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IS THE PREDICTION OF TOXICITY OF A TANK MIXTURE TO NON-TARGET SPECIES THROUGH CONCENTRATION ADDITION MODEL DEPENDENT ON THE TEST ORGANISM GROUP?

Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal orientada pelo Prof. José Paulo Filipe Afonso de Sousa e pelo Dr. Tiago Manuel Natal-da-Luz e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2021

Faculdade de Ciências e Tecnologia
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Abstract

The use of Plant Protection Products (PPPs) in agricultural fields to protect crops against weeds, pests and diseases in plants, have been an essential part of crop management over the years. The simultaneous occurrence of a large variety of harmful organisms in the same field has led farmers to adopt new ways of crop management such as mixing pesticides in a tank for application in order to reduce costs**.** Tank mixtures consist in mixing two or more chemicals (e.g. pesticides or fertilizers) in the tank of a sprayer equipment and applying them simultaneously in the fields. The mixture can be composed of PPPs with single or multiple active ingredients (a.i). Current Environmental Risk Assessment (ERA) at the European Union, ecotoxicological data are only required for pure a.i and for individual PPPs, with the combined risk of multiple pesticides being not considered in the pre-authorization process. However, the exposure of combined PPPs, usually with more than one a.i. in the mixture, can lead to adverse effects than can deviate from the additive toxicity of single pesticides (i.e. synergistic/antagonistic effects) which may pose a risk to non-target organisms. To predict the behavior between chemicals, models have been used, being the Concentration Addition model (CA) the most used for regulatory purposes. This model assumes that the components of the mixture have similar mode of action, with each component of the mixture being a diluent agent of the other(s), acting in an additive manner. Soil organisms responsible for key roles in the ecosystem, such as earthworms, collembolans and non-target higher plants, are constantly exposed to the application of pesticides mixtures with more than one a.i in the mixture. However, these organisms may have different sensibilities to the exposure of a pesticide mixture, since they represent different routes of exposure.

This study was developed with the aim of: 1) Assess the effect of a tank mixture composed of PPPs with different modes of action to non-target soil organisms from different ecological groups, representative of different routes of exposure and 2) Evaluate if the CA model is adequate to predict the mixture's toxicity independently of the test organism group. Based on these objectives, two working hypotheses were considered: 1) The tank mixture selected for the study is toxic for all non-target species used in the experiments even at concentrations lower than the respective recommended doses of each PPP that composes the tank mixture and 2) The Concentration Addition model is adequate to predict the toxicity of the pesticide mixture to the test organisms and the deviations to the CA model is independent on the organisms group.

To attain these purposes, in Chapter II it was assessed the single and combined effects of three PPPs with different modes of action, using the CA model, on non-target soil species. PPPs of pendimethalin as Podium® (herbicide), chlorantraniliprole as Coragen® (insecticide) and mancozeb+metalaxyl-M as Ridomil Gold Mz Pépite® (fungicide) were used. For this purpose, standardized laboratory tests were conducted using artificial soil and four non-target species representing different routes of exposure to PPPs: two plants (monocotyledon *Avena sativa* and eudicotyledon *Brassica rapa*) and two invertebrates (Arthropod *Folsomia candida* and Oligochaete *Eisenia andrei*). Phytotoxic response in emergence and dry weight to the plant species and mortality and reproduction to soil invertebrates were chosen as endpoints to assess the single and combined effects of PPPs. In individual tests, the PPP of chlorantraniliprole was toxic only to *F. candida*, while the PPP of mancozeb+metalaxyl-M was more toxic to *F. candida*, but also revealed toxicity to *E. andrei*. The PPP of pendimethalin was more toxic to *A. sativa*, followed by *E. andrei, F. candida* and *B. rapa*. The toxic doses for each PPP individually were always higher than the highest field doses of the respective products. The mixture of the three PPPs revealed additivity when tested in *A. sativa* and *B. rapa*. However, deviations from the conceptual model were translated into antagonism when tested in the *E. andrei* species and synergism to *F. candida* species. The different sensitivities of the species to the PPP mixture suggest that the reaction to the test mixture depends on the route of exposure of the test organism.

This study reinforces the need to better understand the toxicity associated with the use of combined PPPs and its risk to non-target organisms of different ecological groups, including the adequacy of the CA model to predict the toxic effects.

Key words: Tank mixtures, Non-target organisms, Ecotoxicology, CA model, PPPs

Resumo

O uso de Produtos de Proteção de Plantas (PPPs) em campos agrícolas para proteger as lavouras contra ervas daninhas, pragas e doenças nas plantas tem sido uma parte essencial do manejo da lavoura ao longo dos anos. A ocorrência simultânea de uma grande variedade de organismos prejudiciais em um mesmo campo, tem levado os agricultores a adotarem novas formas de manejo da cultura, como a mistura de produtos fitossaniários em um tanque para aplicação, a fim de reduzir custos. As misturas em tanques consistem em misturar dois ou mais produtos químicos (e.g. pesticidas ou fertilizantes) no tanque de um equipamento pulverizador e aplicá-los simultaneamente no campo. A mistura pode ser composta de PPPs com um ou vários ingredientes ativos (a.i). A atual Avaliação de Risco Ambiental (ERA) na União Europeia, os dados ecotoxicológicos são necessários apenas para a.i puro e para PPPs individuais, com o risco combinado de vários pesticidas não sendo considerado no processo de pré-autorização. No entanto, a exposição de PPPs combinados, geralmente com mais de um a.i. na mistura, pode levar a efeitos adversos que podem desviar da toxicidade aditiva de pesticidas individuais (i.e. efeitos sinérgicos/antagônicos), podendo representar um risco para organismos não-alvo. Para prever o comportamento entre PPPs, modelos são utilizados, sendo o modelo de adição de concentração (CA) o mais utilizado para fins regulatórios. Este modelo assume que os componentes da mistura têm modo de ação semelhante, sendo cada componente da mistura um agente diluente do outro, agindo de forma aditiva. Organismos do solo responsáveis por papéis-chave no ecossistema, como minhocas, colêmbolos e plantas superiores não-alvo, estão constantemente expostos à aplicação de misturas de pesticidas com mais de um a.i na mistura. No entanto, esses organismos podem ter diferentes sensibilidades à exposição de uma mistura de PPPs, uma vez que representam diferentes vias de exposição.

Este estudo foi desenvolvido com o objetivo de: 1) Avaliar o efeito de uma mistura em tanque composta por PPPs com diferentes modos de ação a organismos não-alvo de solo de diferentes grupos ecológicos, representativos de diferentes vias de exposição; 2) Avaliar se o modelo CA é adequado para prever a toxicidade da mistura, independentemente do grupo de organismos de teste. Com base nesses objetivos, foram consideradas duas hipóteses de trabalho: 1) A mistura do tanque selecionada para o estudo é tóxica para todas as espécies não-alvo utilizadas nos experimentos mesmo em concentrações inferiores às respectivas doses recomendadas de cada PPP que compõe a mistura do tanque e 2) O modelo de adição de concentração é adequado para prever a toxicidade da mistura de pesticidas para os organismos de teste e os desvios para o modelo de CA são independentes do grupo de organismos.

Para isso, no Capítulo II foram avaliados os efeitos individuais e em mistura de três PPPs com diferentes modos de ação, usando o modelo CA, em espécies de solo não-alvo. Foram utilizados PPPs de pendimetalina como Podium® (herbicida), clorantraniliprol como Coragen® (inseticida) e mancozeb+metalaxil-M como Ridomil Gold Mz Pépite® (fungicida). Para isso, foram realizados testes laboratoriais padronizados em solo artificial e quatro espécies não-alvo representando diferentes vias de exposição aos PPPs: duas plantas (monocotiledônea *Avena sativa* e eudicotiledônea *Brassica rapa*) e dois invertebrados (Artrópode *Folsomia candida* e Oligochaete *Eisenia andrei*). A resposta fitotóxica na emergência e peso seco para as espécies de plantas e mortalidade e reprodução para invertebrados do solo foram escolhidos como endpoints para avaliar os efeitos únicos e combinados de PPPs. Nos testes individuais, o PPP de clorantraniliprol foi tóxico apenas para *F. candida*, enquanto o PPP de mancozeb + metalaxil-M foi mais tóxico para *F. candida*, mas também revelou toxicidade para *E. andrei*. O PPP de pendimetalina foi mais tóxico para *A. sativa*, seguido por *E. andrei*, *F. candida* e *B. rapa*. As doses tóxicas para cada PPP individualmente foram sempre superiores às doses máximas de campo dos respectivos produtos. A mistura dos três PPPs revelou aditividade quando testada em *A. sativa* e *B. rapa*. No entanto, desvios do modelo foram traduzidos em antagonismo quando testados em *E. andrei* e sinergismo em *F. candida*. As diferentes sensibilidades das espécies à mistura de PPPs sugerem que a reação à mistura teste depende da via de exposição do organismo testado.

Este estudo reforça a necessidade de compreender melhor a toxicidade associada ao uso de PPPs combinados e seu risco para organismos não-alvo de diferentes grupos ecológicos, incluindo a adequação do modelo de CA para prever os efeitos tóxicos.

Palavras-chave: Misturas em tanque, Organismos não-alvo, Ecotoxicologia, Modelo CA, PPPs.

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of mancozeb + metalaxyl-m), using *Folsomia candida* as test species. *Number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

Figure 8. Visual effects of a pesticide mixture composed of Podium® (herbicide of pendimethalin), Coragen® (insecticide of chlorantraniliprole) and Ridomil Gold Mz Pépite® (fungicide of mancozeb + metalaxyl-m) in *A. sativa* and *B. rapa* at different concentrations (concentration in the bottom right corner of each picture) based on the sum of the Toxic Units (TU) of each pesticide in the mixture (A: *Avena sativa*; B: *Brassica rapa*).

Figure 9. Emergence (dots – mean; *n*=4) and dry weight/plant (bars – mean + standard deviation; *n*=4) observed in replicates of higher plant growth tests using *Avena sativa and Brassica rapa* as test species (graphs A and B) and number of surviving adults (dots – mean) and juveniles (bars – mean + standard deviation) observed in replicates of reproduction tests using *Eisenia andrei* and *Folsomia candida* as test species (graphs C and D) with artificial soil spiked with increasing concentrations of a pesticide mixture composed of Podium® (herbicide of pendimethalin), Coragen® (insecticide of chlorantraniliprole) and Ridomil Gold Mz Pépite® (fungicide of mancozeb + metalaxyl-m). Concentrations are expressed in toxic units. *Mean dry weight/plant or mean number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

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Abbreviations

a.i Active ingredient

CA Concentration addition

DAR Draft Assessment Report

EC Emulsifiable Concentrate

ECx Effect Concentration x. Concentration causing x% effect in a dose-response test

EFSA European Food Safety Authority

ERA Environmental Risk Assessment

EU European Union

FD Field Dose

IA Independent action

ISO International Organization for Standardization

K*oc* Adsorption Coefficient

K*ow* Partition Coefficient

LCx Lethal Concentration X. Concentration causing the death of X% of the exposed population

LOEC Lowest Observed Effect Concentration. The lowest tested concentration that is significantly different from the control

MoA Mode of action

NOEC No Observed Effect Concentration. The highest tested concentration to which no differences are observed when compared to the control

NOEC No-effect concentration

OECD Organization for Economic Cooperation and Development

OM Organic Matter

PPP Plant Protection Product

RMS Rapporteur Member state

SC Suspension Concentrate

TM Tank mixture

TU Toxic Unit

US United States

US EPA United States Environmental Protection Agency

WDG Water Dispersible Granule

WHC Water Holding Capacity

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Chapter I: General introduction

Pesticides can be defined as a product intended for preventing, repelling, controlling, or destroying any harmful organism or pest (US EPA, 2021a). Pesticides include Plant Protection Products (PPPs), which aim to protect crops in pre- and post-harvest losses against harmful organisms (e.g. pests, weed and diseases in plants) and biocides, which are used for non-agricultural purposes (e.g. control of mosquitoes, rats or mice in houses and streets) (EC, 2021). PPPs consist in at least one active substance which is responsible for the properties of the product and may also contain co-formulants such as wetting and anti-foaming agents (EC, 2021). Playing a major role in agricultural output, the use of PPPs has increased due to a rising level of global demand on agricultural production as the population grows over the years. Currently, the amount of pesticides used worldwide is estimated to be approximately 2 million tonnes, where 47.5% are herbicides, 29.5% are insecticides, 17.5% are fungicides and 5.5% are other pesticides and an increase up to 3.5 million tonnes was expected by the last year of 2020 (Sharma et al., 2019).

Over the last 60 years, the use of PPPs have been an essential part of crop management and with its correct use, primary benefits have been achieved, such as increase in agricultural production (Aktar et al., 2009). The pesticide use by farmers in several crops, along with other agricultural practices, such as the selection of plant varieties more adequate to local climatic conditions and the use of machinery, has led to a reduction in weeds, diseases and insect pests that can decrease the amount of harvestable yield and economic margin (Abubakar et al., 2019). Therefore, PPPs play an important role in food production to sustain the high demand by the population worldwide, performing a significant contribution to the increase crop yields and to provide access to a great supply of high-quality food (Tudi et al., 2021).

In agricultural fields it is common the simultaneous occurrence of a large variety of pests and diseases, in the same field, at the time and in the same area. Therefore, this set of problems has led farmers to adopt new ways of crop management such as mixing pesticides in a tank for application (Gazziero, 2015). Tank mixtures (TM) are associations between two or more chemicals (e.g. pesticides or fertilizers) in the container of the applicator equipment, which are performed shortly before applying in the field (Gandini et al., 2020). This technique is often adopted by farmers because when compared to the single product application, it may provide a reduction in costs, fuel saving and labor-hours, reduce soil compaction, less time of exposure of rural workers and an efficient management promoting adequate pest resistance (Tornisielo et al., 2013).

Despite the economic benefits of PPPs tank mixtures, the combination of two or more pesticides as a mixture could pose an addition risk to the environment, especially to non-target

species (Tang and Maggi, 2018). The environment is exposed simultaneously to a wide range of PPPs (alone or in mixtures) that contain numerous active substances with different environmental behavior, fate and persistence in different compartments (i.e. soil, biota and water) (Kienzler et al., 2014). This issue brings a concern because different types of interactions can occur among the components of the mixture, which can lead to enhanced or reduced effects, for instance synergism or antagonism (Hernández et al., 2017; Kienzler et al., 2014). Several studies in ecotoxicology have shown that different effects of combinations of pesticides and metals to non-target organisms such as aquatic and soil invertebrates and plants may be harmful and that the practice of tank mixing deserves more attention (e.g. Amorim et al., 2012; Chen et al., 2020; Santos, et al., 2011a, 2011b).

However, current Environmental Risk Assessment (ERA) of chemicals for PPPs regulatory purposes, does not take into account exposure of combined PPPs simultaneously, but mainly consider the assessment of individual substances (Kienzler et al., 2016). In other words, currently regulation for PPPs introduction on the European market only requires data for pure active ingredients and for individual PPPs (with one or more active ingredients as a mixture), but no ecotoxicological data for tank mixtures are required. In this sense, considering the unpredictability of interactions that can occur between pesticides in a tank mixture may result in synergisms or antagonisms, it is important to access the toxicity associated to the use of TMs and their effects on non-target organisms.

1. Regulatory assessment of pesticides mixtures

European Union (EU)

The introduction of a PPP on the EU market is regulated by EC Nº 1107/2009 which stipulates rules for the evaluation, authorization, placing on the market and control of PPPs and aims at ensuring a *"high level of protection of both human and animal health and the environment at the same time to safeguard the competitiveness of Community agriculture"* (EC, 2009). This regulation applies to both active ingredients (a.i.) and the preparations made with the approved a.i. (i.e. mixtures or solutions composed of two or more chemicals intended for use as PPPs or as an adjuvant) (EC, 2009). To approve an active ingredient at the European Union, a peer-review process should be performed, where a selected EU Rapporteur Member state (RMS) evaluate the risk assessment provided by the applicant in a Draft Assessment Report (DAR), which presents a broad range of data regarding environmental effects, and with the authorization of the active ingredient, a Member State can grant the use in a formulated product (Bopp et al., 2018). The authority responsible for this process in co-operation with EU Member States is the European Food Safety Authority (EFSA), which provides guidances in toxicology, ecotoxicology, fate and behavior (Panizzi et

al., 2017). The risk assessment for PPPs in the EU is presented by the applicant for non-target organisms, in accordance with the regulatory requirements for active substances and formulations described in EC No 283/13 (EC, 2013a) and EC 284/13 (EC, 2013b), respectively. These hazards and/or risk assessment requirements for active ingredients and formulated products on the European market are performed prospectively based on the properties of the individual constituents and are assessed individually. However, when several formulated products are used/applied (i.e. application of PPP mixture in the field), the combined risk is not assessed, with no effect data required in the pre-authorization process (Kienzler et al., 2016).

Over the past decade, concerns about the effects of chemical mixtures have been growing in terms of the scientific and legislative areas (Bopp et al., 2019). In 2012, the European Commission in its Communication on "Combined effects of chemicals – chemical mixtures" (EC, 2012), stated concerns about the current limitations of assessing compounds individually and identified priorities and knowledge gaps to ensure a better comprehension within the risk associated with chemical mixtures. It also stated that current EU legislation does not provide a comprehensive and integrated assessment of cumulative effects of different chemicals considering different routes of exposure and that there is no mechanism for a systematic and integrated assessment of mixture effects. However, different guidance documents and scientific progress has been achieved aiming to understand mixture effects, promoting the development of new models and on the overview of methodology and terminology to assess risk from combined exposures to multiple chemicals (Bopp et al., 2019; Kienzler et al., 2014, 2016; Meek et al., 2011; OECD, 2018; SCHER, SCENIHR, & SCCS, 2012; EFSA, 2018a). Regarding environmental risk, methodologies to deal with combined toxicity of pesticides on different non-target organisms such as in-soil organisms, terrestrial plants, bees, birds, mammals and aquatic species have been discussed in Scientific Opinions by EFSA (EFSA, 2009, 2012, 2013a, 2014a, 2017a). More recently, a joint Workshop "Advancing the Assessment of Chemical mixtures and their Risks for Human Health and the Environment", co-organized with research projects funded by the EU (i.e. Euromix and EU-ToxRisk), was held in 2018 with the aim to create a joint forum for researchers and policy-makers to identify gaps in RA and discuss legislative matters of chemical mixtures (Drakvik et al., 2020).

United States (US)

The US Environmental Protection Agency (US EPA) is the main authority responsible for the authorization and regulation of pesticides in the United States (US EPA, 2021b). EPA regulates pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), conducting the production, distribution, sale and use of pesticides used in the US (US EPA,

2021c). The first step to register a pesticide or active ingredient on US market, applicants must fulfill data required by FIFRA and EPA, regarding the pesticide's effect on both the environment and human health (US EPA, 2021c). In ecological risk assessments to non-target organisms, the EPA's Office of Pesticide Programs, follows the framework for Ecological Risk Assessment of 1992, that was last updated in 1998 into a Guideline (US EPA, 1998). However, as the ERA required by the European Union for pesticides' authorization, the EPA's current process for evaluating pesticides in ERA, mainly have relied on toxicity information from single active ingredients, not considering situations where products may be mixed prior to application (i.e. tank mixtures) (US EPA, 2019a).

