, 1999; ENDLWEBER et al., 2006) and spiders (FOUNTAIN et al., 2007). In addition, laboratory single-species tests have shown that it could reduce survival and reproduction in arthropods like Collembola, mites and isopods (SANTOS et al., 2012; MORGADO et al., 2016; KAMOUN et al., 2017) and Oligochaeta species (ZHOU et al., 2011; YANG et al., 2017).

In European Union the use of PPPs requires a previous ERA. The pesticide regulation from 1991 (EC, 2002) was replaced in 2009, with the publication of the Regulation 1107/2009 (EC, 2009) and the data requirements to non-target organisms (including in-soil fauna) were updated in 2013 (EU 283 and 284/2013). However, the guidance to assess pesticides risk to in-soil organisms is still the same since 2002 (EC, 2002). Because of that EFSA's Panel on PPPs and their Residues (PPR Panel) elaborated recently a revised document about gaps and further development necessaries to improve the approach of assess risks to in-soil fauna (EFSA, 2017). While EU is working to fill the gaps on ERA schemes, other countries, like Brazil, are actually still without a developed risk assessment scheme to control the use and the impact of PPPs in agricultural fields. Brazil is one of the biggest food producers in the world, which consequently applies large amounts of pesticides in agricultural crops (CAMARGO et al., 2017). Currently, the required data to 'approve' pesticides concerning in-soil fauna is an acute lethality test (LC_{50}) with *Eisenia andrei/fetida* with the objective to classify commercial products as slight (LC_{50} > 1000 mg a.i. kg^{-1}) to extremely (LC_{50} < 10 mg a.i. kg^{-1}) toxic (IBAMA, 1996). However, laboratory acute tests have been weakly sensitive to characterize pesticide risk and because of that acute tests were removed from the data requirement in EU legislation since 2013 (EU 284/2013). The inadequacy of this endpoint to predict risks was already pointed also to tropical regions (ALVES et al., 2013). These facts suggested that to establish an ERA to in-soil organisms in Brazil is necessary to guarantee a more ecological and safety use of PPPs.

Among several research needs, the PPR Panel (EFSA, 2017) highlighted the importance of: i) using EC_{10} or EC_{20} from chronic tests values, since more protective and robust endpoints should be used instead of EC_{50} or NOEC and its requirement in the currently regulation (EU, 2013); ii) the demand for more sub-lethal ecotoxicological data both for the standard species, but also increasing the number of species to be tested under laboratory to improve lower tiers; iii) the need of using intermediate tiers (like Species Sensitivity Distribution - SSDs) to integrate lower tier data and better support higher tiers. The SSD approaches assume the principle that the risk posed by a chemical cannot be completely eliminated but should be reduced to an acceptable/low level. From SSDs, threshold limits may be established, the so-called hazardous concentrations (HC) based on a distribution function (ALDENBERG:JAWORSKA, 2002; POSTHUMA et al., 2002; BROCK et al., 2004). SSDs allow the derivation of environmental quality criteria making the bridge between policy makers and single-species toxicity test data for chemicals. This approach has been frequently used, especially for aquatic organisms (RODRIGUES et al., 2017), where a large amount of sensitivity data is available. On the other hand, to in-soil fauna, the short dataset is probably one of the major limitations to use SSDs (FRAMPTON et al., 2006). This fact has resulted in the use of SSDs that integrate toxic values of species weakly related in terms of exposure to the contaminants and ecological niche in the environment. Recent papers have reported SSDs using plants and arthropods in the same curve (Silva et al., 2014) or even plants, oligochaetes, arthropods and algae species (Kwak et al., 2018). However, the high taxonomic distance between species result in probably high differences in terms of sensitivity against the contaminants (DAAM et al., 2011), which may contribute to underestimate the risk doses for the most sensitive groups (MALTBY et al., 2009). Its still unclear how the data toxicity of in-soil organisms could be combined (e.g. for different taxonomic groups), and EFSA (2017) also highlighted that despite of the usefulness of SSDs, this methodology cannot yet be applied to in-soil organisms until further guidance on this subject become available.

In an ERA scheme, besides of toxicity data (EC_s, NOECs or HC_5), the concentrations of PPP in soil to which the species will be exposed, the so called Predicted Environmental Concentration (PEC), have also to be estimated. The toxicity-exposure ratio (TER) is a ratio between toxicity data and the predicted concentrations, that is often used to evaluate the risk involved in the use of a PPP. For this ratio, the trigger value (5) is used in a way that when TER<5 a risk is assumed. EFSA (2017) suggested different PECs depending on the tier of the ERA process. A PEC initial ($PEC_{initial}$) (FOCUS, 1997) is suggested to the lower tier as a worst-

case scenario. On the other hand, to the next tiers all other scenarios the estimation of a PEC to ten years ($PEC_{accumax}$) should be used. This $PEC_{accumax}$ considers a PPP use through good agricultural practices (GAP), taking into consideration specific DT_{50} values for each PPP and soil features where the product will be applied.

Recognizing the issues and gaps in PPPs ecological risk assessment highlighted above, the present work has as main objective to increase the dataset of in-soil fauna sensitivity using Collembola (*Folsomia candida, Folsomia fimetaria, Sinella cuviseta, Protaphorura fimata; Proisotoma minuta*) and Oligochaeta species (*Eisenia andrei, Perionyx excavatus, Dendrobaena veneta, Enchytraeus crypticus, Enchytraeus bigeminus, Enchytraeus dudichi*) in tropical artificial soil (TAS) to perform SSD approaches understanding the importance/relevance of using in-soil species from the same taxonomic group to estimates robust and appropriate HC values in order to support ERA of chlorpyrifos (Lorsban 480®). In addition, toxicity information was applied in European and Brazilian distinguish exposure scenarios assessed though the estimation of the predicted environmental concentrations (PEC) of chlorpyrifos in soil.

3.3 MATERIAL AND METHODS

3.3.1 Test substance

The commercial formulation of the insecticide Lorsban 480BR Dow Agro® (48% a.i.L^{-1}) with the organophosphate Chlorpyrifos as active ingredient (a.i.) was used as test substance. Physical and chemical characterization of this active ingredient are shown in Table 11.

Table 11 - Physicochemical characteristics of Chlorpyrifos. Data collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and IUPAC (https://sitem.herts.ac.uk/aeru/iupac/Reports/).

Characteristic	Chlorpyrifos
CAS	2921-88-2
IUPAC name	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
Empirical formula	$C_9H_{11}Cl_3NO_3PS$
Molecular mass (g mol^{-1})	350.58
Relative density (g cm^{-1})	1.4
Solubility (pH = 7) (mg L^{-1} 20ºC)	1.05
Log K_{ow} (at 20ºC)	4.06
Henry's Law constant (25ºC Pa m3 mol^{-1})	0.478
Degradation Soil (20 °C aerobic) (days)	386
Degradation Soil Field (days)	27.6

3.3.2 Test Soil

Tropical Artificial Soil (TAS) was used in the laboratory experiments. This soil is a modified version of the artificial soil proposed by Garcia (2004) and is composed by 75% of fine sand (washed and dried), 20% of kaolin clay and 5% of coconut coir dust. The pH of the soil was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$.

3.3.3 Test Organisms

Collembola species used in the laboratory tests were *Folsomia candida* Willem, 1902; *Folsomia fimetaria* Linnaeus, 1758 and *Proisotoma minuta* Tullberg, 1971 (Isotomidae); *Sinella curviseta* Brook, 1882 (Entomobryidae) and *Protaphorura fimata* Gisin, 1952 (Onychiuridae). In addition, six Oligochaeta were used: the earthworms species *Eisenia andrei* Bouché, 1972; *Dendrobaena veneta* Rosa, 1886 (Lumbricidae)*; Perionyx excavatus* Perrier, 1872 (Megascolecidae) and the enchytraeids species *Enchytraeus crypticus* Westheide and Graefe, 1992; *Enchytraeus bigeminus* Nielsen & Christensen, 1963 and *Enchytraeus dudichi* Dózsa-Farkas, 1995 (Enchytraeidae). All species were obtained from laboratory cultures except *D. veneta* that was obtained from a vermiculturist.

F. candida is a parthenogenetic and euedaphic species widely distributed and recommended as test species by ISO guidelines (ISO, 2007; ISO, 2011). *F. fimetaria* has sexual reproduction and have a smaller body size than *F. candida*. This species is euedaphic, have common occurrence in European agricultural soils and was already pointed as a suitable species for ecotoxicity tests (FOUNTAIN:HOPKIN, 2005) being included in an OECD guideline for

reproduction tests since 2016 (OECD, 2016). An alternative species recommended by OECD (OECD, 2016), is the edaphic Collembola *S. curviseta*, which colonizes habitats similar to those where *F. candida* is usually found, have sexual reproduction and a bigger size than *F. candida* and *F. fimetaria* (BANDOW et al., 2014). The OCED guideline (OECD, 2016) also suggests the use of the hemiedaphic Collembola *P. minuta*, which also reproduces sexually and is a cosmopolitan species. Despite to be present in large number of habitats (Larsen et al., 2009), it is often found in tropical regions, mostly within agriculture, forestry and pasture areas (Buch et al., 2016). Lastly, the *P. fimata* is an euedaphic species that has sexual reproduction, a wide distribution and a high abundance in soil, leaf litter and composts (FJELLBERG, 1998; MITSCHUNAS et al., 2006).

Belonging to the oligochaete species, *E. andrei* is one of the most commonly studied species for standard ecotoxicological testing, being included in ISO and OECD standards (e.g. ISO 11268-2 2, 2012; OECD 222, 2016). This epigeic species has a relatively small size when compared to *D. veneta*, also an epigeic species. Both species have active role on decaying organic matter process (RORAT et al., 2013). Due to the large body mass of *D. veneta*, researches with this species have mostly investigated its use in vermicomposting (MUYIMA et al., 1994; FAYOLLE et al., 1997; EDWARDS et al., 2010) and its use in laboratory ecotoxicological tests is scarce (VERDÚ et al., 2018). *P. excavatus* is an epigeic non-standard species that have been used in ecotoxicological studies to evaluate ecotoxicity of substances like gasoline (AN:LEE, 2008), metals (REINECKE et al., 2001) and pesticides (REINECKE et al., 2002). Biology and life cycle of this species has been extensively studied (JOSHI:DABRAL, 2008). The enchytraeid *E. crypticus* is a test species commonly used in standardized ecotoxicity tests (ISO, 2004; KUPERMAN et al., 2004; ZHANG et al., 2019). With sexual reproduction by self-fertilization and possibly also by cross-fertilization (SCHMELZ:COLLADO, 2012; GONÇALVES et al., 2016), this species has been largely used in laboratory studies. Notwithstanding, the ecology of this species is still not entirely known, as it has so far been found only in a compost plant (WESTHEIDE:GRAEFE, 1992; CHELINHO et al., 2011). *E. bigeminus* reproduces mostly by fragmentation (Christensen, 1973) but, at low densities, may reproduces sexually. This species usually colonizes soils rich in organic matter (DÓZSA-FARKAS, 1995; SCHMELZ et al. 2000; BANDOW et al., 2013). *E. dudichi* also reproduces by fragmentation and physiologically has some similarities with *E. bigeminus* (NIVA et al. 2012).

All collembolan and oligochaete species were maintained in the laboratory under a photoperiod of 16:8h light:dark and a temperature of 20 ± 2ºC. The exception was for *P.*

excavatus which was kept at 25ºC due to its biological requirements (Edwards et al., 1998; de Silva et al., 2010). Earthworms were kept in boxes (12 L) with perforated lids, containing a moistened mixture of horse manure (previously defaunated through two freeze–thawing cycles of 48 h at -20ºC followed by 48 h at 25ºC) and coconut coir dust with sand (7:2:1, w:w:w) as substrate. The organisms were fed twice a month with oat porridge, except for *D. veneta* which were kept in *Sphagnum* peat (with pH corrected to 6.5 through the addition of $CaCO_3$) and fed weekly with defauned cow manure. The enchytraeids were cultured in plastic vessels with perforated lids, containing moistened TAS as substrate and fed weekly with oat. Collembolan cultures were kept in a mixture of plaster of Paris and activated charcoal (11:1, w:w) as substrate and fed weekly with dried baker's yeast. Synchronized cultures were established before the beginning of the tests to ensure that the organisms were synchronized with the adequate age for the tests. All substrates were moistened with deionized water once a week.

3.3.4 Experimental procedure

For each test, a gradient of laboratory spiked soils with increasing concentrations of chlorpyrifos was achieved. Each gradient of soils was prepared by a stock solution diluting Lorsban 480® in distilled water. The concentrations of each gradient were selected based on literature data and preliminary laboratory tests to assess the full dose-response relationships and to allow the estimation of EC_{10} and EC_{50} values for each species. The range of concentrations used in reproduction tests is presented in table 12. Over the experiments, test containers were opened weekly to allow aeration and weight loss of the replicates was reestablished by the addition of distilled water to compensate water losses. All tests were performed under a photoperiod of 16:8h light:dark and at 20ºC, except tests with *P. excavatus* that were performed at 25ºC.

The procedures adopted in the reproduction tests with collembolans were based on the methods described in the international standards available for *F. candida* and *F. fimetaria* (ISO, 2011, OECD, 2016), and in papers from literature for other collembolan species (BANDOW et al., 2014; BUCH et al., 2016; NAKAMORI et al., 2008). At the end of the experiments, test containers were emptied to larger vessels and flooded with water. After the addition of few drops of blue ink (to increase contrast between collembolans and water surface), and after carefully stirring, the water surface was photographed to allow juveniles counting using the software ImageJ (SCHNEIDER et al., 2012).

Laboratory reproduction tests with Oligochaetes were carried out based on the procedures described in ISO standards 11268-2 (ISO, 2012) for earthworms and on the methods described in the ISO standard 16387 (ISO, 2014) and Bandow et al. (2013) for enchytraeids. At the end of the tests with earthworms (*D. veneta*, *E. andrei* and *P. excavatus*), 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.

After the test period of tests with enchytraeids (*E. crypticus*, *E. bigeminus* and *E. dudichi*) 15 mL of alcohol (95%), few drops of bengal rose (1% in ethanol) and 15 mL of water were added in the test containers to preserve and color the organisms. After a minimum of 48 h, the organisms were counted using a stereomicroscopic microscope (60× of magnification). For further experimental details on laboratory tests see Table 12.

Table 12 - Procedures adopted in laboratory reproduction tests with the collembolans *Folsomia candida, Proisotoma minuta, Sinella curviseta, Protaphorura fimata, Folsomia fimetaria,* and the oligochaetes *Eisenia andrei*, *Dendrobaena veneta, Perionix excavatus, Enchytraeus crypticus, Enchytraeus bigeminus* and *Enchytraeus dudichi* using chlorpyrifos as the model PPP.

Collembola	*F. candida*	*P. minuta*	*S. curviseta*	*P. fimata*	*F. fimetaria*
Range of concentrations used (mg a.i. kg^{-1})	0.00125 – 0.5	0.00125 – 0.1	0.00125 – 0.5	0.00125 – 0.5	0.00125 – 0.5
Test period (days)	28				21
Number of organisms per replicate	10	10	20	20	20
Age of starting organisms (days)	10 - 12	10 - 12	20 - 23	20 - 23	23 - 26
Days of food supply	Weekly				
Number of replicates per treatment	5+1[a]				
Test container (ml)	~150				
Food source	Dry yeast				
Food per test container (mg of FW)	2				
Soil per test container (g of DW)	~30				

Oligochaeta	*D. veneta*	*E. andrei*	*P. excavatus*	*E. crypticus*	*E. bigeminus*	*E. dudichi*
Range of concentrations used (mg a.i. kg^{-1})	0.1 – 218.7	3 - 50	0.5 - 128	0.5 - 300	1 - 600	1 - 600
Test period for adults (days)	56	28	28		21	
Test period for juveniles (days)	84	28	28		21	
Number of organisms per replicate	10					
Days of food supply	Weekly					
Number of replicates per treatment	4				5+1[a]	
Test container (ml)	~2000	~1000	~1000		~150	
Weight of starting organisms (g)	1 - 2	0.3 – 0.6	0.3 – 0.6		n.d.	
Length of starting organisms (mm)	n.d.			n.d.	8 - 12	8 - 12
Food source	Cow manure	Horse manure	Horse manure		Rolled oats	
Food per test container (g of FW)	20	5	5		0.001	
Soil per test container (g of DW)	1000	500	500	30	25	25

n.d. – not determined. [a] - Additional replicate without organisms to control soil pH and moisture content at the end of the test.

3.3.5 Predicted Environmental Concentrations

An initial predicted environmental concentration ($PEC_{initial}$) was estimated considering that all the insecticide applied reached the soil (0% of interception by the crop was assumed - worst case scenario) and was distributed homogeneously in the 5 cm top soil layer in a soil with bulk density of 1.5 (FOCUS, 1997). Additionally, the time-weighted average concentration for one year (PEC_{year}) and ten years (as the maximum accumulated in ten years; $PEC_{accumax}$) were estimated considering percentage of interception by crops, DT_{50} values, product characteristics and environmental data, according to data from EFSA (2015).

Three European (South, Center and North) and six Brazilian scenarios were considered for each predicted value. EU scenarios were stablished for carrying out tier-1 soil exposure assessments (EFSA, 2015). For each region, temperature (12, 10, 7 ºC to South, Center and North, respectively), soil texture (medium fine to South; coarse to Center and North) and the respective DT_{50} values for chlorpyrifos (according to local properties) were taken into consideration for PEC values estimation. These data were obtained from data available in the Report Rapporteur Assessment Report (RAR) of chlorpyrifos (https://www.efsa.europa.eu/en/consultations/call/171018-0). Good application practices (GAP) of chlorpyrifos were assumed according to specific recommendations available for each region (table 17 from annex II).

The crops considerer to estimate PEC values were based on the highest application doses and the lowest crop interception. The crop interception was measured by the Biologische Bundesanstalt, Bundesortenamt und Chemische Industrie (BBCH) code, which is a decimal code ranging from 0 to 99 to characterize the crop development stage (Meier, 2001). Through the BBCH code, its possible estimates the fraction of the pesticide dose that was not covered by the crops and for so, reaches the soil (f_{soil}) (EFSA, 2015). In annex II (table 17), more information on the variables used to estimated PEC_{year} and $PEC_{accumax}$ are available.

For Brazil, GAPs for chlorpyrifos were taken from MAPA database (MAPA, 2019). As performed for EU regions, the worst-case scenario (WCS) for chlorpyrifos persistence in soil was established considering the total number of applications, the highest application doses and higher value of f_{soil}. Information are available in annex II (table 18). Since official DT_{50soil} data for Brazilian soils were not found in open databases, these values were provided by a Brazilian Institute of Environment (IBAMA) through an online government public communication chanal (https://esic.cgu.gov.br/sistema/site/index.aspx). Brazilian legislation accepted DT values measured or DT values converted from the percentage (%) of radiolabeled carbon

dioxide ($^{14}CO_2$) detached (IBAMA, 1996) (IBAMA method of conversion available in annex II, table 19). Since the information provided by IBAMA was $^{14}CO_2$ detached of chlorpyrifos, these data were converted to DT_{50} values. Brazilian law from 1996 requires information on three soils (*Latossolo Vermelho Escuro*, *Latossolo Roxo* and *Glei Húmico*). However, considering the current Brazilian soil classification (Embrapa, 2018), *Latossolo Vermelho Escuro* and *Latossolo Roxo* belong now to the same group (*Latossolo Vermelho*), for so, the evaluation of ERA in this paper was conducted to *Latossolo Vermelho soil*, which have a clay texture. In addition, the *Glei Húmico* soil has a low representativeness for agriculture (Embrapa, 2019) and, because of that an *Argisolo* soil (loamy fine sand texture) was used in alternative, since is representative (Embrapa, 2019) and there are IBAMA official data on this soil.

Thus, data taken from IBAMA ($^{14}CO_2$ converted to DT_{50}, texture and OM) for two soils (*Latossolo Vermelho* and *Argissolo*) were used in three different temperatures (20º, 24º and 28º C representing the annual average temperatures of South, Centre and North of Brazil, respectively; INMET, 2019), making a total of six scenarios.

3.3.6 Data analysis

In each reproduction test, the number of juveniles was compared between treatments and control through one-way ANOVA, followed by Dunnett's post hoc test. Normality and homogeneity of variances of data were verified by Kolmogorov-Smirnov test and Levene's tests, respectively. Statistical differences found in this analysis allowed to establish NOEC concentrations.

Effective concentrations for 10% and 50% of effect (EC_{10}, and EC_{50}) were estimated using nonlinear models (Environmental Canada, 2007) in Statistica 7.0 software (StatSoft, Inc., 2004). The estimation method was Levenberg-Marquardt and the fit of the model was evaluated by the analysis of the normality of the residuals via Q-Q plots.

EC_{10} and EC_{50} values were used to generate SSD curves through ETX 2.2 software (van Vlaardingen et al., 2004) and to calculate hazardous concentration for a protection level of 5 and 50% based on EC_{10} (HC_{5EC10} and HC_{50EC10}, respectively) and EC_{50} values (HC_{5EC50} and HC_{50EC50}, respectively). As proposed by Maltby et al. (2009) the interspecific variation in sensitivity for each SSD curve may be measured though the HC_{50}:HC_5 ratio. The greater the ratio, the shallower the slope of the SSD and hence the greater the interspecific variation.

The toxicity to exposure ratio (TER) is a value estimated by the ratio of the sensitivity (EC_x) to exposure (PEC) and its used in ERA to establish if there is risk. The result should be compared with a trigger value, which is currently 5: if TER is below the trigger value, this indicates risk for the evaluated organisms. According with the current legislation in Europe (EC, 2009), the TER values were estimated, firstly, using the standard species (*F. candida*, *E. andrei*) and $PEC_{initial}$ for the lower tier. Since other species are not required, estimations between other EC_{10} data with $PEC_{initial}$ were not performed. For the intermediate tier proposed in this project, HC_{5EC10} data to Oligochaeta and HC_{5EC10} data to Collembola were compared with PEC_{year} and $PEC_{accumax}$.

3.4 RESULTS

Reproduction tests with Collembola species fulfilled the validity criteria defined in the ISO 11267 for *F. candida* (ISO, 2012). Reproduction tests with the non-standard Collembola species had also an adult mortality <20%, a number of juveniles >100 and a coefficient of variation for reproduction <30% in control. In reproduction tests with oligochaetes, all earthworm species (*E. andrei*, *P. excavatus* and *D. veneta*) fulfilled the validity criteria defined in the ISO 11267 for *E. andrei/fetida* (ISO, 2011). Thus, the earthworms had an adult mortality ≤10%, a number of juveniles ≥30 and a coefficient of variation of reproduction <30% in control vessels. In tests with enchytraeids, the validity criteria defined in ISO 16387 for *Enchytraeus* sp. (ISO, 2013) could not be always confirmed. In tests with enchytraeid species (*E. crypticus*, *E. bigeminus* and *E. dudichi*), the number of juveniles was >25 and the coefficient of variation was <50% always in control vessels. However, an adult mortality <20% could be confirmed only in test with *E. crypticus*. For tests with *E. bigeminus* and *E. dudichi* the number of adults at the end of the test could not be determined as these species may reproduce through fragmentation. Toxic values estimated to each species for chlorpyrifos exposure are presented in table 13.

Table 13 - Reproduction EC10, EC50 (and corresponding 95% confidence intervals) and NOEC values estimated for Collembola and Oligochaeta species when exposed to artificial soil spiked with increasing concentrations of the insecticide Lorsban 480® (with Chlorpyrifos as active ingredient; a.i.). Values are expressed in mg a.i. kg^{-1}. For information about the species used see table 12.

Collembola	EC_{10}	EC_{50}	NOEC
Folsomia candida	0.0023 (0.0016 - 0.0030)	0.010 (0.0091 - 0.011)	0.0012
Folsomia fimetaria	0.0039 (0.0026 - 0.0051)	0.0074 (0.0063 - 0.0086)	0.0025
Sinella curviseta	0.0015 (0.0003 - 0.0027)	0.012 (0.0079 - 0.016)	<0.0012
Protaphorura fimata	< 0.0012 (-)	0.011 (0.0040 - 0.020)	<0.0012
Proisotoma minuta	0.0014 (0 - 0.0044)	0.034 (0.0066 - 0.061)	0.0025
Oligochaeta			
Eisenia andrei	5.20 (4.54 - 5.86)	26.93 (22.74 - 29.32)	15
Dendrobaena veneta	0.89 (0.35 - 1.44)	3.52 (2.59 - 4.46)	0.30
Perionyx excavatus	0.07 (0 - 0.16)	2.69 (1.41 - 3.97)	< 0.50
Enchytraeus crypticus	4.01 (2.70 - 5.31)	31.67 (27.23 - 36.11)	5
Enchytraeus bigeminus	1.67 (0.38 - 3.71)	45.19 (23.64 - 66.74)	< 5
Enchytraeus dudichi	1.92 (0.05 - 3.89)	36.15 (21.29 - 51)	1

The Collembola species were highly sensitive to chlorpyrifos with EC_{10} and EC_{50} values always < 0.005 and < 0.02 mg a.i. kg^{-1}, respectively. This EC_{10} value is 90 times higher than the average of $PEC_{initial}$ estimated to European scenarios (table 15, 0.45 mg a.i. kg^{-1}) and 256 times bigger than the $PEC_{initial}$ estimated to Brazilian scenarios (table 15, 1.28 mg a.i. kg^{-1}). A similar range of toxicity was observed to all species, with similar ECs and NOEC values. Data obtained in tests with *P. fimata* did not allow the estimation of an EC_{10}, due a large reduction in the number of juveniles in the first tested dose (0.00125 mg a.i. kg^{-1}) compared to control. Since the effect observed in the lowest dose was clearly higher than 10% of control, we assumed the lowest test dose as the EC_{10} for the SSD curves. Ordering Collembola species according to their sensitivity against chlorpyrifos for EC_{10} data, from the most to the least sensitive: *F. fimata* ≈ *P. minuta* ≈ *S. curviseta* > *F. candida* > *P. fimetaria.* The oligochaete species were considerably less sensitive to chlorpyrifos compared to collembolans. Among Oligochaeta group, the enchytraeids had a sensitivity within the same order of magnitude (EC_{50} values from 31 to 45 mg a.i. kg^{-1} and EC_{10} values from 1.92 to 4.01 mg a.i. kg^{-1}) overlapping their 95%

confidence intervals. These values were also in the same magnitude of the toxic values estimated for *E. andrei* (EC_{50}: 26.93 mg a.i. kg^{-1} and EC_{10}: 5.20), namely when taking into consideration the 95% confidence intervals. On the other hand, both non-standard earthworm species *P. excavatus* and *D. veneta* were more sensitive than *E. andrei* and Enchytraeid species. *P. excavatus* was 74 times more sensitive (EC_{10}: 0.07 mg a.i. kg^{-1}) and *D. veneta* was six times more sensitive (EC_{10}: 0.89 mg a.i. kg^{-1}) then *E. andrei*. Ordering Oligochaeta species in terms of sensitivity to chlorpyrifos and considering the EC_{10} values estimated, from the most to the least sensitive: *P. excavatus* > *D. veneta* > *E. bigeminus* > *E. dudichi* > *E. crypticus* > *E. andrei*.

