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Further Advances on Soil Microarthropod Community Testing for the Risk Assessment of Plant Protection Products

Dissertação no âmbito do Mestrado em Biologia, orientada pelo Professor Doutor José Paulo Sousa e Doutor Tiago Natal da Luz e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Avanços em Testes de Comunidades de Microartrópodes do Solo para a Avaliação de Risco de Produtos Fitofarmacêuticos

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Resumo

Actualmente, os impactos das actividades antropogénicas na biodiversidade e, consequentemente, na provisão de serviços do Ecossistema são bem conhecidos nas sociedades. Na Europa, os governos e autoridades têm vindo a abordar estes problemas. Projectos como a Estratégia para a Biodiversidade para 2030, construídos pela Comissão Europeia e Estados Membros com o objectivo de '' Trazer de volta a Natureza nas nossas Vidas'' são exemplos disso mesmo. Este projecto reflecte compromissos chave e esforços que devem ser feitos para proteger a Natureza, incluindo terra e mar, das actividades humanas, para além da integração de corredores ecológicos para proteger as espécies e a biodiversidade.

Não obstante, algumas actividades que prejudicam a Natureza ainda são necessárias para o bem-estar e para a humanidade prosperar, tal como o uso dos pesticidas. Os pesticidas têm vindo a ser usados desde há muito tempo, por exemplo, para proteger as culturas contra potenciais pragas que de outra forma poderiam representar uma grande ameaça para as populações humanas ao alimentar-se e, consequentemente, destruindo essas culturas. Porém, os potencias efeitos adversos do uso de pesticidas, nomeadamente, em organismos do solo que não são alvo destes ainda não são bem conhecidos, mas poderão tornar-se contraprodutivos a longo prazo. Estes organismos contribuem para uma variedade de funções do Ecossistema do solo, tal como a decomposição de matéria orgânica que por sua vez influencia a fertilidade do solo, que é vista como um serviço essencial dos Ecossistemas para a Humanidade.

A Autoridade Europeia para a Segurança Alimentar é responsável por abordar estes problemas e, juntamente com os Estados Membros, criaram mecanismos para avaliar o impacto dos pesticidas nos Ecossistemas. De facto, a avaliação do risco ambiental é obrigatória para testar a segurança de contaminantes, incluíndo as substâncias activas presentes nos pesticidas. A avaliação do risco em organismos não-alvo do solo expostos a Produtos Fitofarmacêuticos é feita através de uma metodologia complexa que envolve uma abordagem por vários níveis. Esta avaliação começa com testes laboratoriais padronizados com espécies indicadoras e prossegue com testes mais complexos e análises em mais espécies se o risco calculado para elas for elevado nos níveis mais baixos de testagem.

Ao longo dos anos têm sido feitos esforços para desenvolver novos pesticidas que sejam mais seguros para o Ambiente, entre eles encontram-se o Clorantraniliprole e o Espirotetramato, que são substâncias activas pertencentes a classes químicas desenvolvidas recentemente, as diamidas antranílicas e os cetoenóis cíclicos, respectivamente. Tendo em conta isto, é necessária mais informação acerca dos efeitos destas novas substâncias em organismos não-alvo do solo.

Neste trabalho foram usados dois insecticidas: o Coragen SC 20 e o Movento O-TEQ que têm como substâncias activas o Clorantraniliprole, e o Espirotetramato, respectivamente. Os principais objectivos deste trabalho foram: 1) avaliar os efeitos destes insecticidas em comunidades de microartrópodes usando testes de comunidades, nomeadamente identificar possíveis mudanças na

abundância e na composição das comunidades; 2) avaliar a influência/relevância do modo de acção dos dois insecticidas nos dados de efeitos provocados ao nível da comunidade; 3) perceber até que ponto as doses recomendadas dos dois insecticidas afectam as comunidades do solo; 4) perceber até que ponto os testes de comunidades, comparando com os testes de uma única espécie, permitem ir mais além em termos de dados em relação aos efeitos dos Produtos Fitofarmacêuticos.

O solo e as amostras contendo a comunidade natural de microartrópodes foram colectados na Herdade do Freixo-do-Meio (Alentejo, Sul de Portugal). Dois testes de comunidades foram realizados, um para cada insecticida. Os insecticidas foram misturados no solo e um gradiente de concentrações foi preparado: 0, 0.01, 0.03, 0.1, 0.4, 1.2, 4, 13, 45, 150, e 500 mg i.a./kg. Três amostras contendo os microartrópodes foram extraídos directamente para o solo teste. Após este passo, os microcosmos contendo o solo e os organismos foram incubados em condições de laboratório durante 6 semanas, após as quais, os microartrópodes foram novamente extraídos. Apenas ácaros e colêmbolos foram encontrados em número suficiente para permitir a avaliação dos efeitos. Os colêmbolos foram classificados de acordo com as suas características morfológicas e os ácaros foram divididos em 4 ordens: Oribatida, Astigmata, Prostigmata e Mesostigmata.

Para o teste com o Coragen SC 20, tanto ácaros como colêmbolos foram afectados ao longo do gradiente de contaminação. Isto reflectiu-se em mudanças na abundância de grupos taxonómicos dos ácaros e mudanças na composição funcional dos colêmbolos. Os colêmbolos foram mais sensíveis com base nos valores de toxicidade derivados: EC_{10} = 10.46 mg i.a/kg e NOEC = 4 mg i.a./kg; reflectindo-se também num grande descréscimo na abundância. Por outro lado, os ácaros não foram directamente afectados pela toxicidade com base nos valores: EC_{10} = 497 mg i.a./kg e NOEC = 150 mg i.a/kg; inclusive, os ácaros do grupo Oribatida tiveram um grande aumento de abundância.

Em relação ao teste com o Movento O-TEQ os efeitos foram menos evidentes. Foi observada uma mudança na abundância de grupos taxonómicos dos ácaros ao longo do gradiente de contaminação, nomeadamente em doses mais altas, onde os Astigmata tiveram um grande decréscimo em abundância e os Oribatida tornaram-se mais dominantes. Os ácaros foram bastante mais sensíveis comparando com o primeiro teste com o Coragen SC 20, com base nos valores EC_{10} = 18.98 mg i.a./kg e NOEC = 0.4 mg i.a./kg. Por outro lado, os colêmbolos praticamente não foram afectados com base nos valores EC_{10} = 196.53 mg i.a./kg e NOEC = 150 mg i.a./kg; os seus números até aumentaram em doses mais altas, excepto na última. Não ocorreram mudanças na composição da comunidade de colêmbolos, com base nos valores de mT calculados, o que tem a ver com o facto de que a maioria das espécies presentes na comunidade ter características morfológicas semelhantes.

Palavras Chave: Insecticidas; Clorantraniliprole; Espirotetramato; Testes de Comunidades; Microartrópodes

Abstract

Nowadays the impacts of anthropogenic activities on biodiversity and consequently on the provision of Ecosystem services are well known in societies. In Europe, governments and authorities have been addressing these issues and an example is the Biodiversity Strategy for 2030 constructed by the European Commission and the Member States aiming to ‘’Bring Nature back to our Lives’’. It reflects key commitments and efforts that should be made to protect Nature, including land and sea, from human activities, as well as to integrate ecological corridors to protect species and biodiversity.

Notwithstanding, some activities that harm Nature are still needed for the well-being and to humanity thrive, such as the use of pesticides. Pesticides have been used for a long time, for instance, to protect crops against potentially harmful organisms that in another way could represent a great threat to human populations by feeding on and destructing those crops. However, the potential side effects of using them, for example, on non-target soil organisms are not very clear but they might turn to be counterproductive in the long run. These organisms contribute to a range of Ecosystem functions such as the decomposition of organic matter which in turn influences soil fertility, an essential Ecosystem service for Humanity.

The European Food Safety Authority oversees these issues and along with the Member States created tools to assess the impact of pesticides on ecosystems. Indeed, the environmental risk assessment is mandatory for testing the safety of numerous chemicals. The risk assessment of non-target soil organisms exposed to Plant Protection Products comprises a complex methodology that includes a tiered approach and begins with standard laboratory tests with standard indicator species and moves on with more complex tests and analysis on more species if the risk indicated to them is high in lower tiers.

Throughout the years' efforts to develop new and safer pesticides have been conducted, among them, Chlorantraniliprole and Spirotetramat are active substances belonging to recently developed classes, the anthranilic diamides, and the cyclic ketoenols, respectively.

Considering this, more information on the effects of these new substances on non-target soil organisms are mandatory. In this work, it was used two insecticides: Coragen SC 20 and Movento O-TEQ which have Chlorantraniliprole and Spirotetramat as active substances, respectively. The main objectives were to 1) evaluate the effects of these two insecticides on communities of microarthropods using community tests, including changes in abundance and possible shifts in community composition; 2) evaluate the influence/relevance of the mode of action of the two insecticides in the effect data provoked at the community level; 3) understand how far the recommended doses of both insecticides affect soil communities; 4) understand how far community tests, comparing with single-species tests, permit to go beyond in terms of effect data on PPPs.

The soil and soil cores containing the natural community of microarthropods were collected at Herdade Freixo do Meio (Alentejo, South of Portugal). Two community tests were performed, one

for each insecticide. Insecticides were mixed into the soil and a gradient of concentrations was prepared: 0, 0.01, 0.03, 0.1, 0.4, 1.2, 4, 13, 45, 150, and 500 mg a.i./kg. The microarthropods of three soil cores were extracted directly to test the soil. After this step, the soil microcosms were incubated under laboratory conditions for 6 weeks, after which microarthropods were extracted again. Only Acari and Collembola were present in sufficient numbers to be assessed. Collembolans were classified according to morphological traits and mites according to 4 orders: Oribatida, Astigmata, Prostigmata, and Mesostigmata.

For the test with Coragen SC 20 mites and collembolans were affected along the contamination gradient with shifts in the abundance of taxonomic groups of mites and changes in the functional composition of collembolans. Collembolans were more sensitive based on the toxic values derived: EC_{10} = 10.46 mg a.i./kg and NOEC = 4 mg/kg; reflected by a strong decrease in abundance. On the other hand, mites were not directly affected based on EC_{10} = 497 mg a.i./kg and NOEC = 150 mg a.i./kg; Oribatida had a strong increase in numbers.

Regarding the test with Movento O-TEQ effects were less evident, a shift in the abundance of taxonomic groups of mites was observable, namely at higher doses where Astigmata had a strong decrease and Oribatida become more dominant. Mites were much more sensitive compared with the first test with Coragen SC 20, based on EC_{10} = 18.98 mg a.i./kg and NOEC = 0.4 mg a.i./kg. In contrast, collembolans were practically unaffected based on EC_{10} = 196.53 mg a.i./kg and NOEC = 150 mg a.i./kg; their numbers even increased at higher doses, except at the last. A shift in the community composition of collembolans did not occur based on the mT values derived and this was due to the fact most species had similar traits.

Key Words: Insecticides; Chlorantraniliprole; Spirotetramat; Community tests; Microarthropods

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Chapter 1 - General Introduction

General Introduction

I.1 Soil Ecosystems (biodiversity and the provision of Ecosystem services)

Cardinale et al. (2012) defined biodiversity as the ‘’variety of life’’, including variation among genes, species, and functional traits. Also, it is the mechanism behind the performance of an Ecosystem (Wagg et al., 2014).

Often, biodiversity is associated with the wide range of living organisms that live above-ground, disregarding the fact that a large proportion of the biodiversity within terrestrial ecosystems is hidden below ground in soils (Wagg et al., 2014).

Soils present a large diversity, being estimated that 1g of soil contains up to 1 billion bacteria cells consisting of tens of thousands of taxa, up to 200m fungal hyphae, and a wide range of nematodes, earthworms, mites, and other arthropods (Bardgett, 2005; Roesch et al., 2007; Wu et al., 2014). This great diversity contributes to the total terrestrial biomass and is closely related to the above-ground biodiversity, due to its dependence on the ecosystem functions that the below-ground organisms provide (Fierer et al., 2009; Wardle et al., 2004).

Concepts like natural capital and ecosystem services were developed to raise the attention of organisations, companies, and policymakers on the impacts of anthropogenic activities towards Nature. These activities are necessary to the development of societies, but their relation to the crescent decline of biodiversity affects ecosystem functions, the basis of ecosystem services, that in turn, are essential to human well-being and health (Burkhard and Maes, 2017; Costanza et al., 1997; MEA, 2005). The natural capital represents natural resources such as soils, trees, minerals, water, all living organisms, and so on. For a long time, the value of ecosystems to societies was underrated in public and business decisions, compared to economic services and manufactured capital such as machines and buildings (Burkhard and Maes, 2017; Costanza et al., 1997). Costanza et al. (1997) defined ecosystem services ‘’as a flux of materials, energy, and information from natural capital stocks which combine with manufactured and human capital services to produce human welfare’’. Thus, changes in this flow will certainly have consequences for human populations.