Currently, exposure to pesticides mixtures is considered in many regulatory frameworks and guidelines in the US, but mostly focuses on human health assessments, conducting cumulative risk assessments of several groups of pesticides (US EPA, 2000, 2002, 2003, 2007, 2014, 2016; ATSDR, 2018). In 2016 and 2017, due to problems related to greater than additive effects (GTA) between active ingredients in an herbicide, EPA received petitions asking to require registrants to provide information on potential synergy for consideration in EPA's ecological risk assessments (US EPA, 2019b). Concerned about this problem, EPA has developed an interim process to review data for mixtures of pesticide active ingredients and the potential incorporation of that information into their ecological risk assessment (US EPA, 2019b). The objective of this document is to evaluate the utility of collecting and reviewing information regarding pesticides patents assertations of GTA effects for use in conducting risk assessments and to serve as guidance to registrants regarding related GTA patent claim submissions (US EPA, 2019a).

Brazil

In Brazil, pesticide's regulation and authorization are determined by the law Nº 7.802 from 11 Jul 1989, which lay out research, experimentation, registration, control and inspection of pesticides and their components (BRASIL, 1989). Law decrees Nº 4.704 from 4 Jan 2002 and Nº 5.981 from 6 Dez 2006 regulates the 1989's law (BRASIL, 2006). The registration and approval process of a pesticide in Brazil consist in a set of procedures that are developed within the scope of three Federal Government bodies: Ministério da Agricultura, Pecuária e Abastecimento (MAPA), responsible for evaluating agronomic issues; Ministério do Meio Ambiente (MMA) through Instituto Brasileiro do Meio Ambiente e Recursos Naturais (IBAMA), which is responsible for assessing environmental issues; and Ministério da Saúde (MS) through Agência Nacional de Vigilância Sanitária (Anvisa), responsible for evaluating the effects on human health (BRASIL, 2012). Not differing from US and EU pesticide's regulation, Brazil also requires ecotoxicological data on non-target organisms (e.g. plants, aquatic and

soil organisms and insects) in their ERA from the applicant during the registration process, but no data is required on the effects of mixing different pesticides with same or different MoA (mode of action) as tank mixtures. Currently, pesticide tank mixing is a reality in Brazilian fields and a fundamental practice for phytosanitary management. Research by Gazziero (2015) showed that, among 17 states, 97% of the surveyed farmers used tank mixes and that in 95% of these, between two or five products were used. Even though no data on the possible effects of mixing pesticides on non-target organisms is required during the registration process, the practice of pesticide tank mixing is regulated and permitted through an agronomic prescription in a Normative Instruction Nº. 40 of 11 Oct 2018, from MAPA (BRASIL, 2018). However, no data on how to proceed with the tank mixing is available or required, being the responsibility in charge of an agronomist engineer. Farmers and technicians still lack information regarding the general preparation procedures, the sequence of addition of products and the risk of interactions between the components. More recently, a technical manual on pesticide tank mixture was developed by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) to guide farmers and technicians on how to better manage the mixtures (Gazziero et al., 2021), but no information regarding the possible interactions and its effects on the environment is discussed.

2. Concepts for mixture risk assessment

2.1 Mixtures in regulatory toxicology

To assess the toxicity of mixtures, two approaches can be followed: whole-mixture and component-based approach (Bopp et al., 2018). The whole-mixture approach can be assessed by testing the whole product/mixture itself, as the group of components in the mixture were a single unit (OECD, 2018) and is usually applied to environmental mixtures (e.g. pollutants in river water), where most of times, the composition is unknown (Kienzler et al., 2014). This approach considers interactions among components, but it does not identify the chemicals responsible for interactions and it does not provide information on toxicity of individual mixture components, therefore it is not recommended for use as a general approach (Kienzler et al, 2014; SCHER et al., 2012). The component-based approach is generally used when the individual components of the mixture are known individually, and the combined effect of these components is estimated mathematically using specific predictive models for chemical mixtures (Bopp et al., 2018). To apply a component-based approach, it is required to obtain the relative proportion of the components and their contribution to the overall toxicity of the mixture (OECD, 2018). This approach depends mainly on considerations whether the mixture components act by the same mode of action (MoA) or independently (Kienzler et al., 2016). Also, for its optimal use, it is dependent on the knowledge of the composition of the mixture

and the corresponding MoA of each component (SCHER, SCENIHR, and SCCS, 2012). Within component-based approaches, two predictive additivity models are usually considered depending on the MoA of the components in the mixture: concentration addition (CA), applied to chemicals with a similar MoA; independent action (IA) or response addition (RA) applied to chemicals with a dissimilar MoA (Bopp et al., 2018). The difference between similar and dissimilar action was first introduced by Bliss (1939) and by Plackett and Hewlett (1952) based on statistical principles, and then was extensively accepted for the interpretation of mixture effects (Hernández et al., 2017). Both models are based on the idea that the expected effect of a mixture can be predicted based on the sum of the effects of the mixture's individual components (i.e. additivity) (Rodea-Palomares et al., 2015) and they have been suggested as default approaches in regulatory risk assessments of chemical mixtures (Bopp et al. 2018; Kienzler et al. 2014; De Zwart and Posthuma, 2005).

2.2 Mixture assessment models

Concentration addition (CA)

This model assumes that the effects can be estimated by summing the concentrations of each component of the mixture (assuming similarly acting substances) scaled for their potencies, considering no chemical interaction between the chemical components (OECD, 2018). The CA model implies that the joint effects of the components in the mixture could be expressed as a dilution of each other (Santos et al., 2010) and the contribution of the individual components of the mixture to the overall effect can be added in the form of toxic units (TU), expressed as concentrations (De Zwart and Posthuma, 2005). TU can be defined as the ratio between exposure concentration of each component in the mixture (c) and its effective concentration (EC_x), usually the EC_{50} or LC_{50} ($TU = c/EC_{50}$) (Sprague, 1971). This effect concentration in which TU is based can be selected arbitrarily, however, the EC_{50} is often used because it is the effect concentration that can be estimated with less variability (Jonker et al., 2005). In the CA model, the toxic potential of a mixture is described as the sum of the TUs of the individual chemicals, through the following equation:

$$\sum TU = \sum (ci/EC50, i) \tag{1.1}$$

where, the quotient $c_i / EC_{50,i}$ is the toxic unit of the component i, c_i is the concentration of component i in the mixture (i= 1, 2, …, N), $EC_{50,i}$ is the concentration of component i of the mixture that produces and adverse effect of 50% when applied alone (Natal-da-Luz, 2011b). The concentration additivity concept implies that the expected standard response is expected to occur at TU = 1 (De Zwart and Posthuma, 2005).

The available literature supports the application of CA model as a first approach and it is often used as the default approach to start from in several international recommendations and frameworks, independent of similarity or dissimilarity of the mode of action of the components (Bopp et al. 2018). This model is used to evaluate if a chemical mixture is more or less toxic than the toxicity of the individual components and is not limited to toxicants having similar modes of action (Jonker et al., 2005). The CA model are the most frequently applied for many reasons, but mainly because it is considered to be slightly more conservative than IA model and because CA is a less data demanding tool, requiring only a toxic value from each component in the mixture (Panizzi et al., 2017).

Independent action (response addition) (IA)

The IA model is used for chemicals with dissimilar MoA (independent action between components) and that the sensitives to different toxicants are uncorrelated (OECD, 2018). This model assumes that one chemical does not influence the toxicity of one another and that the combined effect can be calculated though the statistical concept of independent random events (OECD, 2018; Kienzler et al., 2014). It is described as:

$$ECmix = 1 - \prod_{i=1}^{n}(1 - ECi) \quad (1.2)$$

where, EC_{mix} is the effect of the mixture of n compounds and EC_i is the effect of substance *i* when applied alone (Panizzi et al., 2017).

A relevant difference between IA and CA model is that, under CA, mixture components below their individual no observed effect concentration (NOEC) may contribute to the total effect of the mixture, while for IA, since it is based on the effects (response) and not the dose, a mixture component used in a concentration below its NOEC will not contribute to the total effects of the mixture (Rodea-Palomares et al., 2015).

Interaction between chemicals of a mixture: synergism and antagonism

When performing an environmental risk assessment, it is important to consider the occurrence of potential interactions between chemicals in a mixture (Panizzi et al., 2017). These interactions can occur when some or all components of a mixture influence each other's toxicity and the joint effects may deviate from the additive predictions (Hernández et al., 2017). The combined toxicity for multiple pesticides can be categorized as less than additive (antagonistic) or greater than additive (synergistic) and they may vary according to the route(s), duration of exposure, biological target and relative dose (Kienzler et al., 2014). Antagonism

occurs when chemicals interact producing an effect less than predicted by the conceptual model, resulting in a decreased toxicity (Hernández et al., 2017), while synergism occurs when the interaction of the components of a mixture result in effects greater than the estimated for additivity, exerting a larger toxicity than expected (Cedergreen, 2014). When the combined effect of chemicals in a mixture is assumed to deviate from CA and/or IA model, it can be concluded that the models were not adequate to predict the toxicity of the mixture, based on their mathematical assumptions, that is the combined toxicity is not the sum of the individual toxicities of the components in the mixture for CA and the response between chemicals in a mixture are not entirely independent in IA model (Spurgeon et al., 2010)

In the risk assessment to non-target organisms, the result being a decreased toxicity, antagonisms does not represent an issue and the use of additive model could constitute a worst-case (Kienzler et al., 2014; Rodea-Palomares et al., 2015). On the other hand, synergism represents an issue of concern for the public and regulatory authorities, particularly in cases where the sum of the individual effects of each chemical is more toxic than the NOEC of the mixture (Cedergreen 2014; Rodea-Palomares et al., 2015).

3. Pesticide mixture

As mentioned before, mixing PPPs in the container of the applicator equipment (i.e. tank mixing), is a common practice performed by farmers in cultivated areas with the aim to reduce operational costs (Gandini et al., 2020). This combined application of multiple PPPs is frequent in most crops and usually, two to five products with different modes of action (e.g. insecticides, fungicides, herbicides) are mixed in the tank to be further applied in the field (Gazziero, 2015). However, this practice has gained importance in ecotoxicology because interactions between compounds in a mixture may have a greater negative impact to non-target organisms when compared with the individual components (i.e. synergisms) (Hernández et al., 2017).

In a systematic review of mixture toxicity studies, Cedergreen et al. (2014) observed synergisms in pesticides were present in mixtures including cholinesterase inhibitors (i.e. organophosphates and carbamates) or azole fungicides (i.e. ergosterol biosynthesis inhibitors), triazine herbicides (i.e. photosystem II inhibitors) and pyrethroid insecticides (i.e. disrupting sodium channel in insect's nervous system). However, to date most of the available studies in mixture ecotoxicology with soil ecosystems still have only investigated the effects of these types of pesticides, such as dimethoate, spirodiclofen, glyphosate, lindane, acetochlor, chlorpyrifos and others (e.g. Santos et al., 2011a; Santos et al., 2011b; Frampton et al., 2006; Loureiro et al., 2009; Yang et al., 2017). Moreover, most of the studies regarding the effects of pesticides mixtures are in aquatic ecotoxicology (Deneer, 2000; Nørgaard and Cedergreen, 2010) and only few studies with non-target soil organisms have investigated mixtures of PPPs,

being most of these studies with binary mixtures (Santos et al., 2010; Amorim et al., 2012). Considering that mixing more than two PPPs is a reality in the fields (Gazziero, 2015) and the scarce knowledge regarding the effects on non-target terrestrial organisms, there is a need to better understand the toxicity associated with pesticides mixtures, especially with more than two PPPs in the TM.

In many agricultural crops, dinitroaniline herbicides and diamide insecticides such as Pendimethalin and Chlorantraniliprole respectively, along with contact and systemic fungicides, such as Mancozeb and Metalaxyl-m, respectively, have been widely used to control a broad specter of pests. However, the toxic effects of these pesticides individually on non-target soil organisms is still scarce and their effects as a mixture have been not evaluated yet.

3.1. Podium® (a.i pendimethalin)

Podium® (a.i pendimethalin, 330 g a.i/L) is a selective emulsifiable concentrate (EC) herbicide developed by Sapec Agro Business, commonly used to control broadleaf and grassy weeds on a variety of agricultural crops such as maize, carrots, tomatoes, potatoes, onions, garlic, and tobacco (Ascenza, 2021). It can be applied through pre-plant incorporation, pre-emergence, pre-transplanting, or early post-emergence (Ascenza, 2021). Its active ingredient, Pendimethalin, belongs to the dinitroaniline family of herbicides whose mechanism of action is based on mitosis inhibition, where it binds to the major microtubule protein tubulin (Hatzinikolaou et al., 2004). The herbicide-tubulin complex inhibits polymerization of microtubules, leading to a loss of microtubule structure and function, resulting in the absence of the spindle apparatus, preventing the alignment and separation of chromosomes during mitosis (WSSA, 2000). As a result, visual phytotoxic effects such as swelling of root tips in seedlings are observed, since cells in this region neither divide or elongate (WSSA, 2000).

Recommended doses for application of the commercial formulation vary from 4 to 6 L/ha (corresponding to 1.76 to 2.64 mg a.i/kg) (Ascenza, 2021). When applied to crops, residues may reach the soil and water through improper application, drift, leaching or runoff (Strandberg and Scott-Fordsmand, 2004). In soil, pendimethalin adsorbs rapidly and strongly due to its high potential for hydrogen bonding and its persistence is influenced by soil temperature, cultivation practices, soil type and moisture conditions (Sondhia, 2012). In general, the half-live of pendimethalin on soil range from a few days to many months depending on whether conditions and soil type and pH, with residues remaining in soil after 300 days after the initial application (Strandberg and Scott-Fordsmand, 2004). Pendimethalin has low water solubility (0.33 mg/L at 20ºC) (PubChem, 2021a), is immobile in soil and is characterized as lipophilic, with a Log K_{ow} of 5.18, which accounts to strong soil adsorption,

being moderate to high soil persistent (US EPA, 1997). The US Environmental Protection Agency has classified this herbicide as persistent-bioaccumulative toxic (US EPA, 1997).

Data collected from literature on the exposure and risk to humans and the environment, shows that pendimethalin has low acute toxicity, but causes thyroid follicular cell adenomas in rats and it is classified as a possible human carcinogenic (group C) (US EPA, 1997). Also, pendimethalin is found to be toxic to fish (Singh and Singh, 2014; Nassar et al., 2021; Gupta and Verma, 2020) and mammals (Dimitrov et al., 2006).

Regarding the effects of pendimethalin in terrestrial ecosystem, the highest concentrations of this herbicide occur in agricultural soil immediately after application (Strandberg and Scott-Fordsmand, 2004) and its dissipation depends on a range of soil an environmental conditions such as soil texture, moisture and temperature (Belden et al., 2005). Also, when herbicides are applied in combination to other pesticides (e.g. insecticides and fungicides) to the crops, interactions between pesticides are possible in soil (Fogg et al., 2003) and the persistence of the compounds can be affected (Swarcewicz and Gregorczyk 2012), and might affect terrestrial ecosystem species (Swarcewicz et al., 2003).

When applied alone, data collected from literature shows that pendimethalin has toxic effects on non-target terrestrial organisms. Belden et al. (2005) evaluated the toxicity of pendimethalin as pure active ingredient, in four non-target plant species (*Andropogon gerardii*, *Sorghastrum nutans*, *Panicum virgatum* and *Latuca sativa*) and three soil invertebrate species (*Folsomia candida, Eisenia fetida* and *Armadillidium sp.*) using natural soils. At the concentrations 10 mg a.i/kg and below, growth was inhibited for all plant species and germination rates varied among species and tested concentrations, being *S. nutans* the most sensitive plant. For soil invertebrates, *F. candida* significantly decreased reproduction (i.e. number of juveniles) at 90 mg a.i/kg and no mortality was recorded in any treatment. *E. andrei* had significantly decreased biomass of surviving adults, even at the lowest treatment of 10 mg a.i/kg and mortality was observed from 40 mg a.i/kg to 160 mg a.i/kg, with a LC_{50} of 113 mg a.i/kg. For *Armadillidium sp.* a LC_{50} of >200 mg/kg was estimated. Other studies have indicated that pendimethalin can provoke adverse effects, even in the recommended doses of application, on other non-target organisms (Strandberg and Scott-Fordsmand, 2004) such as beneficial ground beetles (Vommaro et al., 2021) and wasps (Oliver et al., 2009).

3.2. Coragen® (a.i chlorantraniliprole)

Coragen® (a.i. chlorantraniliprole, 200 g a.i/L) is a suspension concentrate (SC) of first-generation anthranilic diamide insecticide developed by Dupont, that acts against Lepidopteran and Coleopteran pests (Liu et al., 2018). Its active ingredient, chlorantraniliprole, acts both on larvae and adults by ingestion and contact routes of entry, acting as a ryanodine

receptor (Bentley et al., 2010). After the ingestion or contact with target insects, chlorantraniliprole binds to ryanodine receptors causing a depletion of internal calcium stores in the sarcoendoplasmic reticulum, impairing the muscle contraction, resulting in feeding cessation, lethargy and partial paralysis that leads to death of the insect (Lavtižar et al., 2016). This active ingredient is considered safe to mammals due to its high selectivity for insect over mammalian ryanodine receptors (Cordova et al., 2006). Apart from mammals, chlorantraniliprole is also considered safe to birds and fishes, but it is toxic to aquatic invertebrates (Maloney et al., 2020; US EPA, 2008).

Recommended doses for application of the commercial formulation vary from 50 to 200 mL/ha (corresponding to 0.013 to 0.053 mg a.i/kg of soil) depending on the crop (Bayer, 2021). According to USEPA (2008) the half-life of Chlorantraniliprole in the environment (at 20˚- 25˚C) varies between 30 days on foliage application and 1130 days on bare ground plot application. Also, chlorantraniliprole is poorly soluble in water (0.9-1 mg/L at 20°C, pH 7) and its coefficient of partition (K_{ow}) is 2.76 (PubChem, 2021b). Its persistence, mobility (K_{oc}=329 L/kg) and possibility of accumulation in soil highlight the importance of investigating its toxic effects to non-target soil organisms (Lavtižar et al., 2016; US EPA, 2008).