Using EC_{10} and EC_{50} data of each taxonomic group (Collembola and Oligochaeta) or of both groups, SSDs were performed (Figures 4 -6) and HC_5 and HC_{50} were estimated from each SSD (table 14).

Figure 4 - SSD curves using EC10 and EC50 values of Collembola species to the insecticide Lorsban 480BR Dow Agro® (with Chlorpyrifos as active ingredient). For information about the species used in this SSD see the text.

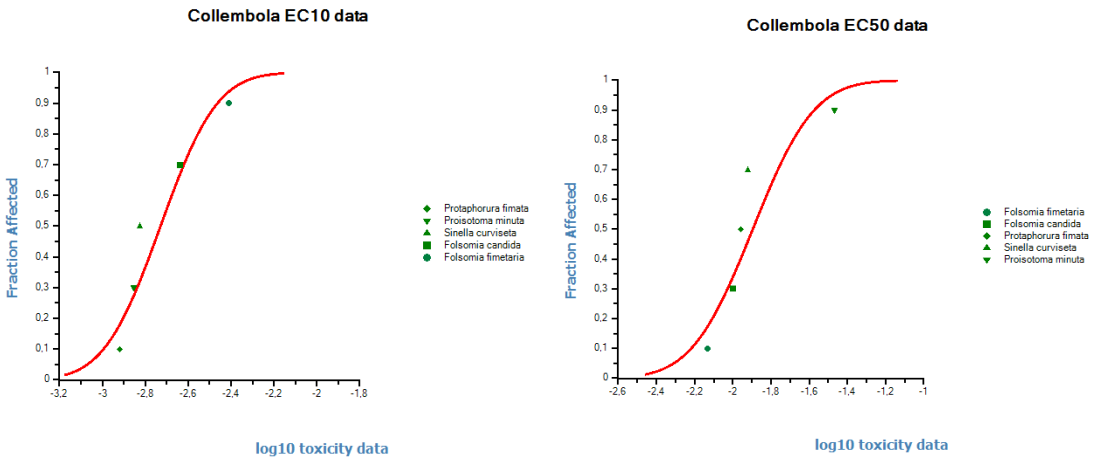

Figure 5 – SSD curves using EC10 and EC50 values of Oligochaeta species to the insecticide Lorsban 480BR Dow Agro® (with Chlorpyrifos as active ingredient). For information about the species used in this SSD see the text.

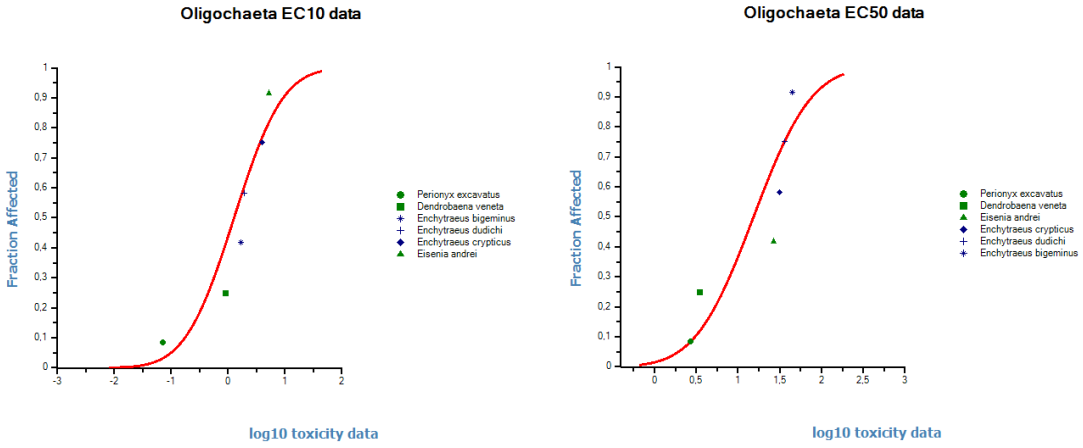

Figure 6 – SSD curves using EC10 and EC50 values of Collembola and Oligochaeta species to the insecticide Lorsban 480BR Dow Agro® (with Chlorpyrifos as active ingredient). For information about the species used in this SSD see the text.

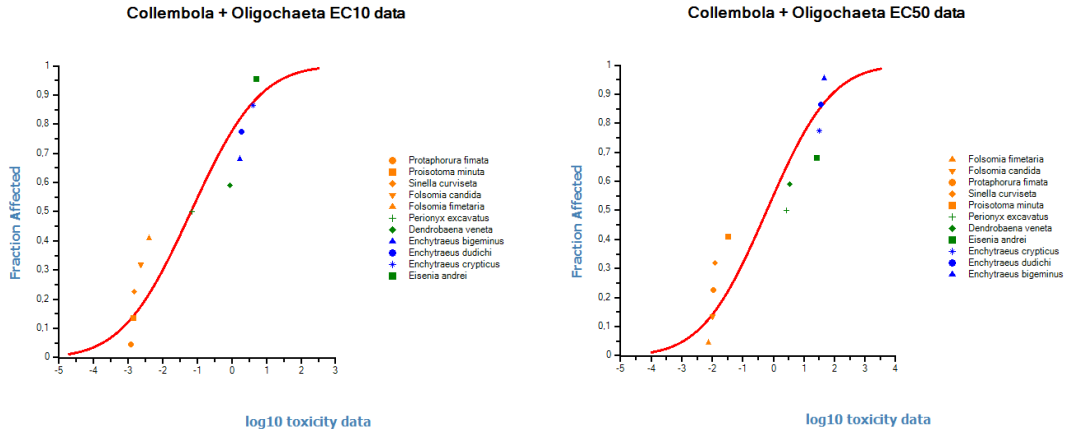

Table 14 - Hazard concentrations (and respective 95% confidence intervals) in mg a.i. kg^{-1} for a protection level of 95% and 50% based on EC_{10} (HC_{5EC10} and HC_{50EC10}, respectively) and EC_{50} values (HC_{5EC50} and HC_{50EC50}, respectively) and estimated through Species Sensitivity Distribution (SSD) curves generated from Collembola species, Oligochaeta species and both Collembola and Oligochaeta species. Chlorpyrifos was used as the model PPP. For information about the species used in each SSD see the text.

	$HC5_{EC10}$	$HC50_{EC10}$	$HC5_{EC50}$	$HC50_{EC50}$
Collembola	0.00081 (0.00026 – 0.0012)	0.0018 (0.0012 – 0.0029)	0.0045 (0.0011 - 0.0079)	0.012 (0.0073 - 0.022)
Oligochaeta	0.084 (0.0040 - 0.32)	1.26 (0.35 - 4.50)	1.69 (0.14 - 5.08)	15.28 (5.43 – 43)
Collembola + Oligochaeta	0.00015 (0.0000027 - 0.0016)	0.066 (0.0092 - 0.47)	0.00092 (0.000013 – 0.011)	0.61 (0.075 – 4.95)

Considering the HC values based on EC_{10} data, the larger value of the HC_{50}:HC_5 ratio (the higher the ratio the higher the discrepancy of sensitivity between species) was estimated for the SSDs with Collembola and Oligochaeta plotted together (440), followed by Oligochaeta (15) and Collembola (2). The same behavior is observed using EC_{50} data (Collembola and Oligochaeta: 663; Oligochaeta: 9 and Collembola: 2.6). The estimation of risk though TER values to different scenarios and PECs according with the tier in ERA is represented in Table 15.

Table 15 - Predicted environmental concentration estimated immediately after pesticide application (PEC_{intial} – Lower tier), and one (PEC_{year}) and ten years ($PEC_{accumax}$ – Intermediate tier) after a scenario of pesticide chlorpyrifos (Lorsban 480®) application. Brazilian (BR 20, BR 24, BR 28) and European scenarios (North, Center and South) were considered (with different temperatures and distinguish to soil type (LS: loamy sand; L/SL: loamy/sand loamy; Clay: clay; LFS: loamy fine sand)). Toxicity exposure ratios (TER) using HC_5 or HC_{50} based on EC_{10} data for Oligochaeta, Collembola and both Oligochaeta and Collembola species in SSDs. * - Risk considered for TER values <5.

Lower tier								
Scenario	Soil	$PEC_{initial}$			TER *F. candida* EC_{10}		TER *E. andrei* EC_{10}	
BR all	-	1.28			0.0018*		4.06*	
EU North	LS	0.51			0.0045*		10.16	
EU Center	LS	0.64			0.0036*		8.13	
EU South	L/SL	0.20			0.012*		26.00	
Intermediate tier								
PEC_{year}			Collembola		Oligochaeta		Collembola + Oligochaeta	
Scenario	Soil	PEC	TER $HC5_{EC10}$	TER $HC50_{EC10}$	TER $HC5_{EC10}$	TER $HC50_{EC10}$	TER $HC5_{EC10}$	TER $HC50_{EC10}$
BR 20	Clay	3.00	0.00027*	0.00060*	0.028*	0.42*	0.000050*	0.022*
	LFS	2.99	0.00027*	0.00060*	0.028*	0.42*	0.000050*	0.022*
BR 24	Clay	2.97	0.00027*	0.00061*	0.028*	0.42*	0.000050*	0.022*
	LFS	2.25	0.00036*	0.00080*	0.037*	0.56*	0.000066*	0.029*
BR 28	Clay	2.24	0.00036*	0.00080*	0.037*	0.56*	0.000067*	0.029*
	LFS	2.22	0.00036*	0.00081*	0.038*	0.57*	0.000067*	0.029*
EU North	LS	0.33	0.0025*	0.0055*	0.25*	3.82*	0.00045*	0.20*
EU Center	LS	0.42	0.0019*	0.0043*	0.20*	3.00*	0.00035*	0.16*
EU South	L/SL	0.15	0.012*	0.012*	0.56*	8.40	0.00099*	0.44*
$PEC_{accumax}$								
BR 20	Clay	3.98	0.00020*	0.00045*	0.021*	0.317*	0.000037*	0.016*
	LSF	3.49	0.00023*	0.00052*	0.024*	0.361*	0.000043*	0.019*
BR 24	Clay	3.18	0.00025*	0.00057*	0.026*	0.396*	0.000047*	0.021*
	LSF	2.98	0.00027*	0.00060*	0.028*	0.422*	0.000050*	0.022*
BR 28	Clay	2.61	0.00031*	0.00069*	0.032*	0.482*	0.000057*	0.025*
	LSF	2.38	0.00034*	0.00076*	0.035*	0.529*	0.000063*	0.028*
EU North	LS	0.61	0.0013*	0.0030*	0.14*	2.07*	0.00024*	0.11*
EU Center	LS	0.66	0.0012*	0.0027*	0.13*	1.91*	0.00035*	0.16*
EU South	L/SL	0.20	0.0041*	0.0090*	0.42*	6.30	0.00075*	0.33*

TER values in the lower tier based on EC_{10} data indicates risk to *F. candida* (TER < 5) in all scenarios. However, *E. andrei* was at risk only in Brazilian scenario and not in European (TER > 5) scenarios. In intermediate tier, regardless the PEC used, TER $HC5_{EC10}$ values indicates risk to Collembola and Oligochaeta in all exposure scenarios. TER $HC50_{EC10}$ to Oligochaeta in Europe South scenario is the only situation that not posed risk. For Brazilian scenarios, PEC values were always higher than those of Europe. Brazilian $PEC_{initial}$ (1.28 mg a.i. kg^{-1}) was two times higher than the higher $PEC_{initial}$ of the European scenarios (Center: 0.64 mg kg^{-1}). Brazilian PEC_{year} and $PEC_{accumax}$ were similar (~ 3 mg a.i. kg^{-1}) while in European Center and North scenarios, $PEC_{accumax}$ were two times higher than PEC_{year}.

3.5 DISCUSSION

3.5.1 *Ecotoxicological data*

Although the most species filled the validity criteria stablished for species from the same taxonomic group, for the enchytraeid species *E. bigeminus* and *E. dudichi* the adult mortality could not be quantified. The ability of these species to reproduce by fragmentation did not allow to distinguish adults from juveniles at the end of the tests. This happened also for chlorothalonil (Chapter II). This fact highlights the need of establishing validation criteria specific for these species. Juveniles number in the end of each test are available in table 20 (annex II).

Results obtained in the laboratory tests evidenced high sensitivity of Collembola species to chlorpyrifos, which could be associated with the mode of action of the product. Despite of has been used to combat pest insects, it affects in a similar way target and non-target organisms (Reigart and Roberts, 1999). Chlorpyrifos acts through the accumulation of the neurotransmitter acetylcholine which causes overstimulation of the neuronal cells, leading to neurotoxicity and eventually death (Karanth and Pope, 2000).

The toxicity of organophosphorus insecticides, as chlorpyrifos, to Collembola has been reported by several authors. Jegede et al. (2017) who performed reproduction tests with *F. candida* using an increase concentration gradient of Pestanal (99% of chlorpyrifos) in artificial soil (with 5% peat) estimated an EC_{50} value of 0.031 mg a.i. kg^{-1}, that was three times higher than the EC_{50} of 0.010 mg a.i. kg^{-1} estimated in the present study. Kamoun et al. (2018), in a study using natural tropical soils from Tunisia (clay = 4.6% and O.C. = 0.29%) and Nigeria (clay = 15% and O.C. = 0.98%) and using the insecticide Pyrica l480 (with 480 g of chlorpyrifos L^{-1}) and *F. candida* as test organism, found EC_{50} values of 0.035 and 0.031 mg a.i. kg^{-1} respectively to Tunisia and Nigeria soils. Santos et al. (2012) tested the effects of the insecticide

Dursban® (with 23.5% of chlorpyrifos) to *F. candida* in a Mediterranean soil (clay = 4.2 %, O.M. = 2.4%, pH H_2O = 7.31) after spraying it with the insecticide in field. The authors estimated an EC_{50} value of 0.045 mg a.i. kg^{-1}, which is even higher than the previous values reported both in the literature and also in the present study.

To the best of our knowledge, until date, the toxicity of chlorpyrifos has been investigated only to *F. candida*. There is a lack of information regarding the toxicity of substances to other species of Collembola, especially PPPs. *F. fimetaria* is the second Collembola species more used in laboratory tests. This species has been used to evaluate toxicity of substances like copper sulfate (Pedersen and Van Gestel, 2001), linear alkylbenzene sulfonate (LAS), the polycyclic aromatic hydrocarbon pyrene (Jensen et al. 2002) and veterinary pharmaceutical products (Jensen et al. 2003). The only PPPs investigated until date were the biocides esfenvalerate, picoxystrobin, triclosan. These substances were tested using a loam soil (clay = 17%, OC = 1.49, pH H_20 = 5.8) and only esfenvalerate presented toxicity (Schnug et al., 2014). Krogh et al. (2009) in a ring test experiment highlighted a similar sensitivity between this species and *F. candida*, which also was observed in Krogh (1995), who reported no crucial differences between both species. Diao et al. (2007) found a difference which proved to be significant, but only for mortality. Pedersen et al. (2000) found that male *F. fimetaria* differed from females in their copper body burden but reported no statistically significant differences between the growth and reproduction endpoints for the two Folsomia species. This similarity between *F. fimetaria* and *F. candida* in sensitivity corroborates with overlaps confidence intervals observed in the present work. In addition, Krogh et al. (2009) concluded that similarities or differences in sensitivity from this both species could be related to the toxic mechanism of the tested substance.

Concerning the other Collembola species, Bandow et al. (2014) investigated whether and how temperature and rain regime could influence the toxicity of an insecticide with lambda-cyhalothrin as a.i. to *S. curviseta* and *F. candida* in artificial soil (5% of peat). These researchers found a steeper dose–response relationship for *S. curviseta* in comparison with *F. candida*. Moreover, *F. candida* was more affected by drought stress in comparison with *S. curviseta*, especially at high temperatures. This fact was also observed for data in the present work, since the EC_{10} values where lower to *S. cuviseta* (0.0015 mg a.i. kg^{-1}) than to *F. candida* (0.0023 mg a.i. kg^{-1}) even that the EC_{50} values were almost the same (0.012 and 0.010 mg a.i. kg^{-1} respectively).

Another study conducted by De Santo et al. (2019), investigated the effect of the herbicide Ally® with the adjuvant Assist® (with metsulfuron-methyl as a.i.) to *P. minuta* in a field study with a haplic cambisol (clay texture, OM = 4.4%). These authors reported EC_{50} values to *P. minuta* considerably lower compared to *F. candida* (0.003 and > 300 mg a.i. kg^{-1}, respectively). The higher sensitivity found to *P. minute* compared to *F. candida* does not agree with the data obtained in the present study where the sensitivity to chlorpyrifos was in the same order of magnitude. A similar sensitivity between these two species was also found by Buch et al. (2016) who investigated the toxicity of mercury in a Brazilian soil (Clay = 35%, OM = 2.6%, pH KCl 1M = 3.98). These data suggest that the relative sensitivity of these two species to soil contaminants might be related to the mode of action of the test substance.

A different study conducted by Sechi et al. (2014), tested the effect of a pyrethroid insecticide with α-cypermethrin as a.i. to a laboratory constructed community composed by one mite, one earthworm, one enchytraeid and five different Collembola species (*Heteromurus nitidus, Mesaphorura macrochaeta, Folsomia fimetaria, Protaphorura fimata, Proisotoma minuta*) in a Danish soil (clay = 9.5%, O.M. = 2.1%, pH H_2O = 6.2). The authors found an EC_{50} of 14 mg a.i. kg^{-1} to *P. fimata,* which was one of the least sensitive species, and attribute this result to their hemiedaphic behavior. In addition, *F. fimetaria* and *P. fimata* were affect in the same order of magnitude, which corroborates to the dataset of the present study.

Regarding the Oligochaeta species, the toxicity of chlorpyrifos to *E. andrei* has been investigated in several previous studies. De Silva et al. (2009), evaluated the effects of chlorpyrifos (98% a.i., Ltd, Denmark) to *E. andrei* at different temperatures, through laboratory reproduction tests, using an artificial soil (OECD, 10% peat) and the natural soil LUFA 2.0 (O.M. = 3.5 – 4.5%, pH = 6.0) at 20ºC to estimate the toxicity under a temperate scenario and the same artificial soil and a Dickwella soil from Matara, Sri Lanka (O.M. = 9%, pH = 6.2) at 26ºC to represents a tropical scenario. At 20º C, the EC_{50} values estimated for artificial soil (7.49 mg a.i. kg^{-1}) were four times greater than in natural soils (1.79 mg a.i. kg^{-1}). The opposite happened at 26ºC, where the EC_{50} values estimated to the artificial soil (3.86 mg a.i. kg^{-1}) was 1.5 times lower than natural soil (5.87 mg a.i. kg^{-1}), which overlaps in confidence intervals.

At 26ºC, the same authors (de Silva et al. 2010) performed laboratory reproduction tests with *P. excavatus* with several pesticides including the insecticide chlorpyrifos (98%) and a commercial formulation (Judo 40 EC, 40% a.i.). These researchers reported EC_{50} values to the commercial formulation of 3 mg a.i. kg^{-1} and argued that it is lower than the usually found to *E. andrei*. The higher sensitivity of *P. excavatus* compared to *E. andrei* was discussed also to carbofuran and mancozeb at temperatures representative of tropical conditions. The high

sensitivity argued by these authors to *P. excavatus* agrees to the data obtained in the present study where EC_{50} for *P. excavatus* was the lowest estimated for Oligochaeta species and about ten times lower than that for *E. andrei*.

Although *Eisenia andrei/fetida* may be representative of Oligochaeta species as a bioindicator in certain cases, there are some studies that evidenced a weak representativeness of this species. Pelosi et al. (2013), performed a meta-analysis selecting 15 publications (11 papers and 4 studies in a book chapter) with several toxicity data of pesticides to earthworms, including chlorpyrifos (Ma and Bodt, 1993). In general, using statistics and modeling tools, the researchers reported that *Eisenia fetida* is less sensitive to pesticides than *Aporrectodea caliginosa* and *Lumbricus terrestris* species. On the other hand, comparing the sensitivity of *Eisenia andrei* with the tropical *Pontoscolex corethrurus*, Buch et al. (2013) reported that both earthworms have similar sensitivity to the pesticides carbendazim, carbofuran and glyphosate in avoidance and acute tests.

Concerning the earthworm *D. veneta*, despite of the lack of information on sensitivity of reproduction of this species to PPPs, this species was already indicated as less sensitive to Bisphenol A than *Eisenia fetida* (Verdú et al., 2018). On the other hand, in a study conducted by Kostecka and Garczyńska (2008), who tested the efficiency of *D. veneta* in vermicomposting with the recommended dose of insecticides with teflubenzuron, diflubenzuron and chlorfenvinfos as a.i., did not find significant changes in vermicomposting activity. Data obtained in the present study shows that both non-standard earthworm species (*P. excavatus* and *D. veneta*) were more sensitive to chlorpyrifos than *E. andrei*.

Enchytraeid species were also affect by chlorpyrifos, but only at higher concentrations (EC_{50}). Rombke et al., (2017) argued that despite of enchytraeids could react to a broad range of pesticides due to their closer contact with the soil pore water, a high ingestion rate and a thin cuticle, few products have been tested with it. In the same literature review, the researchers highlighted that only one study was done with chlorpyrifos and enchytraeids (*E. albidus*) and used avoidance as the endpoint ($EC_{50Avoidance}$ value of 933 mg a.i.kg^{-1} soil) (Amorim et al., 2008). Despite the absence of avoidance behavior in that case, some researches indicated that measure organophosphorus insecticides (e.g. chlorpyrifos) toxicity through this endpoint could not to be appropriated. These substances have effects in nervous system and consequently, it could interfere on ability of choice (Garcia-Santos and Kellen Forrer, 2011).

Results from the present work pointed to a similar range of sensitivity (EC_{50}) between *E. andrei* and the enchytraeids tested. However, tests with more substances are necessary to

investigate this similarity and clarify the inclusion of Enchytraeid species in the data requirements as defended recently by the PPR Panel of EFSA (2017).

3.5.2 Hazardous Concentrations values and Ecological Risk Assessment values

Concerning the SSD curves and the estimated HCs, these reflected the toxic values estimated for the test species (lower and closer from each other in Collembola species and greater and broader in Oligoachaeta species). This was reflected in the interspecific variation of sensitivity that was highest in SSDs based on the toxic values of all species, form which resulted the highest ratio HC50/HC5 of 440.

Diepens et al. (2016) argued that for pelagic organisms the minimum toxicity dataset for an SSD should be composed by eight species, whereas Maltby et al. (2005) fixed this number in six species. Contrasting with aquatic/sediment environment, the available data on in-soil organisms is scarce, which condition the generation of SSD curves to this compartment. Frampton et al. (2006) argued that the majority (96%) of pesticides have toxicity data for less than five in-soil species, which was the minimum number of species used in their work to develop the SSDs. The same researchers found a clear distinction between arthropods and oligochaetes sensitivity to several insecticides, including chlorpyrifos. This agrees to the data obtained in the present study, where Collembola and Oligochaeta species showed distinct sensitivities to a chlorpyrifos-based insecticide. For aquatic organisms, researchers already suggested that the most sensitive group by mode action of the PPPs should be used to guarantee the protection of the whole compartment: Van den Brink et al. (2006) defended the use of plants to test herbicides while Maltby et al. (2009) argued that all major taxonomic groups (vertebrates, invertebrates, and primary producers) to fungicides due the multisite mechanism of action from the products. For insecticides, Maltby et al. (2005) defended that preferentially arthropods should be used, since the HC_5 estimated using data of freshwater arthropods provided the most conservative estimation. These authors argued that the median HC_5 estimated based on acute toxicity data for freshwater arthropods is generally protective when a safety factor of at least five is applied. When different taxonomic groups were used together, the number of species used increased but also increased the uncertainties related to high species variability.