Ecosystem functions depend either on the biotic part (biodiversity) and the abiotic part (e.g. soil properties, geomorphology, climate) of an ecosystem, in order to deliver specific services, which

represent the benefits human societies can obtain, directly or indirectly, from ecosystem functions. For example, primary production and pollination (ecosystem functions) are essential to supply food production (ecosystem service). Water infiltration capacity (function) is necessary to water provision (service), and organic decomposition (function) as an impact on soil fertility (service) (Burkhard and Maes, 2017; MEA, 2005).

The European Food Safety Authority (EFSA) pointed out seven ecosystem services provided by in-soil organisms. They include genetic resources and biodiversity. Nutrient cycling, which refers to the degradation of organic matter by detritivores which in turn is mineralised by microorganisms, making nutrients available for plants to grow. Regulation of pest populations and disease outbreaks on plants, because some in-soil organisms can play a role in controlling pests affecting crops. As well as soil structure formation, water retention and regulation, since the activity of in-soil organisms is related to the aggregate formation, relief of soil compaction, and the regulation of soil water-holding capacity (EFSA, 2017).

Nowadays, there is an increasing concern with the loss of species due to the impact of anthropogenic activities. For instance, agricultural practices that use tillage operations and the application of fertilisers and pesticides may shift the balance of an ecosystem by decreasing species diversity and altering the soil food web **(**Coleman, 2015; Moore and Ruiter, 2012; Rieff et al., 2020**)**. Indeed, land and soil degradation (for example through erosion, loss of structure, acidification, loss of organic matter, and other types of degradation), the decline of biodiversity, and consequently, the loss of ecosystem functioning can have implications on key ecosystem services like food production (Koch et al., 2013).

Tsiafouli et al. (2015) reported that the intensive land use in 4 regions of Europe (Sweden, United Kingdom, Czech Republic, and Greece) resulted in a decrease of community-weighted mean body mass of soil fauna as well as in the average trophic level and diversity among functional groups in the food web. The authors also highlighted the entire loss of some functional groups, as occurred in Greece, in which earthworms and predaceous collembolans disappeared in fields submitted to intensive rotations. Moreover, soil communities had fewer species comparing to land under low intensive farm management, and they were more taxonomically related. On one hand, this may allow a higher functional redundancy, meaning that various species closely related can contribute to the same functions, allowing a higher resilience of those ecosystem functions. On the other hand, the reduction in soil taxonomical diversity, in some cases, can affect soil functioning by decreasing the range of processes provided by the soil ecosystem which form the basis of the provision of soil ecosystem services (Tsiafouli et al., 2015; Vries et al., 2013; Wagg et al., 2014).

The relevance of a functional group is related to the contribution that the activity of each species of that group provides to the performance of a specific function. For instance, earthworms, among other functions, contribute to soil formation by accumulating casts (excretion material containing digested soil, plant material, and microbial agents that proceed with nutrients processing) in soil. They are essential for managing the structure and aeration of soil because they both compact and

loosen soil, creating macropores through burrowing activities (Blouin et al., 2013). These air-filled pores created by earthworms are critical in helping plant roots to thrive. Furthermore, they also play a role in the processes of carbon and nitrogen cycling (Vries et al., 2013). Thus, the loss of a functional group will eliminate the soil function they provide (Tsiafouli et al., 2015).

In Europe, the preservation of biodiversity, ecosystems, and its functions are subjects that are in the order of the day. Efforts from the European Union are reflected through the tentative of implementing a Circular Economy with measures like turning sustainable products the norm in Europe and ensure less waste. Also, the Biodiversity Strategy for 2030 entitled ‘’Bringing Nature back to our Lives’’ is being implemented. In this document, key commitments are addressed in terms of Nature protection: to legally protect a minimum of 30% of the EU’s land area and 30% of the EU’s sea area and integrate ecological corridors, as a part of a Trans-European Nature Network; to strictly protect at least a third of the EU’s protected areas, including all remaining EU primary and old-growth forests; effectively manage all protected areas, defining clear conservation objectives and measures, and monitoring them properly. Moreover, as part of the key commitments by 2030 on the EU Nature Restoration Plan, some measures to be implemented are the reduction by 50% on the use of chemical pesticides and in the risk associated with them. The turning off 25% of agricultural land to organic farming. To reduce nutrient loss from fertilisers by 50%, resulting in the reduction of the use of fertilisers by 20%. The prohibition to use chemical pesticides in sensitive areas (European Commission, 2020).

I.2 Soil Fauna

Soil organisms represent a large part of the world’s biodiversity and are key drivers of ecosystem processes such as nutrients (e.g. nitrogen, carbon, etc) and organic matter recycling (Menta, 2012). Generally, the soil fauna is classified according to their body size into microflora/microfauna (< 0.1 mm), mesofauna (0.1 – 2 mm), and macro/megafauna (> 2 mm). Members of the microflora/microfauna include bacteria, fungi, archaeans, protozoa, small mites, nematodes, rotifers, tardigrades, and some crustaceans. The category of mesofauna includes organisms like most nematodes, microarthropods (mites and springtails), enchytraeids, and also some tardigrades, proturans, diplurans, rotifers, small araneideans, pseudoscorpions, opiliones, some insect larvae, small isopods, and myriapods. Macro/megafauna includes invertebrate organisms like earthworms, gastropods, myriapods, some isopods, some araneideans, and the majority of insects but also some vertebrates like small rodents, reptiles or amphibians are part of this group (Coleman, 2015; EFSA, 2017; Menta, 2012).

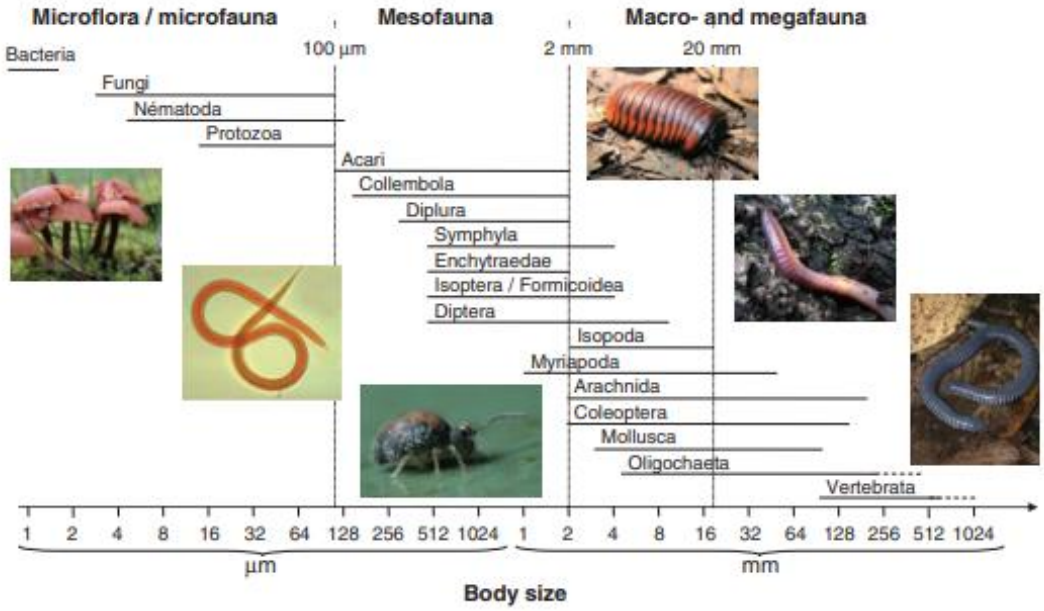

Fig.1 – Representation of the main taxonomic groups of soil organisms based on body-size (Swift et., 1979); Decaens (2010); EFSA (2017) (all photo credits: Flickr, http://www.flickr.com/)

The body size of soil fauna is often related to their (micro)habitats and to the role they play on soil processes. The microfauna inhabit water films, the mesofauna lives in existing air-filled pore spaces and use them for locomotion in soil, and the macrofauna (with a higher locomotor capability), through burrowing, can create their own spaces (Coleman, 2015). Regarding the participation of the different soil fauna to soil functions, three categories have been proposed: ''ecosystem engineers'', like earthworms, ants or termites which play a role on soil structuration and contribute to the flux of nutrients and energy; ''litter transformers'', such as the microarthropods, by fragmenting and decomposing litter, they turn it available for microbes; and ''micro-food webs'', composed by microbes and their direct microfaunal predators (nematodes and protozoans). According to their body sizes, these three groups work on different spatial and time scales (Coleman, 2015; Lavelle et al., 1995; Wardle, 2002).

In the soil food web, mesofauna organisms can be plant feeders, microbial feeders, omnivores, or even predators, covering all the trophic levels. In terms of contribution to soil functions, they are primary consumers because they feed directly on roots but also have an indirect role in primary consumption through their participation in decomposition and nutrient recycling (Crossley et al., 1992; McBrayer et al., 1977; Neher and Barbercheck, 1998).

Soils with high porosity, high organic matter content, and well-structured horizons, generally, have the highest abundance and diversity of mesofauna (Andrén and Lagerlöf, 1983; Neher and Barbercheck, 1998). In agricultural soils, mesofauna (and also microbial) organisms are more abundant within the top 20 cm of soil (usually the tilled layer), on the other hand, in uncultivated

soils, mesofauna is more abundant in the top 5 cm of the soil. Their activity, in the habitable pore spaces, is influenced by the balance between water and air (Neher and Barbercheck, 1998).

Mites, springtails, and nematodes are the most common organisms in the soil in terms of abundance, biomass, and species within the mesofauna (Harding and Studdart, 1974; Samways, 1992).

Within the microarthropods, soil mites belong to the class Arachnida and to the subclass Acari, which are known to be the most abundant organisms in the soil, counting with approximately 55,000 species described and identified. The exact number of mite species may be much larger, given the great abundance that can be found on a small portion of soil and the fact that some ecosystems may be less explored (Walter and Proctor, 2013). The subclass Acari is divided into two superorders: Acariformes and Parasitiformes. Acariformes are represented in three orders which are Oribatida, Prostigmata, and Astigmata. Parasitiformes are mainly represented by a large order, the Mesostigmata (Koehler, 1997).

Oribatida is the most common group of edaphic mites inhabiting forest soils and litter habitats (Behan-Pelletier and Walter, 2000; Hartenstein, 1962). The vast majority are typically saprophages, feeding on vegetal or animal detritus, and mycophages, consuming fungal spores (Krantz and Walter, 2009). Despite this, some authors refer that opportunistic predation on nematodes and other microfauna, as well as necrophagy (scavenging on small dead arthropods) can occur in species from this suborder (Luxton, 1972; Schneider et al., 2004).

Mites from the order Prostigmata can be mycophagous (Kethley, 1990; Lindquist, 1986; Walter and Proctor, 2013), but most of these mite families are predatory, feeding on nematodes, small arthropods (like collembolans), and their eggs (Kethley, 1990). They can also be parasitic or feed on algae (phytophagous) as it happens with species belonging to the family Tarsonemidae (Jeppson, Keifer, and Baker, 1975; Lindquist, 1986).

On the other hand, mites from the order Mesostigmata can be found in a large range of habitats, from the soil, litter and rotting wood, to the manure, carrion, and house dust, going through intertidal areas and the margins of freshwater systems (Krantz and Walter, 2009). Most of the species found in this order are free-living predators, feeding upon nematodes and microarthropods, like mites and collembolans, and their eggs (Evans, Sheals, and MacFarlane, 1961; Krantz and Walter, 2009; Price, 1973). Moreover, some Mesostigmara mites can parasitise or be symbionts of mammals, birds, reptiles, or arthropods (Strandtmann and Wharton, 1958; Walter and Proctor, 1999; Yunker, 1973) and, even though it is less frequent, some can feed on fungi, pollen, or nectar (Walter and Proctor, 1999).

The order Astigmata in terms of habitat can be found in places like decaying logs, fungal fruiting bodies, dung, carrion, and caves, where they are mainly saprophages, feeding on decaying material, bacteria, but preferentially on fungi (Krantz and Walter, 2009; OConnor, 1984). There is also evidence of adaptation to consuming seeds or plant tissues as bulbs and tubers, algae in aquatic species, and some can even predate on nematodes and their eggs (Krantz and Walter, 2009; Muraoka and Ishibashi, 1976).

Soil mites are beneficial to humans because some can prey on invertebrate pests of agricultural and ornamental crops offering, in many cases, an alternative to chemical control measures. As an example, to control synanthropic fly species that breed in dung, some species of the family Macrochelidae (order Mesostigmata) were used successfully as control agents **(**Krantz, 1983, 1998; Rodriguez, Singh, and Taylor, 1970). The predatory mite *Gaeolaelaps aculeifer* (Canestrini) has been also used as a natural control agent in laboratory tests. In the field, this mite is common in soils cultivated with lily bulbs, where it suppressed populations of the plant nematode *Tylenchorhyncus dubius* (Butchli) in an efficient way (Sharma, 1971).