Even though chlorantraniliprole is a new generation insecticide and being on the market for over a decade, data on its possible effects on non-target terrestrial organisms are still deficient. For non-target soil organisms, studies have shown different sensitivities among species. Lavtižar et al. (2016) reported the effects of chlorantraniliprole in springtails (*Folsomia candida*), isopods (*Porcellio scaber*), enchytraeids (*Enchytraeus crypticus*) and oribatid mites (*Oppia nitens*), showing that in sublethal toxicity tests with Lufa 2.2 soil, chronic exposure to this active ingredient in concentrations up to 1000 mg/kg dw did not affect the survival and reproduction of *E. crypticus* and *O. nitens*, nor the survival, body weight and consumption of *P. scaber*. However, for *F. candida*, high toxicity was observed in survival and reproduction with an EC_{50} for reproduction of 0.14 mg/kg dw. Similar results showing high toxicity to *F. candida* were reported by USEPA (2008) and EFSA (2008) with the estimation of EC_{50} values for reproduction of 0.48 mg/kg and 0.85 mg/kg, respectively. For earthworms, Liu et al. (2018) investigated the ecotoxicity of chlorantraniliprole to *Eisenia fetida* using several biomarkers and observed that growth and reproduction are significantly inhibited above 5 mg/kg dw of pure active ingredient, suggesting that this insecticide may have a potential high risk for earthworms. Regarding the effects of chlorantraniliprole to plants, to date, only one study reported the effects of this insecticide to maize. Kilic et al. (2015) found phytotoxic effects on seed germination, stomatal responses in leaves, contents of proline and degradation of photosynthetic pigments of *Zea mays* L. *saccharate* Sturt. Despite being toxic for some non-target terrestrial species, data from literature lead to suppose that chlorantraniliprole is less

toxic to soil microbes and some soil enzymatic activities at low doses (Sahu et al., 2019; Wu et al., 2018), and to parasitoid wasp (Brugger et al., 2010) and bees (Dinter et al., 2010).

3.3. Ridomil Gold Mz Pépite® (a.i maconzeb + metalaxyl-m)

Ridomil Gold Mz Pépite® is a water dispersible granule (WG) systemic and contact fungicide (a.i 64 % (p/p) mancozeb + 4% (p/p) metalaxyl-m) developed by Syngenta and used to prevent types of mildew and black rot mainly in vineyards, being also applied in other cultures such as potatoes, tomatoes, onions, cucumber, melon, and lettuce (Syngenta Portugal, 2021). After applied in plants, it presents a preventive, curative and anti-sporulate effect. With the combination of two active ingredients, mancozeb and metalaxyl-m, the first acts by contact on the fungus, right at the beginning of the germination phase of downy mildew spores. The second is systemic and penetrates plant tissues, circulate in the sap, and protects all parts of the plant, including new growths (Syngenta Portugal, 2021). Recommended doses for application vary from 2.25 kg/ha to 2.5 kg/ha (corresponding to 3 to 3.33 mg formulation/kg of soil) depending on the crop (Syngenta Portugal, 2021).

Mancozeb is a non-systemic fungicide widely used as contact fungicide which belongs to the dithiocarbamate group and has zinc and manganese in its composition (Gullino et al., 2010). Its biological activity has direct effects upon biochemical processes within the fungus, resulting in inhibition of spore germination. This active ingredient displays a multi-site protective effect following application onto the target plant, remaining on the leaf surface and does not penetrate through the cuticle. Used mainly through foliar applications, its mode of action demonstrated activity against a wide range of fungi including ascomycetes, oomycetes and basidiomycetes, controlling diseases such as black rot and mildew in several crops (Gullino et al. 2010). Mancozeb is expected to have low mobility in soil, with an average K_{oc} value of 1000 and the half-life in soil may vary between 1 to 3 days depending on the type of soil (PubChem, 2021c).

Metalaxyl-m is an important phenylamide systemic fungicide applied worldwide to protect crops against pathogenic oocmycetes, such as *Phytophthora* spp. and *Phythium* spp., which causes downy mildews, stem, root and fruit rot and damping-off in several crops (Baker et al., 2010). Metalaxyl is a racemic mixture of two enantiomers (-*S* and -*R* enantiomer). The R-enantiomer is mainly metalaxyl-m and currently the traditional metalaxyl (*rac*-metalaxyl) has been replaced by metalaxyl-m, which has higher efficiency and it is used at a lower dose (Baker et al., 2010; Liu et al., 2014). In plant, it is taken up by roots, leaves, green stems and shoot and transported acropetally, restraining spore formation and inhibiting mycelial growth by selectively disturbing the fungal ribosomal RNA synthesis (Zhang and Zhou, 2019). Its degradation in the environment may vary among the types of soil with a half-life of 0.27 to 38

days, being considered easily degraded within a relatively short time (Baker et al., 2010; He et al., 2021).

No ecotoxicological data on the effects of the formulated product Ridomil Gold Mz Pepíte® to non-target terrestrial organisms could be found to date in the available literature. Therefore, the only source of data available is the Rapporteur Assessment Report (RAR) of the commercial formulation for ecotoxicology (EFSA, 2013b). In accordance with this document, this formulation is safe to bees, with oral LD_{50} > 613 µg Ridomil Gold/bee (*Apis mellifera*, 48h), earthworms (*E. fetida*), with acute toxicity 14-day LC_{50} of > 1000 mg Ridomil Gold/kg of soil and sublethal toxicity 56-day EC_{50} of > 39.06 mg Ridomil Gold/kg of soil, springtails (*F. candida*) with a reproduction EC_{50} of 231 mg Ridomil Gold/kg of soil. For terrestrial plants (*Brassica napus, Avena fatua, Beta vulgaris, Zea mays, Glycine max* and *Allium cepa*) an ER_{50} of > 4500 g Ridomil Gold/ha for seedling emergence and vegetative vigor of the tested plant species was also reported (EFSA, 2013b). Regarding the effects of the active ingredients alone, data on non-target terrestrial organisms are also scarce. On the Rapporteur Assessment Report (RAR) of mancozeb (EFSA, 2017b) data shows that the commercial formulation Dithane (84.6% w/w) is of low toxicity at field doses for earthworms in acute toxicity test (LC_{50} of >190 kg a.i/ha), however, for sublethal test, reproduction was significantly affected, with a NOEC of 20 mg a.i/kg. For springtails, when tested as pure a.i, adults' mortality of *F. candida* showed an increase with increasing mancozeb concentration, with a NOEC for mortality of 18.8 mg a.i/kg and EC_{50} and LOEC for reproduction of 20.1 mg a.i/kg and 17.8 mg a.i/kg, respectively. In higher plants toxicity's tests, the commercial formulation Tridex 75 DG (76,7% w/w), showed no significant difference in fresh and dry weight and no mortality in onion, tomato, soybean, oilseed rape, carrot and cabbage. For metalaxyl-m, on the Rapporteur Assessment Report (RAR) (EFSA, 2013b), toxicity data on the pure a.i was found to earthworms and springtails. In acute toxicity test for *E. andrei*, a LC_{50} of 830 mg a.i/kg was obtained and for sub lethal tests, the EC_{50} for reproduction was >75 mg a.i/kg and NOEC of 75 mg a.i/kg. For springtails, the toxicity data found was NOEC of 125 mg a.i/kg and LC50 and EC_{50} of >500 mg ai/kg.

In addition, studies from other authors have shown that mancozeb is found to be toxic to *F. candida* and *Enchytraeus crypticus* (Carniel et al., 2019) and to *Allium cepa* (Fatma et al., 2018), but harmless to *E. andrei* (Carniel et al., 2019). For metalaxyl-m, toxic effects were observed in aquatic organisms (Yao et al., 2009) and may cause cytotoxic and genotoxic effects on earthworms (Liu et al., 2014; Zhang and Zhou, 2019).

4. The importance of pesticide mixture risk assessment on non-target terrestrial organisms

The soil environment is considered one of the most complex and diverse ecosystems (Cardoso and Nogueira, 2016), which provides support for soil organism community, including plants and micro and macro-organisms that contribute to a range of ecosystem services (Barrios, 2007). These ecosystem services are transformations promoted by plants and other soil organisms that are directly or indirectly responsible for many biochemical and biological processes that, in a certain way, contribute to human well-being (Cardoso and Nogueira, 2016). Soil invertebrates play important roles in the ecosystem functioning, namely in the regulation of microbial activity, soil structuring and decomposition, incorporation, and distribution of organic matter (OM) along soil profiles. Also, their influence on the mineralization of nutrients of soil OM is a fundamental recycling process responsible for a great portion of nutrients required by plants in agricultural and forestry systems (Cardoso and Nogueira, 2016; Stanley and Preetha, 2016). Along with soil invertebrates, plants are essential in nutrient cycling and soil sediment stabilization, being primary producers and recognized as the foundation of terrestrial ecosystems (EFSA, 2014b). In addition, they are useful energy for almost all other life forms and provide food, shelter and nesting habitats for organisms such as invertebrates, fish, birds and mammals (Wang and Freemark, 1995). However, in modern agriculture systems, soil organisms and dynamic soil processes are constantly exposed to pesticides (alone or in tank mixtures) through direct application in soil and the impacts of these pollutants can have major effects on terrestrial ecosystems, ranging from changing the availability of organic matter and soil pH to reducing a species population, affecting many trophic levels (Edwards, 2004).

In ecotoxicology, some studies have shown the effects of different pesticides mixtures in soil organisms. For instance, Santos et al. (2011a) reported the effect of individual and binary combination exposure of spirodiclofen (acaricide) and dimethoate (insecticide) tested as commercial formulations Envidor® and Agror®, respectively, on earthworms (*Eisenia andrei*) and turnip seeds (*Brassica rapa*) in a microcosm-based experiments, using the concept of independent action (IA). For *B. rapa,* there were no significant differences in the fresh weight and length after the exposure to the pesticides in any of the treatments. However, a decrease in approximately 50% in plant length and fresh weight was observed at the highest concentration tested of diomethoate, as well as at 0.3 mg sprirodiclofen/kg of soil. In the binary mixture experiment, no effects on both endpoints were found. However, an antagonistic effect on shoot length and fresh weight of *B. rapa* at all concentrations tested were observed, revealing that the effects predicted from single exposures were much higher than observed upon combined exposure. The exposure of earthworms to the two pesticides caused a

decrease in their weight, but it was not statistically significant. In the binary mixture at field dose (FD) and 5 times de FD, the number of earthworms in the less contaminated soil was lower than expected by the IA model, suggesting that an antagonism has therefore occurred. However, in the mixture tested with 10 times the FD, the observed value of earthworms found in the less contaminated soil (the bottom layer of the chamber test) was higher than the predicted by the IA model, which suggests a synergism when both pesticides are applied in mixture. A previous work from the same authors (Santos et al., 2010), reported different effects of three binary combinations of dimethoate, glyphosate and spirodiclofen (insecticide, herbicide and acaricide, respectively) as commercial formulations (Roundup®, Agror® and Envidor®, respectively), on the avoidance behavior of the terrestrial isopod *Porcellionides pruinosus* and on the reproduction of *F. candida*, using the concentration addition (CA) and independent action (IA) models to predict the toxicity. The results of the mixture exposure to both species differentiated according to the binary mixture and the model used. For *P. pruinosus*, when dimethoate and spirodiclofen were applied together, antagonism was obtained from the two reference models. The dimethoate and glyphosate exposure experiment, an antagonistic deviation from the CA model was observed, but no deviations from the IA model were obtained. The glyphosate and spirodiclofen mixture showed additive effect by the CA model and a dose level deviation from the IA model, with synergism at low doses and antagonism with increasing doses of the two pesticides. For *F. candida*, antagonism was observed by both models in the combination of glyphosate and spirodiclofen and for dimethoate and glyphosate, however, additivity effect when fitted to both models was observed in the binary combination of dimethoate and spirodiclofen. In Amorim et al. (2012), individual and mixture toxicity of atrazine, dimethoate, lindane, zinc and cadmium, tested was pure substances, were studied for *F. candida* in LUFA 2.2 soil, assessing its survival and reproduction using CA and IA models to address the toxic effects in the mixtures. Results showed differences in the response of *F. candida* to the different mixtures in the different endpoints, with synergism being frequently observed upon exposure to the pesticide mixtures of lindane and atrazine, and dimethoate and lindane, or when only one of the components of the mixture was a pesticide (i.e. Cd and dimethoate, for reproduction). Additionally, the authors combined literature reviews from previous papers with their results and concluded that different invertebrate species (*F. candida, Enchytraeus albidus* and *Porcellionides pruinosus*), may respond differently to the same chemical mixtures, highlighting the importance of using different organisms in ecological risk assessment of chemical mixtures. Loureiro et al., (2009), Yang et al., (2017), Wang et al., (2015) and Chen et al, (2018) also reported the effects of pesticide mixtures in soil organisms.

The results obtained in these studies shows that different types of interactions can occur when combining different types of pesticides and depending on the mixture and the

organism (as they represent different routes of exposure), interactions between components may potentiate the toxic effect (i.e. synergistic effect), highlighting the importance of risk assessment on pesticides mixture regarding non-target terrestrial organisms.

5. Ecotoxicology tests and tested species

Ecotoxicology is the scientific area that studies the effects of potentially toxic pollutants (e.g. pesticides) on ecosystems and on non-target species (Hoffman et al., 2003) by assessing the effects on single species of representative organisms and, therefore, trying to establish safe levels for populations and communities (van Gestel, 2012). The study in soil ecotoxicology area began in the middle of the 60s, but it was only in the 80s forward that the area gained more attention (van Gestel, 2012), with the development of the first toxicity test with soil invertebrates standardized by OECD that focused on acute toxicity responses (i.e. survival) of earthworms (OECD, 1984). Over the years, protocols and new methods for ecotoxicological testing with different species such as collembolans, enchytraeids, mites, higher plants, earthworms and others, testing different endpoints (e.g. reproduction, avoidance, biomass etc.) were developed, providing ecotoxicological information required by regulatory authorities prior to the sale of PPPs. The use of standard soil species as bio-indicators to assess the impacts of contaminants in terrestrial ecosystem are an important tool for ERA because they have a high sensitivity and a fast response to contaminants concentrations in the soil. Therefore, assessing the harmful impacts of soil organisms on terrestrial ecosystems may provide early warning of potential threats (Cardoso and Nogueira, 2016). van Gestel (2012) described in his review about the state of the art of soil ecotoxicology that ERA can be divided in two distinct approaches: diagnosis and prognosis. In a diagnosis or retrospective risk assessment the aim is to assess the risk of a soil that is already contaminated with a pollutant (e.g. analyzing samples from a contaminated natural soil with PPPs) and making decisions/actions to remediate and reduce the ecological risk. The prognosis or prospective approach relies on a tired process that aims to assess the possible risks of a contaminant (e.g. PPPs) may have on the environment, regulating their use in the field and providing safe levels for the ecosystem and non-target species. In these two approaches, laboratory and/or field ecotoxicological tests based on dose-response relationship of the contaminant (e.g. PPP), are performed in order to obtain effect data based on endpoints such as exposure time (acute or chronic toxicity), observed effect (e.g. reproduction, mortality, biomass loss, etc.) or effective response (lethal and sublethal) (Cardoso and Nogueira, 2016). This study focused on a prospective approach to evaluate the single and mixture toxicity of a PPP tank mixture to non-target terrestrial species.

The process of a retrospective risk assessment begins with low tier tests (i.e. single species laboratory tests performed under controlled conditions) based on recommended protocols with standard species. Lower tiers tests are considered more conservative, since toxicity are usually higher in the field (EFSA, 2017a). After performing these tests, if the analyzed effect and exposure data found to be unacceptable for risk assessment, more complex and realistic evaluations of the contaminants are performed, namely higher tests (i.e. semi-field or field tests) (EFSA, 2017a). The toxicity data obtained based on the dose-response relationship for the selected endpoint are expressed in parameters such as EC_{10} and EC_{50} (concentrations causing 10% and 50% reduction, respectively in a measured endpoint (e.g. seedling emergence or biomass), LC_{10} and LC_{50} (concentrations killing 10% and 50%, respectively of the exposed organisms) and NOEC and LOEC (no-observable and lowest-observable effect concentration, respectively) (van Gestel, 2012). With these results, thresholds or safe levels of the PPP application or concentration in soil can be established, assessing the potential risk in the environment (Chen et al., 2013).

The selection of organisms to be used in toxicity tests are based on their importance in a community or ecosystem that needs protection (van Gestel et al., 2018). According to van Gestel et al. (1997), it is important to select species with different function and life traits in a trophic chain that represents different taxonomic groups and routes of exposure. Also, practical considerations should be taken into account, such as ease off reproduction under controlled conditions, standardization and fast life cycles (van Gestel et al., 2018). The main methods to evaluate toxicity in non-target soil organisms are the laboratory assays standardized by the norms of the Organization for Economic Cooperation and Development (OECD) and International Organization for Standardization (ISO). These guidelines describe methods used to determine acute and chronic toxicity of chemicals in collembolans, mites, earthworms, higher plants, insects, mollusks, enchytraeids and other organisms used to assess the risk in soil contaminants. Acute toxicity tests are used to assess effects (usually mortality) resulting from a short exposure period, while chronic toxicity tests are used to measure the sublethal effects (such as changes in reproduction and growth) of potentially toxic substances on organisms for a longer exposure period (Cardoso and Nogueira, 2016). In addition, avoidance behavioral tests are also described in guidelines for earthworms and springtails (ISO, 2011, 2008), with the aim to assess the avoidance responses of these organisms to a contaminated soil in a short period, being an endpoint of important ecological relevance that have been studied recently with other soil species (Niemeyer et al., 2018).

Terrestrial plants: *Avena sativa* L. (Poaceae) *and Brassica rapa* (Brassicaceae)

Non-target terrestrial plants can be defined as those growing outside the target area and those growing within the fields that are not target of the pesticide application (i.e. plants growing in borders or between line in fields) (EFSA, 2014b). They display important roles maintaining the biodiversity and ecological balance in agricultural areas such as supporting the food web and attracting and providing habitat for beneficial organisms (e.g. pollinators and other non-target organisms that helps in pest control) (Marshall, 2001). During spray application, non-target terrestrial plants are exposed to pesticides and, in many cases, to more than one active substance, adjuvants and co-formulants simultaneously (EFSA, 2014b). Since plants are primary producers and provide the energetic base for terrestrial ecosystems, it is expected that the adverse effects caused by pesticides may not only affect them, but also the other non-target organisms due to indirect effects (e.g. alterations in food resources and habitat) (Schmitz et al. 2015). In this sense, the main goal in ERA of non-target higher terrestrial plants is to protect not only plants, but also the biodiversity of the terrestrial system.