The SSD approaches present many advantages when compared to methods used in the current ERA of PPPs, which are normally based on few single species tests and field experiments when legally required. Posthuma et al. (2002) highlighted as advantages of the

SSDs: i) their conceptual transparency to decision makers and stakeholders; ii) their general acceptability by regulators and practitioners; iii) and their versatility regarding the possibility to choose percentiles and confidence limits based on the risk manager's preferences. On the other hand, these authors also pointed out the need of requiring relatively large data sets as a disadvantage of the SSD approaches. This issue has been also indicated by other authors (Frampton et al., 2006; Maltby et al., 2009; van Wijngaarden et al., 2010). About issues in SSDs approach statistical methods, Posthuma and collaborators (2002) also pointed that i) there are no mechanistic components, purely empirical; ii) fits of standard functions may be poor; iii) diverse species sets result in polymodal distributions. Wang et al. (2008) argued that, compared to traditional approaches, SSDs have greater statistical significance and ecological meaning. However, the same researches have also highlighted that there is still no uniform standard method to develop SSDs and to estimate HC_5 values. Both parametric and nonparametric methods have been used and to the second, any assumptions about the distribution have been applied. Furthermore, SSD is assumed as a pseudo-continuous distribution: the basic bootstrap method cannot choose values other than original elements in the dataset. The probability of distribution concentrated only in few points may unable the representability of the true distribution.

Wang et al. (2008) also highlighted other uncertainties as the extrapolation from either simple laboratory to complex field environments or from single species to populations and ecosystems. Due to several reasons already presented, SSDs could not be taken as the higher tier of the ERA and should not be taken as the only analysis possible. However, since SSDs represent the probability of effects on a biodiversity of species (HC values) or communities level (fraction affected), reducing uncertainties from lower tier and predicting effects for the higher tier, could be a feasible alternative to an intermediate tier. Other approaches to be used as intermediate tiers have been suggested. Ernst et al. (2015) argued that a two-generation study with *F. candida* could be used as an intermediate tier to improve ERA to Collembola. However, differences between species sensitivity associated with the specific roles in ecosystem services that are performed depending on the Collembola traits must not be overlooked (Eisenhauer et al., 2011; Silva et al., 2016). On the other hand, microarthropods community tests were also highlighted as a possible intermediate tier (Chelinho et al. 2014), but this methodology has some uncertainties, related to representativeness of soil communities used and sampling effort, that needs to be fulfilled before its implementation/recommendation (EFSA, 2017). Moreover, such tests seem more adequate for site-specific scenarios than SSD approaches.

Regardless all PEC values estimated (table 15), those estimated for EU South scenario were the lowest ones. The $PEC_{initial}$ values estimated form Brazilian scenarios were higher than all PEC values estimated for EU scenarios. This fact may be attributed to the higher application rates allowed through the local legislation. The PEC_{year} and $PEC_{accumax}$ estimated for Brazilian scenarios were also higher than the European ones. This due not only to the higher application rates allowed but also due to the DT_{50} values considered in the Brazilian legislation. Despite the expectable higher degradation rates of pesticides in tropical conditions compared to the temperate regions due to the higher average temperatures (Martinez et al., 2008), the official data from IBAMA for chlorpyrifos consider DT_{50} values from 180 to 360 days. These values are substantially higher than the official data considered to the European scenarios. Surprisingly, that was the exact same range observed for chlorothalonil in chapter I, even that both substances have dissimilar characteristics. Moreover, data from literature have reported DT_{50} values from chlorpyrifos far away from the range values considered by IBAMA. Laabs et al. (2000) found DT_{50} values for chlorpyrifos in Ustox and Psamments tropical Brazilian soils of 19.6 and 21.3 days, respectively. However, neither of these soils are required in Brazilian legislation.

Brazil have 13 different soils classes, subdivided in many categories, which makes the choice of representative soil quite difficult (Embrapa, 2019). A better soil characterization in terms of DT_{50} and PECs is necessary, but also a definition of relevant soils to use in the regulation, since one of the suggested soils (*gleisol*) it is not representative for agriculture (Embrapa, 2019). The strategy adopted in Europe by the subdivision of the territory into three regulatory zones could be a suitable alternative to Brazil. The country could be subdivided in regulatory zones taking into consideration local temperature, precipitation, crops and soils properties

Concerning the ERA, TER values estimated for lower tier ($PEC_{initial}$) indicate risk to *F. candida* in all scenarios (TER<5; table 15). The risk remains in the intermediate tier (PEC_{year} and $PEC_{accumax}$). This fact confirms data available in the literature that have reported high risk of organophosphorus insecticides to Collembola species (Santos et al., 2012; Jegede et al., 2017; Kamoun et al., 2018). On the other hand, for *E. andrei*, the lower tier was at risk only in Brazilian scenarios (TER < 5; table 15). In the intermediate tier high risk was found in EU North and Centre scenarios only for TER $HC50_{EC10}$. Nevertheless, to use this hazardous concentration it is not defended because accepted to protect just half of the tested species (HC_{50}) in EC_{10} data (EFSA, 2017).

The use of EC_{10} or EC_{20} and NOEC has been suggested by EFSA (2017), as well as the use of HC_5 instead of HC_{50}. In general, the risk was higher in the intermediate tier (TER < 5) rather than in the lower tier for Oligochaeta species. This indicates that the standard Oligochaeta species used in the current legislation (1107/2009) might be inadequate. The lower tier is supposed to be more protective than the next tiers in ERA scheme, thought that advancing in ERA means increase complexity and realism of measurements. In theory, the risk should be reduced in higher tiers compared with the lower tiers (Muralikrishna and Manickam, 2017). Consequently, data of the present works confirms that the trigger values (or assessment factors) should be refined, as already defended by EFSA (2017).

3.6 CONCLUSION

Collembola species was the most sensitive group to chlorpyrifos in lower and intermediate tier, and *F. candida* allowed to detect risk to other Collembola species. Enchytraeid species and *E. andrei* had EC_{50} values in the same order of magnitude, but the non-standard earthworm species (*P. excavatus* and *D. veneta*) were the most sensitive Oligochaeta species tested. The generation of SSDs individual for each taxonomic group (Collembola and Oligochaeta species) was the most suitable approach rather than using all organisms in the same curve due to avoid high inter-species sensitivity variation. The SSDs work well as intermediate tier to in-soil fauna pesticide risk assessment. The estimation of PEC values seems more realistic for European scenarios than for Brazilian scenarios, due to the most adequate criteria defined by EFSA. In addition, its crucial advance in exposure scenarios to Brazil, considering representative soils and DT_{50} information to improve ERA.

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3.8 ANNEX II

Table 16 - Range of concentrations (mg a.i. kg^{-1}) used in reproduction tests using chlorpyrifos as model PPP (to continue)

Treatment	**Nominal**
Folsomia candida	
C0	0
C1	0.00125
C2	0.0025
C3	0.005
C4	0.01
C5	0.02
C6	0.05
C7	0.1
C8	0.2
C9	0.5
Proisotoma minuta	
C0	0
C1	0.00125
C2	0.0025
C3	0.005
C4	0.01
C5	0.02
C6	0.05
C7	0.1
Folsomia fimetaria	
C0	0
C1	0.00125
C2	0.0025
C3	0.005
C4	0.01
C5	0.02
C6	0.05
C7	0.1
C8	0.2
C9	0.5

Table 16 - Continuation

Protaphorura fimata	
C0	0
C1	0.00125
C2	0.0025
C3	0.005
C4	0.01
C5	0.02
C6	0.05
C7	0.1
C8	0.2
C9	0.5
Sinella curviseta	
C0	0
C1	0.00125
C2	0.0025
C3	0.005
C4	0.01
C5	0.02
C6	0.05
C7	0.1
C8	0.2
C9	0.5
Eisenia andrei	
C0	0
C1	3
C2	5
C3	7
C4	10
C5	15
C6	30
C7	50
Perionyx excavatus	
C0	0
C1	0,5
C2	1
C3	2
C4	4
C5	8
C6	16
C7	32
C8	64
C9	128

Table 16 - Continuation

Dendrobaena veneta	
C0	0
C1	0,1
C2	0,3
C3	0,9
C4	2,7
C5	8,1
C6	24,3
C7	72,9
C8	218,7
Enchytraeus crypticus	
C0	0
C1	0,5
C2	1
C3	5
C4	10
C5	25
C6	50
C7	100
C8	150
C9	200
C10	300
Enchytraeus bigeminus	
C0	0
C1	1
C2	5
C3	10
C4	25
C5	50
C6	75
C7	100
C8	150
C9	300
C10	600

Table 16 - Continuation

Enchytraeus dudichi	
C0	0
C1	1
C2	5
C3	10
C4	25
C5	50
C6	75
C7	100
C8	150
C9	300
C10	600

Table 17 - Good application practices (GAP) of chlorpyrifos according to specific recommendations available for each European region.

Zone	Commercial product (PC)	g i.a.kg or L PC	Crop	rate application (L ha)	dose (mg a.i. ha^{-1})	nº app	interval	BBCH code	reaching soil (fraction)	Interception (%)
EU North	Atena 480 EC	480	Spring oilseed rape	0.6 - 0.8	384	1	-	55 - 59	0.65	35
EU Center	C Y R E N 480 EC	480	Winter rape	0.6 - 1	480	1	-	20-39	0.65	35
EU South	PYRISTAR	250	Spinach	0.6	150	1	-	40	0.75	25

Table 18 - Good application practices (GAP) of chlorpyrifos according to specific recommendations available for Brazil.

Zone	Commercial product (PC)	g i.a.kg or L PC	Crop	rate application (L ha)	dose (mg a.i. ha^{-1})	nº app	interval	BBCH code	reaching soil (fraction)	Interception (%)
BR 1 – BR 6	Lorsban 480	480	Cotton	2	960	2	7	40	0.85	15

Table 19 - Biodegradability of PPPs according with IBAMA legislation (1996).

Biodegradability			Class	Classification	DT_{50} conversion		
0 ≤	% CO_2	< 1	1	Highly Persistent	360 ≤	$T_{1/2}$ (days)	
1 ≤	% CO_2	< 10	2	Very Persistent	180 ≤	$T_{1/2}$ (days)	< 360
10 ≤	% CO_2	< 25	3	Moderately Persistent	30 ≤	$T_{1/2}$ (days)	< 180
25 ≤	% CO_2		4	Low Persistent	0 ≤	$T_{1/2}$ (days)	< 30

Table 20 - Mean number of juveniles (± standard deviation) and coefficient of variation (CV) of control replicates of reproduction tests with Collembola and Oligochaete species. The tested substance was the a.s. chlorpyrifos.

Species	**Mean ± SD in control**	**CV (%)**
Folsomia candida	381 ± 14	3.75
Folsomia fimetaria	168 ± 13	7.89
Sinella cuviseta	291 ± 18	6.31
Protaphorura fimata	253 ± 31	12.32
Proisotoma minuta	158 ± 43	27.07
Dendrobaena veneta	56 ± 3	4.77
Perionyx excavatus	44 ± 2	5.40
Eisenia andrei	60 ± 12	19.72
Enchytraeus crypticus	461 ± 25	5.45
Enchytraeus bigeminus	310 ± 43	13.77
Enchytraeus dudichi	248 ± 29	11.81

4. CHAPTER III: MICROARTHROPOD COMMUNITY TEST IN PESTICIDE ECOLOGICAL RISK ASSESSMENT: THE INTERMEDIATE TIER AS A NEW APPROACH

4.1 ABSTRACT:

The microarthropod community test was used as an intermediate tier approach to assess risks of the fungicide chlorothalonil (Bravonil 500®) and the insecticide chlorpyrifos (Lorsban 480®). Test soil and community were sampled at Portugal and in the end of six weeks the overall fauna groups were identified in major groups. In addition, Collembola species and Acari Orders were assessed through identifications keys. Non-linear regressions, Permanova and Simper analysis were used to estimated toxicity indices. Predicted Environmental Concentrations (PEC) in soil were also estimated to Brazilian and European scenarios. Risk was estimated through toxicity-exposure ratio (TER). Both pesticides had negative effects to in-soil groups. To chlorothalonil, mostly in Brazilian and Portuguese scenarios (TER < 5). To chlorpyrifos, all scenarios estimated risk in the current recommended uses. Microarthropod community tests was a feasible approach to the intermediate tier.

KEY WORDS: Soil ecotoxicology. Pesticides. ecological risk assessment, tiered approach.

4.2 INTRODUCTION

The potential risks of pesticides to in-soil fauna have been assessed mainly through single species ecotoxicity tests (RENAUD et al., 2018; CARNIEL et al., 2019). The current European legislation, for example, requires standard reproduction tests with *F. candida, H. aculeifer* and *E. andrei* which should provide concentrations affecting 10% (EC_{10}) or 20% (EC_{20}) of the respective populations (EU, 2013). This step of the pesticides ecological risk assessment is the lower tier in the tiered approach, which should advance from reproductive and low variability tests to more complex and realistic data (BROCK et al., 2006). The further realistic step (higher tier), is normally performed using field tests, that is required when risk is observed in lower tier. The mainly risk evaluation in the higher step has been performed verifying soil habitat function though the measure of litter mass loss using litter bag test (EC, 2002). Despite this methodology already has successfully used in contaminated areas (NIEMEYER et al., 2015), its use in pesticides ERA have been criticized (EFSA, 2017). Regardless of the evaluated endpoint in the field tests, Schäffer et al. (2011), argued that this methodology is considered a good approach to evaluated PPPs risks, since considerer complex interactions and the natural soil variability, although, these tests requires a high amount of man power, which involves effort, costs and time.

As an alternative to the field tests, the semi-field methods, as the terrestrial model ecosystem (TMEs). This method is designed in a way that the advantages of laboratory tests (e.g., standardization, controlled conditions) are combined with field studies advantages, and for so, was already indicated as a suitable surrogate tier to the higher tier (SCHÄFFER et al., 2010; EFSA, 2017). Nevertheless, despite of the higher ecological realism and several advantages compared with field tests, semi-field methods were already associated with an increasing variability, higher experimental effort and costs (VAN DEN BRINK et al., 2005; SCHÄFFER et al., 2008).

While the single approach does not consider the interactions between species within a community (VAN STRAALEN, 2002; VAN DEN BRINK, 2008), the field and semi-field tests are expensive and time consuming, despite being cost-effective (SCHOLZ-STARKE et al., 2011). So, the intermediate tier as an additional step could provide more information to reduce uncertainties from lower tier and the workloads of the higher tier. To this step, EFSA (2017) suggested the use of species sensitivity distribution curves (SSDs) which could be developed by increasing the number of test species in the lower tier (Chapter I and II).

Another alternative pointed by EFSA Panel for the intermediate tier is the use of community tests. This method has several advantages cited by Chelinho et al. (2014), as a simple, quick, and relatively effortless in the extraction of organisms; no direct handling of animals (and thus, theoretically, diminishing handling related stress); less demanding in terms of space, time and costs compared to field and semi-field studies, and; a presumably lower associated variability. With these tests, besides the intrinsic value of the data gathered with them, the ecological information they introduce in ERA regarding effects on species interactions could help risk assessors and managements to better define the threshold values, as the trigger values (or assessment factors) to apply to lower tier data. An appropriate calibration of this values already was pointed as one of the main gaps in the current Europe pesticides ERA (EFSA, 2017).

Despite of several studies already has investigated effects of pesticides on in-soil fauna (e.g., NATAL-DA-LUZ et al., 2012; JEGEDE et al., 2017; MENEZES-OLIVEIRA et al., 2018), there is still limited experience of assessing effects of chemicals on microarthropods community. Chelinho et al. (2014) assessed carbofuran effects to nematodes and microarthropods. Researchers found significant effects on community composition, favoring Collembola epigeic over euedaphic species, an increase in oribatids as well a decrease in the abundance of predatory mites. Such results showed effects not predicted by single species laboratory tests.

Based on ERA tiered approach proposed by EFSA (2017), the aim of this study was to evaluate risks of chlorothalonil (Bravonil 500®; 500 g a.i. L-1) and chlorpyrifos (Lorsban 480®; 480 g a.i. L-1) through microarthropods community tests using a natural Mediterranean soil and native communities as an intermediate tier. Risk values were compared with predicted environmental concentration (PEC) in soil for European and Brazilian scenarios as in the previous chapters and in addition, with a specific Portuguese scenario were the soil was sampled.

4.3 MATERIAL AND METHODS

4.3.1 Test substances

Commercial formulation of the fungicide isophthalonitrile chlorothalonil (Bravonil 500®; 500 g a.i. L^{-1}) and of the insecticide organophosphorus chlorpyrifos (Lorsban 480®; 480

g a.i. L^{-1}) were used to soil contamination. Physical and chemical characterization of these active ingredients are in table 21. A range of increasing concentrations for both products were used and are available on annex III table 28.

Table 21 - Physicochemical characteristics of chlorothalonil and chlorpyrifos. Chemical characteristics and DT50 data were collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and IUPAC (https://sitem.herts.ac.uk/aeru/iupac/Reports/).

Characteristic	Chlorothalonil	Chlorpyrifos
CAS	1897-45-6	2921-88-2
IUPAC name	tetrachloro-isophthalonitrile	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
Empirical formula	$C_8Cl_4N_2$	$C_9H_{11}Cl_3NO_3PS$
Molecular mass (g mol^{-1})	265.91	350.58
Relative density (g cm^{-1})	1.8	1.4
Solubility (pH = 7) (mg L^{-1} 20ºC)	0.81	1.05
Log K_{ow} (at 20ºC)	2.94	4.06
Henry's Law constant (25ºC Pa m^3 mol^{-1})	2.50 x 10^{-02}	0.478
Degradation Soil (20 °C aerobic) (days)	3.53	386
Degradation/Dissipation Field (days)	17.9	27.6

4.3.2 Test soil and natural community

Test soil and methods are the same described by Renaud et al. (submitted, 2019). Natural soil was collected at Foros do Vale da Figueira, located in the south of Portugal, Alentejo zone (38°41'39.7"N 8°18'27.6"W) from a “Montado” (cork oak forest) area. The soil was sieved (5 mm) and stored until use. Soil properties are shown in table 22.

Microarthropods natural community was collected in the same site. For this purpose, soil cores were collected using pvc rings (5 cm diameter and 5 cm depth), from which the organisms were extracted in Macfadyen system for 72 h at 45ºC directly in the replicates of the experiment (see below). Initial community were also evaluated prior to the microcosm experiment (n: 11).

Table 22 - Properties of the soil used in microarthropods community tests with Bravonil 500® (a.i. chlorothalonil) and Lorsban 480® (a.i. chlorpyrifos).

Soil properties	
Organic matter (%)	4 ± 0.7
Organic Carbon (%)	2.5 ± 0.7
pH	4.02
CEC (meq/100g)	9.2 ± 0.4
WHC (%)	66.9 ± 10.3
Sand	68
Silt	24
Clay	8

4.3.3 Experimental procedure

Tests were performed at Soil Ecology and Ecotoxicology Lab, Coimbra University, Portugal, and followed the procedures described in Chelinho et al. (2014) and Renaud (submitted, 2019). Test vessels received 300 g of contaminated (n: 4) or control (n: 8) soil and the extracted soil invertebrate community.

To perform the extraction, three soil cores with native community were directly extract in a macfadyen system to a portion of soil (10 g) placed in a falcon tube during 24 h. After this period, soil with extracted fauna was added in the correspond treatment and a new falcon, previously conditioned with a portion of soil, was replaced. The procedure was repeated more two times and the total time of the extraction was 72 hours.

Test vessels were placed in an incubation room (21 ± 2 ºC, 16:8 h of light:dark) during six weeks. During test incubation, food in form of granulated dry yeast (approximately 4mg) was provided and soil water content was weekly adjusted by weighting, using distilled water. After test period, organisms were extracted under the same conditions as the initial community into 70% ethanol falcon tubes (30 ml). Soil microarthropods were then identified into major groups using a stereomicroscope (60x). Further, collembolans were identified to species level and mites were identified to the order level (Prostigmata, Mesostigmata, Oribatida) or cohort (Astigmatina) (Lindquist et al., 2009). Table 23 summarize test conditions.

Table 23 - Procedures adopted in laboratory tests with microarthropod community using chlorothalonil (Bravonil 500®) and chlorpyrifos (Lorsban 480®) as the models PPPs in a Mediterranean soil.

Microarthropod community test conditions	
Range of concentrations used (mg a.i. kg^{-1}) in chlorothalonil test	3.75 - 240
Range of concentrations used (mg a.i. kg^{-1}) in chlorpyrifos test	0.008 - 2
Test period (days)	42
Days of food supply	weekly
Number of replicates per treatment	4 + 8 in control
Test container (ml)	1000
Food source	Dry yeast
Food per test container (g of FW)	4 mg
Soil per test container (g of DW)	300
Soil core communities by replicate	3

4.3.4 Predicted Environmental Concentrations

The time-weighted average concentration for ten years (as the maximum accumulated in ten years; $PEC_{accumax}$) were estimated considering percentage of interception by crops, DT_{50} values, products characteristics and environmental data, according to data from EFSA (2015). $PEC_{accumax}$ were used as the exposure to conduce risk assessment, once that was already recommended for intermediate and higher tiers (EFSA, 2015; EFSA, 2017).

To perform the risk assessment of this step, three European exposure assessments scenarios were used (North, Center and South) (EFSA, 2015) and an additional Portuguese scenario were soil and community were sampled. For each region, temperature (7, 10, 12, 14.5 ºC to North, Center, South and Portugal respectively), soil texture (coarse to North and Center; medium fine to South; sandy loam to Portugal) and the respective DT_{50} values for chlorothalonil and chlorpyrifos (according to local properties) were taken into consideration for PEC values estimation. Scenarios data were collected from EFSA (2015) and to Portugal, soil proprieties from table 12 and the annual average temperature online consulted (https://pt.climate-data.org/europa/portugal/alentejo/alentejo-632872/) were used. In addition, DT_{50} data were obtained from the Rapporteur Assessment Report (RAR) of chlorothalonil (https://www.efsa.europa.eu/en/consultations/call/161024) and chlorpyrifos (https://www.efsa.europa.eu/en/consultations/call/171018-0). Good application practices (GAP) to both pesticides were assumed according to specific recommendations available for North, Center, South and Portugal (see annex III, table 29).

The crops considerer to estimate PEC values were based on the highest application doses and the lowest crop interception. The crop interception was measured by the Biologische

Bundesanstalt, Bundesortenamt und Chemische Industrie (BBCH) code, which is a decimal code ranging from 0 to 99 to characterize the crop development stage (MEIER, 2001). Through the BBCH code, its possible estimates the fraction of the pesticide dose that was not covered by the crops and consequently, reaches the soil (f_{soil}) (EFSA, 2015). In annex III (table 29), more information on the variables used to estimated $PEC_{accumax}$ are available.

For Brazil, GAPs for chlorothalonil and chlorpyrifos were taken from MAPA database (MAPA, 2019). As performed for EU regions, the worst-case scenario (WCS) for chlorothalonil and chlorpyrifos persistence in soil was established considering the total number of applications, the highest application doses and higher value of f_{soil}. Information are available in annex III (table 29).

Since official DT_{50soil} data for Brazilian soils were not found in open databases, these values were provided by a Brazilian Institute of Environment (IBAMA) through an online government public communication channel (https://esic.cgu.gov.br/sistema/site/index.aspx). Brazilian legislation accepted DT values measured or DT values converted from the percentage (%) of radiolabeled carbon dioxide ($^{14}CO_2$) detached (IBAMA, 1996) (IBAMA method of conversion available in annex III, table 31). Since the information provided by IBAMA was $^{14}CO_2$ detached of chlorothalonil and chlorpyrifos, these data were converted to DT_{50} values. A *Neossolo quartzarênico* soil (loamy fine sand texture) was used to chlorothalonil and an *Argisolo* soil (loamy fine sand texture) was used to chlorpyrifos, since both are representative in Brazilian agriculture (EMBRAPA, 2019), have the same texture and were available the official information provided by IBAMA. The scenarios were estimated in three different temperatures (20º, 24º and 28º C representing the annual average temperatures of South, Centre and North of Brazil, respectively; INMET, 2019).

4.3.5 Data analysis

Once the identification of microarthropods was completed, the abundance dataset (fourth root) was used to calculate Bray-Curtis distance matrices for the overall microarthropods, for Collembola species and mite orders independently using PRIMER & PERMANOVA 6.0 (CLARKE:GORLEY, 2006). In these matrices, the similarity within control and between control and each concentration were selected to perform dose-response curves. A decrease in similarity is expected to occur when pesticides concentrations increase, if it affects the community. Effective concentrations (EC_{20} and EC_{50}) were estimated using

nonlinear regressions, according to Environmental Canada (2007) and the best fitting model was applied using Statistica 7.0 (STAT. SOFT. Inc., 2004).

Permutational multivariate analysis of variance (PERMANOVA) was used in order to verify significant differences ($p < 0.05$) in similarity between control and treatments and allowed to estimate the non-observed effect concentrations (NOECs). In addition, the groups (overall fauna), species (Collembola) or Order (mites) that contributed to significant differences in Permanova test were observed though the similarity of percentages analysis (SIMPER). These analyses also were performed using PRIMER & PERMANOVA 6.0 (CLARKE:GORLEY, 2006). Furthermore, when possible, EC_{20} and EC_{50} values of the most abundant specie and responsible for most of differences in similarities were calculated (SIMPER analysis).

The toxicity to exposure ratio (TER) was estimated using NOEC values (NOEC / $PEC_{accumax}$) since was the endpoint available to more groups. TER results were compared with a trigger value of 5, as in previous chapters, to indicated presence or absence of risk.

4.4 RESULTS

For the overall microarthropod community exposed to chlorothalonil it was possible to estimate the EC_{50} value (32.26 mg a.i. kg^{-1}) and Permanova analysis pointed to dissimilarities between control from 12.99 mg.kg-1 to the highest concentration ($p < 0.05$, NOEC: 7.63 mg a.i. kg^{-1}). Dissimilarities were mainly explained by mites and collembolans (SIMPER: 85%) (figure 7). Due the high variability in mite data, it was not possible to estimate the ECs or dissimilarities for this group. For collembolans data, values of EC_{20} (5.63 (1.90 - 9.36) mg a.i. kg^{-1}) and EC_{50} (7.42 mg a.i. kg^{-1}) were estimated. Permanova test showed that dissimilarities started at 12.99 mg a.i. kg-1 concentration (NOEC: 7.63 mg a.i. kg^{-1}) explained mainly by the reduction of *H. thermophila* abundance (SIMPER: 51.87%) (Figure 8). For this specie, EC_{20} and EC_{50} were also determinate as 2.47 and 3.75 mg a.i. kg^{-1} respectively. Besides, other Collembola species were found just in control and in lower tested concentrations (*Protaphorura armata, Sphaeridia pumilis*), but the low number of organisms does not permit enough inferences. The toxicity results are summarized in table 24 and SIMPER analysis in table 25.