Moreover, some mites feed on noxious plants and have been used in weed control programs. *Aceria chondrillae* (Canestrini), a Prostigmata, has been used as a biological control agent of *Chondrilla juncea* (L.), commonly named rush skeletonweed (Rosenthal, 1996). In wheat plantations, this weed competes for nutrients and moisture, and the upper parts can obstruct harvesting machinery (Cullen, 1985). *A. chondrillae* form galls in the flower buds, reducing seed production and making the plant more vulnerable, causing early senescence and eventual death (Caresche and Wapshere, 1974). This mite was introduced into Australia in 1971, to control the spread of rush skeletonweed by reducing its seed production (Cullen et al., 1982), where it became well established and within two years was causing plant deaths (Wapshere, 1978). The number of viable flowers produced by this weed were reduced by 95.6% with the high mite treatment (500 or more mites placed on the plant) and by 73.6% in the low mite treatment (10 adult mites per plant) compared to control (Cullen et al., 1982). Another example is the control of *Convolvulus arvensis* (L.), an aggressive perennial weed found worldwide in temperate regions (Holm et al., 1977), by the prostigmatic mite *Aceria malherbae* (Nuzzaci). These mites attack the bud, preventing stem growth and elongation, besides forming galls in young leaves causing them to curl, gaining a yellow-green or reddish appearance, the leaf cells become hypertrophic, forming papillae (Rosenthal, 1983). *A. malherbae* has been released in the United States since 1987, becoming well established in Texas (Boldt and Sobhian, 1993).

Mites that dwell in litter and that are not predators can be effective nutrient recyclers in forest ecosystems by degrading organic litter to a size appropriate for use by other decomposers **(**Krantz and Walter, 2009).

Notwithstanding, some mite species are severe pests of crops and ornamentals since they feed on root tissue, corms, or bulbs in the rhizosphere, causing economic impacts. Species of *Rhizoglyphus* (Astigmata) feed on tulips, onions, hyacinth bulbs, *Gladiolus* corms, and potatoes. The oribatid mite *Perlohmania dissimilis* damages the roots of potatoes, strawberries, and tulips with its feeding activity (Evans, Sheals, and MacFarlane, 1961; Krantz and Walter, 2009).

Concerning Collembola (the most abundant group belonging to the mesofauna, after mites), also known as springtails, they belong to the subphylum Hexapoda and their size ranges between 0.2 to 5 mm of length, making it possible to live in air-filled soil pores and litter, like mites. They are abundant in the soil and soil litter, and the water surfaces can also be colonised, especially when vegetation is present (Hopkin, 1997**)**. Collembolans have a wide global distribution, with species

occurring even in places like Antarctica **(**Salmon, 1962**)**, the Himalayas **(**Yosii, 1971**)**, and the Australian deserts **(**Suhardjono and Greenslade, 1994**)**. Approximately 6500 species have been described, although the real number can be much larger **(**Hopkin, 1997; Rusek et al., 1988**)**.

Functional classification of collembolans (Verhoef and Brussard, 1990**)** can be based on gut content or shape of the mouthparts, which are adapted to the specific feeding habit **(**Swift et al., 1979).

Some authors consider them as generalist feeders (Johnson et al., 2005; Rusek, 1998). Their feeding habits are mostly decaying vegetation and fungi but, in some cases, they can feed on nematodes and plant roots in the soil (Rusek, 1998). Because of that, they can be important biological control agents for crops since they can consume pathogenic fungi. Nakamura et al. (1992) reported that the grazing by *Sinella eurviseta* (Entomobryidae) on the cucumber fungus *Fusarium oxysporum* suppressed the infection and produced healthy plants in comparison to ungrazed controls that were hardly affected. Furthermore, at certain densities of Collembola, grazing of mycorrhizae on roots can stimulate the growth of the symbiont, improving plant growth (Lussenhop, 1996). On the other hand, in consequence of their wide dietary spectrum, they may switch diet based on resource availability and palatability (Endlweber et al., 2009). They may become pests when a preferred food source is absent, as is the case of the root-grazing injury on sugar beet, which is caused by *Onychiurus* spp. (Collembola), rubbing their bodies against roots (Curl et al., 1988). However, if specific weed species and certain amounts of organic matter are present, this type of root injuries can decrease since they become the preferred food source of collembolans.

Collembolans are distributed vertically on the soil profile and can be classified according to that distribution. As described in Martins da Silva et al. (2015), organisms with small antennae, lack of ocelli, and reduced pigmentation are assumed to be more euedaphic and, consequently, with lower dispersion ability, they are more adapted to deeper soil layers. Thus, in soils with thicker litter layers, normally there is a higher richness of euedaphic collembolans (Martins da Silva et al., 2012), having a higher resource availability and the ideal moisture conditions (Hopkin, 1997; Berg and Bengtsson, 2007).

On the other hand, eyed, pigmented organisms, with longer antennae are assumed to move in the upper soil layers, with a higher ability to disperse and colonise new areas (epiedaphic collembolans). Hemiedaphic collembolans usually have mixed characteristics between epiedaphic and euedaphic and are present in the intermediate soil layers.

Collembolans can also be important food sources for predatory mites (Edwards and Thompson, 1973). When conditions are favourable, for instance, if the population of predatory mites decreases, springtail populations usually have a rapid increase in abundance, so they are considered opportunistic microarthropods (Edwards and Thompson, 1973; EFSA, 2017; Sheals, 1953, 1955, 1956).

In terms of functional roles in soil ecosystems, they play a major part in plant litter decomposition processes, with intensive cycling of nutrients during their life cycles, and on forming soil microstructure (Hopkin, 1997; Rusek, 1998).
The number of collembolans and mites in soil has a large fluctuation according to different time seasons, having peak populations in spring and autumn in temperature climates (Edwards and Thompson, 1973).

I.3 Pesticides

Pesticides are mixtures of substances or biological agents that are released into the environment with the purpose of killing/control pests, including insects, rodents, weeds, fungi, and other harmful pests. Thus, according to the organisms to be controlled (the target organisms), pesticides can be insecticides, rodenticides, herbicides, fungicides, bactericides, acaricides, nematicides, molluscicides, insect growth regulators, insect feeding inhibitors, repellents, or biocides. They can be used in public health to kill vectors of disease (the so-called biocides) and in agriculture (the so-called plant protection products; PPPs), in order to kill pests that damage crops (Mahmood et al., 2015; Ravindran et al., 2016; WHO, 2006).
The global use of plant protection products (PPPs) has largely benefited agriculture, through the control of pests, leading to increased yields in crops (Niering, 1968). However, this fact brought environmental constrains which put at stake the sustainability of ecosystems.
The public attention started to be drawn to this matter after the publication of the book ‘’Silent Spring’’ by Rachel Carson, in 1962 (Delaplane, 2000; Palmquist et al., 2012). In her book, she highlighted the consequences of the indiscriminate use of pesticides, such as the sudden death of non-target organisms (most of them beneficial for agricultural crops) either by direct or indirect toxicity, after applications of DDT (Mahmood et al., 2016).
Nowadays in Europe, the use and placement on the market of PPPs are well addressed on Regulation (EC) No 1107/2009, and the Regulation (EC) No 396/2005 covers the legal limits for pesticide residues in food and feed. The European Food Safety Authority (EFSA) evaluates active substances used in PPPs and the 3 regions of Europe (North, Centre, and South) are represented by the specific Member States from each region, that evaluate and approve the use of these products at a national level. This process involves three steps which are the approval of active substances (chemicals or micro-organisms, which are the essential ingredients in PPPs, responsible for the mode of action of the PPPs to the target organisms), authorisation of PPPs, and the monitoring of PPPs (by doing retrospective risk assessments, for example). Therefore, when a manufacturer applies for approval of an active substance, it must follow the agreed

guidance (from EFSA and the European Commission), supporting it with relevant studies and data, laid down in Regulation (EC) No 284/2013. After, a designated rapporteur (the Member State responsible for the evaluation process) conducts an initial draft assessment report (DAR) or a renewal assessment report (RAR) in the case of renewing approved substances. This report is peer-reviewed by EFSA, established by Regulation (EC) No 178/2002, in cooperation with all Member States, and is made public for expert consultations. The next step is performed by EFSA, with a ''Conclusion'' report on the risk assessment of the active substance. Finally, the European Commission in consultation with the Member States and based on EFSA's conclusion report performs the risk management role, taking the final decision on whether to include the active substance on the list of approved substances. This will determine if the substance can be used in PPPs to be placed on the European market.

The renewal of approved active substances on the market follows the same approach. Active substances are approved for 15 years (10 years for first application), and before the expiration time, the manufacturer can apply for renewal. Generally, the replacement of PPPs on the market only happens when environmentally safer alternatives are produced and broadly assessed.

The major classes of conventional insecticides are organochlorines, organophosphates, carbamates, and synthetic pyrethroids (Kodandaram et al., 2010).

Organochlorines, such as DDT, were banned in most of the developed countries in the late 1970s and early 1980s because of their accumulation in the environment and toxicity to humans (Aktar et al., 2009; Moore et al., 2009; Palmquist et al., 2012). After this, the use of organophosphate and carbamate insecticides started to increase. Several studies on non-target soil organisms, such as predatory mites and Collembola, have been conducted with this type of insecticides (Edwards and Thompson, 1973). Edwards et al. (1967) reported very high toxicity to predatory mites and, because of that, the increase of the number of other mite and springtail species was very common after the application of these insecticides on the soil. Moreover, since some uses of these compounds were proven to be toxic to humans, especially infants, a gradual replacement took over and synthetic pyrethroids became more common (Oros and Werner, 2005; Palmquist et al., 2012).

However, insect resistance was an emerging problem associated with pyrethroids. Bed bug resistance could become widespread (Romero et al., 2007) and the increasing resistance of malaria vectors to this class of insecticides sounded alarming (Ranson et al., 2011). Furthermore, their increasing use had consequences to aquatic ecosystems, and their high toxicity to invertebrates and fish generated public concerns (Luo and Zhang, 2011; Palmquist et al., 2011).

New classes of pesticides such as neonicotinoids, oxadiazines, diamides, tetramic/tetronic acid derivates, pyridines, insect growth regulators (IGR's), among others, have been developed (Kodandaram et al., 2010). They present characteristics like a high level of selectivity and greater specificity to target pests, showing good control at low doses of application with low toxicity to non-target organisms and the environment (Hara, 2000; Kodandaram et al., 2010).

However, the recent observation of a great reduction in pollinator populations, especially bees, has been associated with the use of neonicotinoids (Decourtye et al., 2003; Mahmood et al., 2016; Yang et al., 2008). Also, toxicity to earthworms has been associated with the accumulation of neonicotinoids in the soil (Goulson, 2013).

By the light of these issues, the search for and production of better, safe, and environmentally friendly compounds needs to be a common practice, to reduce the harmful effects related to the usage of pesticides (Mahmood et al., 2016).

One of the issues of pesticides use, as referred before, is that it may affect non-target species that are beneficial and have important roles in terrestrial ecosystems (Edwards and Thompson, 1973). In-soil organisms that are not the target pest can be exposed to the active substances and plant protection products, mainly, through contact and oral uptake routes, because they inhabit essentially in the soil and soil litter layer (EFSA, 2017).

The present work is focused on a specific group of in-soil organisms - Microarthropods - that is composed of organisms that are not the target of PPPs but, ultimately, they end up suffering the effects of these chemicals.

The exposure to PPPs may put at risk either these key drivers as the important soil ecosystem functions that they provide. Therefore, increasing concern regarding these issues in environmental risk assessment schemes is in the order of the day.

I.4 Ecotoxicology and risk assessment for in-soil organisms exposed to PPP's

Ecotoxicology is usually based on testing a limited number of species to make inferences about a much larger number of species (EFSA, 2017). This research area aims to characterise the risk of chemicals to non-target organisms in the environment. This characterisation is usually achieved by assessing the effects of contaminants in single standard species of selected test organism's representative of key groups (Alves and Cardoso, 2016).

Risk assessments can be prospective, as it happens in the context of market authorisation of a substance, or aimed to identify the causes of adverse effects that have already occurred associated with used substances - retrospective risk assessment (Callow and Forbes, 2003).

The current prospective risk assessment of PPPs has four main steps which are similar to the general environmental risk assessment. These steps are hazard identification, hazard characterisation, exposure assessment, and risk characterisation (EFSA, 2017). It follows a tiered approach where the aim is to start with simple and conservative evaluations and go through more complex and realistic assessments when the risk calculated in the previous evaluations is

unacceptable (APVMA, 2009; EFSA, 2017; USEPA, 2008a; USEPA, 2008b). As for the retrospective risk assessment, when uncertainties in lower tier studies are shown, we should proceed to higher tier studies, where the tests are more complex but also more ecologically relevant. Concerning exposure in a prospective risk assessment, a broad evaluation of fate and behaviour of the active substance and its metabolites in the affected soil area is performed. Here, initial and maximum PEC's (Predicted Environmental Concentrations) are estimated according to FOCUS (FOCUS, 1997). These estimations take into consideration properties of the active substance such as the degradation rate (persistence, half-life time DT_{50}, biodegradation, hydrolysis, photolysis), mobility in soil (water solubility, soil-water partition coefficient Koc, retardation factor R) and bioaccumulative potential (octanol-water partition coefficient Kow) (FAO, 2000), as well as the intended uses according to the different crop context. For assessment of effects, studies based on dose-response relationships are made where the organisms are exposed to a series of increasing concentrations of the test contaminant (e.g. pesticide). In these studies, the acute and/or chronic effects are measured by assessing different endpoints, such as mortality, reproduction, growth, and feeding activity (Van Gestel, 2012). These experiments allow the estimation of toxic values like EC_{10} (concentration of the active substance causing 10% effect compared to control), EC_{20} (concentration of the active substance causing 20% effect compared to control) - these both are data requirements set out in Regulation (EU) No 284/2013 -, EC_{50} (concentration of the active substance causing 50% effect on tested organisms compared to control), or NOEC (No Observable Effect Concentration) and LOEC (Lowest Observed Effect Concentration). The toxic values are used to establish safe levels of the contaminant for populations and communities. The ecological risk of the tested substance is estimated through the toxic exposure ratio (TER) that is the ration between toxic values with the PEC value (EFSA, 2017; Van Gestel, 2012).