Phytotoxicity can be defined as the capacity of a pesticide or a contaminated soil to cause temporary or permanent damage to plants (Kalsch et al., 2006). Soil toxicity tests using higher plants in ERA usually measure the phytotoxic effects on the early life-stages, from seed germination and emergence to early root and shoot development because they are considered to be more toxicologically sensitive to contaminants (OEHHA, 2009). Phytotoxicity endpoints that are typically analyzed in international protocols include quantitative and qualitive measurements such as fresh and dry weight of above-ground plants, survival and emergence percentual and visual observations of phytotoxic effects on the plants (e.g. leaf chlorosis, reduction in plant growth, wilting, etc.). The main standardized phytotoxic test guidelines used to assess the effects of chemicals on higher terrestrial plants are described by ISO 11269-2 (ISO, 2012a), OECD 208 (OECD, 2006) and US EPA (1996). In a lower tier approach, to perform a pesticide phytotoxicity test, the substance is spiked into a substrate (e.g. artificial or natural soil) on a given concentration range and mixed thoroughly until the substrate obtain a homogenous consistency. Seeds from a plant species are then sown in replicates per treatment and the selected endpoints are evaluated in a 14 to 21-d period under controlled conditions (e.g. in greenhouse, germination room or phytotron). For the selection of a plant species to use in a standard soil toxicity test, these mentioned protocols consider several crop species from two plant groups, monocots and eudicots, such as the common oat (*Avena sativa* L.), maize (*Zea mays*), Soybean (*Glycine* max), lettuce (*Latuca sativa* L.), turnip (*Brassica rapa*) and others. Crop species are usually selected as test species because of the ease to obtain seeds in a good quality, relatively easy maintenance, good homogeneity and because they cover a wide range of plant families (e.g. Poaceae, Leguminosae, Brassicaceae,

Solanaceae, etc.) (Bayer, 2018). However, sensitive non-crop species were recommended in OECD and US EPA guidelines in a list that contains 52 different wild plant species and 32 crop species that could be used in phytotoxicity tests (OECD, 2006; US EPA, 2012) and studies have reported the differences in using crop and non-crop species to assess the potential harmful effects of pesticides on non-target plants and the environment (Clark et al., 2004; Dalton and Boutin, 2010).

Phytotoxic effects of pesticides on non-target terrestrial plants have been reported for many years (Wang & Freemark, 1995; White & Boutin, 2007; Boutin et al., 2012) and the species *Avena sativa* and *Brassica rapa* (Figure-1 A and B) have been widely used in the ecological risk assessment of contaminants in soil (Natal-da-luz et al., 2011a; Wang et al., 2019; Rogacz et al., 2020). However, few studies in this area have reported the effects of pesticides mixtures in non-target plants, with the available literature mainly focusing on the effects of binary mixtures (Santos et al., 2011a; Santos et al., 2011b).

Soil invertebrates: *Folsomia candida* (Collembola: Isotomidae) and *Eisenia andrei* (Oligochaeta: Lumbricidae)

Also known as springtails, collembolans are among the most abundant arthropods on Earth and constitute an important component of soil mesofauna in terrestrial ecosystems (Rusek, 1998). Most Collembola species feed on fungi, soil organic matter and leaf litter and live in both wet and dry habitats, ranging from arctic and alpine tundra to deserts and tropical rain forests (Fountain and Hopkin, 2005). According to their distribution in soil, Collembola species can be divided in epedaphic collembolans (i.e. live on soil surface and among vegetation), hemiedaphic collembolans (i.e. live on soil top layers and lead litter) and euedaphic collembolans (i.e. soil-dweller species that inhabits soil pores) (Rusek, 1998). Their role in ecosystem includes the control of soil microbiota (fungi, bacteria, actinomycetes and algae) by feeding and thus, control of fungal diseases in vegetation, organic matter recycling and nutrient availability in soil and stimulation of mycorrhizae growth modulating nutrient allocation in plants (Rusek, 1998; Ngosong et al., 2014). When exposed to chemicals in soil, Collembolans are particularly at risk because they are exposed through water ingestion or absorption from wet/moist surfaces, food consumption and soil pore air inhalation (Cardoso and Nogueira, 2016). In this sense, they have been used to evaluate the effect of pesticides and other environmental pollutants on non-target soil arthropods for almost four decades with *F. candida* being the most used species as representative of soil mesofauna in standardized ecotoxicological tests (ISO, 1999; OECD, 2016a). This species has been used as a model arthropod due to their wide geographical distribution and for its high sensitivity, short

generation time, high reproduction rate and easy culturing in the laboratory (Cardoso and Nogueira, 2016).

Folsomia candida (Willem 1902) (Figure 1A) is an arthropod belonging to the Isotomidae family that is distributed in soil worldwide and has a high occurrence rate in soil rich in organic matter (Fountain and Hopkin, 2005). Their vertical distribution in soil is euedaphic, inhabiting soil pores (Rusek, 1998). Although this species reproduces primarily by parthenogenetic females, some males may appear (one male can be produced per each 10,000 females, approximately) (Krogh et al., 2008). They are around 2mm long and have no pigmentation or eyes. They are sexually mature at 21 to 24 days of age at 20ºC, and each batch lays about 30 to 50 eggs, which take 7 to 10 days to hatch (Cardoso and Nogueira, 2016). Also, they are classified as microsaprophagous, preferring fungi growing on the surfaces of leaf litter than on soil particles, but in laboratory culture they can be fed with dry yeast (Fountain and Hopkin, 2005). *F. candida has* been used extensively as a model species in soil ecotoxicology tests, along with other groups (i.e. earthworms, enchytraeids, mites, netamtodes) and are the most used species of collembolans in ecotoxicology studies (e.g. Lavtižar et al., 2016; Natal-da-Luz et al., 2011b; Niemeyer et al., 2018; Santos et al., 2012). In 2009, OECD guideline 232 (OECD, 2016a) included another collembolan species as a standard species, *Folsomia fimetaria*. This species has a worldwide distribution in natural and agricultural habitats (Krogh et al., 2008) and the choice of including another collembolan to standardized toxicity tests was mainly because of its sexual mode of reproduction, providing therefore, higher genetic variability compared to *F. candida*. The standardized ecotoxicological tests used to assess the toxic effects of pollutants in *F. candida* are described by ISO 11267 (ISO, 1999) and OECD guideline 232 (OECD, 2016a). Those guidelines test sub-lethal endpoints, namely survival and reproduction of 10 to 12 days old springtails in a 28-day experiment.

Along with the Collembolans, earthworms are considered as a biological indicator of soil health and toxicity (Miglani and Bisht, 2020). Earthworms have been recognized as ‘ecological engineers’ due to their role in soil formation and soil structure maintenance, improving soil physical properties such as bulk density, infiltrability, hydraulic conductivity and porosity (Stanley and Preetha, 2016). Besides that, they have other roles in the ecosystem such as the improvement of soil fertility and nutrient availability, management of organic waste and vermicomposting and bioremediation of polluted environments, which can be considered ecosystem services (i.e. benefits provided by ecosystem to humans as well as other species) (Rodriguez-Campos et al., 2014; Stanley and Preetha, 2016). Earthworms are oligochaete annelids that are segmented and bilaterally symmetrical, with a glandular section called clitellum, which is part of their reproductive system (Edwards et al, 2011). They are hermaphrodites and reproduce normally through copulation and cross fertilization, with mature

individuals being distinguished by the presence of the clitellum (Edwards, 2004). The Lumbricidae is the most studied and geographically scattered earthworm family and based on their ecological characteristics such as burrowing and feeding habits, they can be classified as epigeic, engogeic and aneic species (Paolleti, 1999). Epigeic species such as *Eisenia fetida, Eisenia andrei* and *Lumbricus rubellus* are pigmented, found in the soil surface, and are in general non burrowing and dwell in litter (Paolleti, 1999; Edwards, 2004). Endogeic species produce largely horizontal galleries and live near the soil surface, usually in the 10-15 cm soil layer and consume mineral soil. Species such as *Allolobophora caliginosa* and *Aporrectoedea rosea* are included in this group (Edwards, 2004). The Anecic group of species such as *Lumbricus terrestris* and *Aporrectodea trapezoids* are deep-burrowing large species, that forms vertical burrows, and usually come to soil surface at night to draw litter down (Paolleti, 1999; Edwards, 2004).

When pesticides are applied directly to the soil, the first group of earthworms affected by these contaminants are the epigeics, since they dwell in surface layers. They are exposed to soil contaminants through their exterior epidermis and alimentary surfaces and sense the pesticides by the sensory tubercles on their body surfaces and their sensitivity against chemicals depends on the nature of the pollutant and its concentration (Stanley and Preetha, 2016). Due to their high sensitivity to pollutants in soils and their importance in soil ecosystem, earthworms became a standard organism in terrestrial ecotoxicological tests. The first standardized toxicity guideline with earthworms was the OECD nº 207 (OECD, 1984) that describes two short term tests (acute tests), one using 14 days of exposure in soil and the other one exposing the worms for 2 days in filter paper impregnated with the test chemical, using the number of surviving organisms as endpoint. By the end of the 1990s and early 2000, toxicity tests using sub-lethal endpoints, namely reproduction, were standardized for Collembola, enchytraeids and earthworms by both the OECD and ISO. For earthworms, the available toxicity tests are OECD 207 (OECD, 1984) and ISO 11268-1 (ISO, 2012a) for acute tests (mortality), OECD 222 (OECD, 2016) and ISO 11268-2 (ISO, 2012b) for reproduction and ISO 17512-1 (ISO, 2008) for the avoidance behaviors (sub-lethal endpoint tests).

Due to its short life cycle, world-wide distribution, high reproductive rate and culture easiness, the epigeic earthworm *Eisenia fetida* Savigny 1826 and *Eisenia andrei* Bouché 1972 (Figure 2-B) have been widely used in acute and chronic toxicity tests and they are recommended as model species by the guidelines mentioned above (Cardoso and Nogueira, 2016). Both Oligochaeta model species for toxicity tests, *E. fetida* and *E. andrei* are closed related species. Morphologically, *E. fetida* has the area around the intersegmental groove with no pigmentation or appearing yellowish and with the common name “tiger” earthworm and *E. andrei* is uniformly reddish with the common name “red” worm (Edwards et al., 2010). Despite the differences in pigmentation, the two species are morphologically similar, and their

reproductive performances and lifecycles do not differ significantly, although growth rate and cocoon production are higher in *E. andrei* and they reach sexual maturity more rapidly than *E. fetida* with approximately 35 days after hatching (Reinecke and Viljoen, 1991).

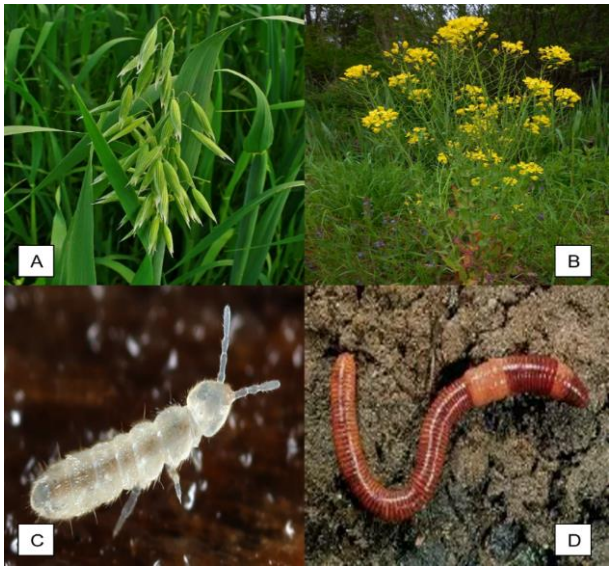

Figure 1. (A) Common oat (*Avena sativa* L.), (B) Turnip (*Brassica rapa*), (C) *Folsomia candida* Willem 1902 (Collembola: Isotomidae) and (D) *Eisenia andrei* Bouché 1972 (Oligochaeta: Lumbricidae). Source: (A) shorturl.at/hoBMT, (B) shorturl.at/gnow5, (C) shorturl.at/elozA, (D) shorturl.at/exPX4.

6. Objectives and working hypothesis

The main objectives of the present study are to assess the effect of a tank mixture composed of PPPs with different modes of action to non-target soil organisms from different ecological groups, representative of different routes of exposure, and to evaluate if the CA model is adequate to predict the mixture's toxicity independently of the test organism group.

To attain this purpose, a laboratory study was conducted and described in Chapter II. This study was composed of standard laboratory tests with artificial soil and using two non-target higher plants (*Avena sativa* and *Brassica rapa*) and two soil invertebrates (*Eisenia andrei* and *Folsomia candida*) to assess the single and combined effects of a pesticide mixture containing one herbicide, one insecticide and one fungicide (pendimethalin, chlorantraniliprole and + mancozeb+metalaxyl-M, respectively).

More specifically, the objectives (O) of the present work are:

OB1. Assess the effect of a tank mixture composed of three PPPs with different mode of actions to four non-target species.

OB2. Evaluate the adequacy of CA model to predict the toxicity of the pesticide tank mixture selected for the study evaluating also if the deviations to the CA model is dependent on the organism group.

The working hypotheses (H) for each objective are:

H1.The tank mixture selected for the study is toxic for all non-target species used in the experiments even at concentrations lower than the respective recommended doses of each PPP that composes the tank mixture.

H2. The Concentration Addition model is adequate to predict the toxicity of the pesticide mixture to the test organisms and the deviations to the CA model is independent on the organisms group.

Chapter II: Effect of a pesticide tank mixture to non-target plants and soil invertebrate species

Abstract

The application of mixtures of two or more plant protection products (PPPs) in agricultural fields is a widely used practice to reduce costs and time in application. The risk assessment of PPPs in the EU is done for each PPP individually, however, when these products are mixed, interactions between them can occur resulting in synergies or antagonisms, which makes it difficult to predict their effects on non-target organisms. The concentration-addition (CA) model has been widely used to assess the toxicity of mixtures of contaminants in the soil, however, this model assumes the absence of interactions between the components of the mixture. In the present work, the individual and combined toxicity (using the CA model) of three PPPs of pendimethalin (herbicide), chlorantraniliprole (insecticide) and mancozeb+metalaxyl-M (fungicide) was evaluated. Standardized laboratory tests were conducted using artificial soil and four non-target species representing different routes of exposure to PPPs: two invertebrates (the Collembola *Folsomia candida* and the Oligochaete *Eisenia andrei*) and two plants (the monocotyledon *Avena sativa* and the eudicotyledon *Brassica rapa*). In individual tests, the PPP of chlorantraniliprole was toxic only to *F. candida*, while the PPP of mancozeb+metalaxyl-M was more toxic to *F. candida*, but also revealed toxicity to *E. andrei*. The PPP of pendimethalin was more toxic to *A. sativa*, followed by *E. andrei, F. candida* and *B. rapa*. The toxic doses for each PPP individually were always higher than the maximum recommended doses of the respective products. The mixture of the three PPPs revealed additivity when tested in *A. sativa* and *B. rapa*. However, deviations from the conceptual model were translated into antagonism when tested in the *E. andrei* species and synergism to *F. candida* species. The different sensitivities of the species to each PPP individually suggest that the interactions in the test mixture depends on the route of exposure of the test organism.

Key words: Tank mixtures, PPPs, CA model, non-target organisms, routes of exposure

1. Introduction

The use of a variety of Plant protection products (PPPs) became an essential tool in agriculture in order to protect crops and enhance yield quantity and quality against undesired pests, weeds and diseases in plants. It is estimated that without PPPs, the loss of fruit production would be 78%, followed by 54% loss in vegetable production and 32% loss of cereal production (Tudi et al., 2021). The intensive use of PPPs estimated in three billion kilograms used worldwide every year (Hayes et al., 2017), includes more than 500 active ingredients belonging to different chemical and functional classes, that are included in several commercial formulations (Tang and Maggi, 2018). In agricultural fields, the use of agrochemicals has a high operational cost and aiming to reduce expenses, farmers mix different PPPs directly in the tank reservoir of the sprayer for application in crops (Gandini et al., 2020). This practice is known as pesticide tank mixing and most of the users usually combine up to two to five products concomitantly, usually using the highest recommended field doses (Gazziero et al, 2015). In current Environmental Risk Assessment (ERA) at the European Union, the approval of a PPP to be released in the market under the regulation EC Nº 1107/2009 (EC, 2009), ecotoxicological data are only required for pure active ingredients and for the respective commercial formulation, with the combined risk of multiple pesticides (e.g. application of pesticide in mixtures) being not considered in the pre-authorization process (Kienzler et al., 2016). However, the exposure of combined PPPs, thus with more than one active ingredient (a.i.) in the mixture, can lead to adverse effects that can deviate from the additive toxicity of single pesticides (i.e. synergistic/antagonistic effects) which may interfere in the risk to non-target organisms (Hernández et al., 2017; Santos et al., 2010).

Soil ecosystems shelter complex and diverse populations of soil organisms that are responsible for key biological processes, accounting for a considerable amount of Earth's biodiversity (Barrios, 2007). Soil invertebrates are responsible for key processes in soil function such as soil structure formation, decomposition of organic matter (OM) and nutrient mineralization of soil OM required by plants (Lavelle et al., 2006). Along with soil invertebrates, non-target plants usually found in bordering areas of crop fields, display essential roles in nutrient cycling and serving as useful energy for almost all other life forms, providing food, shelter and nesting habitats for a variety of organisms, in particular pollinators, which are essential for a good agricultural crop production (Wang and Freemark, 1995; Marshall, 2001). However, in agricultural environments, the constant exposure of several pesticides (alone or in tank mixtures) may have major effects on both target and non-target soil organisms or bordering areas, affecting many trophic levels (Edwards, 2002). Therefore, it is of critical importance the evaluation of possible harmful effects caused by the application of pesticides in soil organisms (including invertebrates and plants) due to their important roles in the soil

ecosystem maintenance. In this sense, earthworms, springtails and several non-target plants have been widely used in ecotoxicology as test organisms along with other organisms' groups (e.g. enchytraeids, mites, nematodes) to assess the impact of pollutants in soil due to their widespread distribution and their ecological relevance (van Gestel, 2012).

To assess the potential toxicity of a chemical mixture, models from pharmacological studies are used, being the concentration addition (CA) the most used for regulatory purposes (Bopp et al., 2018). This model assumes that the effects of a chemical mixture can be estimated by summing the individual effect of each chemical according to its concentrations in the mixture (assuming similarly acting substances) scaled for their potencies (OECD, 2018). The contribution of the individual components of the mixture to the overall effect can be added in the form of toxic units (TUs), expressed as concentrations, and calculated by the sum of the fraction between exposure concentration of each component in the mixture and its effective concentration, usually the EC_{50} (De Zwart and Posthuma, 2005). The CA model is often applied for many reasons, but mainly because requires less data than other models like IA model, being also more easy to be used (Panizzi et al., 2017). However, literature shows that deviations from the CA model predictions may indicate that this model, in some cases, could not be adequate for some mixtures (i.e. the interaction of components in a mixture did not behave in an additive manner, resulting in synergisms or antagonisms) (Cedergreen et al. 2008; Nørgaard and Cedergreen; 2010). Most of the ecotoxicological studies investigating the effect of pesticide mixtures through the CA model using soil organisms, usually use one or two test organisms and evaluate the effect of binary mixtures. (e.g. Amorim et al., 2012; Santos et al., 2011a; Chen et al., 2019; Santos et al., 2011b). However, soil non-target species may respond differently to pesticides mixtures and, therefore, the deviations to the CA model predictions may depend on the species (Santos et al., 2010; Amorim et al. 2012). Moreover, as non-target organisms are usually exposed in the field to mixtures with more than two PPPs (Gazziero et al, 2015), the investigation of the effect of mixtures with more than two PPPs in tank, using different soil species representative of different routes of exposure is needed.