Figure 7 - Overall microarthropods community in increasing concentrations of chlorothalonil (Bravonil 500®) spiked in a natural Mediterranean soil. Points represents the mean values of similarity to control after six weeks

of exposure (±SD). Asterisks (*) indicates differences between each treatment and control (Permanova, $p < 0.05$). In addition, the contribution of Acari, Collembola and Coleoptera to dissimilarities is represented.

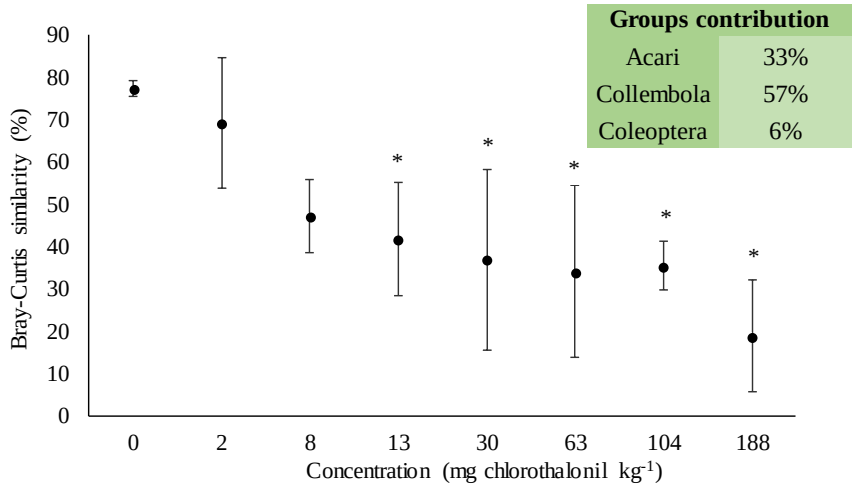
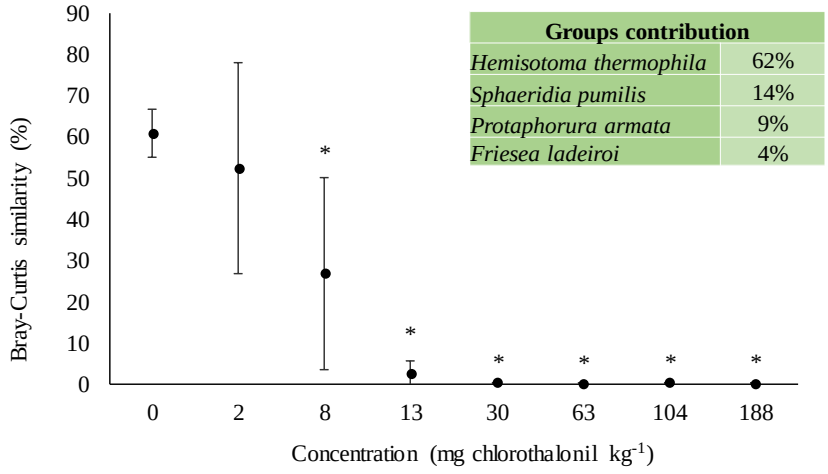


Figure 8 - Collembola group in increasing concentrations of chlorothalonil (Bravonil 500®) spiked in a natural Mediterranean soil. Points represents the mean values of similarity to control after six weeks of exposure (±SD). Asterisks (*) indicates differences between each treatment and control (Permanova, $p < 0.05$). In addition, the contribution of distinguish species to dissimilarities is represented.



For chlorpyrifos data, considering the overall microarthropod community, ECs could not be estimated due to the high variability of the data. However, differences were observed in Permanova ($p < 0.05$) (NOEC: 0.008 mg a.i. kg^{-1}) and dissimilarities were mostly attributed to higher abundance of collembolans in control than in the pesticide concentrations (SIMPER: 50%) (Figure 9). Using Collembola species was possible to estimate the EC_{20} (0.013 mg a.i. kg^{-1}) and EC_{50} (0.031 mg a.i. kg^{-1}) values. Permanova data followed same results as the microarthropod community (NOEC: 0.008 mg a.i.kg^{-1}) and dissimilarities were caused mostly by *H. thermophila* (Figure 10). In mite data, due the high variability was not possible to estimate the ECs. Permanova test showed that dissimilarities started at 0.02 mg chlorpyrifos kg^{-1}

concentration (NOEC: 0.008 mg a.i. kg^{-1}) caused mainly by Oribatida order (SIMPER: 63%) (Figure 11). The toxicity results are summarized in Table 13 and SIMPER analysis in table 26.

Furthermore, the $PEC_{accumax}$ values and estimated TERs based on NOEC data to chlorothalonil and chlorpyrifos are summarized in Table 27.

Figure 9 - Overall microarthropods community in increasing concentrations of chlorpyrifos (Lorsban 480®) spiked in a natural Mediterranean soil. Points represents the mean values of similarity to control after six weeks of exposure (±SD). Asterisks (*) indicates differences between each treatment and control (Permanova, $p < 0.05$). In addition, the contribution of Collembola, Acari and Coleoptera to dissimilarities is represented.

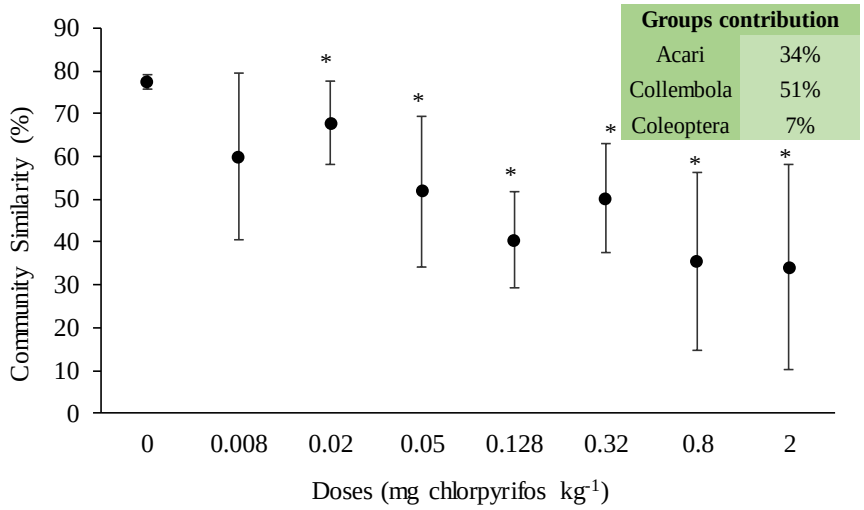


Figure 10 - Collembola group in increasing concentrations of chlorpyrifos (Lorsban 480®) spiked in a natural Mediterranean soil. Asterisks (*) indicates differences between each treatment and control (Permanova, $p < 0.05$). In addition, the contribution of distinguish species to dissimilarities is represented.

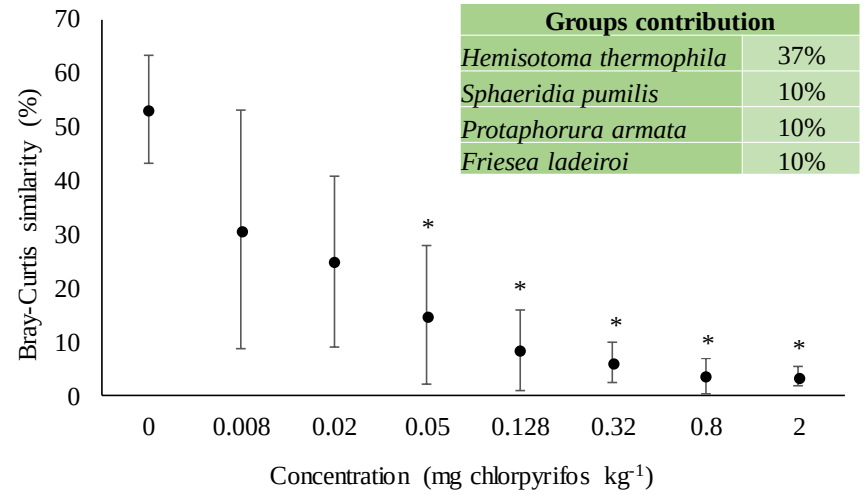


Figure 11 - Acari group in increasing concentrations of chlorpyrifos (Lorsban 480®) spiked in a natural Mediterranean soil. Asterisks (*) indicates differences between each treatment and control (Permanova, $p < 0.05$). In addition, the contribution of Oribatida and Mesoastigmata to dissimilarities is represented.

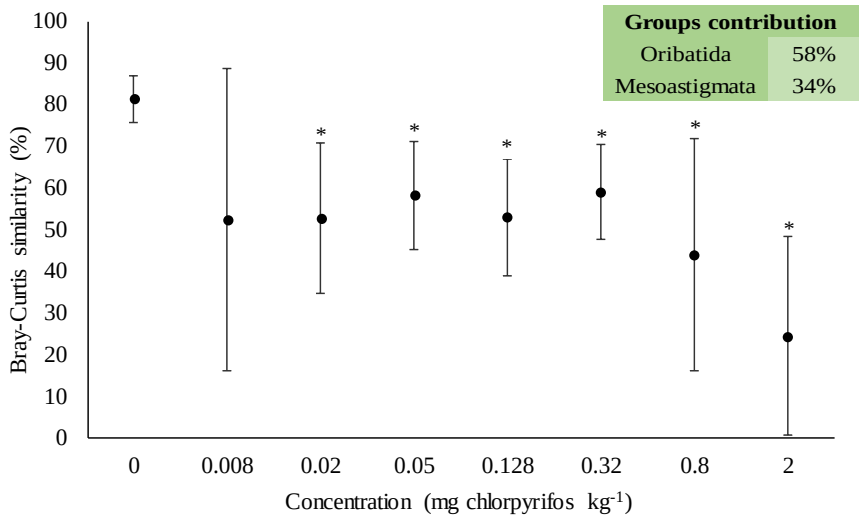


Table 24 - Toxicity values (EC_{20}, EC_{50} and NOEC) expressed in mg a.i.kg^{-1} to the PPPs chlorothalonil (Bravonil 500®) and chlorpyrifos (Lorsban 480®) estimated through dissimilarities between control and treatments in microarthropods community tests.

Chlorothalonil			
Organisms	EC_{20}	EC_{50}	NOEC
Overall microarthropods	-	32.26 (2.06 - 62.46)	7.63
Collembola	5.63 (1.90 - 9.36)	7.41 (5.80 - 9.02)	7.63
H. thermophila	2.47 (0.79 - 4.15)	3.75 (1.50 - 5.99)	2.24
Mites	-	-	-
Chlorpyrifos			
Organisms	EC_{20}	EC_{50}	NOEC
Overall microarthropods	-	-	0.008
Collembola	0.013 (0.0020 - 0.024)	0.031 (0.0065 - 0.055)	0.02
H. thermophila	-	-	-
Mites	-	-	0.008

Table 25 - Percentage dissimilarities (SIMPER) to statistically differentiate chlorothalonil (Bravonil 500®) treatments from control (Permanova, $p < 0.05$). The average dissimilarity for each tested treatment x control (%); fauna groups that most contributed to dissimilarities to overall fauna (%) and Collembola species that most contributed to dissimilarities to Collembola group. In addition, the total explained by the groups or species (%).

Chlorothalonil								
Evaluated group	Difference between		Average dissimilarity		Contribution (%) of fauna groups			*Total (%)*
					Collembola	Mites	Coleoptera	
Overall fauna	Control x D3		58.16		56.29	30.48	6.48	93.25
	Control x D4		62.94		59.17	29.18	5.7	94.05
	Control x D5		65.83		55.52	32.11	6.08	93.71
	Control x D6		64.54		63.55	23.95	6.14	93.64
	Control x D7		81.16		50.07	38.96	5.43	94.46
			Contribution (%) of Collembola species					
Evaluated group	Difference between	Average dissimilarity	*Hemisotoma thermophila*	*Sphaeridia pumilis*	*Protaphorura armata*	*Friesea ladeiroi*	*Ceratophysella gibbosa*	*Total (%)*
Collembola	Control x D3	89.37	50.91	14.89	12.42	7.08	5.41	90.71
	Control x D4	99.66	54.88	9.62	12.58	7.38	6.24	90.7
	Control x D5	99.83	50.01	9.23	11.18	6.66	5.2	82.28
	Control x D6	99.66	54.88	9.62	12.58	7.38	6.24	90.7
	Control x D7	93.55	48.70	9.62	11.36	6.77	6.07	82.52

Table 26 - Percentage dissimilarities (SIMPER) to statistically differentiate chlorpyrifos treatments from control (Permanova, $p < 0.05$). The average dissimilarity for each tested treatment x control (%); fauna groups that most contributed to dissimilarities to overall fauna (%); Collembola species that most contributed to dissimilarities to Collembola group and mites Order that most contributed to the dissimilarities to Acari group. In addition, the total explained by the groups, species or Order (%).

Chlorpyrifos

Evaluated group	Difference between	Average dissimilarity	Contribution (%) of fauna groups			
			Acari	Collembola	Coleoptera	*Total (%)*
Overall fauna	Control x D2	32.11	41.35	32.78	11.93	86.06
	Control x D3	48.21	35.94	49.04	7.29	92.27
	Control x D4	59.59	28.32	58.61	6.36	93.29
	Control x D5	49.75	30.22	55.05	7.15	92.42
	Control x D6	64.64	32.21	55.39	6.06	93.66
	Control x D7	65.84	35.13	52.55	6.02	93.70

Evaluated group	Difference between	Average dissimilarity	Contribution (%) of Collembola species				
			Hemisotoma thermophila	*Sphaeridia pumilis*	*Friesea ladeiroi*	*Protaphorura armata*	*Total (%)*
Collembola	Control x D3	70.98	33.33	8.85	5.93	5.76	53.87
	Control x D4	42.88	40.24	12.79	9.82	8.42	71.27
	Control x D5	78.73	35.81	6.97	10.29	6.24	59.31
	Control x D6	66.81	37.42	8.21	6.30	21.41	73.34
	Control x D7	64.63	38.68	10.66	12.93	5.59	67.86

Evaluated group	Difference between	Average dissimilarity	Contribution (%) of mite groups		*Total (%)*
			Oribatida	Mesoastigmata	
Mites	Control x D2	47.53	53.83	38.65	92.48
	Control x D3	41.8	65.12	26.66	91.78
	Control x D4	45.84	55.33	36.97	92.3
	Control x D5	41.1	59.22	32.52	91.74
	Control x D6	56.16	56.21	33.42	89.63
	Control x D7	75.72	60.25	34.12	94.37

Table 27 - Predicted Environmental Concentration in total soil ($PEC_{accumax}$) estimated values to chlorothalonil (Bravonil 500®) and chlorpyrifos (Lorsban 480®). Toxicity exposure ratio (TER) estimated using NOEC values (Table 24) to overall fauna community, Collembola, *H. thermophila* and mites. Asterisks (*) indicated that the TER is lower than the current trigger value (5), which means that risk is predicted.

Scenarios	$PEC_{accumax}$ (mg.kg^{-1})		TER (NOEC)							
			Chlorothalonil				Chlorpyrifos			
	Chlorothalonil	Chlorpyrifos	Overall	Collembola	*H. thermophila*	Mites	Overall	Collembola	*H. thermophila*	Mites
Brazil 20	16.64	3.49	0.46*	0.46*	0.13*	-	0.0023*	0.0057*	-	0.0023*
Brazil 24	14.03	2.98	0.54*	0.54*	0.16*	-	0.0027*	0.0067*	-	0.0027*
Brazil 28	12.08	2.38	0.63*	0.63*	0.19*	-	0.0034*	0.0084*	-	0.0034*
EU North	1.14	0.61	6.69	6.69	1.96*	-	0.013*	0.033*	-	0.013*
EU Center	3.59	0.66	2.13*	2.13*	0.62*	-	0.012*	0.030*	-	0.012*
EU South	1.50	0.20	5.09	5.09	1.49*	-	0.040*	0.10*	-	0.040*
Portugal	3.79	0.094	2.01*	2.01*	0.59*	-	0.090*	0.21*	-	0.090*

The $PEC_{accumax}$ values of chlorothalonil were higher in Brazilian scenarios (12 ~ 16 mg kg^{-1}) than in European scenarios (1 ~ 4 mg a.i. kg^{-1}). Portugal value (3.74 mg a.i. kg^{-1}) was more similar with EU Center (3.59 mg a.i. kg^{-1}) than with South (1.50 mg a.i. kg^{-1}). $PEC_{accumax}$ values of chlorpyrifos were also higher to Brazilian (~ 3 mg a.i. kg^{-1}) than European scenarios (0.2 – 0.6 mg a.i. kg^{-1}). Portugal in this situation presented a $PEC_{accumax}$ value (0.094 mg a.i. kg^{-1}) seven times lower than EU center (0.66 mg a.i. kg^{-1}) and two times lower than EU South (0.20 mg a.i. kg^{-1}).

Almost all scenarios presented risk to microarthropods (TER < 5). The exceptions were to chlorothalonil observing Overall group and Collembola species at EU North and South scenarios (TER > 5). To chlorpyrifos all scenarios putted in-soil organisms at risk (TER < 5).

4.5 DISCUSSION

Concerning the effects of chlorothalonil on the overall in-soil fauna community, there is a lack of information to compare the sensitivity in similar tests. However, in chapter I, if the HC_{5EC50} of 7.52 (4.05 - 9.61) mg a.i. kg^{-1} for Collembola is observed, it is possible to realize that the range of sensitivity is the same of the NOEC in the community test to overall community (7.63 mg a.i.kg^{-1}), as well to the Collembola (7.63 mg a.i.kg^{-1}). In this case uncertainties were reduced about chlorothalonil effects to in-soil fauna, since two different methods reached the same range of results.

Despite the absence of similar microarthropods tests with this active ingredient, Simões et al. (2019a) testing toxicity of Bravo (40% chlorothalonil w/w) in a Portuguese soil (pH: 7.1) of similar texture (62:28:10 %w:w sand, silt, clay, respectively) to *Folsomia candida* in standard test under laboratory conditions found an EC_{50} of 41.3 (30.9 –51.7) mg a.i. kg^{-1}. Moreover, Leitão et al. (2014) using the same product and organism, estimated an EC_{50}: 31.1 (24.7–37.5) mg a.i. kg^{-1} in also a Portuguese soil (sandy clay loam, pH = 5, OM = 5.7%). These values are higher than the observed in the present work with microarthropod community test. Besides the absence of studies until the present using fungicides and *H. thermophila*, Greenslade et al. (2010) evaluated in a field experiment in Australia, the herbicides bromoxynil ($C_7H_3Br_2NO$) and hoegrass (diclofop-methyl), on the activity of surface-dwelling Collembola. The only sensitive species were *H. thermophila* and *B. platensis*, which has a more simplified cuticle structure than the other three species evaluated. Renaud et al. (submitted, 2019) also described in a community test using the same soil contaminated with metal mixtures the

dominance of *H. thermophila*, which appears to have an intermediate sensitivity – it was less sensitive than *Ceratophysella gibbosa* but more sensitive than *P. armata*.

There is a lack of studies involving mites and chlorothalonil. However, some effects of fungicides on mites, mainly on Oribatida, already were described in literature. Al-Assiuty et al. (2014) investigated the effects of fungicides and biofungicides on population density and community structure of soil oribatid mites, having found significant influence of these compounds in terms of shifts occurring among individual species. In addition, complementary laboratory tests with *Hypoaspis aculeifer* using contaminated soil (chlorothalonil, Bravonil 500®) and clean prey or contaminated soil plus contaminated prey (cheese mites) (annex III, figure 12) pointed to the absence of sensitivity to both methods (EC_{50} > 420 mg a.i. kg^{-1}). Soil organisms usually show a heterogenous spatial distribution and mites are no exception. Reis et al. (2016) already associated some results in Collembola studies with a heterogeneous structure of the habitat and suggested that to attach an adequate sampling effort some of the studied sites needed a larger number of samples (over 12). This could to indicate that to propose community tests as a requirement in pesticides ERA some requirements in sampling needs to be discussed.

Effects of chlorpyrifos on the overall community estimated a NOEC of 0.008 mg kg^{-1}, which was mostly explained by Collembola. Despite of the NOEC of mites was lower (0.008 mg a.i. kg^{-1}) than to Collembola (0.02 mg a.i. kg^{-1}), the average of dissimilarity between control and concentrations was higher to Collembola (mean: 64.80 %) than to Acari (mean: 51.35%) which implies effects not estimated by NOEC values. The average of dissimilarities estimated by Bray-Curtis distances was already used to assess effects of pesticides to non-target fauna (FRANCO et al., 2016; ATWOOD et al., 2018). This approach could be a useful tool in ERA to observed more specific effects at community level.

Chelinho et al. (2014), evaluated effects of the carbamate insecticide carbofuran to soil organisms through community tests for native fauna in Brazil, spraying the pesticide in the field, and in Portugal, where the soil was spiked in laboratory conditions. The Portugal community was larger in number of total collected fauna than in the present study and presented organisms in all concentrations. The Collembola and Acarina were also the groups that most contributed to dissimilarity between control and treatments. Sechi et al. (2014), tested the pyrethroid insecticide α-cypermethrin through a different methodology of a community test. The researches performed a soil multi-species (SMS) test systems using not natural communities, but laboratory species (one mite, one earthworm, one enchytraeid and five different collembolans) in a Denmark soil (clay = 9.5%, O.M. = 2.1%, pH H_2O = 6.2). They argued that oligochaetes gave rise to dramatically different community responses to the

insecticide as well as creating different conditions resulting in different degradation dynamics of α-cypermethrin. In addition, values of EC_{10} and EC_{50} were estimated to the species used. Despite of the relevance of the SMS approach, mostly if it is compared with single species tests, to use laboratory species instead of natural communities will always bring uncertainty about the field sensitivity. Perhaps this method is more comparable with an SSD approach than with a community test, as was described.

Chlorpyrifos, as also other organophosphates insecticides, already was pointed as a highly toxic pesticide to arthropods as mites and isopods (MORGADO et al., 2016; KAMOUN et al., 2017), mostly Collembola (SANTOS et al., 2012; CARNIEL, 2019) in laboratory standard tests. *F. candida*, as the most used standard Collembola, is also the species with more available literature data. Jegede et al. (2017) using Pestanal (chlorpyrifos, 99%) in an OECD soil (5% peat) observed EC_{50} values to *F. candida* (0.031 mg a.i. kg^{-1}) and concluded a high toxicity of this product. Tests performed in tropical soils and Mediterranean soils corroborated with this data and information (SANTOS et al., 2012).

The higher $PEC_{accumax}$ values of chlorothalonil in Brazilian scenarios (12 ~ 16 mg a.i. kg^{-1}) than in European scenarios (1 ~ 4 mg a.i. kg^{-1}) was already discussed in Chapter I, as well chlorpyrifos values were discussed in Chapter II.

The intermediate ERA assessed through microarthropods tests indicates chlorothalonil risks in all Brazilian scenarios, EU Center and Portugal. This confirmed the risk indicated in the previous chapters and indicates that this approach could be an alternative to specific scenarios as a complementary test, maybe to test commercial products in different regulatory zones by finding representative soils and respective communities.

4.6 CONCLUSION

The intermediate ERA assessed through microarthropods tests indicates chlorothalonil risks in all Brazilian scenarios, EU Center and Portugal. This confirmed the risk indicated in the previous chapters and indicates that this approach could be an alternative to specific scenarios as a complementary test, maybe to test commercial products in regulatory zones. However, since the high variability, mainly to Acari group, a standardization of the method is necessary before a legislation recommendation. In addition, advance deeper in soil fauna groups identification would help to better access possible pesticides effects on communities.

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4.8 ANNEX III:

Table 28 - Nominal and actual concentrations of chlorothalonil and chlorpyrifos (mg a.i.kg^{-1}) in a Mediterranean soil in the microarthropods community test.

Treatment	**Nominal**	**Actual**	**% of nominal**
Chlorothalonil			
C0	0	0	
C1	3.75	2.24	59.67
C2	7.5	7.63	101.77
C3	15	12.99	86.59
C4	30	30.01	100.04
C5	60	63.18	105.30
C6	120	103.58	86.32
C7	240	188.14	78.39
Chlorpyrifos			
C0	0	-	
C1	0.008	-	
C2	0.02	-	
C3	0.05	-	
C4	0.128	-	
C5	0.32	-	
C6	0.8	-	
C7	2	-	

Table 29 - Good application practices (GAP) of chlorothalonil and chlorpyrifos according to specific recommendations available for European North, Center, South and Portugal (PT) scenarios, based on the worst-case scenario.