For risk characterization, toxicity exposure ratios (TERs) are calculated (SANCO/10329/2002) and then compared with trigger values to determine if the existing risk is acceptable. For soil and aquatic organisms, the current trigger value is 5, which means that if the TER values are below this number, the risk is unacceptable, and further higher tier tests must be performed (Christl et al., 2015; EFSA, 2017; European Commission, 2011).

$$\text{TER} = \text{toxicity value } (EC_{50}, \text{NOEC}) / \text{exposure (PECsoil)}$$

$$\text{Acceptable risk to soil organisms is indicated if TER} \geq 5$$

In the current risk assessment for in-soil organisms exposed to PPPs, the different groups of organisms are assessed individually, at lower tiers. The first tier is usually composed of single-species tests with selected invertebrate model species (e.g. *Eisenia fetida/andrei*, *Folsomia*

candida and *Hypoaspis aculeifer*) and one microorganism-mediated process (N transformation). Therefore, the current standard species may not represent other relevant groups of in-soil invertebrates, such as isopods that the main route of exposure to PPPs is the consumption of litter and, currently, there are no standardised tests to assess effects on this type of exposure route (EFSA, 2017).

The Health and Consumer Protection Directorate General of the European Commission (DG-SANCO) is responsible for the implementation of European Union laws on the safety of food and other products, on consumers' rights, and considering the protection of people's health. In 2002, this working group released guidance documents on terrestrial ecotoxicology (SANCO/10329/2002) to address the ecotoxicological risk assessment of these chemicals. However, and as it is highlighted in the regulatory framework for plant protection products (PPP's) deliberated in Commission Regulation (EC) No 1107/2009 and Commission Regulation (EU) No 283/2013 and 284/2013, the assessment of effects on biodiversity is not well defined. Indeed, in this regulatory framework, data on the impacts of PPPs to non-target species, on their ongoing behaviour, as well as on biodiversity and the ecosystem are required. These include possible indirect effects through alteration of the food chain web (EFSA, 2017). Because of this, in 2008, EFSA requested the Panel on Plant Protection Products and their Residues (PPR) to calibrate and reinforce the guidance documents mentioned previously to establish and define a proper risk assessment methodology. In that review, key driver organisms that sustain important in-soil ecosystem services in agricultural landscapes have been identified and specific protection goals (SPGs) for in-field and off-field areas were proposed (EFSA, 2017)

The risk assessment scheme for in-soil organisms proposed by EFSA (2017) comprehends two categories. One is the measurement of effects following a tiered approach, with laboratory tests (low and intermediate tiers) and semi-field or field studies (higher tiers). The second is the assessment of species recovery and long-term impacts of PPPs using population modelling, to ensure that the lower tier is the most conservative. This methodology implies the existence of a reference tier which demonstrates the real situation for the community of in-soil organisms in the agricultural landscape, and a surrogate reference tier which refers to population models to integrate long-term effects on populations and the measurement of effects on field or terrestrial model ecosystems studies using in-soil populations, communities and processes (EFSA, 2017). Furthermore, EFSA PPR Panel (2010) proposed a relation between the tier of risk assessment and the protection goals. Here, a general protection goal is identified, as well as specific protection goals consistent with the general ones. The surrogate reference tier allows to link the environmental risk assessment with the specific protection goal and enables the calibration of lower tiers (EFSA, 2017). Concerning the measurement of effects in macrofauna, mesofauna, microfauna, the scheme comprehends a lower tier where single species are tested and an intermediate tier (A), where more single species are tested using SSDs (species sensitive distribution). However, combined toxicity data for different groups of in-soil organisms is currently lacking to perform relevant SSDs analyses (EFSA, 2017). Thus, an alternative

intermediate tier (B) with assembled multispecies tests, covering different groups, and where interactions between species and functional responses of endpoints for communities can be assessed (EFSA, 2017; Schäffer et al., 2008). The higher tier refers to natural assemblage tests with soil organism communities in semi-field or field conditions.

I.5 Ecotoxicological tests with in-soil organisms for risk assessment of plant protection products

Nowadays, the sale of PPPs in the European Union is covered by the Regulation (EU) Nº 284/2013 that requires a list of effect data taken from standardised ecotoxicological tests to assess the impact of these substances to non-target soil organisms (Alves and Cardoso, 2016). The Organisation for Economic Cooperation and Development (OECD), the International Organisation for Standardisation (ISO), and the International Organisation for Biological Control/West Palearctic Regional Section (IOBC/WPRS) are examples of International Institutions that work for the establishment of these guidelines with a battery of standardised tests available, assessing acute and chronic toxicity, and the effects on the behaviour of organisms like earthworms, collembolans, enchytraeids, and mites (Van Gestel, 2012). The objective of this standardisation is to make these methods repeatable in different laboratories across the world, allowing data comparisons, and increasing the reliability of the established toxicity values. To accomplish this, standard species have been chosen based on ecological relevance, ease maintenance in laboratory conditions, and short-generation time (Fountain et al., 2005; Ronday et al., 1996). Hence, selected test organisms need to be identified as key drivers of ecosystems, meaning that they have to represent key functional and taxonomic groups with a variety of biological traits in order to be considered models, sensitive enough to be able to establish guidance values sufficiently protective to the majority of species (EFSA, 2017).

The most common invertebrate species used in these tests are earthworms, collembolans, mites and, enchytraeids (Alves and Cardoso, 2016). Having different morphological, physiological, and behavioural habits, these species represent different taxonomic and functional groups with different exposure routes to chemicals, like the water and air present in the soil pores, and the ingestion of food and soil particles (Pejnenburg et al., 2012).

The tests included in the data requirements on non-target arthropods (NTA's) exposed to PPPs intend to evaluate acute (mortality) and chronic effects (on reproduction, if assessed) on the parasitic wasp *Aphidius rhopalosiphi* (IOBC/WPRS Mead-Briggs et al., 2000) and the predatory mite *Typhlodromus pyri* (IOBC/WPRS Blümel et al., 2000). Legislation clear states that for PPPs

applied as a foliar spray, data on these two indicator species might be considered for a preliminary risk assessment. However, if effects occur on either species or if data are not available, testing effects on reproduction (chronic toxicity) of the collembolan *Folsomia candida* (OECD TG 232/ISO 11267) and the predatory mite *Hypoaspis aculeifer* (OECD TG 226) is required. Moreover, for PPPs applied as soil treatments directly to soil as a spray or as a solid formulation, testing on both *F. candida* and *H. aculeifer* is mandatory. Regarding effects on other non-target soil meso-macrofauna, such as earthworms, the data requirements include testing on the earthworm *Eisenia fetida* to assess both acute toxicity (OECD TG 207/ISO 11268-1) and chronic toxicity, evaluating sub-lethal effects on growth and reproduction (OECD TG 222/ISO 11268-2) (EFSA, 2017; Regulation (EU) No 284/2013). The standard guidelines, both from ISO and OCDE, for temperate regions recommend the use of artificial soil (70% sand, 20% kaolinite clay, 5-10% ground *Sphagnum* peat, and pH adjusted to 6), which has the advantage of being relatively well reproducible, permitting data comparisons among different laboratories (Van Gestel, 1992). On the other hand, the use of natural soils in laboratory tests beyond making the data more site-specific (i.e. less comparable with other studies with the same test substance), its use improves the ecological relevance of the data obtained as the soil properties influencing test organisms and chemicals behaviour are from a realistic scenario (Alves and Cardoso, 2016; EFSA, 2017).

The natural soils to be used in the tests should be adequate for the test organisms that must be able to survive and reproduce within the validity criteria established for the tests in the absence of the test chemicals. To allow data comparisons between different laboratories, natural soils with similar properties must be used (Alves and Cardoso, 2016). Some examples of reference natural soils that have been used in laboratory ecotoxicological tests are LUFA soils (Lokke and Van Gestel, 1998), EURO soils (Kuhnt et al., 1994), and standard soils selected for specific regions, such as some Mediterranean soils (Chelinho et al., 2011).

In the section below, a brief description of some of the different types of experiments to assess the effects of contaminants on soil meso-macrofauna (focusing on NTA's) in the different tiers of a tiered approach is presented.

I.5.1 Lower tier tests

The experimental procedure is the one usually applied in laboratory ecotoxicological tests: the PPP is incorporated into soil aliquots (natural or artificial) to obtain a gradient of homogenous soil portions with increasing concentrations of the PPP; the toxicity and sub-lethal effects (on growth and reproduction) are assessed and determined by a dose-response relationship which

permits to derive the EC_{10}, EC_{20} and NOEC and LOEC values. The tests consider also chemical (e.g. pH, organic matter content, cation-exchange capacity) and physical (e.g. texture, aggregate stability, soil porosity) parameters of the soil and properties of the active substance in the PPP such as the degradation rate (e.g. half-life time DT_{50}), mobility in soil (e.g. soil-water partition coefficient - Koc), and the potential for bioaccumulation (e.g. octanol-water partition coefficient – Kow).

Regarding earthworms, the chronic toxicity test has a duration of 8 weeks and the study includes the observation of behaviour and morphology, the measurements of the number and final weight of the adult surviving worms after the first four weeks of exposure and the number of juveniles hatched at the end of the second four-week period. The acute toxicity test has a duration of 14 days, in which mortality is assessed at day 7 and day 14, and sublethal endpoints such as the mean body weight and behavioural abnormalities can be recorded. Both tests usually include 5 treatments (8 for ECx-design) with 4 replicates (8 for control) and 10 adult earthworms (not older than 1 year) per replicate. The test substance can be applied either by mixing it into the soil (natural or artificial) or by spraying onto the soil surface. Besides mortality and reproduction tests, there are standard guidelines to evaluate bioaccumulation (OECD TG 317) and avoidance behaviour (ISO 17512-1:2008) at lower tiers.

The chronic toxicity test with collembolans has a duration of 28 days and the number of test concentrations and replicates depends on the experimental design. Usually, 5 concentrations (8 for ECx-design) with 4 replicates for each concentration as well as 8 control replicates are used. In each test vessel, 10 sub-adults *F. candida* are placed on 30g of modified OECD artificial soil using a 5% organic matter content (OECD, 2016). During the test, collembolans are fed with dry yeast and at the end of it, surviving adults and the number of offspring produced are counted. Similarly to earthworms, there is an available protocol for testing avoidance behaviour on collembolans (ISO 17512-2:2011).

Chronic toxicity test with mites consists of exposing adult females of similar age to a range of concentrations of the test substance mixed into 20g dry mass of soil 28-35 days after the start of the egg-laying period. Concentrations tested may range from 5 to 12 depending on the toxicity values to be measured (ECx, NOEC, or both). In terms of the number of replicates, 2 to 4 for each test concentration and 6 to 8 control replicates with 10 females each are recommended. The duration of the test is 14 days and the number of surviving females and juveniles per test vessel is determined. The fecundity of the mites exposed to the chemical is compared to that of controls to derive the ECx or the NOEC. Any differences regarding the behaviour and the morphology of the mites between controls and treated vessels should be reported (OECD, 2008). Contrary to earthworms and collembolans, currently, there is no standardised protocol for testing avoidance on mites. Owojori et al. (2014) used an adaptation of the earthworm and collembolan avoidance test to evaluate the avoidance response of the predatory mite *H. aculeifer* and reported a strong avoidance to 3 of 5 chemical groups tested, reflecting the first step to develop a standardised avoidance guideline on this group of organisms.