Considering that pesticide mixtures may have a greater detrimental impact on non-target organisms than the additivity of individual components when acting alone, the combined toxic effects of mixtures have been identified as an issue of environmental concern (Stepić et al., 2013). In this sense, the present study aimed to evaluate the toxicity of a ternary pesticide mixture composed of three PPPs with different modes of action, using four non-target soil organisms as test species representative of different routes of exposure (*Avena sativa*, *Brassica rapa*, *Eisenia andrei* and *Folsomia candida*), through the CA model. The pesticide mixture used in the study was composed of an herbicide of Pendimethalin, an insecticide of Chlorantraniliprole and a fungicide of Mancozeb+Metalaxyl-m. The three PPPs are applied to a variety of agricultural crops, however most of their single effects on non-target soil organisms

are still scarce and their effects as a mixture have not been evaluated yet. For this purpose, two working hypotheses were considered: 1) the tank mixture selected for the study is toxic for all non-target species used in the experiments even at concentrations lower than the respective recommended doses of each PPP that composes the tank mixture and 2) the Concentration Addition model is adequate to predict the toxicity of the pesticide mixture to the test organisms and the deviations to the CA model is independent on the organisms group.

2. Material and Methods

The present study was composed of 4 steps. The first step consisted in collecting toxicity data from the existing literature on the three pesticides selected for the test mixture for each of the non-target test species selected (see Table 5 in Results). The second step comprised the performance of single species laboratory tests with each pesticide individually for each of the non-target species selected and to which toxicity data could not be found in the literature. As for *A. sativa and B. rapa* no toxicity data was found in the available literature to any of the selected pesticides, higher plant growth tests with the three pesticides individually and for each plant species were performed. Since toxicity data for *E. andrei* and *F. candida* to the selected herbicide and insecticide were found in the literature, only laboratory tests with the fungicide (Ridomil Gold Mz Pépite®) were performed for both invertebrate species. An additional test with the concentration of 1000 mg a.i/kg of soil of the insecticide Coragen® was performed to confirm that no toxic effects are found by this pesticide at concentrations below or equal to this dose as supported in the data from literature (EFSA, 2008). The third step was composed of laboratory experiments with the pesticide mixture for each test organism. The fourth and last step integrated the data analyses of the toxicity data collected in the literature search (step 1) and obtained in the laboratory tests (steps 2 and 3) by the light of the CA model for each organism.

2.1 Test chemicals and test soil

Three commercial formulations were used in the laboratory tests: the pre-emergence herbicide Podium® with pendimethalin as a.i. (330 g a.i/L), the dinitroaniline insecticide Coragen® with chlorantraniliprole as a.i. (200 g a.i/L) and the systemic and contact fungicide Ridomil Gold Mz Pépite® with mancozeb (64%, w/w) and metalaxyl-m (4%, w/w) as a.i. The pesticides were purchased at Cooperativa Agrícola de Coimbra. Pendimethalin is a selective dinitroaniline herbicide commonly used in pre-emergence to control broadleaf and grassy weeds on a variety of agricultural crops (Ascenza, 2021), acting on mitosis inhibition, causing a disruption on microtubule protein tubulin process that leads to death of weed seedlings (Hatzinikolaou, et al., 2004). Chlorantraniliprole is a first-generation anthranilic diamide

insecticide that acts both on larvae and adults of Lepidopteran and Coleopteran pests by ingestion and contact, acting as a ryanodine receptor, causing a depletion of internal calcium stores, impairing muscle contraction, leading to the death of the target insect by feeding cessation, lethargy and partial paralysis (Bentley et al., 2010). Ridomil Gold Mz Pépite® is a systemic and contact fungicide of the two active ingredients, Mancozeb and Metalaxyl-m, used to prevent types of mildew and black rot in many crops (Syngenta, 2021).

The pesticides application doses recommended by the manufacturer were converted to mg of active ingredient per kg of soil assuming a homogeneous distribution of the chemical in the top 5-cm soil layer and a soil density of 1.5 g/cm^3. For the fungicide Ridomil Gold Mz Pépite®, application doses were converted to mg of formulated product per kg of soil. Therefore, the concentrations used in the tests were based on the highest recommended field dose of 2.64 mg pendimethalin/kg of soil for the herbicide, 0.057 mg chlorantraniliprole/kg of soil for the insecticide and 3.33 mg Ridomil/kg of soil for the fungicide. For each laboratory test, a stock solution was prepared by diluting a volume of the respective commercial formulation or mixture of commercial formulations in distilled water. Soil was then spiked with increasing volumes of the stock solution to obtain the increasing nominal concentrations of the active ingredients desired (See Table 1, 2 and 3). Volumes of stock solution, water and soil were made compatible with 50% of the soil water holding capacity (WHC), with an exception in the *F. candida* assay with the pesticide mixture, where the WHC was adjusted to 40%.

In the laboratory test with the pesticide mixture, the toxic effects of the ternary combination (herbicide + insecticide + fungicide) were evaluated in the selected species. After obtaining all the individual toxicity values of each PPP for each species (from the literature and individual tests), Toxic Units (TU) of each product for each species were determined using the equation $TU = Ci/EC_{50}$, where Ci was the highest recommended dose of the PPP applied in the field and EC_{50} was the effect concentration of the PPP acting alone to reduce 50% of the species compared to control. To obtain the potential toxic effect of the mixture for each species, the TU of each product was summed ($\sum TU = \sum Ci/EC50$).

To calculate the concentration gradients of the pesticide mixture to use in the laboratory tests, concentrations higher and lower than the ones corresponding to the ∑TU of each species were used. Therefore, the concentrations of the pesticide mixture included doses higher and lower than the highest recommended field dose of each product. To prepare the pesticides mixture for soil spiking, a stock solution for each pesticide was prepared according to the highest value of the concentration gradient of each pesticide and mixed taking into consideration the solubility of the products, being mixed first the fungicide (WG), second the insecticide (SC) and lastly the herbicide (EC). This order of addition was followed to avoid incompatibility or precipitation of the chemicals (Gazziero et al, 2021). Once prepared the stock solution of the pesticide mixture, this solution was diluted in distilled water in different

proportions according to the concentration desired. By this way, soil was then spiked with increasing volumes of the mixture stock solution to obtain the increasing nominal concentrations of the pesticide mixture for each test (Table 4).

The soil used in the laboratory tests was an artificial soil composed of a mixture of 5% *Sphagnum* sp. peat (air dried and sieved at 5 mm), 20% of Kaolin clay and 75% of fine sand (Natal-da-luz et al., 2019). The choice of artificial soil as test substrate in the laboratory tests had the purpose to allow comparability of effect data between the existing data in the DAR or RAR documents, and in data from scientific literature (as the most effect data available were generated in artificial soil). The dry constituents were blended in the correct proportions and mixed thoroughly and the soil pH was adjusted in to 6.0 ± 0.5 through the addition of $CaCO_3$. Soil pH was determined following the methods described in ISO 10390 (ISO, 2005) and the soil water holding capacity (WHC) was determined according to ISO 11274 (ISO, 2019).

Table 1. Nominal concentrations (in mg a.i/kg of soil) of the gradients of the commercial formulation Podium® with pendimethalin as active ingredient, used in the laboratory tests with *Avena sativa* and *Brassica rapa*.

Podium® (34% (w/w) pendimethalin)		
A. sativa	*B. rapa*	
0	0	0
0.08	0.08	5
0.17	0.17	10
0.33	0.33	20
0.67	0.67	40
1.33	1.33	80
2.67	2.67	160
5.34	5.34	
10.68	10.68	
21.35	21.35	

Table 2. Nominal concentrations (in mg a.i/kg of soil) of the gradients of the commercial formulation Coragen® with chlorantraniliprole as active ingredient, used in the laboratory tests with *Avena sativa, Brassica rapa* and *Eisenia andrei*.

Coragen® (18% (w/w) chlorantraniliprole)				
A. sativa		*B. rapa*		*E. andrei*
0	0	0	0	0
1.5	197.5	1.5	197.5	1000
6	296.3	6	296.3	
12	444.4	12	444.4	
24	666.7	24	666.7	
48	1000	48	1000	
96		96		
192		192		

Table 3. Nominal concentrations (in mg Ridomil/kg of soil) of the gradients of the commercial formulation Ridomil Gold MZ Pépite® used in the laboratory tests with *Avena sativa, Brassica rapa, Eisenia andrei and Folsomia candida*.

Ridomil Gold Mz Pepite® (64% (w/w) Mancozeb + 4% (w/w) Metalaxyl-m)					
A. sativa		*B. rapa*		*E. andrei*	*F. candida*
0	0	0	0	0	0
1.5	197.5	1.5	197.5	2	2
6	296.3	6	296.3	6	6
12	444.4	12	444.4	18	18
24	666.7	24	666.7	50	50
48	1000	48	1000	100	100
96		96		150	150
192		192		250	250
				300	300
				450	450
				900	

Table 4. Nominal concentrations of the pesticide mixture for *Avena sativa, Brassica rapa, Eisenia andrei* and *Folsomia candida* toxicity experiments based on the sum of the TU of each pesticide (values are expressed in TU).

Pesticide mixture			
A. sativa	*B. rapa*	*E. andrei*	*F. candida*
0	0	0	0
0.03	0.02	0.02	0.01
0.06	0.04	0.04	0.02
0.13	0.07	0.09	0.05
0.26	0.14	0.17	0.09
0.52	0.28	0.34	0.18
1.03	0.56	0.69	0.37
2.06	1.13	1.38	0.74
4.13	2.25	2.76	1.48
		5.51	2.96
			5.91

2.2 Test organisms

The selected species for the test were two plant species, oat (*Avena sativa*) and turnip *(Brassica rapa*) and two soil invertebrates, the earthworm *Eisenia andrei* and the springtail *Folsomia candida*. These species were selected because they are standardized species described in international protocols (e.g. ISO and OECD) to evaluate the chemical exposure response in soil and due to the prominent literature on the effects of several chemicals on the four species (Santos et al., 2011a; Santos et al., 2012; Amorim et al., 2012; Rogacz et al., 2020).

Plant species

The monocotyledonous *A. sativa* (Poaceae) and dicotyledonous *B. rapa* (Brassicaceae) were used in the higher plant growth tests. *A. sativa* seeds were obtained from a local supplier and *B. rapa* seeds were obtained from a commercial brand. All experiments were conducted in an acclimatized room which was maintained at 25 ± 5°C, under a photoperiod of 16:8h light:dark with a light intensity of about 7000 lx on the soil surface. The relative air humidity was between 40% and 60%.

Soil invertebrate species

The collembolans *Folsomia candida* (Isotomidae: Collembola) and the earthworms *Eisenia andrei* (Lumbricidae: Oligochaeta) were used as test organisms in laboratory reproduction tests. Both organisms belong to standard species advised for laboratory test in ISO guidelines (ISO, 1999; ISO, 2012b).

Both earthworms and Collembola were obtained from the laboratory cultures of the Soil Ecology and Ecotoxicology Laboratory of the University of Coimbra (Portugal). Both species were bred under a photoperiod of 16:8h light: dark cycle at 20 ± 2ºC. Rearing procedures were in accordance with the methods described in the standardized guideline ISO 11267 (ISO, 1999) for the springtails and ISO 11268-2 (ISO, 2012b) for the earthworms.

The collembolans were bred in plastic containers with the bottom covered with a thin layer of mixture of plaster of Paris and activated charcoal (11:1 mass ratio) saturated with distilled water. They were fed with granulated dry yeast and aerated regularly. Once or twice a week, the springtails were transferred to fresh containers, by tapping, to induce oviposition. The earthworms were kept in plastic boxes (36 cm length, 22 cm width, and 11 cm height) using a mixture of *Sphagnum* sp. Peat and cow manure previously defaunated as substrate. Fresh cow manure was given as food once a week (the manure was obtained from cows free of medications or other chemical treatments).

2.3 Experimental procedures

Higher plant growth tests with *Avena sativa* and *Brassica rapa*

Higher plant growth tests were performed following the procedures described in the ISO guideline 11269-2 (ISO, 2012). Four replicates per concentration were prepared, each one in a plastic container (12 cm length, 8.5 cm width and 5.5 cm height) with 400 g of contaminated soil (dry weight equivalent). The soil in the control replicates was not contaminated. Both pH and soil moisture were determined immediately before the start of the tests and at the end in all test treatments and control. The test treatments used in the laboratory tests are presented in Tables 1, 2, 3 and 4. A plastic container with the same measures filled with deionized water mixed with a commercial fertilizer an NPK ratio of 7:3:6 (approximately 5 ml/L) was placed underneath each test container to keep an adequate moisture of the soil during the test. The connection between soil and fertilizer solution is established through a twine that is introduced in the bottom of each replicate and dipped in the solution of the underneath container. Ten seeds were sown in a uniform distribution in each test container to a depth of about 0.5 cm. Seed germination was determined by visual seedling emergence and was recorded daily. After 50% of the seed in the control pots had germinated, the test started

for a period between 14 to 21 days. Visual detrimental effects (e.g. chlorosis, mortality, leaf and wilting necrosis) were also recorded. In the end of each test, the seedling shoots were cut above the soil surface, and the fresh biomass was immediately weighed. After that, the seedling shoots were dried in an oven at 70°C for 16h and then weighted to obtain the dry weight biomass per replicate.

Reproduction tests with *Eisenia andrei*

The methodology applied in all earthworm's reproduction tests followed the methods advised in ISO 11268-2 (2012b). Four replicates per concentration were prepared, each one consisting of cylindrical glass containers (8.6 cm diameter and 16 cm height) with approximately 500 g of contaminated soil (dry weight equivalent). The soil in the control replicates was not contaminated. Both pH and soil moisture were determined immediately before the start of the tests and at the end in all test treatments and control. The test treatments used in the laboratory tests are presented in Tables 2, 3 and 4. In the test, adults between two months and one year old with a developed clitellum were used and acclimatized for 7 days in the artificial soil and fed with cow manure in a sufficient amount before being used. Ten worms previously washed and gently wiped with absorbent paper, with a wet mass of 347.7 ± 57 mg (average ± standard deviation, *n*= 880), were selected and randomly introduced in the test containers after the soil contamination in each treatment. After this, approximately 15 g of wet cow manure were added to each replicate. During the first four weeks of the test, food was given once a week and the test containers were reweighed periodically to control water losses. At the 28th day of the test, adults of each container were removed and the total number and biomass of living adults were recorded. For the other four weeks of the test, the cocoons were kept for the development of the offspring. At the beginning of this period, juveniles were fed once with approximately 15 g of wet cow manure. At the end of the experiments, the test containers were placed in water bath at a temperature of 50 ºC to 60ºC for 20 min, to enable the offspring to appear at the substrate surface, and to be further collected and counted.

Reproduction tests with *Folsomia candida*

Collembola reproduction tests followed the methodology defined in the standard protocol ISO 11267 (ISO, 1999). Juveniles of *F. candida* with an age of 10-12 days were taken from synchronized culture and used in the tests. To attain this purpose, freshly laid eggs were isolated in fragments of substrate, using a paintbrush and a pipette, and placed in fresh containers (5 cm diameter and 10 cm height) with substrate. After incubation, eggs hatched, and fragments of substrate were removed two days after the first hatch. Then, instars were fed, watered, and aerated until they attained the specific age to be used in the test. Each

treatment consisted of five replicates with 30 g (fresh weight equivalent) of contaminated soil. The soil in the control replicates was not contaminated. Both pH and soil moisture were determined immediately before the start of the tests and at the end in all test treatments and control. The test treatments used in the laboratory tests are presented in Tables 3 and 4. Extra replicates were used to measure soil pH and water content of each treatment both at the beginning and at the end of the test. Ten healthy springtails with synchronized ages were selected and introduced to each replicated. Before introducing them in the test vessels, they were first transferred to a Petri dish to confirm that they were in a good condition. Replicates were kept in the culture room, at 20±2ºC, 40-50% air humidity and 16:8h light:dark photoperiod. They were aerated twice a week and the collembolans were fed at the 0 and 14th day of testing. The test had the duration of 28 days. At the end of the test, the content of each replicate was transferred to an individual plastic container and flooded with water colored with dark ink to facilitate springtails counting. Each plastic container was photographed from the top and individuals were counted using ImageJ software. The number of surviving adults was also recorded in each replicate.

2.5 Statistical analysis

For all experiments, One-way ANOVA was performed to analyze the difference between with the independent variable (i.e. the pesticides or mixture concentration) and the dependent variable (i.e. the measured endpoints for each species). Data normality were checked through Kolmogorov-Smirnov and Shapiro-Wilk's W test and the homogeneity of data variances was confirmed though Levene's test. Differences between each treatment and the control were assessed by performing *post-hoc* Dunnett test. When homogeneity of data variances was not validated and the differences between treatments was evaluated through the nonparametric Kruskal-Wallis test.

The Lowest Observed Effect Concentration (LOEC) was defined as the lowest test concentration significantly different from control and the No Observed Effect Concentration (NOEC) as the highest tested concentration that had no significant difference when compared to control. To estimate the EC_{50}, EC_{20} and EC_{10} values (Effect concentration where there is a 50%, 20% and 10% reduction in the measured endpoint when compared to control) and the respective confidence intervals of 95% for reproduction of *E. andrei* and *F. candida* and biomass production (in dry weight) of *A. sativa* and *B. rapa*, dose-response non-linear regressions were performed based on dose-response models (using Logistic, Gompertz, Hormesis or exponential models). The most adequate model was guided by the better fit to the model and by the higher coefficient of regression (r^2). Statistica software (version 7) was used to perform these statistical analyzes. The median lethal effect (LC_{50}, Lethal concentration

capable of killing 50% of the individuals) was assessed by applying the mortality data of the soil invertebrates in a linear probit regression, using PriProbit software. All experiments were performed considering a significance of 5% (α=0.05).

The Concentration Addition (CA) model was used to predict the joint effects of the pesticides in mixture (Bliss, 1939). The CA model assumes that the mixed chemicals have the same mode of action and the contribution of the individual components of the mixture to the overall effect can be added in the form of toxic units (TUs), expressed as concentrations (De Zwart and Posthuma, 2005). This model is mathematically described by the following equation:

$$\sum TU = \sum (ci/EC50, i) \tag{1.1}$$

where, the quotient c_i / $EC_{50,i}$ is dimensionless the toxic unit of the component *i*, c_i is the concentration of component *i* in the mixture (*i*= 1, 2, …, *N*), $EC_{50,i}$ is the concentration of component *i* of the mixture that produces an adverse effect of 50% when applied alone (Natal-da-Luz et al., 2011b). For the interpretation of the data obtained in the experiments with the pesticide mixture it was assumed that i) for an EC_{50} = 1, there was an additive effect; ii) for an EC_{50} > 1, there was an antagonism and; iii) for an EC_{50} < 1, there was a synergism.

3. Results

3.1 Data collected from the from the existing literature on the three pesticides selected for the test mixture

The results found in the first step of the work, which consisted in collecting toxicity data from the existing literature on the three pesticides selected for the test mixture for each of the non-target test species selected are described in Table 5.