Zone	Active ingredient	Commercial Product	g i.a.kg CP	Crop	rate application (l. ha^{-1})	Dose (g.ha^{-1})	nº app	Interval application (days)	BBCH code	reaching soil (fraction)	Interception (%)
North	Chlorothalonil	Amistar Opti	400	Spring barley	2 – 2.5	1000	1	-	30-59	0.7	30
Center	Chlorothalonil	Arastar Twin 480 SC	480	Carrots	2 – 2.5	1200	2	10	40-89	1	0
South	Chlorothalonil	Fongil FL	500	Tomato	2	1000	3	10	40	0.7	30
PT	Chlorothalonil	Bravo 500 gL	500	Tomato	3	1500	3	7	40-89	0.8	20
North	Chlorpyrifos	Atena 480 EC	480	Spring oilseed rape	0.6 – 0.8	384	1	-	55 - 59	0.65	35
Center	Chlorpyrifos	Cyren 480 EC	480	Winter rape	0.6 - 1	480	1	-	20-39	0.65	35
South	Chlorpyrifos	Pyristar	250	Spinach	0.6	150	1	-	40	0.75	25
PT	Chlorpyrifos	Pirinex 5% p/p	5	tomato	15	75	1	-	40-89	0.8	20

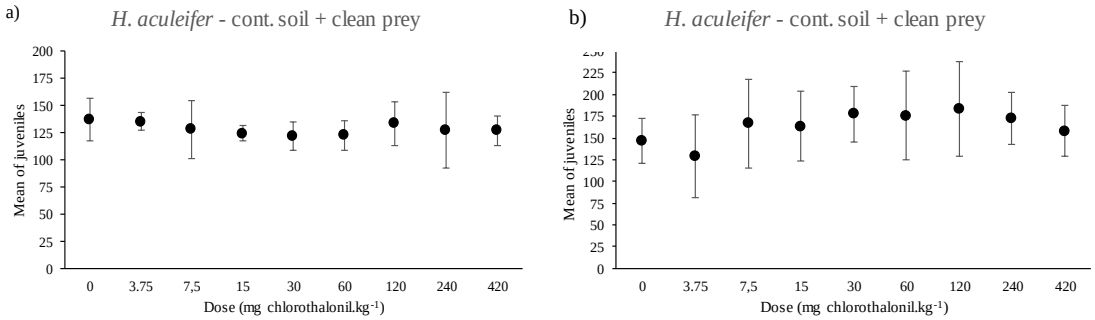
Table 30 - Good application practices (GAP) of chlorothalonil and chlorpyrifos according to specific recommendations available for Brazil, based on the worst-case scenario.

Zone	Active ingredient	Commercial Product	g i.a.kg PC	Crop	rate application (L.ha^{-1} or kg.ha^{-1})	Dose (g.ha^{-1})	nº app	Interval application (days)	BBCH code	reaching soil (fraction)	Interception (%)
Brazil	Chlorothalonil	Bravonil 500	500	Potato	2.5 – 3.0	1500	8	10 d	40	0.85	15
Brazil	Chlorpyrifos	Lorsban 480	480	Cotton	2	960	2	7	40	0.85	15

Table 31 - Biodegradability of PPPs according with IBAMA legislation (1996).

Biodegradability			Class	Classification	DT_{50} conversion		
0 ≤	% CO_2	< 1	1	Highly Persistent	360 ≤	$T_{1/2}$ (days)	
1 ≤	% CO_2	< 10	2	Very Persistent	180 ≤	$T_{1/2}$ (days)	< 360
10 ≤	% CO_2	< 25	3	Moderately Persistent	30 ≤	$T_{1/2}$ (days)	< 180
25 ≤	% CO_2		4	Low Persistent	0 ≤	$T_{1/2}$ (days)	< 30

Figure 12 - Additional tests with *Hypoaspis aculeifer* (OECD, 2016) using chlorothalonil (Bravonil 500®) in tropical artificial soil (TAS). A scenario with contaminated soil and clear prey (a) and other scenario with contaminated soil and contaminated prey were performed (b). NOEC > 420 mg a.i.kg^{-1}.



5. CHAPTER IV: SEMI-FIELD METHODS AS A SURROGATE HIGHER TIER: THE LAST STEP IN ECOLOGICAL RISK ASSESSMENT OF PPPS.

5.1 ABSTRACT:

The higher tier step of the Ecological Risk Assessment (ERA) for chlorothalonil (Bravonil 500® g a.i. L^{-1}) and chlorpyrifos (Lorsban 480® 480 g a.i. L^{-1}) to in-soil fauna organisms was performed through terrestrial model ecosystem (TME) semi-field tests as a surrogate tier to field tests. Two distinguish exposure scenarios were evaluated: 1) continued application (2x) based on soybeans crop application and; 2) single application, modelling based on Europe ERA instructions for higher tier. To both scenarios, three different doses were used. The abundance of overall soil fauna groups (major taxonomic groups), Collembola (morphotypes), Mites (Order, suborder and cohort) and Enchytraeids (genus) were evaluated and significative differences were estimated using PERMANOVA analysis, followed by SIMPER. In addition, the Bray-Curtis dissimilarity was used to verify potential risks to each evaluated group. The bait lamina method was used to estimate the feeding activity. Bait lamina results were corelated mostly with overall fauna and mites, mostly in chlorothalonil experiments. Increasing doses of both products reduce the abundance of populations compared with control, even at the lowest tested concentrations, regardless the exposition scenario. Results indicated an absence of recovery even eight weeks after the contamination (Bray-Curtis dissimilarity > 35%) to Collembola morphotypes to both pesticides regardless the exposure scenario. Effects on earthworms in TMEs were not verified due the low number of organisms. No effects have been observed in enchytraeids using this methodology. The previous tiers were capable of predicted risks, which were not reduced from lower to higher tier, mostly for collembolans. Semi-field tests showed to be useful as a surrogate higher tier in ecological risk assessment.

KEY WORDS: Terrestrial Model Ecosystems. Subtropical soil. Chlorothalonil. Chlorpyrifos.

5.1 INTRODUCTION

The use of Plant Protection Products (PPPs) may provide immediate advantages to crop production, although, the adverse effects provoked by these products to the environment (AKTAR et al., 2009; LARSEN et al., 2017) affect the health of the terrestrial system, compromising crop production in the sub-sequent years. Beyond other factors, PPPs often lead to spatial and temporal changes on soil biological communities in the agricultural landscape. These changes have impact on the provision of soil ecosystems services (ES), namely on food production (MEA, 2005). The in-soil fauna organisms have a crucial role in the provision of ES and the maintenance of this provision is highly dependent on the diversity of species that compose such communities. Because of that, the preservation of biodiversity in soil systems has been seen as a priority and specific protection goal (MEA, 2005; EFSA, 2010). Thus, in order to estimate the potential hazard of PPPs, threshold values are stablished by performing an ecological risk assessment (ERA). An ERA scheme should be refined from the lower to the higher tiers, making protection values realistic and protective for soil communities (EC, 2002; EC, 2009; EU 2013).

For in-soil fauna, the current ERA guidance in Europe (EC, 2002) comprises single laboratory tests with three invertebrate species (*Eisenia andrei/fetida*, *Folsomia candida*, *Hypoaspis aculeifer*) at the lower tier followed directly to field tests (higher tier). This approach has been criticized in a recent EFSA Scientific Opinion (EFSA, 2017), which highlights the need of improving actual Guidance to ERA of PPPs to in-soil fauna. This improvement has to fulfill actual gaps as the inclusion of additional steps in ERA schemes, namely between laboratory (lower tier) and field tests (higher tier). These additional steps (intermediate tiers) may comprise SSD approaches or laboratory community tests as discussed in the previous chapters (I, II, III). Then, if protection levels are not met, risk should be refined in higher tiers by performing, e.g., semi-field tests through terrestrial model ecosystem (TMEs) experiments that may work as a surrogate of the higher tier. Furthermore, these surrogate higher tier assessments can help to calibrate the threshold values (or the assessment factors) from lower tiers, making them more protective, as desired. The use of TMEs allow to have the controlled conditions of a laboratory tests combined with the natural communities, complex interactions between species and soil structure typical of real scenarios. Despite the increasing variability, higher experimental work and costs compared to laboratory single-species tests (VAN DEN BRINK et al., 2005; SCHÄFFER et al., 2008), TMEs permit to reduce sampling effort and man power generally needed in field experiments (SCHOLZ-STARKE et al., 2011).

Furthermore, ERA does not take into account only toxicity data, but also the organisms exposure to estimate possible risks. The predicted environmental concentration (PEC) of PPPs in soil estimated the presence of the active ingredients in soils over time, taking into consideration different factors/properties (both environmental and intrinsic of the substance) from which depend the persistence of PPPs in soil, like DT_{50}, rate and number of applications, soil properties and average temperature (EFSA, 2015). In Europe, the exposure scenarios consider the PEC estimated to occur in ten years ($PEC_{accumax}$) for the higher tier. This value takes into account the cumulative persistence of the PPP estimated for ten years of applications. However, it is still unclear if this model allows to estimate realistic predictions of effects to in-soil organisms. The main question is to know if, modelling the total final concentration through $PEC_{accumax}$, it is possible to have realistic prediction of the risk in scenarios that consider continuous PPP applications.

While in Europe the toxicity data requirements have been updated (EC, 2009; EU, 2013), in other countries legislation for ERA of PPPs is still inadequate to protection the environment and the research to support the improvements required in ERA schemes are clearly insufficient. Eijsackers et al. (2017) have defended that, in South Africa, data requirements for ERA should include local species, typical from this environment and representative of dominant groups like ants and termites. These researchers argue that ERA scheme adopted in Europe and United States are often inapplicable to African tropical environmental conditions. This fact emphasizes the need of increasing research on this issue for different biomes of the globe and especially to the regions where the use of PPPs is more implemented like countries of Latin America. For example, Brazil is one of the biggest food producers in the world and applies a large amount of PPPs annually in the agricultural field (CAMARGO et al., 2017). The regulation of PPPs for in-soil fauna in Brazil is based uniquely in acute lethality test (LC_{50}) with *Eisenia andrei/fetida*. These acute tests are the only requirement needed to classify pesticide commercial formulations from slight (LC_{50} > 1000 mg a.i.kg^{-1}) to extremely (LC_{50} < 10 mg a.i.kg^{-1}) toxic (IBAMA, 1996). The earthworm acute tests are actually rarely used in ecotoxicology studies due to its low sensitivity to soil contaminants, namely pesticides. This weak sensitivity has been reported as even higher in tropical conditions (Alves et al., 2013). For this reason, earthworm acute tests were removed from the data requirements in EU legislation since 2013 (EU 2013). In addition, *Eisenia andrei* has been reported as a species with low sensitivity to pesticides compared to other soil invertebrates in two different subtropical soils from South of Brazil (CARNIEL et al., 2019). Beyond the gaps in data

requirements to in-soil organisms in Brazil, there are also lacks and inadequacies of criteria defined to use in the estimation of PEC and exposure scenarios.

In Brazilian legislation, to estimate the products permanence time in soils it is accepted the use of DT_{50} values measured in three specific soil classes or converted from the percentage of radiolabeled carbon dioxide ($_{14}CO^2$) detached (IBAMA, 1996). This second methodology is based on a protocol (Art. 21, IBAMA, 1996) which was suspended in 2001 for review (Art. 1, IBAMA, 2001). Despite that, the data generally used for substances is based on the $_{14}CO^2$ detached. Moreover, since official DT_{50soil} data for Brazilian soils are not available in open databases, these values had to be required to the Brazilian Institute of Environment (IBAMA) through an online government public communicate channel (https://esic.cgu.gov.br/sistema/site/index.aspx).

Beyond the specific Brazilian problems, mostly in estimate PECs and exposure scenarios, a global issue has been that the toxicity of several pesticides remains unclear, or few assessed, at least to in-soil fauna. Fungicides, for example, are an emerging chemical class of concern, which has deserved little attention compared to herbicides or insecticides (ELSKUS, 2012). A non-systemic fungicide which is very effective for agricultural usage around the world due to its multi-site contact-activity mode of action is the active ingredient chlorothalonil (tetrachloroisophthalonitrile; CAS 1897-45-6) (SIMÕES et al., 2019a). The fungicides based on chlorothalonil are highly efficient and, because of that, widely commercialized (ZHANG et al., 2016). As occur with other PPPs, there is still few information available about the toxicity of chlorothalonil to soil fauna. Tu et al., (2011) reported that chlorothalonil applied through Daconil Ultrex, may reduce feeding activity and abundance of earthworms in the field and Leitão et al. (2014) estimated reproduction EC_{20} values of 18.2, 39.4 and 20.8 mg a.i.kg^{-1} to *F. candida*, *E. crypticus* and *E. andrei*, respectively, using gradients of increasing concentrations of Bravo® (40% w:w of chlorothalonil) in a natural soil. More recently, Simões et al (2019a) evaluated genomic alterations caused by chlorothalonil in *F. candida* also using Bravo® and a natural soil. The present study is integrated in a broader research that evidenced toxic effects of the fungicide Bravonil 500® (with chlorothalonil as a.i.) in Collembola and Oligochaeta species in laboratory reproduction tests (chapter I).

Among insecticides, the organophosphorus compounds (like chlorpyrifos) have been used in agriculture as a substitute for organochlorine insecticides due to its lower cost, easy synthase and lower persistence in the environment (SOLOMON et al., 2014). It can enter in the animal body mainly via contact with ingestion of contaminated matrices, and its toxic mode of action involves acting in the nervous system by inhibiting the synthesis of cholinesterase,

causing muscular paralysis by excess of acetylcholine (SAVOLAINEN, 2001). Chlorpyrifos [O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate], has been reported as toxic for collembolan communities (FRAMPTON, 1999; ENDLWEBER et al., 2006) and spiders (FOUNTAIN et al., 2007). Toxic effects on survival and reproduction of Collembola, mite, isopod and Oligochaeta species have been also reported in laboratory tests (SANTOS et al., 2012; MORGADO et al., 2016; KAMOUN et al., 2017; ZHOU et al., 2011; YANG et al., 2017 and chapter II). Although chlorpyrifos is actually banned in several European countries (DE; DK; SI; FI; SE; IE; LV; LT) due to its risks to human health (RAUH et al., 2012), this a.i. is still allowed in some European countries like Portugal and Spain (EC, 2019) and countries from Latin American like Brazil, where almost eight thousand tons of chlorpyrifos are sold per year (IBAMA, 2018).

Framed on this reality, the objectives of the present work were i) to investigate the risk posed to in-soil organisms by the fungicide Bravonil 500® (500 g chlorothalonil L^{-1}) and the insecticide Lorsban 480® (480 g chlorpyrifos L^{-1}) in semi-field tests; ii) to evaluate the adequacy of the threshold values determined in lower and intermediate tiers developed in the previous chapter (I, II and III) for the fungicide Bravonil 500® (500 g chlorothalonil L^{-1}) and the insecticide Lorsban 480® (480 g chlorpyrifos L^{-1}); ii) to evaluate if the use of application doses based on $PEC_{accumax}$ (as actually used in ERA of PPPs in EU) are reliable for multiple application scenarios of Bravonil 500® and Lorsban 480® considering soy beans agriculture recommendations. The fulfill these objectives, two TME experiments were performed using a subtropical Brazilian Nitosol in different exposure scenarios and pesticides doses evaluating potential effects on in-soil fauna (overall groups, Collembola, Acari and Enchytraeids) and in-soil feeding activity though bait lamina method in two sampling times (immediate effects and recovery).

5.2 MATERIAL AND METHODS

5.2.1 Test Substances

Commercial formulation of the fungicide Bravonil 500® with isophthalonitrile chlorothalonil as active ingredient (500 g a.i. L^{-1}) and the insecticide Lorsban 480® with the organophosphate chlorpyrifos as active ingredient (480 g a.i. L^{-1}) were used. Physical and chemical characterization of these active ingredients are described in table 32.

Table 32 - Physicochemical characteristics of chlorothalonil and chlorpyrifos. Chemical characteristics and DT_{50} data were collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and IUPAC (https://sitem.herts.ac.uk/aeru/iupac/Reports/).

Characteristic	Chlorothalonil	Chlorpyrifos
CAS	1897-45-6	2921-88-2
IUPAC name	tetrachloro-isophthalonitrile	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
Empirical formula	$C_8Cl_4N_2$	$C_9H_{11}Cl_3NO_3PS$
Molecular mass (g mol^{-1})	265.91	350.58
Relative density (g cm^{-1})	1.8	1.4
Solubility (pH = 7) (mg L^{-1} 20ºC)	0.81	1.05
Log K_{ow} (at 20ºC)	2.94	4.06
Henry's Law constant (25ºC Pa m^3 mol^{-1})	2.50 x 10^{-02}	0.478
Degradation Soil (20 °C aerobic) (days)	3.53	386
Degradation/Dissipation Field (days)	17.9	27.6

5.2.2 Experimental procedure

A TME experiment was performed for each commercial formulation using the same procedure and a similar strategy for both Bravonil 500® and Lorsban 480BR Dow Agro®. A total of 72 TMEs (40 cm height; 17.5 cm diameter) were collected for each experiment in a grassland area in Concordia, Santa Catarina, Brazil (-27.311710, −51.990814). This area is composed by a dystroferric Red Nitosol (EMBRAPA, 2018) with the physical and chemical properties presented in table 33.

Table 33 - Properties of soil used in terrestrial model ecosystem tests with Bravonil 500® (a.i. chlorothalonil) and Lorsban 480® (a.i. chlorpyrifos).

Soil properties	
Organic matter (%)	4.7
CEC pH 7 (cmolc/dm³)	19.1
pH	5.50
WHC (%)	48.1
Sand	35
Silt	32
Clay	33

TME extraction followed methods described by Ng et al. (2014). TMEs were distributed by 6 carts (12 TMEs per cart) that were maintained under controlled temperature (25 ± 2°C) and photoperiod (16:8 hours light:dark). The temperature inside the carts was kept at 12º C in

order to simulate the real temperature below the soil surface in each TME. Each TME experiment had a test period of 16 weeks (four months).

The TMEs had an acclimation period of 14 days. During this time, soil moisture was monitored in each TME during the week immediately after TME extraction using hydrofarm equipment in order to establish a rain regime capable to maintain soil moisture between 50 and 60% of its water holding capacity (WHC). The rain regime established was 5.42 mm/48 h and artificial rain (VELTHORST, 1993) was used. In addition, still during the acclimation period, the natural vegetation present in the soil was cut. At the end of this period, soya beans (Nidera 5445 - Gelfix 5® - 5 x 109 UFC/ml *Bradyrhizobium elkaniibr*) were sown (4 seeds/TME) and a week after the germination, only one plant was selected to kept in each TME.

Two contamination scenarios were simulated in each experiment:

1) GAP scenario – simulated according with Good Agricultural Practices (GAP) using three different tested doses (0.25xGAP, GAP and 10xGAP) composed by two applications separated by an interval of two weeks. To contaminated soil, pesticides were spiked homogeneously directing on the soil surface. The GAP dose applicated, considering the crop interception (see below) to chlorothalonil was 1125 g a.i. ha^{-1} (2.70 mg a.i.TME^{-1}) in the first application and 975 g a.i. ha^{-1} (2.34 mg a.i. TME^{-1}) in the second application. To chlorpyrifos, GAP dose was 408 g a.i. ha^{-1} (0.98 mg a.i. TME^{-1}) in the first application and 384 g a.i. ha^{-1} (0.92 mg a.i.TME^{-1}) in the second application.

2) PEC scenario – was based on the maximum Predicted Environmental Concentrations ($PEC_{accumax}$) estimated at ESCAPE (Klein, 2015) and follow the described in EFSA (2015) to perform the exposure in ERA higher tier step. This scenario was composed by one application that happened in the same day of the second application of GAP scenario (figure 13). $PEC_{accumax}$ values were calculated based on the Good Agricultural Practices for each pesticide in three different doses (0.25xPEC, PEC, 10xPEC). To contaminated soil, pesticides were spiked homogeneously directing on the soil surface. The PEC dose applicated, considering the crop interception (see below) to chlorothalonil was 1809 g a.i. ha^{-1} (4.35 mg a.i. TME^{-1}). To chlorpyrifos, PEC dose was 612 g a.i. ha^{-1} (1.47 mg a.i. TME^{-1}).

Nominal concentrations estimated to occur in soil after these applications in both scenarios were used to evaluate the results and are presented in table 34. Moreover, the Figure 13 represents the scenarios contamination and exposure time.

Since both commercial formulations used in the experiments are usually applied over plants, part of the pesticide applied is intercepted by plant (crop interception) and does not reach

the soil. Thus, the pesticide fraction that reaches the soil (f_{soil}) depends on the development stage of the crop and this fact influences the concentration to which soil organisms are exposed (EFSA, 2015). For the estimation of the f_{soil}, the BBCH code for soya beans was used (Meier et a., 2001; EFSA, 2015). In addition, despite of the rate application has been estimated based on Brazil recommendations to soy beans crops (MAPA, 2019), molecules DT_{50} information used were based on EFSA available data (EFSA 2016, EFSA 2017), since these results were closer to the literature than IBAMA information. Data used to estimate values are available in annex IV table 42.

Table 34 - Nominal concentrations of chlorothalonil and chlorpyrifos estimated to occur in TMEs (thorugh Bravonil 500 and Lorsban, respectively) in two different scenarios (GAP and PEC). Estimations performed through ESCAPE (Klein, 2015) considering depth soil (5 cm), bulk density (1.5 g cm^3) and the organic matter content (4.7%). The f_{soil} considered to both pesticides were 0.80% to the first application in GAP and 0.75% to the second GAP and PEC scenario application. Values are expressed in mg $a.i.kg^{-1}$.

GAP Scenario	First application			Second application		
a.i.	0.25xGAP	GAP	10xGAP	0.25xGAP	GAP	10xGAP
Chlorothalonil	0.67	2	20	0.67	2	20
Chlorpyrifos	0.14	0.64	64	0.14	0.64	64
PEC Scenario				Single application		
a.i.				0.25xPEC	PEC	10xPEC
Chlorothalonil				0.80	2.40	24.10
Chlorpyrifos				0.20	0.81	8.16

Ten replicates for each treatment and controls were used and two sampling dates were considered to evaluate not only the impact of PPPs application but also the recovery of soil communities. Therefore, 5 TMEs of each treatment and control were destructively sampled two weeks (T1) after the last contamination in the GAP scenario and the single contamination in the PEC scenario (n = 5). The remaining 5 TMEs of each treatment and control (n = 5) was destructively sampled eight weeks (T2) after the last pesticide application in the GAP scenario and the single application in the PEC scenario. At each sampling date the top 10-cm soil layer was divided in half into two portions for the analyses described below. The 10–40 cm soil layer of each TME sampled was hand-sorted to collect macrofauna. Figure 13 represents a scheme of the experimental design.

5.2.3 Bait lamina test

A total of 1440 bait lamina sticks were prepared per each TME experiment using a mixture of finely ground oat (0.106 mm sieved), activated charcoal powder and cellulose powder in a 1:5:14 ratio (w:w:w) as bait (ISO, 2016). Four bait lamina sticks were placed in each TME in different times over the experiment, as represented in Figure 13. Bait-lamina remained in the soil for ten days per each time. The first bait-lamina test was introduced 24 h after the first pesticide application (GAP scenario) and without pesticide application in PEC scenario. It was evaluated for the feeding activity ten days after (72 h before the next contamination); the second test was introduced on TMEs 24 h after the second pesticide application (GAP scenario) and the single application (PEC scenario). It was evaluated for the feeding activity ten days after (72 h before T1); the third bait lamina test was introduced 13 days before the last destructively sampled and remained in TMEs during ten days (the evaluation of the feed activity was 72 h before the destructively sampled in T2).

After the 10 days of exposure, bait lamina sticks were carefully dislodging from soil adhering with tap water. Each stick was assessed visually by holding the sticks against a light to count the number of eaten holes. The feeding activity per sample (group of four sticks) at each TME was expressed in percentage of eaten holes.

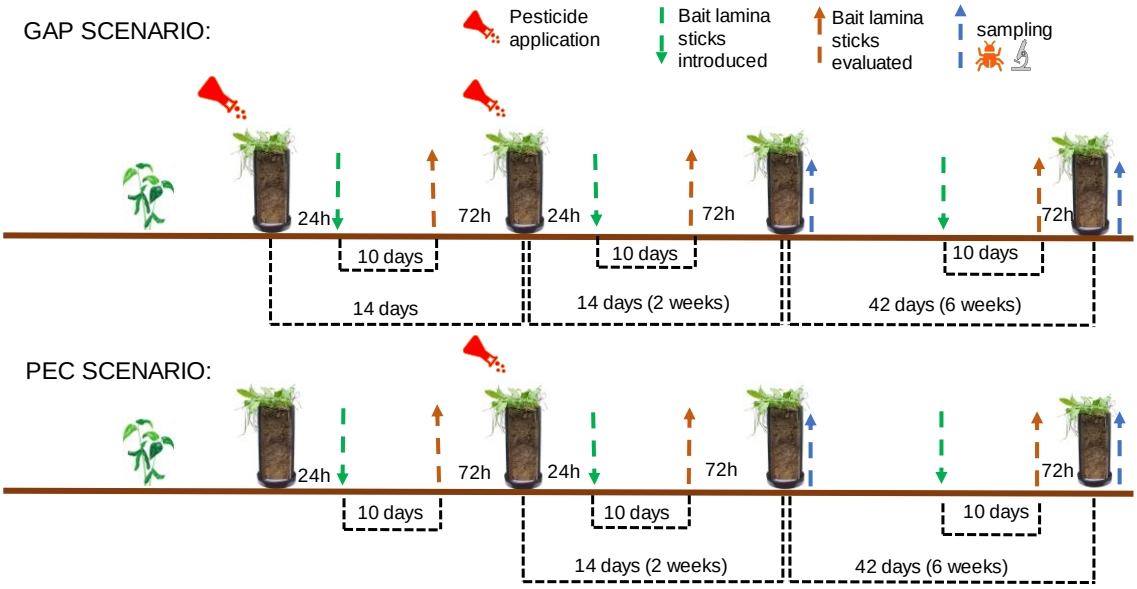
5.2.4 In-soil fauna

Half of the top 10-cm soil layer of each TME was used to extract mesofauna communities. This extraction was performed through a modified Berlese funnel (SOUTHWOOD, 1968) to a detergent solution (2%) for seven days. After that period, the extracted fauna was transferred a 70% ethanol solution. Then, the organisms were counted and sorted into higher taxonomic entities under a stereomicroscope (60× magnification) according to Minor and Robertson (2006). Collembolans were identified in morphotypes according to their morphological characteristics (traits). Afterwards, collembolans were grouped then into life-form groups according to their individual traits (epigeic, hemiedaphic and euedaphic). The traits considered were presence of ocelli, antenna length, development of furca, presence or absence of body hairs/scales, and pigmentation (VANDEWALLE et al., 2010). Mites were sorted into four main groups: Oribatida (suborder), Mesostigmata (order), Prostigmata (suborder) and Astigmata (cohort), according to Lindquist et al. (2009).