I.5.1.1 Limitations of lower tiers

Some of the limitations of lower tier tests include the fact that the sensitivity of the selected test species may not be representative of other species among the same group of organisms. For instance, *E. fetida* is an epigeic earthworm species and does not reflect the traits of endogeic earthworm species in terms of lifestyle, exposure routes, and life cycle. Frampton et al. (2006) reported that *E.fetida* was less sensitive than other earthworms to 13 substances based on acute toxicity endpoints. Regarding mites and collembolans, studies on the representativeness of these organisms to the same groups are few. Schnug et al. (2014) reported that the collembolan *Protaphorura firmata* was more sensitive (in 5 orders of magnitude represented by LC_{50}) compared to *Folsomia fimetaria* after exposure to picoxystrobin, but argued that differences could be due to food and habitat preferences. Lokke and Van Gestel (1998) tested the sensitivity of the oribatid mite *Platynothrus peltifer* to copper chloride, LAS, and dimethoate compared with *H. aculeifer*, but the EC_{50} results for reproduction did not differ by more than a factor of 2. On this matter, EFSA (2017) reported that no conclusion could be drawn on the representativeness of *H. aculeifer* to other mites because hardly any data were found and further research was required. Although *H. aculeifer* seems less sensitive to chemicals compared to other soil invertebrates (Huguier et al., 2014; Owojori et al., 2014) its importance in tier 1 tests is recognised since it is the only predatory mite species which has a standardised protocol available (EFSA, 2017). Moreover, Natal da Luz et al. (2019) performed reproduction tests with *H. aculeifer* and reported that the predatory mite was more sensitive to copper (Cu) contamination after fed with mites that were previously exposed to Cu. The current protocol with this mite does not take into consideration the oral exposure route and it has been discussed the implementation of this route to better estimate the toxicity of contaminants on non-target predatory mites (EFSA, 2017; Natal da Luz et al., 2019).

Furthermore, these types of tests do not take into account possible interactions among various stress factors of different origins that may occur in the ecosystem (Vighi et al., 2013). For instance, the use of single-species tests does not consider the interactions between species (e.g. predation, competition) within a community, including potential indirect effects via alteration of the food web (Chelinho et al., 2014; EFSA, 2017). Trophic interactions are essential in ecological processes and they affect the occurrence and performance of many organisms at the individual, population, community, and ecosystem levels (Thébault and Loreau, 2006). Moreover, these tests are performed under stable and controlled laboratory conditions that do not resemble natural

conditions of field tests, and the distribution and degradation of chemicals are ignored (Van Gestel et al., 2018). Indeed, environmental conditions of ecosystems affect the community structure, species combinations of traits, and life history strategies among invertebrates (Callejas-Chavero et al., 2015).

I.5.2 Intermediate tier tests

To better understand the impact of chemicals on soil organisms and to increase ecological relevance of the effect data, the identification of responses at higher levels of biological organisation (e.g. communities) is required. This can be accomplished using multispecies tests which, contrary to the single-species laboratory tests, consider the interactions and relationships between species and contaminants. (Cardoso et al., 2012; Van Gestel, 2012). EFSA proposed the implementation of community tests and species sensitive distributions as intermediate tiers in the risk assessment of soil organisms exposed to PPPs (EFSA, 2017).

I.5.2.1 Community tests

This type of tests intends to assess the effect of contaminants at the community level of specific groups of soil organisms, either cultured in the laboratory (i.e. artificially built communities), as is the case of gnotobiotic tests (Morgan and Knacker 1994; Schäffer et al., 2010; Scott-Fordsmand et al., 2008), or using natural communities extracted from the field (Chelinho et al., 2011, 2014; Parmelee et al., 1993). In both cases, communities are added to micro or mesocosm set ups containing soil spiked with increasing concentrations of the substance to be assessed. After the test period, organisms can be counted and identified at species, life-form group level, or any other trait-based typology of interest in the different test treatments (EFSA, 2017).

The advantages of assessing effects of chemicals at this level of organisation are that beyond the effects provoked directly by the exposure to the test chemical, potential indirect effects, such as the effects resulting from interactions between organisms (e.g. competition, predator-prey relationships) can be studied. Also, these types of systems allow to correlate the reproducibility

of laboratory single species tests with a gain in ecological realism (EFSA, 2017; Schäffer et al., 2010), and compared to full-scale field tests they are less complex and less expensive (Van Gestel et al., 2018). On the other hand, when using site-specific soil communities, and by simulating a more natural environment, the replication and standardisation of these tests becomes more difficult (Van Gestel et al., 2018), thus complicating the comparability between laboratories. Bellow, some examples of studies using community tests are described.

Baatrup et al. (2006) used a microcosm set up with the species *Hypoaspis aculeifer* and *Folsomia fimetaria* to test effects on predator-prey interactions of the organophosphorus insecticide dimethoate. By using communities artificially built, parameters like the age of the organisms at the beginning of the experiment are known (Krogh, 1995). In this study, Baatrup et al. (2006) argued that dimethoate increased the locomotory capacity of the *H. aculeifer* mite, in contrast with a reduction in locomotion of the collembolan *F. fimetaria* (after 6 days of exposure at 20ºC), which ended up favouring the capacity of the mites to prey upon the collembolans.

Also, Pernin et al. (2005) used a laboratory mesocosm experiment to test the effects of copper-enriched sewage sludge on a mesofauna community partly composed of organisms collected from the field and other part composed of organisms from laboratory cultures (*P. armata, F. fimetaria, Mesaphorura macrochaeta, Enchytraeus crypticus, and H. aculeifer*). In this study, changes in species composition and trophic structure of the mesofauna community were reported (monitored for 12 weeks), such as the increased abundance of the predatory mite *H. aculeifer* in all treatments tested. The authors argued that such an increase could be related to the retention of Collembola on fungal mycelium and/or to their intrinsic sludge-dose-sensitivity, turning to be an easily available food source for the predatory mites.

More recently, Chelinho et al. (2014) assessed the effects of a commercial formulation of carbofuran (pesticide) on soil microarthropods using this type of approach, but with natural communities. A reduction in the abundance and species richness was reported, as well as the prevalence of epigeic species of Collembola over euedaphic ones along the contamination gradient. This affected the abundance of competitor-mite species, with an increase of oribatids and the decrease of predatory mites.

The advantage of using natural communities instead of artificially built communities in microcosms is that it resembles a more natural system, often with more species, which makes the interactions of tested organisms more ecologically relevant. Also, by testing the effects of PPPs on natural soil using natural communities from that soil, the ecological relevance is increased because the community is adapted to that soil (EFSA, 2017; Parmelee et al., 1993).

I.5.2.2 Species Sensitive Distributions

Species Sensitive Distribution (SSD) is a probabilistic procedure which intends to evaluate the probability of a species to be affected by a given concentration of a toxic substance (Vighi et al., 2013). This approach may be very useful to address the uncertainty which comes from the differences in sensitivity of the current standard laboratory single-species tests towards chemicals, such as PPPs (EFSA, 2017). Indeed, SSDs allow us to estimate the number of species affected at a specific concentration. In this way, confidence limits can be estimated according to the percentage of species to be protected in the community (EFSA, 2013). More specifically, the calculation of a hazardous concentration for 5% of species (HC_5) can be achieved based on the SSD approach, to protect 95% of the species present/considered in the curve (Wang, 2008).

In risk characterisation, this approach uses the concept of Potentially Affected Fraction (PAF) instead of the traditional Toxicity Exposure Ratio (TER). PAF considers several different species within the community of interest and not only a small number of model species, supposedly, representatives of the ecosystem to protect (Sala et al., 2012).

However, the lack of effect data on a large number of species, other than the commonly used in standard laboratory tests, remains a major limitation for the use of this type of approach in environmental risk assessment for in-soil organisms, since an SSD with few species lacks statistical power (EFSA, 2017; Frampton et al., 2006). Also, by using species belonging to completely different groups (e.g. microarthropods, microorganisms, plants) the SSD becomes less assertive, because an important group of organisms may be eliminated from the equation even when considering a high percentage of species protected (EFSA, 2017; Sala et al., 2012). They have been mostly used in the environmental risk assessment of aquatic ecosystems (Sala et al., 2012; Van den Brink et al., 2006; Wang, 2008)

I.5.3 Higher tier tests

These types of tests are required whenever an unacceptable risk is indicated in lower tiers following the relevant risk quotient analysis (e.g. TER calculation). Bellow, higher tier tests are briefly described.

I.5.3.1 Terrestrial Model Ecosystems

Terrestrial Model Ecosystems (TMEs) are a semi-field test system composed of intact soil cores (16.5 cm diameter and 40 cm height), with the original communities and the soil structure conserved, that is performed under laboratory conditions. These types of tests attempt to take into account processes and interactions between components (e.g. soil properties and the natural community of microorganisms, animals, or plants) in portions of intact terrestrial systems (Knacker et al., 2004; Sheppard, 1997). Effects on the abundance of most species, taxonomic diversity, and functional endpoints (such as feeding activity or organic matter breakdown) can be assessed because TMEs can last and remain stable for up to 6 months (EFSA, 2017; Knacker et al., 2004). Besides, TMEs can mimic the variability of the data found in the field and can show comparable response patterns and responses (measured through the estimation of toxic values) within the same order of magnitude of those obtained in the field (Knacker et al., 2004).

The TME system was ring-tested and field-validated by a project entitled ‘’The use of Terrestrial Model Ecosystems to assess environmental risks in ecosystems’’ performed by four partners from Europe (ECT Oekotoxikologie GmbH in Germany, Vrije University in The Netherlands, the University of Coimbra in Portugal, and the University of Wales in Bangor, UK) and protocols for these tests are available (ASTM, 1993; Knacker et al., 2004; Schaeffer et al., 2008). Moreover, TMEs fulfill a range of ecological criteria (such as relevance, because they use natural communities and all endpoints parameters can be measured) and performance criteria (in terms of practicability, reproducibility, experience, and standardisation) which makes them a promising method to use on assessing the effects of pesticides at higher tiers in ecotoxicological risk assessment of soil fauna (EFSA, 2017; Schaeffer et al., 2008; Scholz-Starke et al., 2011). Bellow, some examples of studies from the literature using TMEs are described.

Bandow et al. (2015) tested the effects of the fungicide pyrimethanil combined with different moisture regimes on enchytraeids, using TMEs. Two TME semi-field studies were conducted, one in Portugal and the other in Germany. The main objective was to assess the possible effects of the interaction between pyrimethanil and different levels of soil moisture (mimicking irrigation events of different intensity to simulate extreme rain events related to global climate change) on enchytraeids. Three levels of moisture were studied (low, medium, and high). Both studies were similar in application rates but differed in experimental design (in Portugal, a NOEC design with two concentrations, 1.82 mg/kg dry soil, and 9.09 mg/kg dry soil; in Germany, an EC_{χ} design, using 11 concentrations). The TME sampling was done at two and eight weeks after the fungicide application, through the collection of soil cores with 5.6 cm diameter in TMEs from both studies. In the Portuguese study, no risk was found to enchytraeids at field and fivefold field rates of the fungicide, but in the German study, a reduced sensitivity of enchytraeids was associated with soil moisture, causing effect concentrations 2.6-fold higher than the lowest environmentally relevant concentration. The authors concluded that different climatic factors (caused by global climate

change and natural variations between different climate zones) may influence the toxicity of PPPs and should be considered in environmental risk assessments.

More recently, Rieff et al. (2020) used TMEs to study the consequences of four different types of soil management in maize crops (conventional tillage, conventional tillage with insecticide, no-tillage soil with herbicide, and no-tillage soil with herbicide and insecticide, using undisturbed soil as control) on soil mesofauna and the way soil communities recovered after these disturbances. The duration of the test was eighty-nine days and the measured endpoints were taxonomic diversity and abundance of collembolans, mites, and enchytraeids. The insecticide used was Judo® (with lambda-cyhalothrin as active substance) and the herbicide was Montana® (with glyphosate as active substance). The authors reported that soil tillage did not affected soil communities, whereas the insecticide application did. Moreover, the impact of the insecticide on collembolans and enchytraeids had no relation with the type of soil management (tillage or no-tillage). Also, changes in Collembola abundance did not affect functional diversity. As for mites, insecticide impact was greater in conventional tillage compared to no-tillage system. The authors concluded that soil fauna was better protected under no-tillage management compared to conventional tillage because the destruction of soil structure (promoted by soil tillage) amplified the effect of the insecticide (in the case of mites). Furthermore, they argued that different soil disturbances (physical or chemical) acting sinergistically, can have higher impacts on soil fauna.

I.5.3.1.1 Advantages and limitations of TMEs approaches

TMEs add knowledge to the ecological relevance of effects obtained in lower tier studies because they can assess the recovery potential and indirect effects at the soil community level. Moreover, they reproduce realistic exposure conditions, a broad range of species-specific sensitivities are covered, they can be applied in different environments (such as pasture, grassland, etc) and may include many replicates for higher statistical power (Bandow et al., 2015; EFSA, 2017). On the other hand, TMEs have limited size so they are unable to predict or mimic the field situation (at least for the macrofauna) and the number of samples depends on the surface area of the soil cores (Schaeffer et al., 2008). Also, since TMEs are closed systems, the possible recolonisation by species from the adjacent non-impacted areas cannot occur and this phenomenon is often what happens in field situations. Therefore, the impact of recolonisation by species from surrounding areas is usually disregarded in TMEs (Rieff et al., 2020).