Table 5. Data collect from the existing literature on the toxicity of the herbicide Pendimethalin, the insecticide Chlorantraniliprole and the fungicide formulation Ridomil Gold Mz Pépite® (with mancozeb + metalaxyl-m as a.i.) for the plant species *Avena sativa* and *Brassica rapa* and the invertebrate species *Eisenia andrei* and *Folsomia candida*. Toxicity data are expressed in mg a.i/kg for Pendimethalin and Chlorantraniliprole and in mg formulated product/kg for Ridomil Gold Mz Pépite®.

Species	A.i./Formulation	Toxicity data (mg/kg)	Reference
A. sativa	Pendimethalin	N.D.A	
	Chlorantraniliprole	N.D.A	
	Ridomil Gold Mz Pépite®	N.D.A	
B. rapa	Pendimethalin	N.D.A	
	Chlorantraniliprole	N.D.A	
	Ridomil Gold Mz Pépite®	N.D.A	
E. andrei	Pendimethalin (as Podium®)	EC_{50} = 33.1	Patrício Silva et al. *in prep.*
	Chlorantraniliprole (as DPX-E2Y45 35 WG)	NOEC = 350	EFSA, 2008
	Ridomil Gold Mz Pépite®	EC_{50} = > 39.06	EFSA, 2018b
F. candida	Pendimethalin (as Podium®)	EC_{50} = 37.1	Patrício Silva et al. *in prep.*
	Chlorantraniliprole (as Coragen®)	EC_{50} = 0.91	Ferreira (2020)
	Ridomil Gold Mz Pépite®	EC_{50} = 231	EFSA, 2018b

N.D.A = No data available in the literature.

3.2 Laboratory tests exposing pesticides individually

All laboratory tests complied with the validity criteria defined in standardized guidelines for each species (ISO, 1999, 2012b, 2012c). In the higher growth plant tests, emergence of seeds in control replicates was always higher or equal to 7. In laboratory test with *F. candida*, in the control replicates, adult mortality was ≤ 20%, the average number of instars per replicate was 227±45 (average ± standard deviation, *n*=5) and coefficient of variation was 19.84%. For tests with *E. andrei* adult mortality was 0%, the average number of juveniles per replicate was 101.5±14 (average ± standard deviation, *n*=4) and coefficient of variation was 15.31% in replicates of control.

Formulation of pendimethalin (herbicide)

Single exposure tests with the herbicide were only performed for the two plant species due to lack of data on literature. Toxicity data for *E. andrei* and *F. candida* from a paper that is still under preparation were considered (see Table 6). When *A. sativa* and *B. rapa* were exposed to pendimethalin in single laboratory tests, different sensitivities among the plant species were observed (Figure 2). Firstly, both species were tested within a concentration range of 0.08 to 21.35 mg ai/kg of soil in order to test concentrations up to 8x the highest field application dose (i.e. 2.64 mg a.i/kg of soil). A significant decrease of dry biomass production was observed to *A. sativa* at the concentrations 5.34, 10.68 and 21.35 mg a.i/kg of soil and an EC_{50} of 10.24 mg ai/kg of soil (95% CI: 3.38 - 17.11) was estimated (Table 6). For *B. rapa*, dry biomass production was not significantly affected by the herbicide, but visual phytotoxic effects were observed when compared to control, such as visual reduction in shoot length, root and hypocotyl swelling and stem injury (i.e. stem necrosis) at 21.35 mg a.i/kg of soil. Injuries were also observed in *A. sativa* at concentrations of 10.68 and 21.35 mg a.i/kg of soil, with leaf chlorosis, decrease in shoot length and root and hypocotyl swelling in some plants.

To allow the estimation of an EC_{50} for *B. rapa*, it was decided to perform a single experiment only with *B. rapa* within a higher concentration range of the herbicide of pendimethalin (5 to 160 mg ai/kg of soil). In this test, turnip dry weight per plant significant differ from the control at concentrations higher or equal to 40 mg ai/kg of soil. The same visual phytotoxic effects previously observed at 21.35 mg a.i/kg of soil were observed at concentrations higher or equal to 20 mg a.i/kg of soil. The estimated EC_{50} value for dry biomass production of *B.* rapa was 83.0 mg a.i/ kg of soil (95% CI: 40.77 – 125.23) (Table 6).

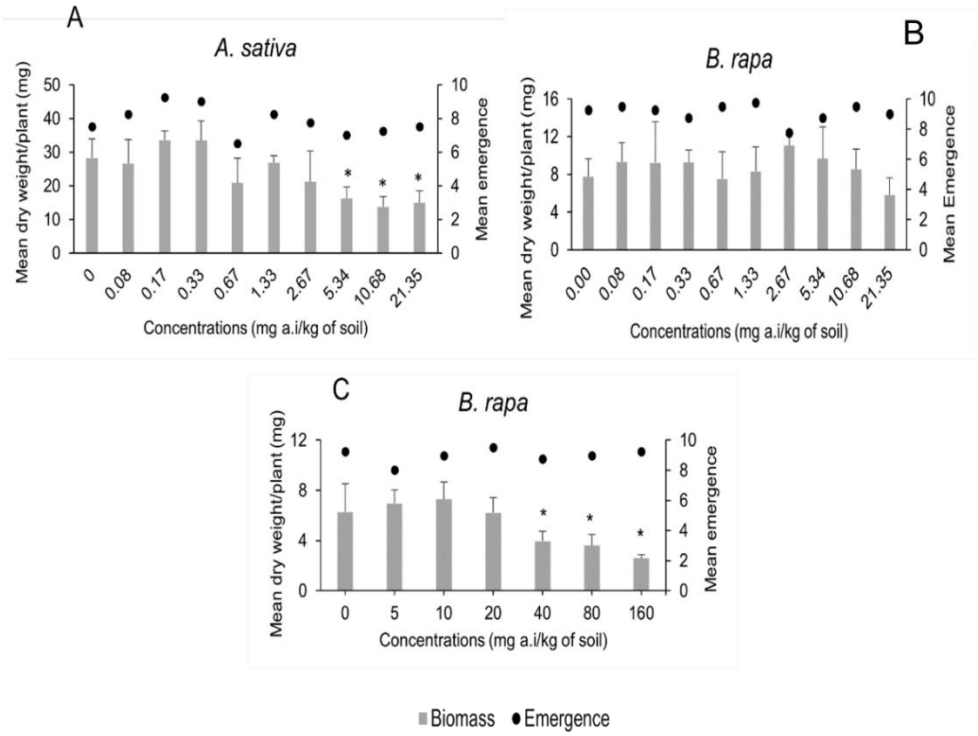

Figure 2. Emergence (dots – mean; *n*=4) and dry weight/plant (bars - mean + standard deviation; *n*=4) observed in replicates of higher plant growth tests with artificial soil spiked with increasing concentrations of Pendimethalin used as the commercial formulation Podium®, using *Avena sativa and Brassica rapa* as test species. *Mean dry weight/plant significantly different compared to the respective control ($p \leq 0.05$).

Formulation of Chlorantraniliprole (insecticide)

In Chlorantraniliprole's single experiments, tests with *A. sativa*, *B. rapa* and *E. andrei* were performed. For *F. candida* it was considered the data available in Ferreira (2020) (See Table 6). Oat and turnip were tested in two different assays, first in a concentration range of 1.5 to 192 mg a.i/kg of soil and secondly in 197.5 to 1000 mg a.i/kg of soil to allow the estimation of the toxic values. When tested in the first concentration range, no significant effects were observed for both species, neither in dry biomass production nor in emergence. When tested within the gradient composed of higher concentrations (concentrations up to 1000 mg a.i/kg of soil), also no significant differences in both evaluated endpoints for *A. sativa* and *B. rapa* were observed (Figure 3). Since no significant differences were obtained, the NOEC value was 1000 mg a.i/kg of soil for both species (Table 6).

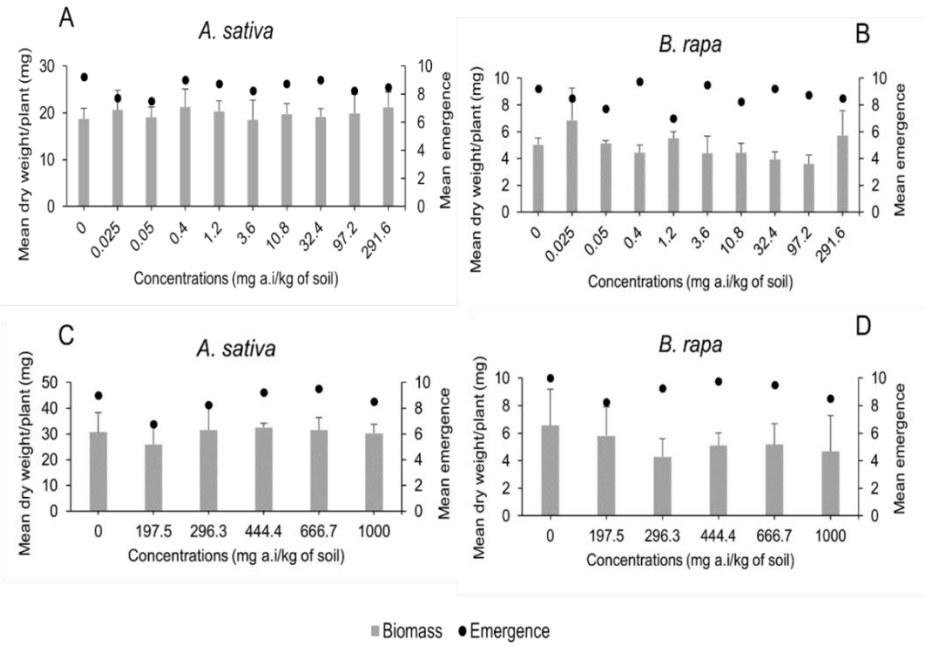

Figure 3. Emergence (dots – mean; *n*=4) and dry weight/plant (bars – mean + standard deviation; *n*=4) observed in replicates of emergence and growth tests with an artificial soil spiked with increasing concentrations of Chlorantraniliprole used as the commercial formulation Coragen®, using *Avena sativa and Brassica rapa* as test species.

As observed in plant tests, the exposure of Chlorantraniliprole to *E. andrei* resulted in no effects in reproduction and mortality of adult earthworms, even when tested at 1000 mg a.i/kg of soil. No significant difference was observed between this concentration and the control (Figure 4). The NOEC and LC_{50} values were 1000 and >1000 mg a.i/kg of soil, respectively (Table 6).

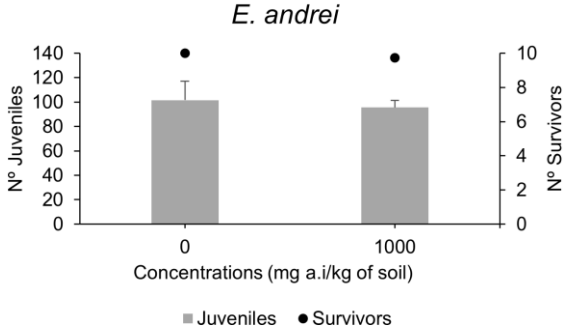

Figure 4. Number of surviving adults (dots – mean; *n*=4) and juveniles (bars – mean + standard deviation; *n*=4) observed in replicates of reproduction tests with artificial soil spiked with 1000 mg Chlorantraniliprole/kg of soil, used as the commercial formulation Coragen® and *Eisenia andrei* as test species. *Number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

Ridomil Gold Mz Pépite® (fungicide)

There were no significant differences in dry weight/plant and in emergence of *A. sativa* in any of the treatments when tested with Ridomil Gold Mz Pépite®, even 1000 mg Ridomil/kg of soil (Figure 5 A and C). Similar results were observed with *B. rapa* at the same concentrations (Figure 5 B and D). Both plant species were not affected by the fungicide at the recommended dose of 3.33 mg Ridomil/kg of soil. No visual detrimental effects were observed in any treatments for both species. The NOEC for both species was 1000 mg Ridomil/kg of soil (Table 6).

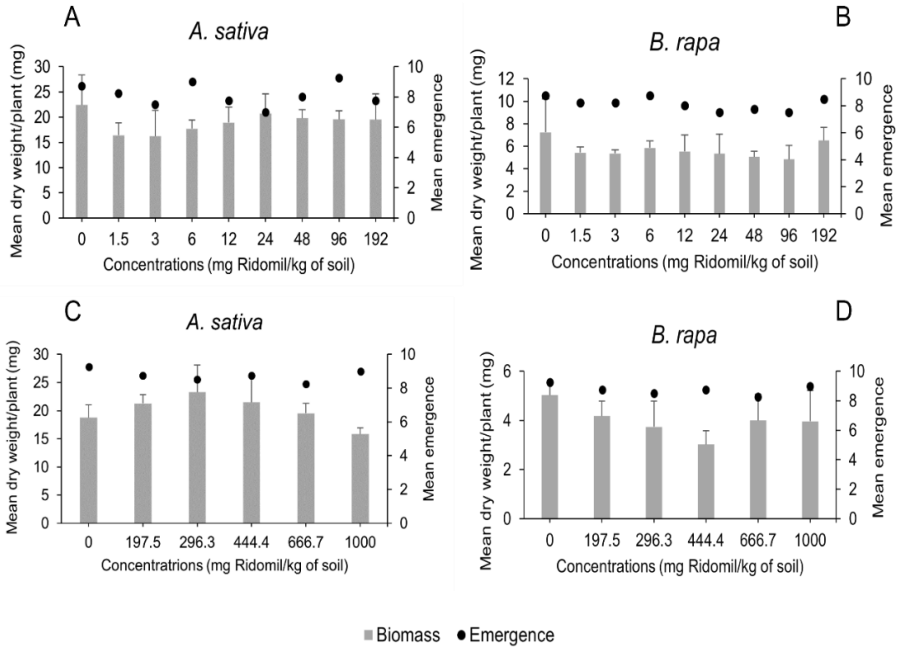

Figure 5. Emergence (dots – mean; *n*=4) and dry weight/plant (bars – mean + standard deviation; *n*=4) observed in replicates of higher plant growth tests with artificial soil spiked with increasing concentrations of the commercial formulation Ridomil Gold Mz Pépite®, using *Avena sativa and Brassica rapa* as test species.

In earthworm reproduction tests, the number of surviving adults was not affected by Ridomil Gold Mz Pépite® (Figure 6). Despite that, the number of juveniles was significantly reduced at concentrations higher or equal to 300 mg Ridomil/kg of soil, which represents ≥ 90 times the fungicide dose recommended by the manufacturer. The reproduction EC_{50} was 536.3 mg Ridomil/kg of soil (95% CI: 394.4 – 678.3) (Table 6). There were no effects on mortality or reproduction at the recommended dose (3.33 mg Ridomil/kg of soil).

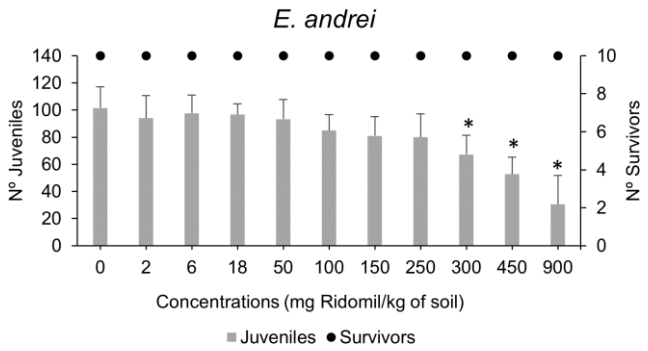

Figure 6. Number of surviving adults (dots – mean; *n*=4) and juveniles (bars – mean + standard deviation; *n*=4) observed in replicates of reproduction tests with artificial soil spiked with increasing concentrations of the commercial formulation Ridomil Gold Mz Pépite® (Mancozeb + Metalaxyl-m), using *Eisenia andrei* as test species. *Number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

For *F. candida*, the number of surviving adults decreased with increasing doses of the fungicide formulation (Figure 7). This exposure resulted also in a clear reduction in the number of juveniles produced. Significant differences in the number of juveniles were observed when testing concentrations corresponding to ≥15 times the recommended dose (≥50 mg Ridomil/kg of soil) and a substantial decrease in both number of juveniles and adults was observed from 30 times the recommended dose to the highest concentration tested (100 to 400 mg Ridomil/kg of soil). The reproduction EC_{50} was 60.1 mg Ridomil/kg of soil (95% CI: 50.08 – 69.1) (Table 6). No significant effects were found in mortality and reproduction at the recommended dose (3.33 mg Ridomil/kg of soil).

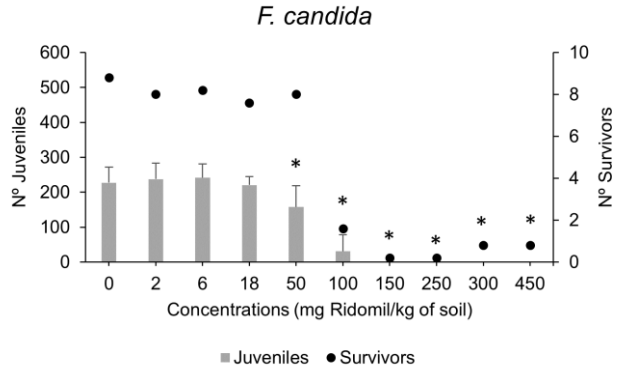

Figure 7. Number of surviving adults (dots – mean; *n*=5) and juveniles (bars – mean + standard deviation; *n*=5) observed in replicates of reproduction tests with artificial soil spiked with increasing concentrations of the commercial formulation Ridomil Gold Mz Pépite® (fungicide of Mancozeb + Metalaxyl-m), using *Folsomia candida* as test species. *Number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

3.3 Laboratory tests exposing pesticides in mixture

All laboratory tests complied with the validity criteria defined in standardized guidelines for each species (ISO, 1999, 2012b, 2012c). In the higher growth plant tests, emergence of seeds in control replicates was always higher or equal to 7. In laboratory test with *F. candida*, in the control replicates, the adult mortality was ≤ 20%, the average number of instars per replicate was 501.8 ± 72 (average ± standard deviation, *n*=4) and coefficient of variation was 14.34%. For the test with *E. andrei* adult mortality was 0%, the average number of juveniles was 105.6 ± 13.6 (average ± standard deviation, *n*=3) and coefficient of variation was 18.03% in replicates of control.

Effects of the pesticide mixture to *A. sativa and B. rapa*

When plant species were exposed to the pesticide mixture, similar sensitivity against test mixture in both species was observed, with a reduction in biomass production with increasing concentrations of the mixture and no significant differences in emergence (Figure 8 and 9 in graphs A and B). The ∑TUs of the pesticide mixture for each species was 0.26 for *A. sativa*; 0.04 for *B. rapa*. The visual effects observed during the experiment period were, reduction in the shoot heights for both species, along with leaf chlorosis and stem rot. These visual effects were more evident in *B. rapa* than in *A. sativa* (Figure 8). The EC_{50} values estimated were 1.82 (95%CI: 0.55 - 3.10) for *A. sativa* and 0.91 (95%CI: 0.30 - 1.52) for *B. rapa* (Table 7). Considering predictions of toxicity through the CA model, an additive effect between the pesticides was observed for both species, even though the EC_{50} value for *A. sativa* was higher than one and for *B. rapa* lower than 1. This interpretation was drawn because in this work, we considered as an additive response, if the value of TU = 1 was present in the confidence interval.