In the other half of top 10-cm soil layer, soil samples were taken using plastic cores (5 cm depth, 5 cm diameter) to extract enchytraeid community. These soil samples were stored at

15º C until being submitted to a hot wet extraction (O'CONNOR, 1955). Enchytraeids identification was performed to genus level following methods described by Schmelz and Collado (2010).

Figure 13 - Experimental design of TME experiments with Bravonil 500® (with chlorothalonil as a.i.) and Lorsban 480® (with chlorpyrifos as a.i.) applied 0.25, 1 and 10 times the recommended dose considering two scenarios: Scenario 1: based on Good Agricultural Practices (GAP) composed by two applications and Scenario 2: based on $PEC_{accumax}$ estimations with a single application. For additional information regarding the $PEC_{accumax}$ estimation see in the text. Different dashed and colored arrows correspond to different steps of the experiment: Red figure mean pesticide applications; green arrows mean introduction of bait lamina sticks; orange arrows mean bait lamina sticks evaluation; blue arrows mean TME sampling time (T1 and T2).



5.2.5 Data Analysis

For bait lamina test, feeding activity was determined through the mean percentage of empty holes per stick (%) in each TME. Differences between control and treatments at each sampling time in each scenario were assessed through one-way ANOVA followed by Dunnett's pos hoc test. Normality and homogeneity of variances were assessed with Kolmogorov–Smirnov and Bartlett's tests, respectively.

As performed with data from Chapter III, the abundance of microarthropods dataset (fourth root) was used to calculate Bray-Curtis distance matrices for the overall mesofauna, for Collembola morphotypes and for mite groups, using PRIMER & PERMANOVA 6.0 (CLARKE:GORLEY, 2006). Permutational multivariate analysis of variance (PERMANOVA) was used to verify significant differences ($p \leq 0.05$) in similarity between control and treatments at each sampling time and at each scenario.

In the new EFSA Scientific Opinion (EFSA, 2017), specific protection goals are proposed for in-soil organisms as drivers of particular ecosystem services. To an in-field situation, effects of 10 to 35% compared to control on abundance/biomass of earthworms, enchytraeids, microarthropods, and/or some other communities happening within few months may be acceptable. Effects of 35 to 65% to the same communities but happening in a shorter period of time (within few weeks) may be also accepted. Based on these assumptions, the dissimilarities (%) estimated by Bray-Curtis distance between control and treatments were used as an alternative to total abundance, since a decrease in similarity is expected when pesticide concentration increased and affects community (making the community more dissimilar to that of control). Since the TME sampling to assess community recovery was performed after eight weeks of the first pesticide application (T2), a risk was assumed when the dissimilarities were above 35% in this period.

In addition, in the treatments were there was significant differences from control detected through PERMANOVA, a similarity of percentages analysis (SIMPER) was performed to establish the groups that most contribute for these differences. This was performed to overall fauna groups, Collembola species and mite groups.

5.3 RESULTS

Chlorothalonil. Feeding activity measured through bait lamina sticks in the different treatments for both scenarios is presented in annex IV and figures 14 to 19. Statistical differences compared to control were found in GAP scenarios only for the highest application dose (20 mg a.i. kg^{-1}) in all of the three bait lamina evaluations (Dunnett, $p < 0.05$), where the feeding activity was reduced. For PEC scenario, the second evaluation indicates a decrease in feeding activity for all doses compared to control (Dunnett, $p < 0.05$). These differences were not found neither in the first nor in the third evaluations (Dunnett, $p > 0.05$; table 35).

Table 35 - Statistical differences of feeding activity measured through bait lamina sticks (p values) in three evaluation times between control and treatments of a TME experiment with Bravonil 500 (with chlorothalonil as a.i.) considering two scenarios: GAP scenario: based on Good Agricultural Practices (GAP) composed by two applications and PEC scenario: based on $PEC_{accumax}$ estimations with a single application. For additional information regarding the timing of each bait lamina evaluation see in the text. * - indicates statistical differences between control and treatments (through one-way ANOVA followed by a Dunnett post hoc test, $p \leq 0.05$). Doses are expressed in mg a.i.kg^{-1}.

GAP scenario			
Control x doses	First evaluation	Second evaluation	Third evaluation
0.67	0.35	0.14	0.88
2	0.07	0.47	0.22
20	0.0017*	0.036*	0.030*
PEC scenario			
Control x doses	First evaluation	Second evaluation	Third evaluation
0.67	0.87	0.034*	0.68
2	0.75	0.0038*	0.90
20	0.93	0.0011*	0.59

Data of in-soil fauna obtained in TME experiment of chlorothalonil regarding dissimilarities compared to control is summarized in Tables 22 (GAP scenario) and 23 (PEC scenario). Enchytraeid genus data did not differed from control to treatments in any sampling time, scenario or concentration (PERMANOVA, $p > 0.05$) and because of that, these data are not shown. In GAP scenario (table 36), T1 did not have significant differences between control and application doses to overall soil fauna groups. On the other hand, Collembola community was highly sensitivity being significantly affected by chlorothalonil since the lowest concentration (0.67 mg a.i. kg^{-1}). In general, epiedaphic Collembola were the species that mostly contributed to explain differences between control and contaminated soils. Mites were significantly affected only in the highest concentrations (20 mg a.i. kg^{-1}). For T2, neither fauna groups nor mites were affected by chlorothalonil. Collembolans had significant differences between control and all test doses. Considering risk scenarios according to EFSA (2017), there were no risks to overall fauna groups and mite orders (dissimilarity < 35%). On the other hand, to Collembola morphotypes, there was risk in all doses (dissimilarity ≥ 35%).

In PEC scenario (table 37) significant differences between control and treatments were detected to soil fauna groups and to mite orders in T1 sampling for two application doses (2.40 and 24.10 mg a.i. kg^{-1}). Collembola species were again the most sensitive organisms with significant differences found between control and treatments influenced mostly by epigeic and hemiedaphic species. For T2 sampling, Collembola species showed significantly differences between control and all doses that were mostly explained by the abundance decrease of epiedaphic and hemiedaphic species. The risks observed were similar to those of GAP scenario.

No risks were found to fauna groups and mite Orders, but for Collembola morphotypes there were risks in all application doses.

Table 36 - SIMPER analysis to groups that most contributed to dissimilarities in chlorothalonil GAP treatments in the first (T1) and second (T2) sampling time. * - indicates significant dissimilarity. ** - indicates significant dissimilarity and an assumed risk (dissimilarity ≥ 35%). Doses are expressed in mg a.i. kg^{-1}.

Chlorothalonil – GAP								
Overall fauna			*Average of Dissimilarities (%)*					
	control x doses		T1			T2		
	0.67		23%			19%		
	2		22%			16%		
	20		28%			19%		
Collembola			Contribution of Collembola group by each morphotype (%)					
			Epigeic			Hemiedaphic	Euedaphic	
Sampling	control x doses	Average of Dissimilarities	*M 75*	*M 88*	*M 98*	*M 29*	*M 12*	*M 1*
T1	0.67	29%*	0%	45%	25%	13%	10%	0%
	2	39%*	0%	40%	7%	19%	19%	8%
	20	50%*	0%	35%	0%	29%	16%	13%
T2	0.67	42%**	19%	31%	18%	18%	0%	15%
	2	35%**	20%	36%	21%	13%	0%	10%
	20	45%**	18%	26%	17%	2%	0%	12%
Acarina			Contribution of Acarina Orders (%)					
Sampling	control x doses	Average of Dissimilarities	Astigmata		Oribatida	Mesoastigmata	Prostigmata	
T1	0.67	15%	41%		27%	18%	13%	
	2	15%	37%		25%	20%	17%	
	20	29%*	25%		27%	16%	31%	
T2	0.67	32%	41%		27%	18%	13%	
	2	26%	37%		25%	20%	17%	
	20	22%	31%		27%	16%	25%	

Table 37 - SIMPER analysis to groups that most contribute to dissimilarities in chlorothalonil PEC treatments. in the first (T1) and second (T2) sampling time. * - indicates significant dissimilarity. ** - indicates significant dissimilarity and an assumed risk (dissimilarity ≥ 35%). Doses are expressed in mg a.i. kg^{-1}.

Chlorothalonil – PEC								
Overall fauna			*Average of Dissimilarities (%)*					
	control x doses		T1			T2		
	0.80		23%			18%		
	2.40		22%*			16%		
	24.10		25%*			16%		
Collembola			Contribution of Collembola group by each morphotype (%)					
			Epigeic			Hemiedaphic	Euedaphic	
Sampling	control x doses	Average of Dissimilarities	*M 75*	*M 88*	*M 98*	*M 29*	*M 12*	*M 1*
T1	0.80	39%*	0%	36%	0%	25%	20%	12%
	2.40	54%*	0%	34%	0%	32%	16%	13%
	24.10	53%*	0%	34%	0%	30%	16%	14%
T2	0.80	39%**	19%	28%	18%	23%	0%	11%
	2.40	35%**	21%	36%	21%	0%	0%	13%
	24.10	39%**	17%	31%	18%	14%	0%	19%
Acarina			Contribution of Acarina Orders (%)					
Sampling	control x doses	Average of Dissimilarities	Astigmata		Oribatida	Mesoastigmata	Prostigmata	
T1	0.80	16%	37%		21%	18%	25%	
	2.40	21%*	32%		20%	17%	31%	
	24.10	27%*	27%		36%	17%	20%	
T2	0.80	33%	30%		24%	24%	22%	
	2.40	31%	30%		25%	22%	22%	
	24.10	29%	30%		27%	24%	19%	

Chlorpyrifos. Feeding activity measured through bait lamina sticks in both GAP and PEC scenarios is presented in annex IV and figures 20 to 25. Significant differences were detected in all application doses and bait lamina evaluations of GAP scenario (table 38), with a reduction of feeding activity. In PEC scenario, no significant differences in feeding activity were found in the first bait lamina evaluation since there was not pesticide application in this period. The second bait lamina evaluation showed significant decreases of feeding activity in 0.81 and 8.16 mg a.i. kg^{-1} doses, while on the third bait lamina evaluation, significant differences were found in all doses.

Table 38 - Statistical differences of feeding activity measured through bait lamina sticks (p values) in three evaluation times between control and treatments of a TME experiment with Lorsban (with chlorpyrifos as a.i.) considering two scenarios: GAP scenario: based on Good Agricultural Practices (GAP) composed by two applications and PEC scenario: based on $PEC_{accumax}$ estimations with a single application. For additional information regarding the timing of each bait lamina evaluation see in the text. * - indicates statistical differences between control and treatments (through one-way ANOVA followed by a Dunnett post hoc test, $p \leq 0.05$). Doses are expressed in mg a.i.kg^{-1}.

GAP scenario			
Control x doses	First evaluation	Second evaluation	Third evaluation
0.14	0.0099*	0.0028*	0.0000080*
0.64	0.000096*	0.0017*	0.000013*
64	0.00013*	0.00018*	0.0000080*
PEC scenario			
Control x doses	First evaluation	Second evaluation	Third evaluation
0.14	0.99	0.10	0.0000080*
0.64	0.98	0.030*	0.000014*
64	0.48	0.024*	0.0000070*

The dissimilarities of in-soil fauna data between control and test doses are summarized in table 39 for GAP scenario and 40 for PEC scenario. Enchytraeid genus data did not show significant differences between control and treatments in any sampling date or scenario. Because of that these data are not show. For T1 TME sampling of GAP scenario differences between control and doses (0.64 and 64 mg a.i.kg^{-1}) were observed to soil fauna groups and mites. Collembola species had significant differences in all application doses and these differences were explained by a decrease in the abundance of species from all morphotypes. In T2 sampling of GAP scenario, significant differences were observed just between control and the high tested dose (64 mg a.i.kg^{-1}) for fauna groups and mites. Collembola species significantly decreased in all doses (table 39).

In PEC scenario, T1 TME sampling showed significant differences between control and all doses for fauna groups. Mites were significantly affected in 0.81 and 8.16 mg a.i. kg^{-1} doses. Collembolans had significant morphotypes decreases in all doses. For T2 TME sampling, fauna

groups had significant differences in 0.81 and 8.16 mg a.i. kg^{-1} doses, while mites did not show differences between control and application doses. Collembola species showed significant differences between control and all test doses (table 40).

Table 39 - SIMPER analysis to groups that most contribute to dissimilarities in chlorpyrifos GAP treatments in the first (T1) and second (T2) sampling time. * - indicates significant dissimilarity. ** - indicates significant dissimilarity and an assumed risk (dissimilarity ≥ 35%). Doses are expressed in mg a.i. kg^{-1}.

Chlorpyrifos – GAP											
Overall fauna			*Average of Dissimilarities (%)*								
	Control x doses		T1					T2			
	0.14		33%					33%			
	0.64		29%*					27%			
	64		38%*					30%			
Collembola			Contribution of Collembola group by each morphotype (%)								
			Epigeic	Hemiedaphic				Euedaphic			
Sampling	Control x doses	Average of Dissimilarities	M88	M62	M55	M50	M41	M24	M12	M3	M1
T1	0.14	60%*	17%	8%	15%	14%	11%	9%	0%	9%	9%
	0.64	56%*	18%	8%	15%	14%	12%	9%	7%	10%	0%
	64	69%*	20%	7%	14%	13%	11%	9%	0%	9%	11%
T2	0.14	49%**	29%	8%	23%	0%	12%	0%	0%	11%	13%
	0.64	48%**	29%	8%	24%	0%	13%	0%	0%	11%	11%
	64	43%**	30%	8%	21%	0%	14%	0%	0%	14%	10%
Acari			Contribution of Acarina Orders (%)								
Sampling	Control x doses	Average of Dissimilarities	Astigmata		Oribatida			Mesoastigmata		Prostigmata	
T1	0.14	19%	31%		17%			11%		41%	
	0.64	16%*	40%		14%			32%		13%	
	64	35%*	21%		0%			34%		36%	
T2	0.14	14%	41%		24%			19%		17%	
	0.64	14%	42%		29%			18%		11%	
	64	35%	22%		29%			26%		23%	

Table 40 - SIMPER analysis to groups that most contribute to dissimilarities in chlorpyrifos PEC treatments in the first (T1) and second (T2) sampling time. * - indicates significant dissimilarity. ** - indicates significant dissimilarity and an assumed risk (dissimilarity ≥ 35%). Doses are expressed in mg a.i. kg^{-1}.

Chlorpyrifos – PEC											
Overall fauna			*Average of Dissimilarities (%)*								
	Control x doses		T1					T2			
	0.20		33%*					26%			
	0.81		32%*					33%			
	8.16		46%*					34%*			
Collembola			Contribution of Collembola group by each morphotype (%)								
			Epigeic	Hemiedaphic				Euedaphic			
Sampling	Control x doses	Average of Dissimilarities	*M88*	*M62*	*M55*	*M50*	*M41*	*M24*	*M12*	*M3*	*M1*
T1	0.20	52%*	19%	8%	14%	13%	12%	9%	8%	11%	0%
	0.81	61%*	20%	8%	13%	14%	12%	9%	0%	10%	7%
	8.16	70%*	19%	7%	14%	13%	10%	8%	0%	9%	13%
T2	0.20	47%**	29%	8%	23%	0%	11%	0%	0%	14%	10%
	0.81	51%**	26%	8%	24%	0%	10%	0%	0%	12%	17%
	8.16	67%**	25%	0%	20%	0%	9%	0%	0%	10%	26%
Acari			Contribution of Acarina Orders (%)								
Sampling	Control x doses	Average of Dissimilarities	Astigmata		Oribatida		Mesoastigmata		Prostigmata		
T1	0.20	15%	41%		22%		22%		16%		
	0.81	23%	28%		18%		32%		22%		
	8.16	37%*	17%		17%		30%		36%		
T2	0.20	16%	39%		18%		18%		25%		
	0.81	28%	29%		23%		29%		19%		
	8.16	18%	34%		16%		26%		23%		

5.4 DISCUSSION

The feeding activity measured by bait lamina sticks is still scarcely used in pesticides studies, despite the ISO standard developed since 2014 (ISO 2014). This method has the advantage of assessing effects on the feeding activity of soil communities belowground by analyzing the feeding profiles (LARINKA: SOMMER, 2002). In a field study using metsulfuron-methyl based herbicides, de Santo et al. (2019) did not find significant differences in feeding activity using this method. On the other hand, Niemeyer et al., (2018) highlighted functional impacts of glyphosate evidenced by bait lamina test, which showed low consumption from 4.5 to 7 cm depth. In addition, these authors concluded that avoidance tests were not enough to predict these impacts suggesting that bait lamina sticks may be more sensitive than avoidance tests. Besides the absence of a large dataset on bait lamina evidencing effects of pesticides, this method has been used to verify effects of other contaminants to soil feeding activity. Jensen and Scott-Fordsmand (2012), using a multi-species test system to evaluate the impact of pharmaceutical ivermectin argued that the structural endpoints were more sensitive than functional ditto measured by bait lamina. In areas contaminated by heavy metals, Niemeyer et al. (2010, 2012) already concluded that this method could be a good approach to distinguish the level of soil contamination, since it was significantly correlated with metal loadings.

Despite some researchers have criticized the method of bait lamina sticks, arguing that its often shows inconsistences of data provided (KLIMEK et al., 2015), the feeding activity measured by this method have shown positive correlations with the abundance of in-soil groups like microarthropods (FILZEK et al. 2004; ROMBKE et al. 2006). Data obtained in tests with chlorothalonil of the present study, mostly for the PEC scenario, indicates a possible relationship between feeding activity and the overall fauna and mites since the reduction in feeding activity found in the T1 sampling agrees with the significant effects found for these evaluated groups. Moreover, in the third bait lamina evaluation, the absence of differences in feeding activity agrees with the absence of dissimilarities between control and treatments to all fauna groups and mites. To chlorpyrifos, however, these correlations are not so clear. Bait lamina sticks indicated a significant reduction in feeding activity of all tested doses in GAP and PEC scenarios, except in in the lowest tested dose of GAP (0.14 mg a.i. kg^{-1}) where no statistical differences were found. Perhaps the in-soil group associated to the feeding activity in this experiment changed. Collembola (through morphotypes) was the most sensitive group

evaluated and was positively correlated with feeding activity measured through bait lamina method (HELLING et al., 1998).

Results evidenced a high sensitivity of Collembola in TMEs tests of both pesticides, even in the lowest dose (25% of the recommended dose), regardless the scenario. Despite the scarce information regarding fungicide effects on this group, carbendazim, which was considered a reference substance by ISO (ISO, 1999), was used in a TME project to evaluate effects in earthworm and Collembola communities in different European scenarios (RÖMBKE et al., 2004). In that study, carbendazim did not affect Collembola species diversity (KOOLHAAS et al., 2004). The authors considered that the absence of effects of carbendazim agreed with the lack of effects also found in standard laboratory tests with collembolan performed in the same project. More recently, Simões et al. (2019b), evaluating effects of chlorothalonil through molecular markers in *Folsomia candida*, also observed high correlations between laboratory and in-field experiments. In fact, laboratory results obtained in Chapter I suggest that Collembola species are considerably sensitive to chlorothalonil (through SSD approach - HC_{5EC10}: 0.83 mg a.i. kg^{-1}). This agrees to the data observed in the present chapter where an absence of recovery was observed eight weeks after chlorothalonil application (T2), regardless dose or scenario.

In general, epiedaphic and hemiedaphic morphotypes seem to be more affected by chlorothalonil and chlorpyrifos than euedaphic Collembola. This agrees to a study conducted by Martikainen et al. (1998) who investigated the effect of the insecticide dimethoate by performing a microcosm experiment with soil collected from a pesticide-free area in central Finland. The authors found higher effects (reduction of microarthropod abundances) on the upper than in the lower soil layer. On the other hand, Sechi et al. (2014), in a soil multi-species test constructed with seven cultivated species in the laboratory, contaminated with the pyrethroid insecticide α-cypermethrin, observed that the epigeic and hemiedaphic species *Heteromurus nitidus* and *Proisotoma minuta* were the least affected. This was the opposite result found in the present results for epigeic and hemiedaphic Collembola. However, it should be taken into account that, besides the use of laboratory species, researchers added food weekly giving moistened dried cattle manure, which could serve as a non-contaminated habitat for the upper soil layer organisms. In addition, a deeper Collembola identification for TMEs experiments (e.g. species level) would provide a more precise information on this group sensitivity. However, since there is a lack of available taxonomists, mostly in Brazil

(ZEPPELINI FILHO:BELLINI, 2004), effects on collembolans have been investigated through functional traits (OLIVEIRA-FILHO et al., 2016).

Despite the sensitivity of epiedaphic Collembola at laboratory level has been scarcely addressed until date, some species, like *Sinella curviseta*, have been indicated by OECD (OECD, 2016) as alternative test species. Bandow et al (2014) tested at different temperatures and moisture regimes the pyrethroid insecticide lambda-cyhalothrin and highlighted that, compared to *F. candida, S. curviseta* might be more sensitive to small variations in lambda-cyhalothrin when soil water content differs.

Ideally, the predictive ability of the laboratory (lower-tier) studies should be validated against pesticide effects data obtained under more ecologically realistic (higher-tier) conditions. However, there is still a lack of data to validate the predictions of lower-tier tests, to many PPPs mostly to microarthropods. The toxicity of organophosphorus insecticides to arthropods has been largely discussed. Despite that, there is still limited information on semi-field and field tests regarding the effect of this compound in soil fauna (FOUNTAIN et al., 2007). Chlorpyrifos data obtained in chapter II indicated a high sensitivity of all Collembola species to this compound similarly to that observed in the TME experiments of the present chapter. Although it has been reduced, the sensitivity remains in the community test (chapter III), mostly explained by *H. thermophila*. In TME experiment of the present chapter, significant dissimilarities were observed in all doses regardless the scenario.

For mites, additional laboratory tests were performed to achieve the requirements for the lower tier in Europe (EC, 2009), and could be used for a comparison. As previously discussed for chlorothalonil (chapter III) the EC_{50} estimated in additional laboratory tests to *Hypoaspis aculeifer* was > 420 mg a.i. kg^{-1}, despite of using a different methodology for exposure method (through contaminated prey). Risks in community tests (chapter III) to mites could not be verified, since there was a high variability in dataset. However, in the present chapter, effects on mites measured through significative dissimilarities (PERMANOVA, $p < 0.05$) were observed in GAP scenario in a concentration 20 times lower (control x 20 mg a.i. kg^{-1}) than the estimate in lower tier. A decrease on Oribatida suborder explained 27% of this dissimilarity and its cohort Astigmata, 25%. This group is composed by microbial feeders that are able to chew vegetable material and fungi (PHILIPS, 1990) so it plays an important role in decomposition (COLEMAN et al., 2004).

Since chlorothalonil is a fungicide, the absence of its toxicity to mites in lower tier regardless of the exposition method used could be related with the absence of sensitivity of the predatory species for this mechanism of action. Moreover, the decrease of Oribatida could be

related with possible effects of chlorothalonil on non-target fungi. This corroborates with a research with the fungicide carbadenzim from Koolhaas et al. (2004), which was used for the field validation of semi-field tests. The researchers observed a significant decrease of mite abundance at the two highest concentrations tested (13 and 77.8 kg/ha for Amsterdam and Bangor soil TME pre-tests, respectively). To this both experiments Astigmata were the most sensitive mite to the treatments. Despite of some research have been performed to Oribatida mites in the laboratory, mostly with *Oppia nitens* (PRINCZ et al., 2010; HUGUIER et al., 2014), and even with the tropical *Muliercula inexpectata* (OWOJORI et al., 2019) to our knowledge, no study reported until the present the toxicity of fungicides to this Order of mites.

For chlorpyrifos, additional tests with *H. aculeifer* were also performed. Results based on the standard OECD test (OECD, 2016, figure 26) estimates an EC_{50} value of 1.41 (1.16 – 1.66) mg a.i. kg^{-1} while TMEs in GAP scenario T1 sampling showed significative differences to mites between control and 0.64 mg a.i.kg^{-1} (PERMANOVA, $p \leq 0.05$). The differences were explained by an increase in the Astigmata abundance (40%) and the reduction of Mesoastigmata Order (32%). A low sensitivity of Oribatida to insecticides was observed in ecotoxicity laboratory tests with *O. nitens*, which was the least sensitive invertebrate species in reproduction tests to imidacloprid (NOEC: 100 mg a.i.kg^{-1}) and thiacloprid (NOEC > 100 mg a.i.kg^{-1}) when compared with *E. andrei* (NOEC: 0.3 mg a.i. kg^{-1} to both products)*; F. candida* (NOEC: 0.1 and 1.1 mg a.i. kg^{-1} respectively) and *E. crypticus* (NOEC: 1 and 3 mg a.i. kg^{-1} respectively) in a LUFA 2.2 soil (SILVA et al., 2017). Despite of the Mesostigmata Order has some few polyphagous species (e.g. Uropodidae), feeding on fungi, nematodes, and juvenile insects (GERSON et al., 2003), this group is composed mostly by predatory mites (COLEMAN et al., 2004) as *H. aculeifer*, used in laboratory tests. This species was capable of predict chlorpyrifos risks in lower tier to mites when the same did not occur in chlorothalonil experiments.