I.5.3.2 Field tests

These tests represent the most realistic exposure scenario. Until now, the only widely used field test that has been standardised for soil organisms is the earthworms field study (ISO 11268-3, ISO 2014; EFSA, 2017). This field study uses natural populations of earthworms where adults are identified to species level and juveniles to 2 morphological groups (tanylobous and epilobous). The study design involves 3 treatment groups: a test item treatment, the water treated control, and the toxic reference item treated with 6 to 10 kg/ha carbendazim. Each treatment has 4 replicates (plots) arranged in a randomised plot design (plot size is 10m x 10m). The distance between plots is at least 3 m and approximately 10 m distance to fields next to the test site. The sampling plots are chosen from grassland or arable field sites with homogeneous conditions. Moreover, the application of test substances is performed with a calibrated mobile plot sprayer or granule applicator. The number of test substance applications and the application rates are in accordance with good agricultural practices (GAP) considering realistic conditions of application, product persistence and possible interception by vegetation. Throughout the study environmental conditions are recorded (e.g. soil characterisation, climate data, microclimate). Before applying the test substance(s) a pre-sampling on the test site is carried out to assess earthworm abundance and the presence of important ecological groups (e.g. anecic, endogeic species). Artificial irrigation may be necessary if rainfall is less than 10 mm within 3 days after substance application to ensure exposure of earthworms. Within the next year, 3 additional earthworm samplings are carried out (usually 1, 4/6, and 12 months after the last test substance application). The duration of this test depends on the characteristics of the test substance, the endpoints that are being assessed, as well as the specific protection goals that are to be accomplished (e.g. recovery of populations) but is usually 1 year in this field test. Usually, the endpoints measured here are abundance and biomass of adult and juvenile earthworms which are monitored (in this case) over 1 year. Data of the most abundant species are analysed in detail and the results are described taking into consideration possible differences of the controls compared to the test and reference item treatment.

Römbke et al. (2009) designed a field study with microarthropods that intended to evaluate the impact of pesticides on the biodiversity of the soil organism community. The study site was in grassland and the environmental conditions (air temperature at 2 m height, soil temperature at 5 cm depth and rainfall) were recorded throughout the test. Characterisation of soil (e.g. measurement of pH, maximum water holding capacity, total organic carbon content, etc) and microbiological activity were performed as well. The study design consisted of 3 treatments: a water control, a test substance treatment (a new pesticide that the authors did not mention due to confidential reasons), and a reference item treatment, consisting in a mix of 2 reference substances, the fungicide Benomyl and the insecticide Chlorpyrifos(ethyl). Each treatment had 6 replicate plots (size: 3 m x 7 m) arranged in a randomised block design, separated by 3 m between

each and, with at least 5 m distance to the edge of the field. Two applications of the test substance were carried out with a 14-day interval to simulate a realistic exposure scenario. The duration of this test was 1 year and sampling of organisms occurred 2 days before substance application (mainly to assess the presence of organisms and to create a species list), after 1 month, 3 months, 6 months and, 12 months after substance application. The taxonomic groups assessed were enchytraeids, soil mites, collembolans, and nematodes. Results regarding the test substance, according to the authors, did not show significant effects to tested organisms (results on collembolans were not covered in the study), except for a significant reduction on enchytraeids at the 4th sampling date which could not be explained. Moreover, the authors reported that the number of enchytraeids and nematodes was reduced in the reference plots, confirming the spray application on grass as a valid route of exposure and the validity of the study, at least for these organisms. On the other hand, mites were not affected by the reference substances (Benomyl and Chlorpyrifos(ethyl)), thus not validating the study for these organisms and, suggesting that another compound should be identified for this objective. In conclusion, this field test lacks standardisation (EFSA, 2017; Römbke et al., 2009), and the authors recommended to conduct more studies similar to this one to improve the design and performance of the test, to reach this goal.

Field tests use naturally occurring communities and are open systems (allowing recolonisation) placed outdoors (so effects of environmental conditions are covered). Thus, field tests allow the assessment of long-term effects and recovery at the community level (Jong et al.,2010; Schäfer et al., 2007). On the other hand, field tests have some disadvantages in comparison with laboratory studies. For instance, the application rates of test substances in the field cannot be higher than the legal limits. Also, unauthorised substances cannot be tested in the field, presenting a constraint to the approval of new substances on the market (European Commission, 2009).

I.6 Ecotoxicological profile of Chlorantraniliprole

Chlorantraniliprole, whose chemical name is 3-bromo-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloro-2-pyridine-2-yl)-1H-pyrazole-5-carboxamide and the empirical formula $C_{18}H_{14}N_5O_2BrCl_2$ (USEPA, 2008a) was introduced by DuPont Crop Protection in 2007, with different formulations according to different end uses. Registered uses are for pome fruit, stone fruit, leafy vegetables, *Brassica* leafy vegetables, cucurbit vegetables, fruiting

vegetables, cotton, grapes, potatoes, rice, and ornamentals, and turfgrass growing in residential, commercial, and public landscape areas. Application rate for food crops is 0.2 lb a.i./acre (~224.2 g/ha), except for rice, which is 0.13 a.i./acre/year (~145.71 g/ha). For turfgrass, the application rate is 0.5 lb a.i./acre (~560.4 g/ha) and for ornamentals it can range between 0.33 to 0.5 lb a.i/acre (~560.4 g/ha) (USEPA, 2008a). In this work, we focused on the formulated product DuPont Coragen 20 SC (200g/L; 18.4% a.i. suspension concentrate) which is meant for Agricultural uses (USEPA, 2008a).

Chlorantraniliprole is an active substance belonging to the anthranilic diamide class of chemistry and is usually adopted to control Lepidopteran, Coleopteran, and some Dipteran pests in both perennial and annual crops. Insects are usually exposed to the active substance by oral uptake or contact routes, being controlled through unregulated activation of ryanodine receptor channels, located in the sarcoendoplasmic reticulum, after Chlorantraniliprole binds to these receptors. Consequently, this will lead to internal calcium store depletion into the cytoplasm that impairs the regulation of muscle contraction. Insects exposed to Chlorantraniliprole exhibit general lethargy and muscle paralysis followed ultimately by death. One interesting particularity is that Chlorantraniliprole exhibits strong differential selectivity for insect over mammalian Ryanodine Receptors (RyRs), which is assumed to be related with the observed low mammalian toxicity (Lahm et al., 2007). Indeed, comparative studies with mammalian cell lines that endogenously express RyRs demonstrate that the strongest anthranilamide tested exhibits more than a 500-fold differential selectivity for insect over mammalian receptors (anthranilamides have much more affinity for insect receptors comparing to mammalian ones) (Cordova et al., 2006).

In Tables 1 and 2, an overview of the toxicity of both the formulation product Coragen SC 20 (which was used in this work) and the active substance Chlorantraniliprole, respectively, is present. Data were taken from the literature (APVMA, 2008; EFSA, 2013; NFSA, 2010; USEPA, 2008a) and regards to non-target terrestrial invertebrates, focusing on NTA's.

Table 1: Toxicity of Coragen SC 20 towards non-target terrestrial invertebrates in tier 1 and extended laboratory tests (tier 2).

Test species	Test type (endpoint)	Toxic value
Aphidius rhopalosiphi (parasitic wasp)	Mortality	LR_{50} > 750 g cpa/ha
	Reproduction	ER_{50} > 750 g cpa/ha
Apis mellifera (honeybee)	Acute oral toxicity (48h)	LD_{50} > 114.1 µg cpa/bee
	Acute contact toxicity (48h)	LD_{50} > 100 µg cpa/bee
Chrysoperla carnea (green lacewing larvae)	Mortality (extended test)	EC_{50} = 120 g cpa/ha
	Reproduction (extended test)	LOEC = 120 g cpa/ha
Coccinella septempunctata (lady bird beetle)	Mortality (extended test)	LOEC = 60 g cpa/ha
	Reproduction (extended test)	LOEC = 60 g cpa/ha
Eisenia fetida (earthworm)	Acute toxicity	LC_{50} > 200 mg cpa/kg dry weight soil

Typhlodromus pyri (mite)	Mortality	LR_{50} > 750 g cpa/ha
	Reproduction	ER_{50} > 750 g cpa/ha

Table 2: Toxicity of the active substance Chlorantraniliprole towards terrestrial invertebrates in tier 1 standard laboratory tests.

Test species	Test type (endpoint)	Toxic value
Apis mellifera (honeybee)	Acute oral toxicity (48h)	LD_{50} > 4 µg cpa/bee (in acetone)
	Acute contact toxicity (72h)	LD_{50} > 104 µg cpa/bee (in acetone)
Eisenia fetida (earthworm)	Acute toxicity	LC_{50} > 1000 mg cpa/kg d.w.s.
Folsomia candida (collembolan)	Reproduction	EC_{50} = 0.48 mg cpa/kg d.w.s. NOEC = 0.39 mg cpa/kg d.w.s.
Hypoaspis aculeifer (mite)	Reproduction	NOEC = 100 mg cpa/kg d.w.s.

According to the Conclusion on the peer review of the pesticide risk assessment of the active substance, Chlorantraniliprole exhibits low risk to birds, mammals, fish, soil microorganisms, non-target plants, and sewage treatment organisms (EFSA, 2013a) (data not shown here).

Regarding terrestrial invertebrates, in both acute oral and contact toxicity studies with Chlorantraniliprole and the formulation Coragen SC 20 on honeybees, effects such as apathy, moribundity, and uncoordinated movements were observed. Such effects occurred initially (4 hours) at all the tested doses for the contact toxicity but, by the end of the study, bees were no longer affected at lower doses. As for the oral toxicity study, bees were initially affected at higher doses, but they all recovered by the end of the study. Based on the toxic values LD_{50} > 114.1 µg/bee and LD_{50} > 100 µg/bee, Coragen SC 20 has low oral and contact toxicity, respectively, towards bees (Table 1). On the other hand, the active substance Chlorantraniliprole exhibits low contact toxicity to bees based on LD_{50} > 104 µg/bee but is toxic to bees through oral exposure route based on LD_{50} > 4 µg/bee (Table 2). As for the soil meso-macrofauna, both Chlorantraniliprole and the formulation Coragen SC 20 have low risk to earthworms based on LC_{50} > 1000 mg/kg and LC_{50} > 200 mg/kg, respectively, for acute toxicity (Tables 1 and 2). Regarding non-target arthropods, both Chlorantraniliprole and Coragen SC 20 have low toxicity to predatory mites based on LR_{50} > 750 g/ha (mortality) and ER_{50} > 750 g/ha (reproduction) derived with *T. pyri*, and NOEC = 100 mg/kg (reproduction) derived with *H. aculeifer* (Tables 1

and 2). Parasitoids are not affected as well, based on LR_{50} and ER_{50} > 750 g/ha (mortality and reproduction, respectively) derived using *A.rhopalosiphi*. However, high chronic toxicity of Chlorantraniliprole was identified for the collembolan *F. candida* based on the EC_{50} = 0.48 mg/kg and NOEC = 0.39 mg/kg values derived (Table 2).

Other organisms that suffered significant effects, and are not represented in Tables 1 and 2 above, are aquatic invertebrates where Chlorantraniliprole showed a very high acute and chronic risk. These risks were identified based on LC_{50} = 0.0116 mg a.i./L and NOEC = 0.00447 mg a.i./L, respectively, derived with the water flea *Daphnia magna*. The LC_{50} = 0.0859 mg a.i./L (acute) and NOEC = 0.005 mg a.i./kg (chronic) toxicity values with the sediment-dwelling organism *Chironomus riparius*. And also, the eastern oyster *Crassostrea virginica*, an estuarine/marine invertebrate, which was highly sensitive to CPA with an EC_{50} = 0.0399 mg a.i./L derived for acute toxicity (APVMA, 2008; EFSA, 2013a; USEPA, 2008a).