Figure 8. Visual effects of a pesticide mixture composed of Podium® (herbicide of pendimethalin), Coragen® (insecticide of chlorantraniliprole) and Ridomil Gold Mz Pépite® (fungicide of Mancozeb + Metalaxyl-m) in *A. sativa* and *B. rapa* at different concentrations (concentration in the bottom right corner of each picture) based on the sum of the Toxic Units (TU) of each pesticide in the mixture (A: *Avena sativa*; B: *Brassica rapa*).

Effects of the pesticide mixture to *E. andrei and F. candida*

When the soil invertebrates were exposed to the pesticide mixture, different effects were observed between them. Significant reduction in the number of juveniles and adults compared to the control were only observed for earthworms in the three last concentrations tested, where high pesticides concentrations were applied together. The ∑TUs of the pesticide mixture for each species was 0.08 *for E. andrei* and 0.18 for *F. candida*. The mixture estimated EC_{50} for *E. andrei* was 1.30 (95%CI: 1.15 - 1.46) (Table 7), with an antagonism predicted by the CA model. For *F. candida*, reduction in both endpoints could be observed with increasing concentrations, with significant differences in reproduction and mortality, starting from the concentration 0.18, which represent the sum of the TUs of the recommend dose applied in the field, to the last concentration tested. The mixture estimated EC_{50} for *F. candida* was 0.22 (95%CI: 0.10 - 0.33) (Table 7), with a synergism predicted by the CA model.

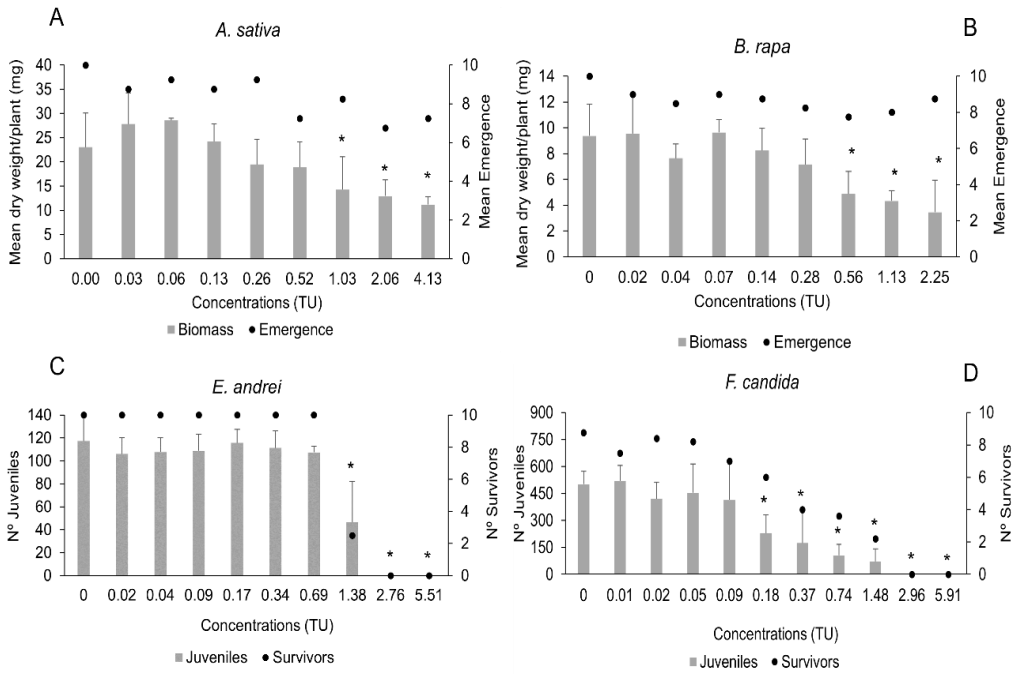

Figure 9. Emergence (dots – mean; *n*=4) and dry weight/plant (bars – mean + standard deviation; *n*=4) observed in replicates of higher plant growth tests using *Avena sativa and Brassica rapa* as test species (graphs A and B) and number of surviving adults (dots – mean) and juveniles (bars – mean + standard deviation) observed in replicates of reproduction tests using *Eisenia andrei* and *Folsomia candida* as test species (graphs C and D) with artificial soil spiked with increasing concentrations of a pesticide mixture composed of Podium® (herbicide of pendimethalin), Coragen® (insecticide of chlorantraniliprole) and Ridomil Gold Mz Pépite® (fungicide of Mancozeb + Metalaxyl-m). Concentrations are expressed in toxic units. *Mean dry weight/plant or mean number of juveniles significantly different compared to the respective control ($p \leq 0.05$).

Table 6. EC_{50} (Effective concentration causing 50% reduction), NOEC (no-observable effect concentration) LOEC (lowest-observable effect concentration) and LC_{50} (Lethal concentration for 50% of mortality) values in mg a.i or p.f/kg of soil and 95% confidence intervals for the effects of the single-exposure pesticides on *A.sativa, B. rapa, E. andrei* and *F. candida* on artificial soil.

Pesticide	Species	EC_{50}	EC_{20}	EC_{10}	LC_{50}	NOEC	LOEC	Reference
Podium® (a.i pendimethalin)	*A. sativa*	10.24 (3.38 – 17.11)	1.55 (-)*	0.51 (-)*	NE	2.67	5.34	
	B. rapa	83 (40.77 – 125.23)	25.8 (-)*	13.05 (-)*	NE	20	40	
	E. andrei	33.1 (1.5 - 64.7)	NE	29.5 (23.9 – 35.2)	33.6 (-)*	30	50	Patrício Silva et al. *in prep.*
	F. candida	37.1 (32.5 - 41.6)	25.80 (21.96 – 29.65)	21.09 (16.94 – 25.24)	52.96 (47.74-58.77)	< 20	20	Patrício Silva et al. *in prep.*
Coragen® (a.i chlorantraniliprole)	*A. sativa*	> 1000	> 1000	> 1000	NE	1000	> 1000	
	B. rapa	> 1000	> 1000	> 1000	NE	1000	> 1000	
	E. andrei	> 1000	> 1000	> 1000	> 1000	1000	> 1000	
	F. candida	0.91 (0.60 - 1.21)	NE	0.33 (0.06 - 0.61)	0.17 (0 - 1.09)	0.10	0.40	Ferreira (2020)
Ridomil Gold Mz Pépite® (a.i mancozeb+metalaxyl-m)	*A. sativa*	> 1000	> 1000	> 1000	NE	1000	> 1000	
	B. rapa	> 1000	> 1000	> 1000	NE	1000	> 1000	
	E. andrei	536.3 (394.4 – 678.3)	205.6 (118.3 – 292.9)	117.3 (43.9 – 190.7)	> 900	250	300	
	F. candida	60.1 (50.08 – 69.1)	42.4 (33.7 – 51.03)	34.4 (24.7 – 44)	30.09 (6.65 – 83.17)	18	50	

NE = Not estimated; * - Data did not allow to estimate 95% confidence intervals

Table 7. EC_{50} (Effective concentration causing 50% reduction), NOEC (no-observable effect concentration) LOEC (lowest-observable effect concentration) and LC_{50} (Lethal concentration for 50% of mortality) values and 95% confidence intervals for the pesticide mixture obtained through the CA model estimation and 95% confidence intervals on *A. sativa, B. rapa, E. andrei* and *F. candida* in artificial soil.

Species	EC_{50}	EC_{20}	EC_{10}	LC_{50}	NOEC	LOEC
A. sativa	1.82 (0.55 - 3.10)	0.30 (-)*	0.10 (-)*	NE	0.52	1.03
B. rapa	0.91 (0.30 - 1.52)	0.18 (-)*	0.07 (-)*	NE	0.28	0.56
E. andrei	1.30 (1.15 - 1.46)	1.03 (0.61 - 1.45)	0.90 (0.37 - 1.43)	1.32 (1.38 - 1.36)	0.69	1.38
F. candida	0.22 (0.10 – 0.33)	0.07 (0.01 – 0.13)	0.04 (-)*	0.20 (0.10 – 0.38)	0.09	0.18

NE = Not estimated; * - Data did not allow to estimate 95% confidence intervals

4. Discussion

4.1 Single exposures

Podium® (herbicide formulation of pendimethalin)

When evaluating the toxicity of an herbicide on soil organisms, it is expected that primary producers (plants) are going to be more affected than organisms from other groups (e.g. soil invertebrates), due to the its mode of action (Vighi et al., 2017). In this study, different sensitivities between the tested species to pendimethalin were observed, being *A. sativa* the most sensitive species. The order of sensitivity to pendimethalin based on the EC_{50} values estimated, from the most sensitive species to the least sensitive, *A. sativa* > *E. andrei* > *F. candida* > *B. rapa* (Table 6). At the highest field recommended dose of Podium® (i.e. 2.64 mg a.i/kg of soil) no toxic effects were observed in any of the species. However, pendimethalin at a concentration 3.9x above the recommended dose reduced by 50% the dry biomass production of *A. sativa*, at 12.5 and 14x reduced by 50% the reproduction of *E. andrei* and *F. candida*, respectively and 31.4x reduced by 50% the dry biomass production of *B. rapa*. These results indicate that a tight safety margin exists for *A. sativa*, and if a contamination in soil

occurs within concentrations up to 31.4x the recommended dose, pendimethalin could impact non-target plant and invertebrate communities. Belden et al. (2005) observed similar sensitivities between plants and soil invertebrates when evaluating the toxicity of pendimethalin as pure active ingredient, in four non-target plant species (three grasses species *Andropogon gerardii*, *Sorghastrum nutans*, *Panicum vergatum* and one eudicot species *Latuca sativa*) and three soil invertebrates (*Folsomia candida, Eisenia fetida* and *Armadillidium sp.*) using a natural soil. Similarly to our results, pendimethalin was less toxic to the eudicot species *L. sativa*, followed by *Armadillidium sp.*, *F. candida*, *E. andrei* and the monocots species. The authors reported EC_{50} values of 7.74, 6.23, 1.09 and 1.33 for *L. sativa*, *P. vergatum*, *A. gerardii*, *S. nutans*, respectively and LC_{50} of 47, 113 and >200 mg ai/kg for *F. candida*, *E. andrei* and *Armadillidium* sp., respectively. However, all plant species were affected by the herbicide with significant decreases in biomass production in concentrations >10 mg a.i/kg.

When comparing the sensitivities between *A. sativa* and *B. rapa*, it is not well understood why oat was more sensitive than turnip. Pendimethalin is a selective herbicide designed to control both herbaceous grass and broadleaf weeds in different crops and research in literature shows different sensitivities between monocot and eudicot crops. Hatzinikolaou et al. (2004) studied the influence of pendimethalin applied as the commercial formulation Stomp 330 EC (330 g a.i/L) ® in a silty clay loam soil on the root growth response in maize (*Zea mays*), oat (*Avena sativa*), sorghum (*Sorghum bicolor*) and sugar beet (*Beta vulgaris*). In their results, oat and sugar beet was more affected in root length than sorghum and corn, with reporting EC_{50} values of 0.34, 0.35, 0.54 and 2.74 µg a.i/g for oat, sugar beet, sorghum and corn, respectively. Smith et al. (2004) reported the phytotoxicity of pendimethalin applied in higher and lower doses in a sandy clay-loam soil (also using the commercial formulation Stomp 330 EC®) using Indian spinach *Basella alba.* The authors observed high phytotoxicity in 0.33 to 1.98 kg a.i/ha concentrations (equivalent to 0.44 to 2.64 mg ai/kg of soil) affecting root dry matter, shoot biomass and plant height. Visual phytotoxic effects in concentrations >0.33 kg a.i/ha exhibited stunted, dark-green, swollen stem and shrunken mottled leaves. However, seedling emergence was not affected in any of the treatments.

In our results, both oat and turnip were not affected in emergence and with increasing pendimethalin's concentrations, visual injuries such as reduced shoot length, root and hypocotyl swelling and stem injury (i.e. stem necrosis) were observed. These symptoms can be explained by the herbicide's mode of action. Dinitroaniline herbicides acts as microtubule disruptor, preventing tubulin from polymerizing into microtubules, inhibiting mitosis in sensitive monocots and eudicot weeds (Hatzinikolaou et al., 2004). The symptoms of this interference of microtubule development are cessation of root growth along with swelling of hypocotyl and root tips (Glover and Schapaugh, 2002). This interference in seedling developments, results in the decreases of plant height, biomass production and seedling mortality (El-Nady and Belal,

2013). However, pendimethalin is not expected to prevent seedling emergence (Smith 2004). Similar crop phytotoxicity in non-target crops have been observed in cucumber (El-Nady and Belal, 2013), rice (Ahmed & Chauhan, 2015), indian spinach (Smith, 2004), oat, sugar beet, sorghum and maize (Hatzinikolaou et al., 2004).

In this work, it was considered the toxicity values for soil invertebrates from a previous study still under preparation (Table 6). This study reports similar sensitivities for *E. andrei* and *F. candida* when exposed to pendimethalin in artificial soil, with reproduction EC_{50} values of 33.1 mg a.i/kg of soil (95% CI: 1.5 - 64.7) for *E.* andrei and 37.1 mg a.i/kg of soil (95% CI: 32.5 - 41.6) for *F. candida* (Table 6). Herbicides are often assumed to be less toxic to soil invertebrates compared to other groups of pesticides due to specific modes of action (Velki and Ečimović, 2017). However, depending on the type of soil, exposure time and type of exposure (e.g. active ingredient or product formulation), the toxicity of herbicides to non-target soil invertebrates may vary (Santos et al., 2012; Niemeyer et al., 2018b; Correia and Moreira, 2010; Brooks et al., 2005). Regarding the effects of dinitroaniline herbicides on soil biota, few studies are available in literature. Chakravorty et al. (2015) evaluated the toxicity of pendimethalin as a commercial formulation (Kristop 30EC) in a natural soil using *Cyphoderus javanus* (Collembola: Hexapoda) as test species under laboratory conditions. In this study, the authors obtained a 24h LC_{50} value of 581 g a.i/ha (0.77 mg ai/kg) and observed a high toxicity compared to the recommend dose (i.e. 1250 g a.i/ha or 1.67 mg a.i/kg). The eggs hatching success was also affected in sublethal doses below the LC_{50}, where significant decrease was observed from 72.6 to 290.5 g ai/ha (0.10 to 0.39 mg a.i/kg). In other study, Haque et al. (2011) using pendimethalin also as the commercial formulation Kristop 30EC and in a natural soil, verified that this herbicide is also toxic to *Xynylla welchi* (Hexapoda: Collembola) with a 24h LC_{50} value of 190 g a.i/ha (0.25 mg a.i/kg), also impacting the hatching success in doses below the LC_{50}. In addition to springtails, earthworms are also affected by the herbicide pendimethalin. Belden et al. (2005) observed significantly decreased biomass in surviving adults of *E. andrei* when exposed to pendimethalin as pure active ingredient, even at the lowest treatment of 10 mg a.i/kg and mortality was observed from 40 mg a.i/kg to 160 mg a.i/kg, with a LC_{50} of 113 mg a.i/kg. Adverse effects of pendimethalin were also observed in beneficial ground beetles (Vommaro et al., 2021) and wasps (Oliver et al., 2009). These studies evidenced that pendimethalin may adversely affect non-target soil biota, however the toxicity intensity might be variable depending on the type of soil, commercial formulation or active ingredient.

Coragen® (insecticide formulation of chlorantraniliprole)

When the test organisms were exposed to chlorantraniliprole, the only sensitive species was *F. candida* (Table 6). The tests performed in this study showed no toxic effects in the evaluated parameter up to 1000 mg a.i/kg of soil for plant and earthworm species.

The high sensitivity of *F.* candida to chlorantraniliprole was expected since insecticide's mode of action are design to affect arthropods (i.e. the target group) (Frampton et al., 2006; Wiles and Frampton, 1996; van Gestel et al., 2017; Hennig et al., 2020). In this study, it was considered the toxicity data of chlorantraniliprole to *F. candida* obtained in Ferreira (2020), where the reproduction EC_{50} in a natural soil was 0.91 mg a.i./kg (Table 6). This author assessed the effect of two insecticides (Chlorantraniliprole and Spirotetramat) on survival and reproduction of different species of springtails in a natural soil, being *F. candida* the most sensitive species to chlorantraniliprole (tested as Coragen®). Similar results were found by Lavtižar et al. (2016), who exposed *F. candida* to chlorantraniliprole (as pure active substance) to assess its effect on reproduction in four different natural soils (Lufa 2.2 and three other natural soils) with different properties. An avoidance behavior test was also performed using Lufa 2.2. The effects on reproduction varied according to the soil organic matter content, with EC_{50} values between 0.14 and 0.76 mg a.i/kg of soil. For the avoidance test, springtails seemed to be more affected by concentrations up to 1 mg Chlorantraniliprole/kg dw. In addition, toxicity data of *F. candida* to chlorantraniliprole (applied as pure active ingredient) reported by US EPA (2008) presents an EC_{50} of 0.48 mg a.i/kg dry soil and a NOEC of 0.39 mg a.i/kg of dry soil for reproduction, however, no information was provided regarding the type of soil. These differences in EC_{50} values of Chloratraniliprole to *F. candida* found between authors may be due to the nature of tested material (i.e. exposed to pure active ingredient or commercial formulation) and the type of soil and its properties used in the experiments.

The reason why chlorantraniliprole is found to be toxic to *F. candida* is well discussed by Lavtižar et al. (2016). The literature shows that crustaceans and other non-target insects are very sensitive to chlorantraniliprole (US EPA, 2008) and since springtails are suggested to be closely related to these taxa, the authors suggest that this might be the reason why they are highly sensitivity to chlorantraniliprole. Also, a comparison among the sensitivities of different non-target species to chlorantraniliprole available in literature was done and the authors showed that some non-target insects (caddisflies and mayflies) are highly sensitive to this insecticide, but honeybees (*Apis mellifera*), lady birds beetles (*Coccinella septempunctata*) and parasitoid wasps (*Aphidius rhopalosiphi, Aphelinus mali, Dolichogenidea tasmanica, Diadegma semiclausum* and *Trichogramma* spp.) are less affected, suggesting that the effect of chlorantraniliprole depends on the insecticide specific binding receptors and, therefore, this

seems to confirm the presence of high affinity of the ryanodine receptor for chlorantraniliprole in *F. candida*.