In terms of risk assessment, despite the significant dissimilarities found in the TME data (PERMANOVA, $p < 0.05$), only Collembola morphotypes were at unacceptable risk at the T2 sampling. As suggested by EFSA (2017), the magnitude/temporal scale of acceptable effects are small effects/up to months for a percentage of effect < 35. However, since this 35% value seems weakly supported by real data, the temporal scale looks inaccurate (the precise number of weeks to define the evaluation time is not provided) and few endpoints are considered (abundance/biomass), these results should be carefully evaluated. For example, if instead of 35%, a value of 30% was considered as the limit of acceptable effects, for chlorpyrifos the all

fauna groups would be at risk in the highest dose (64 mg a.i. kg^{-1}) of GAP scenario (30%) and in the recommended dose (0.81 mg a.i. kg^{-1}) in PEC scenario (33%). In addition, as already highlighted in chapter III, dissimilarity values were not proposed as an endpoint by EFSA in the Scientific Opinion (2017) but could be a valid endpoint for the risk assessment since it's a way to measure shifts in community composition. Of course, if this becomes a reality, perhaps the threshold levels for acceptable effects have to be revised.

Concerning differences between GAP and PEC scenarios, both pesticides presented similar behavior. For chlorothalonil, all fauna groups and mites were not at risk in T2 sampling. Collembola morphotypes in both scenarios presented risk, although, if the percentage of the dissimilarities is observed, values are higher in GAP than in PEC scenario. Since the dissimilarity might increase when the differences between control and treatments are larger, the effects observed in Collembola were high in GAP (dissimilarity control x 20 mg a.i. kg^{-1} = 45%) than in PEC scenario (dissimilarity control x 24.10 mg a.i. kg^{-1} = 39%). On the other hand, for chlorpyrifos, a similar behavior to both scenarios was observed considering the limit of 35% of dissimilarity for risk characterization. However, there was other differences, mainly to all fauna groups. In the T2 sampling, despite the absence of risk (dissimilarity < 35%), there was differences in similarities (PERMANOVA, $p < 0.05$) between control and the highest dose of PEC scenario (8.16 mg a.i. kg^{-1}). This is not considered as risk (34%) because it was lower than the accepted range (35%), which highlighted the issues in the ranges suggested of acceptable risks (EFSA, 2017). For Collembola, a risk was also observed to both scenarios. However, dissimilarities were higher in PEC (67%) than in GAP (49%). Even with the uncertain in this comparison between scenarios, since this was not a pattern to all in-soil fauna organisms evaluated, apparently both PEC and GAP were able to accurately predicted risks, which suggests that both strategies are adequate to predict risks of real application scenarios. To both PPPs, regardless the scenario (GAP or $PEC_{accumax}$) Collembola group does not recovery (dissimilarity > 35%). For the other evaluated group, even when differences between control and doses happened in the first sampling (T1), there was no risk in the second sampling (T2) (dissimilarity < 35%) indicating recovery.

The problem of using $PEC_{accumax}$ in Brazilian scenarios is more evident in this chapter. If the PEC was calculated based on the DT_{50} IBAMA range information (data not show) values to both pesticides would be in average two times larger than the ones used in the experiment. As discussed before, the DT_{50soil} of the products have been not very well addressed in Brazil, and in order to use the tiered approach, or even the toxicity-exposure ratio (TER) values in

preliminary tiers, it is crucial to have a reliable estimation of the exposure. Otherwise, the toxicity could be assessed, but not the potential real risk.

5.5 CONCLUSION

Both chlorothalonil and chlorpyrifos showed risk in TME experiments even at the lowest tested doses (25% of recommended dose) regardless the exposure scenario, mostly for Collembola. Despite the differences between control and treatments in the T1 sampling, data suggest that a recovery is possible to all fauna groups and mites, since the dissimilarities compared to control decreased over time (T2). The use of dissimilarity as endpoint seems adequate to assess pesticide risk in TMEs, similarly as was previously shown to community tests (chapter III) but to establish more specifics thresholds for acceptable risks is necessary. GAP and PEC scenarios presented similar results, which suggests that the modeling used in Europe corroborates the agronomy practices. Predicted pesticide risks to Brazilian scenarios using European ERA tiered approach is still a challenge. The uncertainties associated to the prediction of the exposure is the major issue to access risks in Brazilian scenarios. Semi-field tests showed to be useful as a surrogate higher tier in ecological risk assessment.

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5.7 ANNEX IV

Table 41 - Biodegradability of PPPs according with IBAMA legislation (1996).

Biodegradability			Class	Classification	DT_{50} conversion		
0 ≤	% CO_2	< 1	1	Highly Persistent	360 ≤	$T_{1/2}$ (days)	
1 ≤	% CO_2	< 10	2	Very Persistent	180 ≤	$T_{1/2}$ (days)	< 360
10 ≤	% CO_2	< 25	3	Moderately Persistent	30 ≤	$T_{1/2}$ (days)	< 180
25 ≤	% CO_2		4	Low Persistent	0 ≤	$T_{1/2}$ (days)	< 30

Table 42 - Good Agricultural Practices (GAP) in Brazil to chlorothalonil and chlorpyrifos used in TMEs scenarios in crop of soybean. To estimate PEC values, a depth soil of 5 cm and a bulk density of 1.5 $g.cm^{-3}$ were considered.

Product			Exposure scenario		Data used						
Active ingredient	**Commercial Product**	**g i.a.L^{-1}**	**Scenario**	**Application**	**rate application ($L.ha^{-1}$)**	**Dose ($g.ha^{-1}$)**	**nº app**	**Interval (days)**	**BBCH code**	**reaching soil (fraction)**	**Interception (%)**
Chlorothalonil	Bravonil 500	500	GAP	1	3	1500	2	14	40 - 89	0.75	25
				2					90 - 99	0.65	35
			PEC	-					90 - 99	0.65	35
Chlorpyrifos	Lorsban 480	480	GAP	1	1	480	2	14	10 - 19	0.85	15
				2					20-39	0.80	20
			PEC	-					20-39	0.80	20

Figure 14 - Chlorothalonil bait lamina results (feeding activity, %) in GAP scenario, for the first evaluation, to Control, 0.25xGAP, GAP and 10xGAP

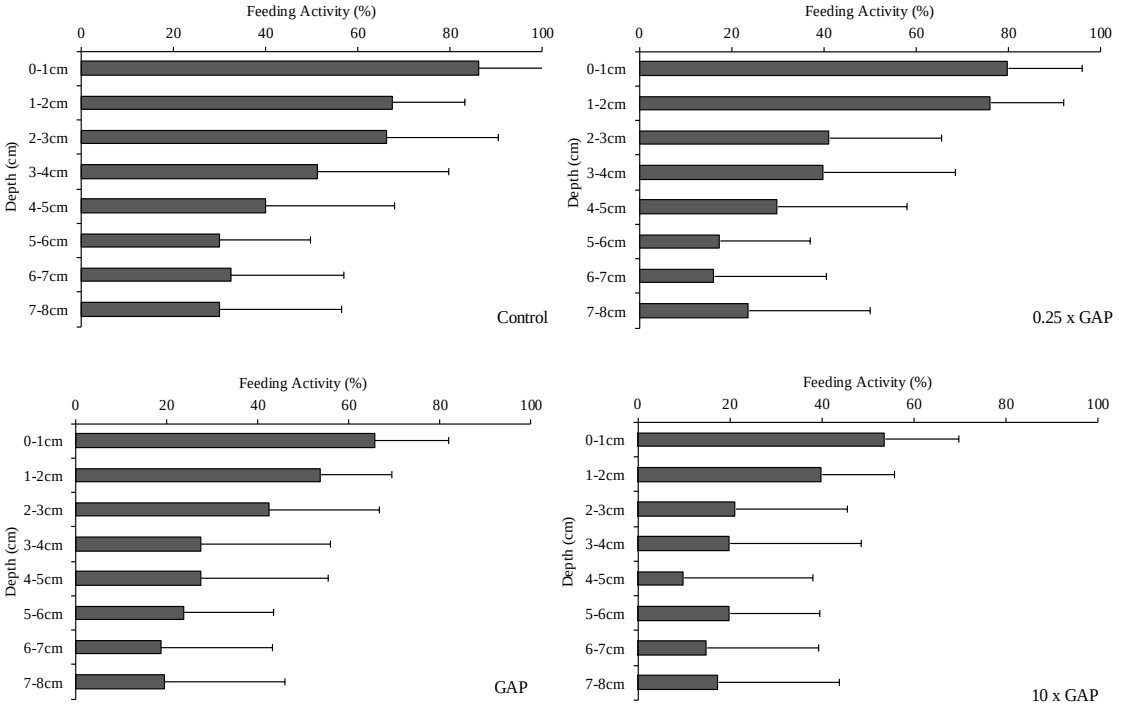


Figura 15 - Chlorothalonil bait lamina results (feeding activity, %) in GAP scenario, for the second evaluation, to Control, 0.25xGAP, GAP and 10xGAP

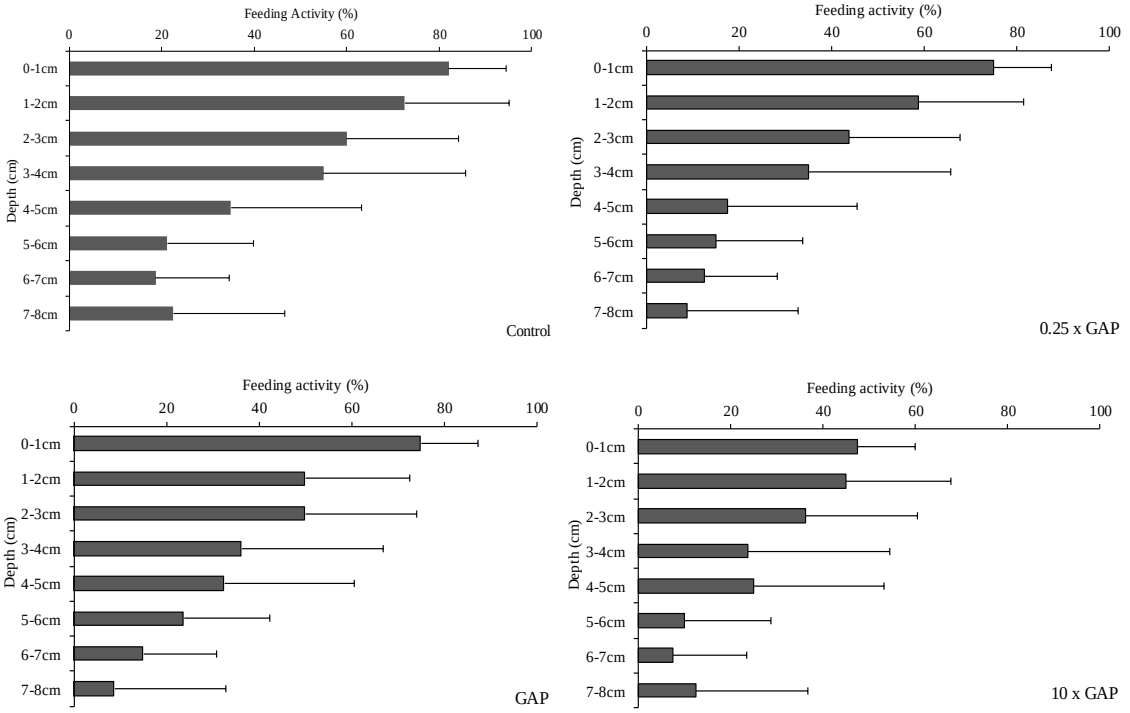


Figura 16 - Chlorothalonil bait lamina results (feeding activity, %) in GAP scenario, for the third evaluation (recovery), to Control, 0.25xGAP, GAP and 10xGAP.

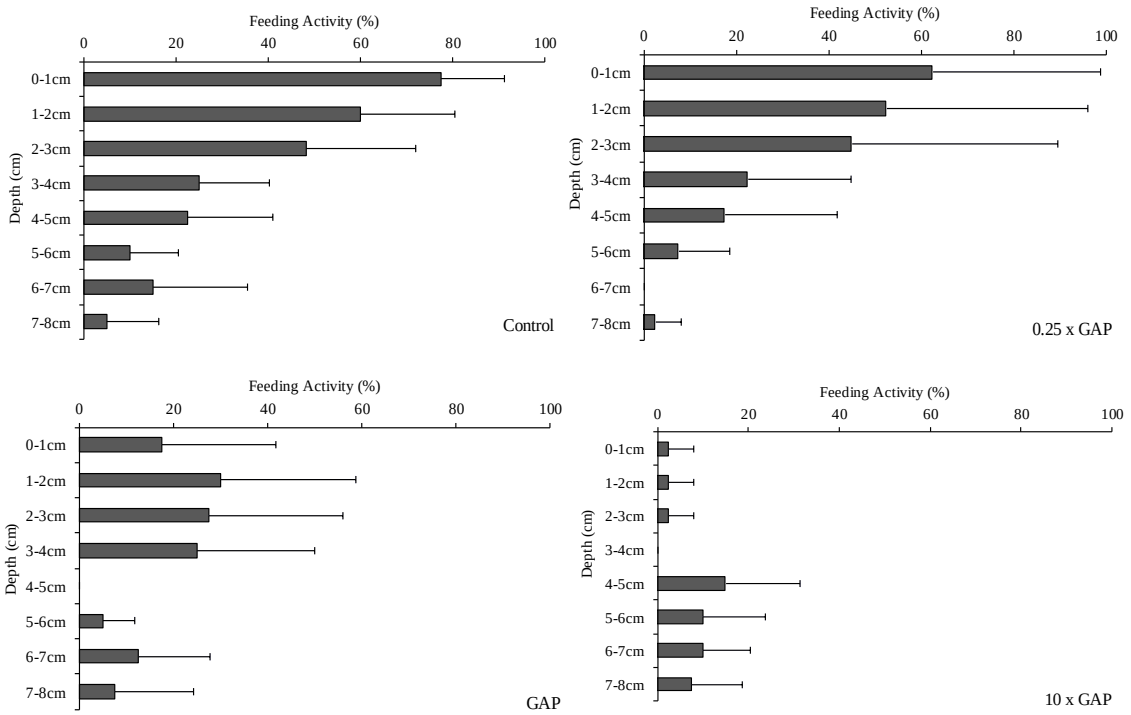


Figura 17 - Chlorothalonil bait lamina results (feeding activity, %) in PEC scenario, for the first evaluation, to Control, 0.25xPEC, PEC and 10xPEC

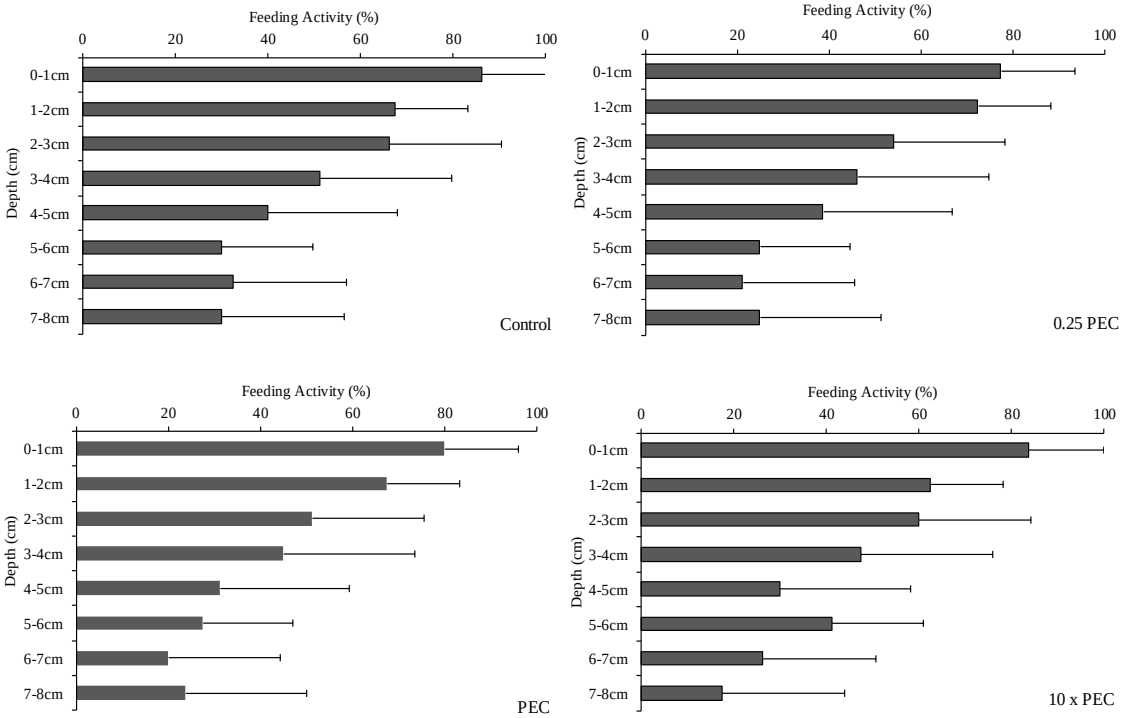


Figura 18 - Chlorothalonil bait lamina results (feeding activity, %) in PEC scenario, for the second evaluation, to Control, 0.25xPEC, PEC and 10xPEC.

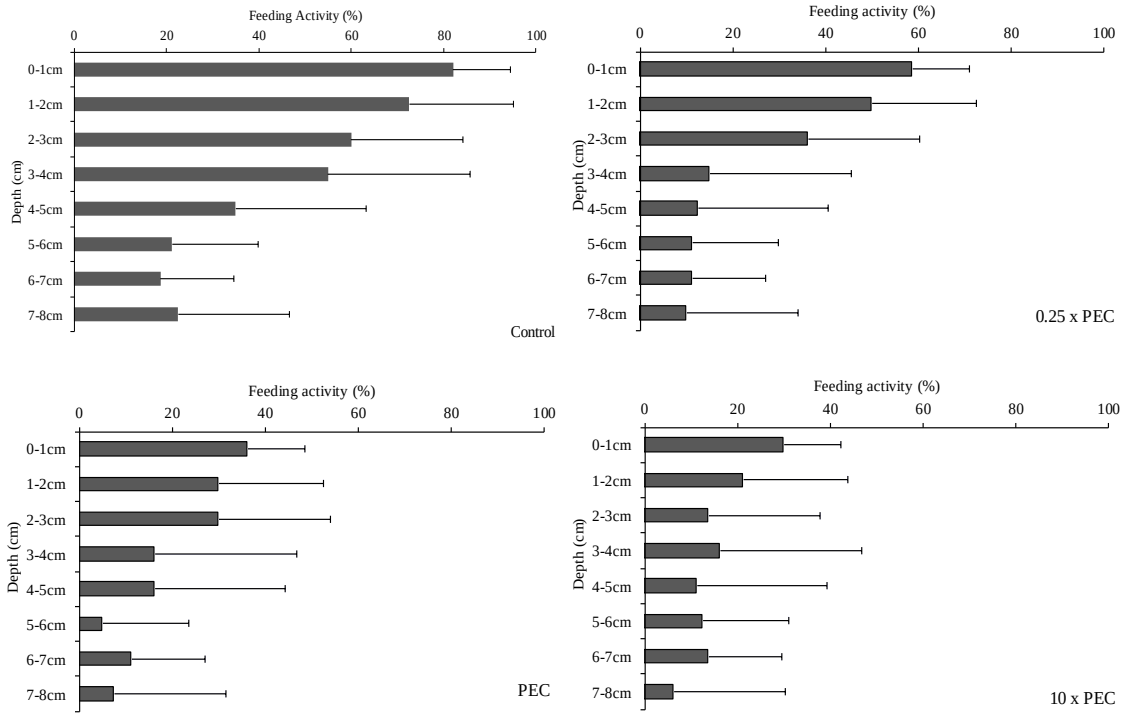


Figura 19 - Chlorothalonil bait lamina results (feeding activity, %) in PEC scenario, for the third evaluation (recovery), to Control, 0.25xPEC, PEC and 10xPEC.

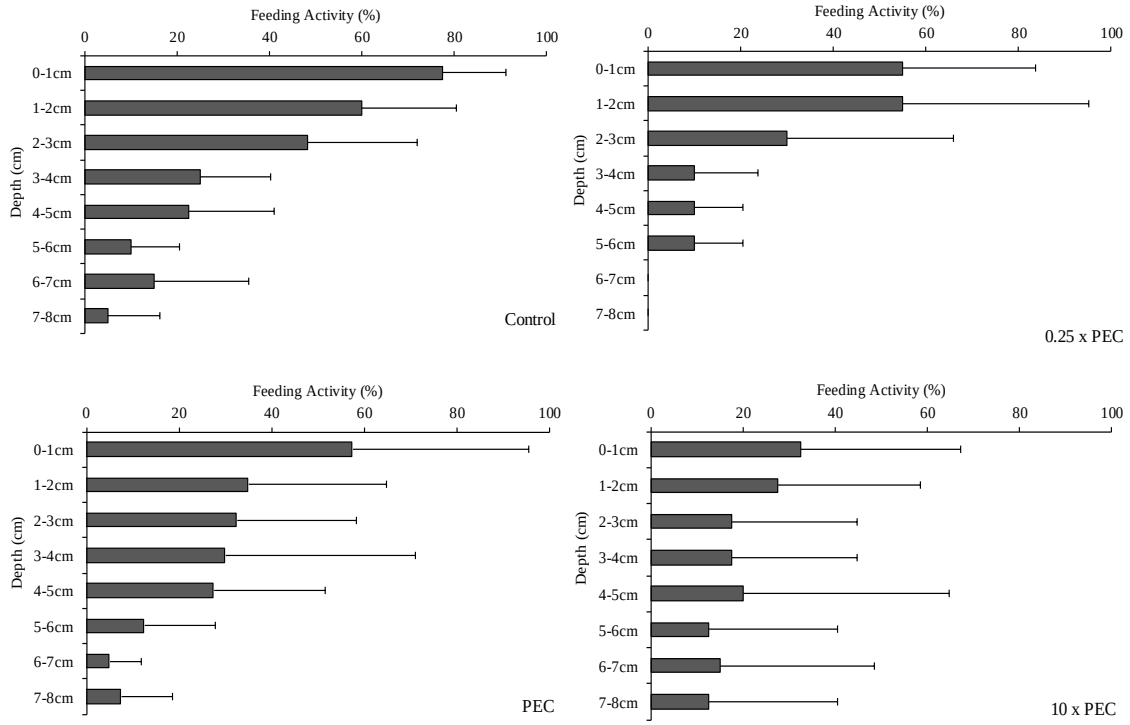


Figura 20 - Chlorpyrifos bait lamina results (feeding activity, %) in GAP scenario, for the first evaluation, to Control, 0.25xGAP, GAP and 10xGAP.

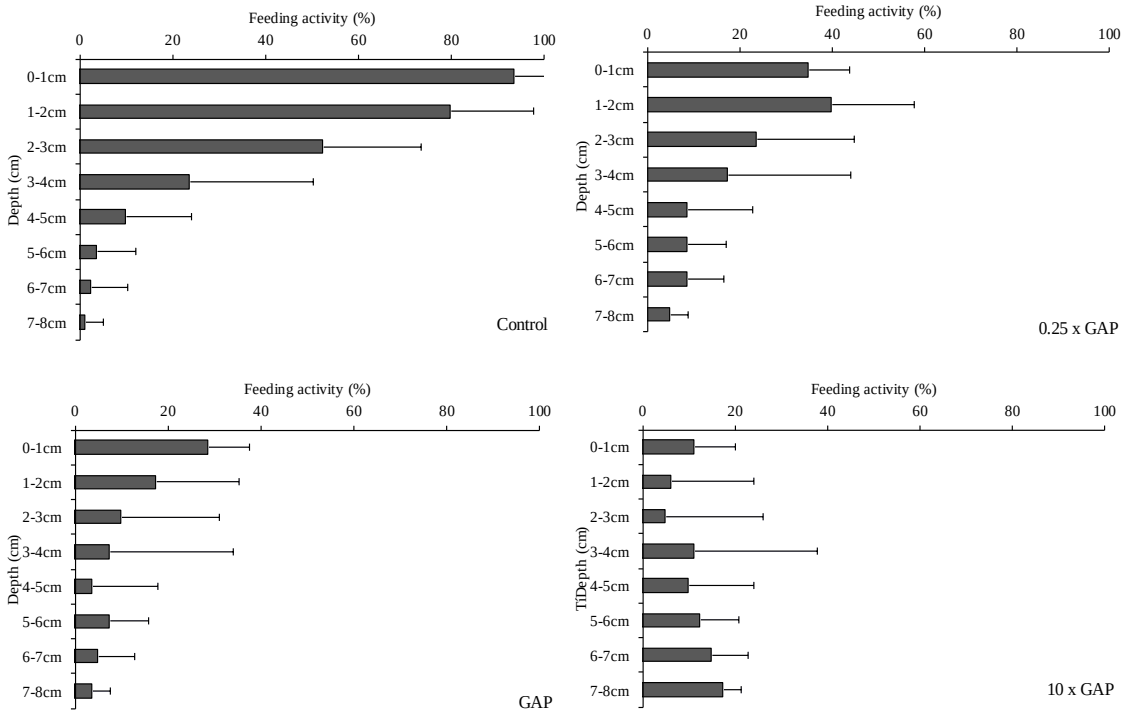


Figura 21 - Chlorpyrifos bait lamina results (feeding activity, %) in GAP scenario, for the second evaluation, to Control, 0.25xGAP, GAP and 10xGAP.