I.7 Ecotoxicological profile of Spirotetramat

In June 2008, the United States Environmental Protection Agency (USEPA) issued the pesticide fact sheet for Spirotetramat conditional registration. This insecticide was developed by Bayern CropScience and its chemical name is cis-3-(2,5-dimethlyphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl-ethyl carbonate, with the empirical formula $C_{21}H_{27}NO_5$ (USEPA, 2008b). As it is common with pesticides, Spirotetramat is included in different commercial formulations and product names, with different end-uses. The formulations commercially available are Movento (22.4% a.i.; suspension concentrate), Ultor (14.5% a.i.; suspension concentrate), BYI 8330 OD Insecticide/Spirotetramat 150 OD (15.3% a.i.; oil dispersion) for agricultural uses. For Nursery/Greenhouse/Interior Plantscapes uses, the product developed was Spirotetramat 240 SC Greenhouse and Nursery Insecticide (22.4% a.i.; suspension concentrate) (USEPA, 2008b). Spirotetramat belongs to the recent chemical class of insecticides of tetramic acid derivate (ketoenole) and the mode of action consists in the inhibition of lipogenesis in treated insects (Nauen et al., 2007). More specifically, the primary metabolite Spirotetramat-enol inhibits insect acetyl CoA carboxylases by interacting with the carboxyltransferase domain resulting in decreased lipid contents (Lumen et al., 2014). Growth inhibition on young insects occurs, and adult insects have their ability to reproduce reduced because fecundity and fertility are affected in female insects. As a consequence, pest insect populations decrease (Nauen et al., 2007). The registered uses for the commercial formulations are for citrus, cucurbit vegetables, fruiting vegetables, grape, hops, leafy *brassica* vegetables, leafy non-*brassica* vegetables, pome fruit, potato, and other tuberous and corm vegetables, stone fruit, tree nuts, onions, strawberries, livestock commodities, and greenhouses/nurseries (USEPA, 2008b). The commercial formulations based on Spirotetramat are used to control several sucking pests, including aphids, whiteflies, scales, mealybugs, psylla, phylloxera, thrips, and mites (USEPA, 2008b). These pests are among the most problematic and injurious for crops, some even have developed resistance to older classes of chemicals, besides the regulatory restrictions of some chemicals that were intended to control these pests (Brück et al., 2009). Therefore, Spirotetramat was developed to surpass these issues and is expected to be a major alternative to carbamate and organophosphate pesticides (USEPA, 2008b). Until the date, the most systemic insecticides commercially available (where protection is conferred after chemical uptake by plants, leading to transportation of the ingredients throughout its tissues) were one-way systemic. It means that after leaf absorption, they are primarily translocated in the xylem of plants in an upward movement (Brück et al., 2009). One of the outstanding properties of Spirotetramat is that it acts as a two-way systemic where, after foliar spray and uptake, Spirotetramat is hydrolysed to its enol form, which permits the compound to move upwards through the xylem and downwards through the phloem (Nauen et al., 2008). This allows protection against hidden pests, such as root aphids, as well as the protection of new shoots and newly grown leaves that will eventually develop after the foliar

spray application (Brück et al., 2009). The application rate for food crops is 0.4 lb a.i./acre (~ 448.3 g/ha, seasonal maximum) and for greenhouse/nursery is 0.39 lb a.i./acre (~ 437.1 g/ha, seasonal maximum).

Below, Tables 3 and 4 give an overview of the toxicity of Movento O-TEQ and the active substance Spirotetramat, respectively, towards non-target terrestrial invertebrates. Data taken from the literature (APVMA, 2009; EFSA, 2013b; Maus et al., 2008; USEPA, 2008b) is focused on NTA's.

Table 3: Toxicity of Movento O-TEQ to non-target terrestrial invertebrates in tier 1 and extended laboratory tests (tier 2).

Test species	Test type (endpoint)	Toxic value
Aphidius rhopalosiphi (parasitic wasp)	Acute contact toxicity (48h)	LR_{50} > 114.7 g spt/ha
	Acute contact toxicity (extended test 48h)	LR_{50} > 228 g spt/ha
		NOEC = 228 g spt/ha
Apis mellifera (honeybee)	Acute oral toxicity (48h)	LD_{50} > 91.7 µg spt/bee
	Acute contact toxicity (48h)	LD_{50} > 162 µg spt/bee
Chrysoperla carnea (green lacewing larvae)	Mortality (24d extended test)	LR_{50} = 288 g spt/ha
	Reproduction (24d extended test)	NOEC = 288 g spt/ha
Coccinella septempunctata (lady bird beetle)	Mortality (24d extended test)	LR_{50} = 288 g spt/ha
	Reproduction (24d extended test)	NOEC = 288 g spt/ha
Typhlodromus pyri (mite)	Acute contact toxicity (7d)	LR_{50} = 0.33 g spt/ha
	Acute contact toxicity (7d extended test)	LR_{50} > 1.59 g spt/ha
		NOEC = 0.15 g spt/ha

Table 4: Toxicity of the active substance Spirotetramat to non-target terrestrial invertebrates in tier 1 laboratory studies.

Test species	Test type (endpoint)	Toxic value
Apis mellifera (honeybee)	Acute oral toxicity (48h)	LD_{50} > 107.3 µg spt/bee
	Acute contact toxicity (48h)	LD_{50} > 100 µg spt/bee
Eisenia fetida (earthworm)	Acute toxicity (14d)	LC_{50} > 1000 mg spt/kg d.w.s.
		NOEC = 1000 mg spt/kg d.w.s.
Hypoaspis aculeifer (mite)	Mortality and Reproduction[a] (28d)	LC_{50} > 1000 mg metabolite/kg d.w.s.
		NOEC = 316 mg metabolite/kg d.w.s.

[a] – This study used the metabolite Spirotetramat-enol as test substance.

Following the Conclusion on the peer review of the pesticide risk assessment of the active substance Spirotetramat (EFSA, 2013b), low risk was indicated for the acute and short-term toxicity to birds and for the acute and long-term toxicity to mammals. However, a long-term risk was identified for birds regarding the representative field uses (data not shown here). Moreover, the risk to soil microorganisms, terrestrial non-target plants, and sewage treatment organisms was considered low (data not shown here). For aquatic organisms (fish, invertebrates, sediment-dwelling organisms, and algae) the acute and chronic risks were derived by endpoints calculated on *Chironomus riparius* (the most sensitive aquatic species with EC_{50} = 1.3 mg/L for mortality and NOEC = 0.1 mg/L for emergence rate). At FOCUS step 3, the chronic risk to aquatic organisms was considered low and at FOCUS step 4 the acute risk was low but considering mitigation measures such as a 5-meter no-spray buffer zone for the use in citrus crops.
For terrestrial invertebrates, acute oral and contact studies with honeybees showed low toxicity of both the product Movento O-TEQ and the active substance Spirotetramat (Tables 3 and 4, respectively). Considering soil meso-macrofauna, earthworms were not acutely sensitive to the active substance Spirotetramat based on LC_{50} > 1000 mg/kg d.w.s. toxic value. Effects on mortality and body weight were not observed, resulting in a NOEC = 1000 mg/kg d.w.s. (Table 2). However, Zhang et al. (2015) used multiple biomarkers to assess toxic effects on *E. fetida* and reported that at doses ≤ 2.5 mg/kg, Spirotetramat induced reversible oxidative stress and DNA damage. Regarding non-target arthropods, studies on the two standard test species (*A. rhopalosiphi* and *T. pyri*) exposed to dried residues of spirotetramat OD 150 (the formulation present in the product Movento O-TEQ) showed clear dose-responsive mortality. In tier 1 acute contact toxicity tests, mites with an LR_{50} = 0.33 g a.i./ha were much more sensitive comparing to parasitoid wasps with an LR_{50} = 114.7 g a.i./ha (Table 3). Further extended laboratory tests (tier 2) with the same species but exposed to freshly dried residues of spirotetramat OD 150 on a natural substrate (barley leaves for mites and bean leaves for wasps) were conducted. For the wasps, the LR_{50} raised to > 288 g/ha, and reproduction was not affected resulting in a NOEC = 288 g/ha (Table 3). As for mites, a great effect on mortality was still observed with an LR_{50} = 1.59 g/ha, and a NOEC < 0.15 g/ha was derived based on significant negative effects on reproduction (Table 3).

I.8 Experimental work

In the present work, two laboratory community tests were performed as potential tool to be used in the risk assessment of PPPs, possibly filling the gap existing between lower and higher tiers, working as an intermediate tier. An evaluation of the effects of two PPPs (Coragen SC 20 and Movento O-TEQ), assessed separately, on natural microarthropod communities using a natural reference soil, both collected in Alentejo, Portugal, was performed. The experimental set up consisted of 10 treatments with 5 replicates and 1 control with 8 replicates and the natural community of microarthropods was added to the microcosm after extraction from soil cores using a high gradient Macfadyen extractor. Afterwards, microcosm test vessels were incubated at 20ºC±2º under a photoperiod of 16h light and 8h dark, for 6 weeks.

After the incubation period, the organisms present in the microcosm were extracted using the same procedure directly to ethanol. Identification of the organisms present in each treatment and control was the next step, using a binocular magnifier. The data generated were used to calculate EC_{20}, EC_{50}, and NOEC's toxic values using Statistica 7.0 (Stat.Soft.Inc., 2004). PRIMER & PERMANOVA 6.0 (Clarke and Gorley, 2006) was also used to see which organisms could contribute more to the significant differences found between control and treatments. The assessment of effects on mites was based on taxonomic approaches such as changes in abundance and richness of taxonomical groups. As for collembolans, effects were assessed based on a functional approach, called Trait-Based Risk Assessment (TERA) where morphological characteristics of the organisms can be used to describe those effects (Baird et al., 2008).

I.9 Objectives

The general aims of this work were:

1. Evaluate effects of Coragen SC 20 and Movento O-TEQ on communities of microarthropods.
2. Evaluate the influence/relevance of the mode of action of two insecticides in the effect data provoked at community level.
3. Understand how far the recommended doses of two insecticides affect soil communities.
4. Understand how far community tests, comparing with single-species tests, permit to go beyond in terms of effect data on PPP's.

Chapter II – Effects of Coragen SC 20 and Movento O-TEQ on microarthropod communities

Abstract

Chlorantraniliprole and Spirotetramat, present in the insecticides Coragen SC 20 and Movento O-TEQ, respectively, which were used in this work, are examples of recently developed chemistry classes of active substances.

Normally, laboratory single-species tests are performed as a first step to assess the toxicity of chemicals to non-target species. However, single-species tests do not allow to investigate indirect effects that may conditionate the toxicity of, for example, commercial formulations of pesticides on non-target species. This is a reason why it is important to conduct tests, such as community tests, that permit to assess this kind of effects. The main objectives were to - 1) evaluate the effects of these two insecticides on communities of microarthropods using community tests, including changes in abundance and possible shifts in community composition; 2) evaluate the influence/relevance of the mode of action of the two insecticides in the effect data provoked at the community level; 3) understand how far the recommended doses of both insecticides affect soil communities; 4) understand how far community tests, comparing with single-species tests, permit to go beyond in terms of effect data on PPPs. To achieve these objectives, two community tests were performed, one for each insecticide using natural soil and following a concentration gradient composed of 0, 0.01, 0.03, 0.1, 0.4, 1.2, 4, 13, 45, 150, and 500 mg a.i./kg.

For the test with Coragen SC 20 mites and collembolans were affected along the contamination gradient with shifts in the abundance of taxonomic groups of mites and changes in the functional composition of collembolans. Collembolans were more sensitive based on the toxic values derived (EC_{10} = 10.46 mg a.i./kg, EC50 = 35.59 mg a.i./kg, and NOEC = 4 mg a.i./kg), reflected by a strong decrease in their abundance starting from dose 13 mg a.i./kg. On the other hand, mites were not directly affected (EC_{10} = 497 mg a.i./kg, EC50 > 500 mg a.i./kg, and NOEC = 150 mg a.i./kg) and Oribatida had a strong increase in abundance at the two highest doses tested (150 mg a.i./kg and 500 mg a.i./kg) compared to control.

Regarding the test with Movento O-TEQ, effects were less evident compared to the test conducted with Coragen SC 20. Still, a shift in the abundance of taxonomic groups of mites was observed namely, at doses 45 mg a.i./kg and 150 mg a.i./kg, where Astigmata had a strong decrease and Oribatida became more dominant. At 500 mg a.i./kg both these two groups of mites were practically absent. Mites were much more sensitive compared with the test with Coragen SC 20 (EC_{10} = 18.98 mg a.i./kg, EC50 > 500 mg a.i./kg and NOEC = 0.4 mg a.i./kg). In contrast, collembolans were practically unaffected in terms of direct toxicity (EC_{10} = 196.53 mg a.i./kg, EC50 > 500 mg a.i./kg and NOEC = 150 mg a.i./kg) and their abundances even increased at higher doses, except at the highest one.

The use of community tests in this study allowed us to assess the effects on communities of microarthropods. Along the contamination gradient with Coragen SC 20 and Movento O-TEQ,

indirect effects were identified on these groups of in-soil organisms. Based on the results obtained in this study it was possible to evaluate how the mode of action of both insecticides was related to the data obtained at the community level. Moreover, it was concluded that the recommended doses of both pesticides should be safe for these soil communities and that the toxicity measured in single-species tests often can be higher compared to community tests.

Key Words: Insecticides; Chlorantraniliprole; Spirotetramat; Community tests; Microarthropods

II.1 Introduction

The increasing concern with possible direct and indirect effects on Ecosystems linked to the usage of pesticides has promoted the development of new classes that are supposed to be safer to the environment in general and specifically to key non-target species (Kodandaram et al., 2010; Mahmood et al., 2016; Palmquist et al., 2012).

Certain non-target species are key drivers of ecological processes that are the basis of the provisioning of key Ecosystem services (Tsiafouli et al., 2015). For instance, earthworms are called ‘’ecosystem engineers’’ because, through burrowing, they compact and loosen soil, contributing to soil structure and formation (Blouin, 2013). These air-filled pores that they create are essential for plants to grow or to organisms like collembolans or mites to live in (Coleman, 2015). If key species start to decline it is most likely that important functions they provide, will also start to disappear. For instance, the use of neonicotinoids has been described as toxic to earthworms, putting at stake the functions referred before (Goulson, 2013) and very toxic to bees (Decourtye et al., 2003; Yang et al., 2008) with great impacts on pollination (Decourtye et al., 2003; Yang et al., 2008)

Because of these issues, the implementation of new pesticides on the market is well regulated (European Commission, 2009, 2013). The European Food Safe Authority (EFSA) is responsible for evaluating and deciding the approval of Plant Protection Products (PPPs), as well as the active substances within them, on the European market according to their effects on non-target species. This process follows a tiered approach starting with laboratory single-species tests and going through with more complex assays if an unacceptable risk is indicated at lower tiers (EFSA, 2017; Van Gestel, 2012).