The results obtained when the plants were exposed to chlorantraniliprole were also expected due to insecticides' mode of action to be highly specific to control insect species. Research in literature regarding the effects of chlorantraniliprole on non-target plants are still scarce. EFSA (2008) reported toxicity data of Coragen 20SC for corn (*Zea mays* L.), oat (*Avena sativa*), onion (*Allium cepa*), perennial ryegrass (*Lolium perenne*), cucumber (*Cucumis sativa*), oilseed rape (*Brassica napus*), pea (*Pisum sativum*), soybean (*Glycine max*), sugar beet (*Beta vulgaris*) and tomato (*Lycopersicon esculenium*) regarding emergence and early seedling growth using an artificial soil mixture as substrate. Within concentration ranges between 2.34 and 300 g a.i/ha (equivalent to 0.003 and 0.4 mg a.i/kg of soil), no toxic effects were observed, with EC_{50} values always >300g a.i/ha. Our results are in accordance with these late, also evidencing that *A. sativa* and *B. rapa* are not affected by chlorantraniliprole. On the other hand, Kilic et al. (2015) observed phytotoxicity of chlorantraniliprole (as Altacor 35WG) in seed germination, stomatal responses in leaves, photosynthetic and proline content of maize plants (*Zea mays*) in concentrations between 0.08 and 0.5 ppm. Maize seeds were pre-treated with chlorantraniliprole in these concentrations for 72 h and then placed in a Petri dish for 7 days for germination in controlled conditions. After that, the germinated seeds were transferred to pots filled with perline for anatomical and physiological observations for 45 days. This study indicates that seeds treated with chlorantraniliprole may have germination and further plant development affected. Contrarily to that suggested by Kilic et al. (2015), our study and EFSA (2008) evidenced that, when chlorantraniliprole is applied directly in soil, in the recommend field doses (i.e. 0.053 mg a.i/kg of soil) and in higher concentrations, no phytotoxicity is observed at least concerning germination and seedling growth.

As occurred for plants, *E. andrei* did not show significant differences in the reproductive performance and in adults mortality compared to control when tested at 1000 mg a.i/kg of soil, evidencing that chlorantraniliprole is not toxic to *E. andrei* even when applied in high concentrations. Our results agree with the toxicity data reported by EFSA (2008), where in a 14-day acute toxicity with artificial soil, test exposing *E. andrei* to chlorantraniliprole as pure active ingredient, a NOEC of 1000 mg a.i/kg of soil and a LC_{50} >1000 mg a.i/kg of soil was found. In terms of chronic toxicity, chlorantraniliprole applied as the commercial formulation DPX-E2Y45 35WG (350 mg chlorantraniliprole/kg dry soil) in an artificial soil, also did not revealed toxicity in reproduction and mortality of *E. fetida*, with a NOEC of 1000 mg formulation product/kg or 350 mg a.i/kg (EFSA, 2008). On the other hand, Liu et al. (2016) evaluated the toxicity of chlorantraniliprole, as pure ingredient, in growth, reproduction and biochemical state (i.e. impact on ROS level, antioxidant enzyme activities and oxidative damage degree) of *E. fetida* in a 42-day experiment, using artificial soil and a concentration range between 0 and 10

mg a.i/kg. The study reported a significant downward trend in cocoon production and number of juveniles at 5 and 10 mg a.i/kg in the end of the test, and during the entire exposure period, no adult's mortality found in any treatment. Oxidative damage to biomacromolecules was also observed in concentrations 5 and 10 mg a.i./kg due to an excess production of reactive oxygen species (ROS). Despite the toxic effects of chlorantraniliprole in *E. fetida* found by Liu et al. (2016), this finding seems to disagree not only with our data, but also with the ones of EFSA (2008), where both commercial formulation and active ingredient were tested up to 1000 mg a.i/kg in artificial soil, and no toxic effects were observed in *E. andrei/fetida* reproduction and survival.

Ridomil Gold Mz Pépite® (fungicide of mancozeb + metalaxyl-m)

As occurred for the insecticide, considering the EC_{50} values, *F. candida* was the most sensitive species to the fungicide, followed by *E. andrei* and *A. sativa* and *B. rapa* (Table 6). The fungicide formulation Ridomil Gold Mz Pépite® significantly decreases reproduction and adult survival of *F. candida* at concentrations corresponding to 15, 30, 45, 75, 90 and 135 times the recommended dose. Several studies have reported negative effects of different type of fungicides to *F.* candida and other Collembola species (Simões et al., 2019; Bandow et al., 2014; Frampton & Wratten, 2000). However, few studies to date have evaluated the effects of Ridomil Gold Mz Pépite® and/or its active ingredients to *F. candida*. In the Rapporteur Assessment Report (RAR) of Metalaxyl-m for ecotoxicological effects on non-target soil macro-organisms (EFSA, 2013b), it is reported a study conducted in artificial soil that estimated an EC_{50} of 231 mg Ridomil Gold/kg soil and a NOEC for both reproduction and mortality of 125 mg Ridomil Gold/kg soil for *F. candida*. These findings are contradictory to those found in this work. Our results evidence that the toxicity of Ridomil Gold is considerably higher to *F. candida* than the ones presented in the RAR, with an EC_{50} of 60.1 (95% CI: 50.08 – 69.1) mg Ridomil Gold/kg of soil and NOEC of 18 mg Ridomil Gold/kg of soil (Table 6). Since both studies were conducted following the same artificial soil (i.e. same components and ratio of elements) and the same product formulation with the same a.i composition, there is no explanation to justify the appearance of a higher toxicity to *F. candida* in our study. A test repetition would be desirable to confirm this high sensitivity, which, if confirmed, should trigger an improvement in the risk assessment data considered in the Ridomil RAR document. When analyzing effects of the single active ingredients of Ridomil Gold Mz Pépite®, Mancozeb have been reported as more toxic to *F. candida* than Metalaxyl-m. Carniel et al. (2019) evaluated the risk of Mancozeb (tested as the commercial formulation Dithane NT) for *F. candida, E. andrei* and *Enchytraues crypticus* in two different natural soils from Brazil. The sensitivity of Mancozeb varied with the species, being *F. candida* and *E. crypticus* the most sensitive, followed by *E. andrei*. Toxicity of Mancozeb to *F. candida* was different between the two soils for reproduction, with an EC_{50}

of 2.72 mg a.i/kg for Oxisol soil and an EC_{50} of >100 mg a.i/kg for Ultisol soil. Concerning Metalaxyl-m, EFSA (2013b) reported, for the pure active ingredient, and considering reproduction and mortality of *F. candida*, a LC_{50} and an EC_{50} > 500 mg ai/kg and a NOEC of 125 mg ai/kg.

Negative effects of fungicides to earthworms have been reported in literature (Yao et al., 2020; Bart et al., 2017;). However, in the present study, for *E. andrei,* the commercial formulation Ridomil Gold Mz Pépite® significantly affected reproduction and mortality only at concentrations of about 90 to 270x the highest recommended dose (i.e. 300 to 900 mg Ridomil/kg of soil). As for *F. candida*, data in literature is still scarce regarding the effects of this fungicide and its active ingredients to earthworms. According to EFSA (2013b), *E. fetida* is not significantly affected in mortality, biomass or reproduction when exposed to doses of Ridomil Gold up to 39.06 mg Ridomil/kg (EC_{50} >39.06 mg Ridomil/kg), which agrees with the results obtained in our study. The active ingredients present in Ridomil Gold Mz Pépite® seems also to be toxic to earthworms only in higher concentrations than the recommended field doses. Carniel et al. (2019) did not find toxic effects of Mancozeb (tested as the commercial formulation Dithane NT) in adult survival and reproduction of *E. andrei* using a natural soil, presenting a LC_{50} > 1000 mg a.i/kg and an EC_{50} > 500 mg a.i/kg. EFSA (2011) reported an EC_{50} >75 mg a.i/kg for reproduction and an LC_{50} of 830 mg a.i/kg in acute toxicity test when exposing *E. fetida* to metalaxyl-m as pure active ingredient. However, a study have shown that metalaxyl-m applied as pure a.i, may cause genotoxic effects in *E. andrei* in lower concentrations (i.e. 0.1 to 3 mg a.i/kg) when exposed to this a.i in artificial soil for 28 days (Liu et al. 2014).

Similarly to that observed with the insecticide, plant species were not affected by the fungicide formulation where neither biomass production nor emergence were significantly different from that of replicated from control treatments, even at 1000 mg a.i/kg of soil. In literature, data on the effects of Ridomil Gold Mz Pépite® to non-target plants are only available through EFSA (2013b), that report a study where the formulation Ridomil Gold Mz (64% Mancozeb + 4% Metalaxyl-m) was tested to evaluate its effects in seedling emergence and vegetative vigor toxicity for onion (*Allium cepa* L.), wild oat (*Avena fatua* L.), suger beet (*Beta vulgaris* Mill.), oilseed rape (*Brassica napus* L.), maize (*Zea mays* L.), soybean (*Glycine max* L.) in a concentration range between 140.63 and 4500 g Ridomil Gold/ha (equivalent to 0.19 and 6 mg Ridomil Gold/kg of soil). In that study, no significant effects between treatments were observed, with an estimated EC_{50} for both parameters> 4500g Ridomil Gold/ha (equivalent to >6 mg Ridomil Golg/kg of soil or almost 2x the maximum recommended dose). Our results are in accordance with these late as also showed a low sensitivity of *A. sativa* and *B. rapa* to Ridomil Gold Mz Pépite®, even at 1000 mg Ridomil/kg of soil. Effects of the active ingredients alone in literature are only available for mancozeb. In a

study reported by EFSA (2017b), onion, oat, tomate, soyben, oilseed rape, carrot and cabbage were exposed to the commercial formulation Tridex 75DG (76.7% mancozeb) in a natural soil in a vegetative vigor test. No significant effects were found in shoot height and fresh and dry shoot weight and also no mortality was observed.

4.2 Mixture exposure

The exposure of the tested species to the pesticide mixture in our study, showed different responses between them after fitting the data in the CA model. Tests with *A. sativa* and *B. rapa* exposed to the mixture evidenced additivity. Therefore, the tested pesticides in mixture do not interact between each other and, because of that, their effects in mixture can be predicted by CA model using toxicity data from each pesticide when acting alone (Faust et al., 1993). These data confirm our second working hypothesis that assumed that CA model was adequate to predict the toxicity of the mixture and contradict the first working hypothesis that assumed the tank mixture is toxic for all non-target species, even at concentrations lower than the respective recommended doses of each PPP in the tank mixture. Additivity have been reported in other studies for other species and other pesticide mixtures. Liu et al. (2013) observed additive effects through CA model in the green algae *Chlorella pyrenoidosa* and the photobacteria *Vibrio qinghaiensis* when exposing these species to multi-component mixtures of more than two components with the active ingredients simetryn, bromacil and hexazinone (herbicides), dodine (fungicide), and proporxur (insecticide). In another experiment conducted by Santos et al. (2011b) the plant species *Triticum sativum* (wheat) and *Brassica rapa* (turnip) were exposed to binary mixtures of glyphosate (herbicide; as Roundup®), dimethoate (insecticide; as Agror®) and spirodiclofen (acaricide; as Envidor®), and the CA model evidenced that the additive effect was observed only for the shoot length of *T. sativum* when exposed to the mixture of dimethoate and spirodiclofen. The other binary mixtures showed antagonisms to the fresh weight and shoot length of both species. An antagonistic effect was also observed in *B. rapa*, although using the Independent Action model (i.e. a predictive additivity model used for chemicals with dissimilar MoA), by Santos et al. (2011a) in a microcosm experiment with the same binary mixture of dimethoate and spirodiclofen. Several studies have defended the accuracy of the CA model to predict toxicity of chemical mixtures. In a review conducted by Belden et al. (2007) on aquatic ecotoxicological studies of mixtures, mentioned that CA model was able to predict 88% of effects in 207 mixtures (194 binary and 13 consisted of more than two pesticides) of 37 studies. Deener (2000) reported an accuracy of the CA model higher than 90% for 202 mixtures, in 26 studies of aquatic systems. On the other hand, Cedergreen et al. (2008) supported that the CA model adequately predicts only 10% of 158 binary mixtures obtained from studies with seven test species (*Vibrio fischeri*,

activated sludge microorganisms, *Daphnia magna*, *Pseudokirchneriella subcapitata, Lemna minor, Tripleurospermum inodorum*, or *Stellaria media*). More recently, Martin et al. (2021) reviewed 1220 studies in both human and environmental mixture toxicology and reported an approximately equal proportion of studies showing additivity by both CA and IA models (28.3%), synergisms (24.3 %) and antagonisms (19.2%).

In the present study, differently from the additive behavior of the pesticide mixture for plants, deviations from the CA model were observed for soil invertebrates with antagonistic effects (i.e. less toxicity than it would be expectable by additivity) to earthworms and synergistic effects (i.e. higher toxicity than it would be expectable by additivity) to collembolans. This means that the additive effect predicted by the CA model was not corroborated for earthworms and collembolans (Spurgeon et al. 2010). The exposure of the pesticide mixture to *E. andrei* resulting into antagonism means that the toxic effect of the three pesticides decreases when acting simultaneously (in mixture). Antagonistic deviations in pesticides mixtures with herbicides, insecticides and/or fungicides have been observed by other authors in *E. fetida*. Most of the studies used binary mixtures to assess the effects. For instance, Chen et al. (2014) observed antagonism using the CA model in an acute toxicity test with *E. fetida* in artificial soil with 10% of *Sphagnum* peat, when the earthworms were exposed to binary mixtures of butachlor (herbicide), imidacloprid and chlorpyrifos (insecitides) for 7 days. In the 14th day, however, the pesticides mixtures conformed to the CA model, showing additivity. In other study, Chen et al. (2018) also observed an antagonism pattern in *E. fetida* when the species was exposed to mixture composed of the herbicide tribenuron-methyl and the fungicide tebuconazole in artificial soil with 10% of *Sphagnum* peat. However, the authors used the Additive Index method (Marking, 1977) to predict the mixture toxicity. In another study, Wang et al. (2016) also reported antagonistic effects in *E. fetida* when exposed to a ternary mixture composed of atrazine (herbicide), chlorpyrifos and lambda-cyhalothrin (insecticides). Antagonisms through the CA model have been also observed for other soil invertebrates, such as the isopod *Porcellionides pruinosus* when exposed to a mixture of dimethoate and spirodiclofen and the collembolan *F. candida* when exposed to a mixture of dimethoate and glyphosate (Santos et al., 2010).

In the terrestrial environment, antagonism do not pose an additional risk to non-target species due to a decreased toxicity than the predicted by an additivity model. However, synergisms are a matter of concern, as it increases the risk expected for the pesticides when applied in mixture. In the present study, when the collembolan *F. candida* was exposed to the pesticide mixture, a synergism was observed and the pesticide mixture evidenced significant toxicity at the concentration which represents the mixture with each pesticide at the respective recommended doses (i.e. 0.18). Synergisms between PPPs to *F. candida* have been reported in literature. Amorim et al. (2012) reported a synergism in *F. candida* (by measuring

reproduction and adult survival) when exposing collembolans to a pesticide mixture of dimethoate (insecticide) + atrazine (herbicide) and dimethoate + lindane (insecticides), through the CA model. In other soil invertebrates, such as earthworms, the occurrence of synergisms has been also reported. Santos et al. (2011a) observed a synergistic effect in the avoidance behavior of earthworms in a microcosm experiment with *B. rapa* and *E. andrei*, when exposed the organisms to a binary mixture of dimethoate (insecticide) and spirodiclofen (acaricide) at concentrations 10 times higher than the recommended doses of each pesticide of the mixture. Ternary and quaternary mixtures of pesticides (comprising, at least, one insecticide) have also presented synergistic effects to *E. fetida* in acute experiments in artificial soil (Yang et al., 2017). Synergetic effects between chemicals are likely to be promoted by alterations in processes that may influence toxicity of mixtures towards the organisms, namely processes interfering in chemicals bioavailability (i.e. one chemical may affect the availability of the other to the organisms), in uptake and internal transportation of chemicals in the organisms body (e.g. competition by biological ligands or competitive inhibition of transport proteins) and chemical competition to the target site and detoxification processes in the organisms (Cedergreen, 2014). More research would be needed to understand the reason that made the toxicity of the pesticide mixture higher for *F. candida* (compared to that of the other test species) and to understand what was the pesticide of the mixture that mostly contributed to the high toxicity and the synergism observed for *F. candida*.

This species-specific mixture toxicity response found between *E. andrei* and *F. candida* contradict our second hypothesis that assumed deviations to the CA model is independent on the organisms group. Some authors found that the accuracy of CA model is dependent on the tested species and on the elements of the mixture. For instance, Santos et al. (2010) investigated the effects of binary mixtures with glyphosate (herbicide), dimethoate (insecticide) and spirodiclofen (acaricide) to the collembolan *F. candida* and the isopod *Porcellionides pruinosus*. The authors found that the accuracy of CA model depends on the species and on the elements of the mixture when observing different effects (i.e. additivity and antagonism) for the species in the same mixture. Amorim et al. (2012) and Loureiro et al. (2009) also observed different responses patterns through CA model in the effects of binary mixtures with Zinc, Cadmium, Lindane (insecticide), Dimethoate (insecticide) and Atrazine (herbicide) to *F. candida*, *Enchytraeus albidus* and *P. pruinosus* sensitivity. The data reported by these authors support our findings in the present study.

5. Conclusions

At the recommended doses of each pesticide, no effects were observed in any of the species used in the experiments. However, toxicity of the pesticide mixture varied between species, which was evidenced by the CA model.

Pesticide mixture showed additivity response for *A. sativa* and *B. rapa*, antagonism to *E. andrei* and synergism to *F. candida*. The pesticide mixture in a dose corresponding to the mixture of each pesticide in the respective recommended doses was significantly toxic only to *F. candida*.

The results found in this work highlights that species in soil ecosystems respond differently to the combined application of different PPPs as a mixture, with deviations from the CA varying according to the route of exposure of the test organism.

These findings reinforce the need to better understand the toxicity associated to the use of PPPs simultaneously (i.e. in mixture) and its risk to non-target organisms. Data obtained also reinforce that the CA model can be an important tool to predict and characterize the toxic effects of mixtures to non-target organisms.

Chapter III: Main Findings

In the laboratory tests exposing the PPPs individually, different responses between the four non-target species were observed to each of the three PPPs. The insecticide Coragen® (PPP of chlorantraniliprole) was toxic only to *F. candida*, while the fungicide Ridomil Gold Mz Pépite® (PPP of mancozeb + metalaxyl-m) was more toxic to *F. candida*, but also revealed toxicity to *E. andrei*. The herbicide Podium® (PPP of pendimethalin) was more toxic to *A. sativa*, followed by *E. andrei, F. candida* and *B. rapa*. However, the toxic doses for each PPP individually were always higher than the highest recommended doses of the respective products.

When the tested species were exposed in laboratory tests to the pesticides in mixture, the toxicity varied between species, which was evidenced by the CA model. For *A. sativa* and *B. rapa,* the pesticide mixture showed additivity response, while for *E. andrei* an antagonism was revealed and synergism to *F. candida.* An important finding was that the pesticide mixture in a dose corresponding to the mixture of each pesticide in the respective recommended doses was significantly toxic only to *F. candida*.

These findings demonstrates that species in soil ecosystems respond differently to the combined application of different PPPs as a mixture, with deviations from the CA varying according to the route of exposure of the test organism.

This study contributes to a better understanding of the toxicity associated to the use of PPPs simultaneously (i.e. in mixture) and its risk to non-target organisms. The results obtained also reinforce that the CA model can be an important tool to predict and characterize the toxic effects of mixtures to non-target organisms.

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