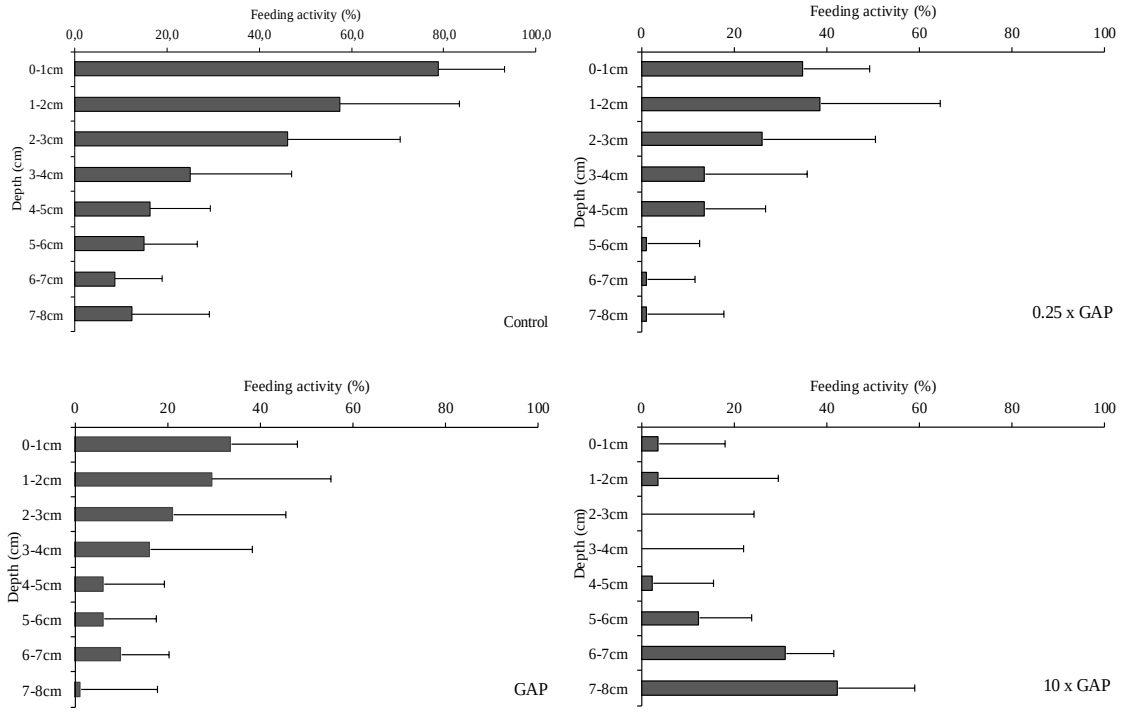


Figura 22 - Chlorpyrifos bait lamina results (feeding activity, %) in GAP scenario, for the third evaluation (recovery), to Control, 0.25xGAP, GAP and 10xGAP.

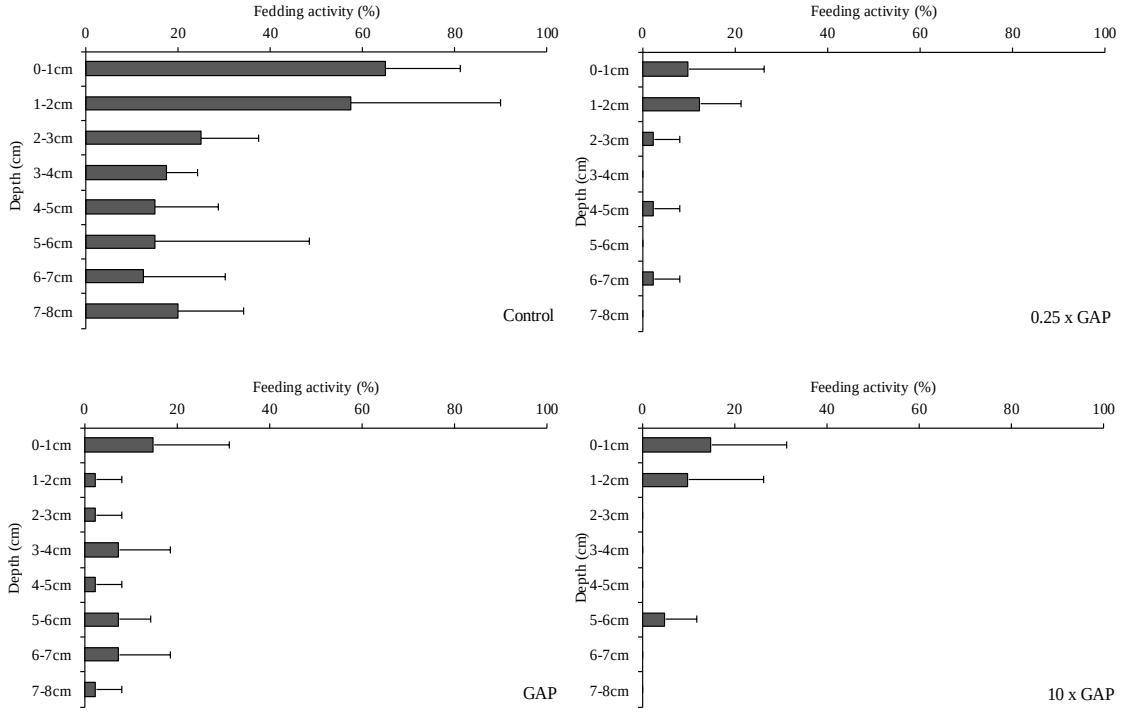


Figura 23 - Chlorpyrifos bait lamina results (feeding activity, %) in PEC scenario, for the first evaluation, to Control, 0.25xPEC, PEC and 10xPEC.

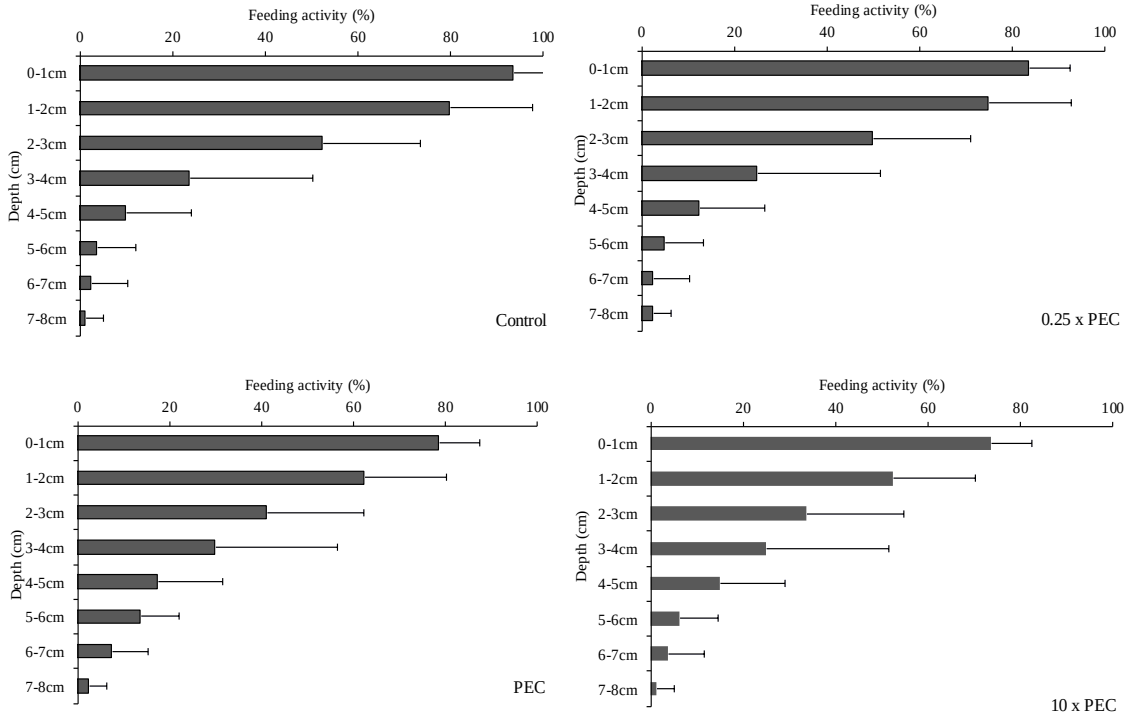


Figura 24 - Chlorpyrifos bait lamina results (feeding activity, %) in PEC scenario, for the second evaluation, to Control, 0.25xPEC, PEC and 10xPEC.

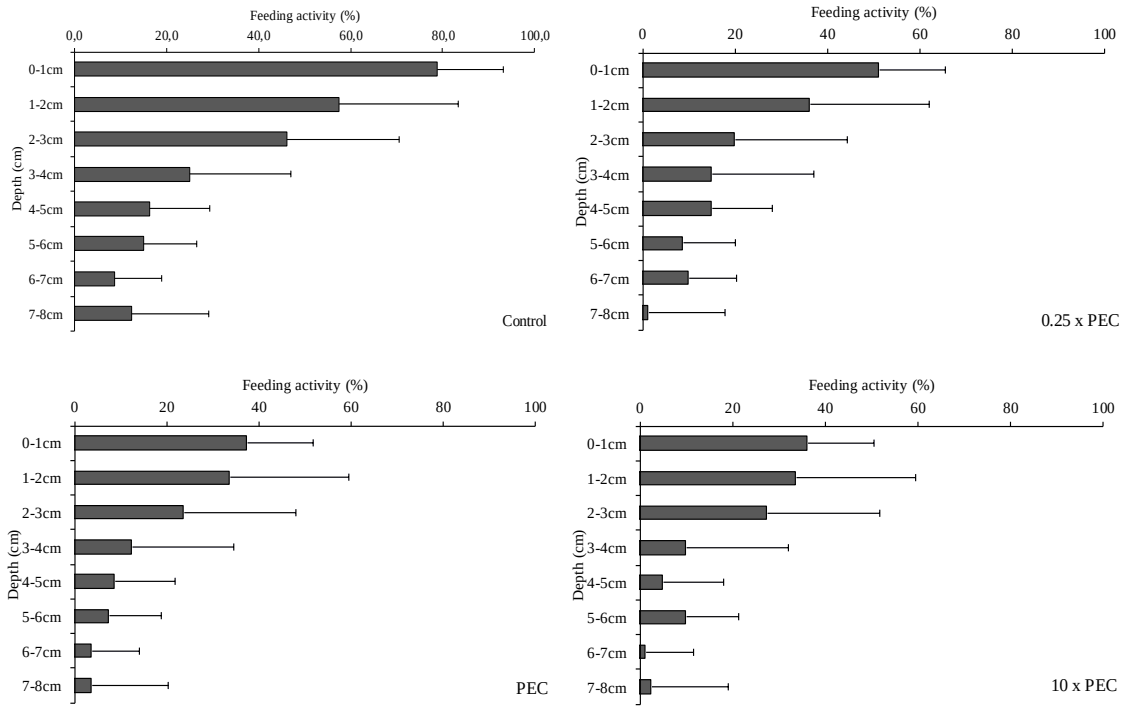


Figura 25 - Chlorpyrifos bait lamina results (feeding activity, %) in PEC scenario, for the second evaluation, to Control, 0.25xPEC, PEC and 10xPEC.

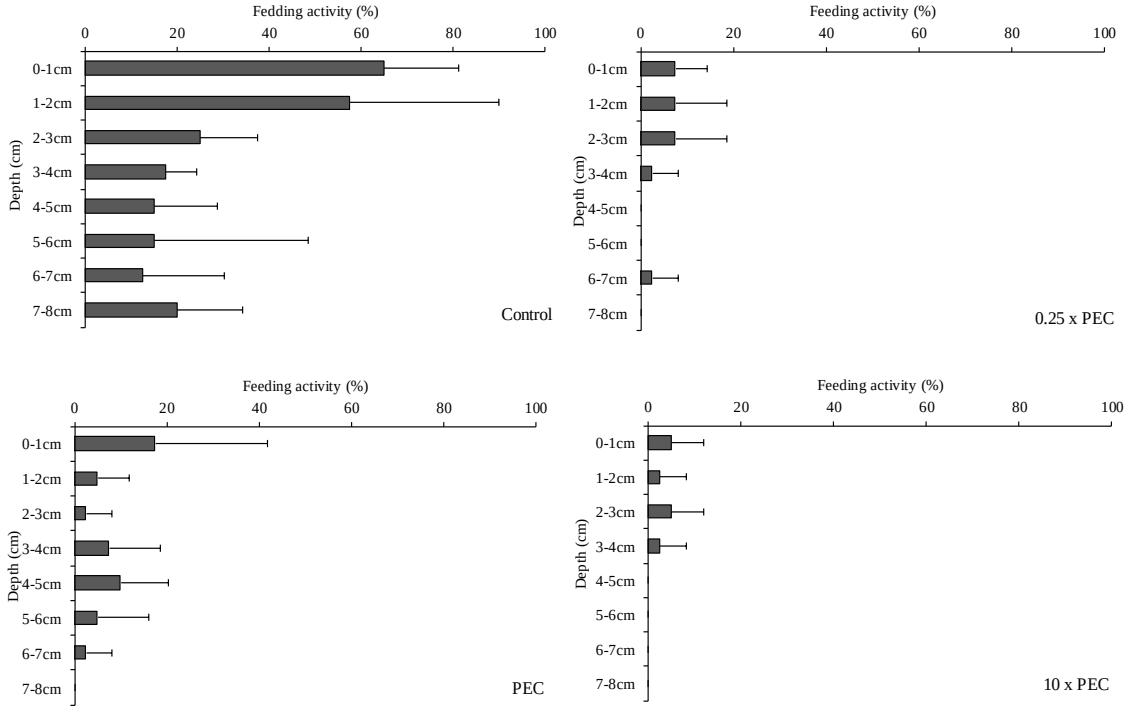
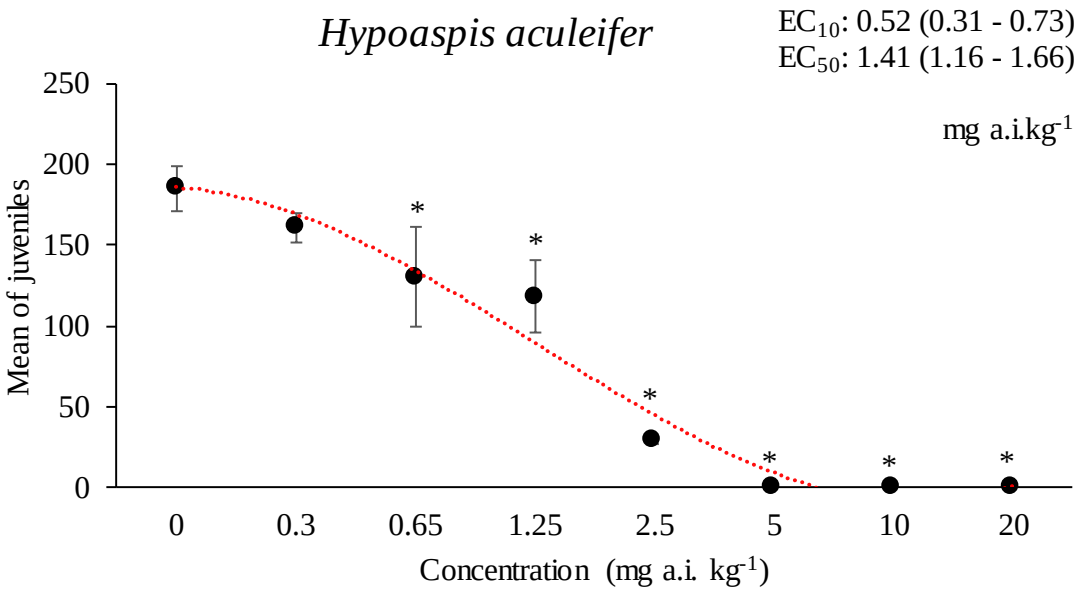


Figura 26 - Reproduction tests (OECD 226, 2016) using increasing concentrations of chlorpyrifos (Lorsban 480®). EC_{10} and EC_{50} are represented, as curve with the sensitivity response for the mean number of juveniles. Asterisks (*) indicated significative different of the concentration from control (Dunnett test, $p < 0.05$). NOEC value estimated is 0.3 mg a.i. kg^{-1}.



6. GENERAL DISCUSSION AND MAIN CONCLUSIONS

Ecotoxicological tests to in-soil fauna with pesticides have been used for several years (KROGH, 1995; BAUER: RÖMBKE, 1997; FRAMPTON, 2000; JÄNSCH et al., 2005; FILSER et al., 2008; NATAL-DA-LUZ et al., 2012; ALVES et al., 2013; RENAUD et al., 2018; CARNIEL et al., 2019). The Ecological Risk Assessment has using this tool since 2002 in Europe regulation (EC, 2002). However, despite being present in regulation for almost 20 years, and has been updated since than (EC 2009, EU 2013) the dataset provided by literature is still limited, and mostly based on earthworms toxicity (PELOSI et al., 2014) and more recently Collembola (JEGEDE et al., 2017; SIMÕES et al., 2019) which prompts the need for further advances in some issues and to fulfill some research gaps to cope with the current general protection goal established in the Regulation 1107/2009.

To improve ERA in Europe following the methodology recommended in EFSA (2017), increasing the number of test species in the lower tier is essential. For instance, in the current legislation the only Oligochaeta species required is *Eisenia andrei*, which was not representative to sensitivity of many Oligochaeta species tested to chlorothalonil in chapter I (*P. excavatus, E. crypticus, E. bigeminus, E. dudichi*) and chlorpyrifos in chapter II (*P. excavatus, D. veneta*). On the other hand, among Collembola species, *F. candida* seems to be a suitable bioindicator of this group (chapter II and III), since all species has a similar dose-response range to both pesticides. Unfortunately, the lack of information for other species besides *F. candida* to more PPPs maintains some uncertainties to Collembola ERA.

This lack of information also reflects in uncertainties to use the SSDs approach in in-soil ERA. SSDs of aquatic organisms already suggested that it could be profitable in practical SSDs applications to distinguish between species groups prior to constructing curves. The use of species not so sensitive with the highly sensitivities changes the curve and consequently leaves to an underestimation of the field effects (POSTHUMA et al., 2002). For both pesticides tested in this thesis there was a separation between Collembola and Oligochaeta species. Increasing tested Oligochaeta species could provide more information for this group, since variability of the data was higher than to Collembola species. Furthermore, SSDs separating enchytraeids and earthworms could be a valuable approach, provide a dataset with closer values, since the taxonomic distance between groups interferes on sensitivity (DAAM et al., 2011). Nevertheless, following the principle defended by several researchers in using the most sensitive group by mechanism of action (MALTBY et al., 2005; VAN DEN BRINK et al.,

2006; MALTBY et al., 2009) maybe insecticides studies, at least the organophosphorus, to develop or improve ERA schemes should be more focused on Collembola than on Oligochaeta species. The high effects of this PPPs to arthropods has been largely discussed and was confirmed in lower tier and intermediate tier with SSDs (chapter II); in the intermediate tier though microarthropod community test (chapter III) and finally in the higher tier through TMEs tests (chapter IV).

However, the same approach could not to be verified for chlorothalonil. Even if Collembola species were more sensitive than Oligochaeta species (chapter I), other fungicides already were pointed as more toxic to earthworms than to Collembola species (FRAMPTON et al., 2006). In addition, studies with chlorothalonil and in-soil fauna are limited, the literature data does not corroborate the data from the present study. The hypothesis for this were the difference in tested substrates and in the commercial product, which give strength to increase the studies with not only this product but also other commercial formulations to realize if the inert ingredients could also have adverse effects, as already pointed by de Santo et al. (2019).

Maltby et al. (2009) already verified that the best approach for SSDs of fungicides and aquatic organisms would be plotted all the major taxonomic group together. Due the multisite mechanism of action of many products, the researches highlighted that it similarly affected the tested groups. However, to in-soil organisms, perhaps the most sensitive group of organisms to fungicides was not tested yet. Among the necessary improvements argued by the EFSA Scientific Opinion (EFSA, 2017), tests with mycorrhiza were also defended. In fact, besides of crucial to food production, this group is also sensitive to environmental changes and contaminants (MALLMANN et al., 2018) and have a direct relationship with soil fauna, mostly Collembola species (BAKONYI et al., 2002; NGOSONG et al., 2014).

Beyond the use of more species and additional laboratory effort, maybe another option to improve the risk assessment would be a better calibration of the trigger value, or assessment factor, as already defended by EFSA (2017). For this propose, community tests might be useful, mainly to local scale.

To integrate SSDs approach and community tests could be a powerful tool in a risk assessment scheme. For instance, when an active ingredient is registered in Europe to be commercialized to all European Union, data from laboratory and artificial soil could be used in a first step, followed by an SSD and a field, or semi-field test if it is required. However, to commercialize specific commercial formulation in each European zone (EFSA, 2015), the community tests could be used to investigate the risk of the products in local scale. Thus, risk managements would have not only global, but also specific data about effects in local soils and

communities to decide if the product risk is acceptable or not. In addition, over time, this dataset will permit a calibration of the lower tier data to each zone though derivation of more feasible trigger values.

Regarding the semi-field TME tests, these proved to be a good tool to act as a surrogate reference tier in ERA. In the present study, TMEs confirmed the risk to both pesticides to Collembola, even eight weeks after the last contamination (chapter V). In both cases, TMEs also confirmed that a trigger value of 5 was enough to protect this group. However, for the earthworms this was not possible to be verified. Unfortunately, the low number of earthworms do not allow a powerful analysis of this data.

In order to summarize the results and to compare the risks for both pesticides in currently approach and the risk estimate in this thesis, the last table (table 42) recapitulates the risk observed in each different step to chlorothalonil (chapters I, III and IV) and chlorpyrifos (chapters II, III, and IV).

Table 43 - Summarized results of the risk observed in previous chapters to chlorothalonil (Bravonil 500®) (chapters II, IV,V) and chlorpyrifos (Lorsban 480®) (chapters (III, IV, V) and the future steps in the ERA that should be followed if risk was pointed.

	Chlorothalonil	**Chlorpyrifos**	**Next step (if there is risk)**
Current approach			
		Lower tier	
Folsomia candida	yes	yes	Field tests
Eisenia andrei	no	no	Litter bag
Hypoaspis aculeifer	no	yes	Field tests
Field tests[a]	-	-	-
Suggested approach on the thesis:			
		Lower tier	
Folsomia candida	yes	yes	Intermediate tier
Folsomia fimetaria	yes	yes	
Prothaphorura fimata	yes	yes	
Sinella curviseta	yes	yes	
Proisotoma minuta	yes	yes	
Hypoaspis aculeifer	no	yes	
Eisenia andrei	no	no	
Perionyx excavatus	yes	yes	
Dendrobaena veneta	yes	yes	
Enchytreus crypticus	yes	yes	
Enchytraeus bigeminus	yes	yes	
Enchytreus dudichi	yes	yes	
		Intermediate tier	
SSD Collembola – HC_{5EC10}	yes	yes	Community test or Semi-field test
SSD Oligochaeta – HC_{5EC10}	yes	yes	Semi-field test or Field test
Community - Collembola	yes	yes	Semi-field test or Field test
Community - Mites[b]	-	-	Semi-field test or Field test
		Higher tier	
TMEs – Overall fauna	no	no	Product not authorized
TMEs – Enchytraeids	no	no	
TMEs – Collembola	yes	yes	
TMEs - Mites	no	no	

[a] Information not provided by the present work;

[b]Due the high variability was not possible to estimate.

Concerning the ERA in Brazil, several improvements are necessary before conducting risk assessment using an ERA scheme at least similar to the European. Firstly, it is utterly urgently to move from acute earthworm data to sub-lethal tests with this species. There is enough published information, including with Brazilian soils (ALVES et al., 2013; CARNIEL

et al., 2009) and even legislations (EU, 2013) evidencing that lethality it is not a sensitive endpoint. Its public knowledge that companies which put products on market in Brazil are quite the same that sells products in Europe. Consequently, they probably already have data using reproduction as an endpoint to many products for a lower tier in Europe, which uses artificial soil and so, could be used also in Brazil. In addition, the EC_{10} and EC_{20} to *F. candida* and *H. aculeifer* mite has been required in Europe since 2013 (EU, 2013) and so, many companies already have this data too. If the Brazilian Environment Agency starts to require this data to companies – or even to Europe Environmental Agencies, it is not so hardy reaches the same toxicology information that is available in Europe.

However, there is a second gap not quite simple. Even that the toxicity data would be accessed, the exposure methods in Brazil are poor and non-realistic. Data accepted by IBAMA is provided by a methodology ($^{14}CO_2$) (IBAMA, 1996) which is not accepted anymore… by IBAMA (IBAMA, 2001). Obviously, the regulation needs an urgent update, and DT_{50} to Brazilian soils must be required instated of the $^{14}CO_2$ detached. Otherwise, will be possible to estimates the toxicity values, but never the risk, since the exposition is unknown.

Concerning the DT_{50}, another question remains: for which soils this information should be required? The obsolete legislation (IBAMA, 1996) defines some type soils that are not the same anymore. While the Brazilian Soil Society (SBCS) advance in research and frequently update the Brazilian soils maps (IBGE, 2019) and soils classification (Embrapa, 2018) there is still a huge issue in defining representative scenarios to ERA. Considering the size and diversity of the country, maybe the existence of regulatory zones as adopted in Europe could be an approach to start. In any case, pesticides regulation must use representative soils for agriculture, which is not the case in the actual Brazil scenario, since the type of soil required to derive the fate parameters (*gleisolo*) present several disadvantages to agriculture (Embrapa, 2019).

Some of ERA issues in Brazil were already discussed in a review of Niva et al. (2016). The researchers highlighted that while in North America and Europe, for example, a well-established legal framework requiring ecotoxicological studies does exist, these requirements are either not available at all or they exist but are not relevant in daily reality in Brazil. Authors still pointed out that in Brazil, the legal requirements for soil conservation move in a very slow pace as well as their implementation.

The data available in this thesis were generated in order to help in clarify part of these gaps and mainly could be an useful tool to help in establish, or start to establish, an ERA in

Brazil. Despite of the European models could not be specifically imitated, its provided information enough to perform a Brazilian adequate one.

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