In general, the first tier toxicity tests consist in exposing standard species to a range of increasing concentrations of the PPP or the active substance to be tested, assessing acute and/or chronic effects on them (Vighi et al., 2013).

As a higher tier, a very common method is the Terrestrial Model Ecosystems (TMEs) which contrary to lower tier, and among other advantages, allow to assess potential indirect effects of pesticides such as changes in the community composition, and assess community recovery (Schaeffer et al., 2008). Also, field studies have been used, representing the most realistic exposure scenario because they are open systems that allow recolonisation, assessing long-term effects and recovery at the community level (EFSA, 2017; Jong et al., 2010; Schäfer et al., 2007). Moreover, the variability of environmental conditions is covered in field tests (Schäfer et al., 2007).

Recently, EFSA (2017) considered soil community tests as a good candidate to make part of an intermediate tier to fill the gap existing between lower and higher tiers. Whenever risk is identified in the lower tiers (e.g. single-species tests, extended laboratory tests), higher tier studies (e.g. field tests) must be conducted to address this risk (EFSA, 2017; European Commission, 2013). However, the process of developing a field test is very expensive and time consuming. By considering intermediate tiers (e.g. community tests) in the risk assessment methodology, there may be no need to perform such expensive tests, if the risk can be addressed in the intermediate tiers. Thus, the existence of intermediate tiers (e.g. community tests) allows a more complete methodology in terms of assessment of effects on in-soil organisms exposed to PPPs. Although community tests are still performed under laboratory conditions, they can be more ecologically relevant than single-species tests. This is because indirect effects like competition, predator-prey relationships, mutualism, or commensalism can be detected using communities of in-soil organisms (Sechi et al., 2014). They are also less expensive and time-consuming than higher tier tests, like TMEs or field tests (Sechi et al., 2014).

In this study, two community tests were conducted, separately, to evaluate the effects of two new generation pesticides, Coragen SC 20 and Movento O-TEQ, on natural communities of microarthropods.

Regarding Chlorantraniliprole, the active substance present in the insecticide Coragen SC 20, it belongs to the new class of anthranilic diamides and is used to control Lepidopteran, Coleopteran, and some Dipteran pests (USEPA, 2008a). The mode of action consists of binding to RyR's (Ryanodine Receptors) resulting in unregulated activation of the receptor channels. The regulation of muscle contraction is impaired because calcium accumulates in the cytoplasm, and consequently, insects exhibit lethargy, general paralysis and eventual death (Lahm et al., 2007). One feature that makes Chlorantraniliprole to be viewed as a potential safe substance is the fact that presents differential selectivity for insect receptors over mammalian ones (Cordova et al., 2006). Moreover, Chlorantraniliprole has been used efficiently and has been seen as a promising alternative to carbamates, organophosphates, pyrethroids, and neonicotinoids (Arnaudov and Petkova, 2020; Mahmood et al., 2016).

Spirotetramat is the active substance present in the insecticide Movento O-TEQ which is a member of the new class cyclic ketoenols and is used to control sucking pests essentially (e.g. aphids, whiteflies, scales, thrips, mites). Spirotetramat inhibits lipogenesis, resulting in fewer lipid

contents, growth inhibition of young insects, and affecting fertility and fecundity of female insects, thus reducing pest populations (Nauen et al., 2008). Spirotetramat is unique because it acts as two-way systemic moving upwards (xylem) and downwards (phloem), allowing protection against hidden pests and protecting new shoots and leaves (Nauen et al., 2008).

The assessment of the effects of both Chlorantraniliprole and Spirotetramat on in-soil organisms has been performed mainly on acute and/or chronic toxicity through single-species tests. Worms (e.g. *Eisenia fetida*), mites (e.g. *Typhlodromus pyri*, *Hypoaspis aculeifer*), and collembolans (e.g. *Folsomia candida*) are examples of key groups of in-soil organisms that have been assessed separately in these tests (APVMA, 2008, 2009; EFSA, 2013a; EFSA, 2013b; Latvizar et al., 2016; Maus et al., 2008; NFSA, 2010; USEPA, 2008a; USEPA, 2008b) which goes in agreement with the data requirements laid down in Regulation (EU) Nº 284/2013 for the meso-macrofauna organisms exposed to PPPs. Community tests assessing the effects of these substances on microarthropods have never been performed so this work intends to contribute with more data information regarding these chemicals and groups of organisms.

The main aims of this work were:

1 - to evaluate effects of both Coragen SC 20 and Movento O-TEQ, assessed separately, on communities of microarthropods, covering indirect effects as well, including changes in abundance and possible shifts in community composition;

2 - to evaluate the influence/relevance of the mode of action of two insecticides in effect data at the community level;

3 - to understand how far the recommended doses of the two insecticides affect soil communities;

4 – to understand how far community tests, comparing with single-species tests, permit to go beyond in terms of effect data on PPPs.

II. 2 – Material and methods

II.2.1 Test soil

The soil used in the laboratory tests was a natural soil collected in Herdade do Freixo-do-Meio, Municipality of Montemor-o-Novo, Alentejo, Portugal (38°41'39.7"N 8°18'27.6"W). This area is a typical cork oak forest (the so-called ‘’Montado’’) where biological agriculture was established since 1997 with sustainable management (untilled soil and without pesticides application), which contributes largely to the fact of being considered a reference site with an undisturbed agro-ecosystem. Once collected, the soil was sieved at 5 mm and stored in the laboratory at 20±3ºC in the dark until use. The soil was not defaunated because the objective was to use the natural community present in it. Soil properties of the test soil were measured following standard procedures and are presented in Table 5.

Table 5 – Characterisation of the natural soil of Herdade do Freixo-do-Meio used in this study.

Soil properties	
pH	4.99
WHC (%)	101.48
OM (%)	15.73 ± 0.096
Texture	
Sand (%)	68 ± 1
Silt (%)	24 ± 0.6
Clay (%)	8 ± 1
	Sandy Loam

II.2.2 Test substances

Two commercial formulations of insecticides were used as test substance: CORAGEN® with Chlorantraniliprole as active ingredient (a.i.; 200g/L; 18.4%, w/w) usually used for pome fruit, stone fruit, leafy vegetables, *Brassica* leafy vegetables, cucurbit vegetables, fruiting vegetables, cotton, grapes, potatoes, rice crops with recommended doses between 250 and 375 ml cpa/ha or 0.067 and 0.1 mg cpa/kg (considering a similar distribution of the pesticide over the top 5 cm soil layer and a soil density of 1.5g/cm³); MOVENTO® with Spirotetramat as active ingredient (150g/L; 15.3%, w/w) usually used for citrus, cucurbit vegetables, fruiting vegetables, grape, hops, leafy *brassica* vegetables, leafy non-*brassica* vegetables, pome fruit, potato and other tuberous and corm vegetables, stone fruit, tree nuts, onions, strawberries crops with recommended doses between 100 and 600 ml spt/ha or 0.02 and 0.12 mg spt/kg (considering a similar distribution of the pesticide over the top 5 cm soil layer and a soil density of 1.5g/cm³).

II.2.3 Laboratory community tests

Laboratory community tests followed the procedures described by Chelinho et al. (2014) with some changes. Soil contamination with Chlorantraniliprole was performed by preparing a stock solution (8.7g of Coragen SC 20 was weighted into a 1L volumetric flask and the volume was fulfilled with distilled water) that was mixed with the soil in different proportions with different water volumes to obtain the following concentration gradient of spiked soils: 0, 0.01, 0.03, 0.1, 0.4, 1.2, 4, 13, 45, 150 and 500 mg Chlorantraniliprole/kg) making a total of 11 treatments.
Regarding soil contamination with Spirotetramat, the only difference to the steps mentioned above was the preparation of the stock solution, where 10.46 g of Movento O-TEQ were added to a 1L volumetric flask and the remaining volume was fulfilled with distilled water.
Eight replicates for the control (0 mg/kg) and 5 replicates for each of the other test treatments were used. Each replicate consisted of cylindrical glass pots (9 cm diameter and 11 cm height) and 300g of a soil treatment (dry-weight equivalent; DW) was weighted into each pot. After preparing all units, the soil invertebrate community of three cores freshly collected from the field was extracted to each test vessel. These soil cores were also collected from the same site of the test soil using pvc tubes (5cm diameter and 5 cm depth). The extraction of soil cores from the field occurred on the 28th of November for the test with Coragen SC 20 and on the 7th of January

for the test with Movento O-TEQ. After collection, soil cores were stored at 20±2ºC until use, but for a period no longer than 1 week.

Also, to characterise the initial community, freshly soil cores were extracted directly to 8 falcon tubes containing ethanol 70%. For these steps, a high gradient Macfadyen extractor was used for 72h at 45ºC, to permit the extraction of soil fauna into test vessels. This was performed by extracting soil communities from cores directly into a portion of soil from a particular treatment placed in a falcon tube (with an approximate volume of 10 ml of fresh soil from the respective treatment). During the 72h of extraction, after each period of 24h, extracted invertebrate fauna (mostly microarthropods) were transferred to the respective test units and new falcon tubes with fresh soil were placed. This procedure was adopted to avoid excessive exposure of the extracted organisms to heat and eventually forced predation between the extracted organisms due to space limitation in the falcon tubes. Finalized this step, all test vessels were covered with plastic caps (perforated with small holes for air circulation) and placed in an incubation chamber at 20 ± 2º and under a photoperiod of 16h light and 8h dark for six weeks. During this period, food in the form of granulated dry yeast was provided weekly (2 mg approximately), and the water losses were compensated with the addition of distilled water individually for each replicate. After six weeks of incubation, soil invertebrates were extracted under the same conditions as the initial community extraction but directly to a 70% ethanol solution.

To check if the soil stored still had fauna present in it, randomly selected portions of the sieved soil, previously stored in the chamber room, were extracted to 8 falcon tubes containing a 70% ethanol solution, using the same heat extraction process.

II.2.4 Microarthropods identification

The next step in the procedure was to identify soil microarthropods extracted into major groups using a binocular magnifier. Collembolans were separated in morphotypes following the method described by Martins da Silva et al. (2015). Morphotypes were attributed considering the following traits: ocelli, furca, antenna, pigmentation, and the presence of hairs and scales. These traits are morphological characteristics connected to the adaptation of each collembolan species to the soil environment and were then combined to create the ‘’Life-form’’ trait (Vanderwalle et al., 2010). Traits that define life-forms were analysed using a combination of morphological traits based on the eco-morphological index (EMI; Vanderwalle et al., 2010). EMI separates collembolan species according to their degree of adaptation to the soil profile. In this work, we used the adaptation of trait codification as it is mentioned in Martins da Silva et al. (2015). After attributing the partial EMI scores (for each trait), the final calculation of the EMI value is the sum

of all the scores from each individual trait. A morphotype was attributed to each combination of different traits. Thus, morphotypes that had an EMI punctuation between 2 and 8 were denominated Ep1, Ep2, Ep3, etc (epiedaphic organisms), morphotypes with EMI between 10 and 12 were designated with He1, He2, He3, etc (hemiedaphic organisms) and morphotypes with EMI score between 14 and 20 were named Ed1, Ed2, Ed3, etc (representing euedaphic organisms) (Table I.1 and I.2 – Annex I).
Mites were identified to the order level (Prostigmata, Mesostigmata, Oribatida and Astigmata) through the identification keys (Guia morfosp Covas (PT)_UDP; Guia morfosp Brôa (BR)).

II.2.5 Calculations and data analysis

Data on Collembola morphotypes and mite orders, extracted in the different treatments were organized in different excel sheets and were statistically analysed individually. The abundance data type of each group (collembolans and mites) were transformed through square root to build Bray-Curtis similarity matrices for each of the two groups mentioned above using PRIMER & PERMANOVA 6.0 (Clarke and Gorley, 2006). To have dose-response curves, the similarity within the controls (i.e. between control replicates) and between controls and each concentration dose were selected. Here, a reduction in similarity is expected to occur between higher doses of the pesticides and the controls as the effects of contamination take place. After this step, effective concentrations for 10 and 50% of effect (EC_{10} and EC_{50}, respectively) were calculated applying nonlinear regressions (Environmental Canada, 2007) and the best-fitting model was chosen using Statistica 7.0 (Stat. Soft. Inc., 2004).
Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed to evaluate significant differences ($p \leq 0.05$) of similarity between control and treatments using PRIMER & PERMANOVA 6.0 **(**Clarke and Gorley, 2006). These differences allowed us to determine the non-observed effect concentrations (NOECs) and the lowest observed effect concentrations (LOEC's) for each test substance. After this, a SIMPER analysis was performed to support the previously PERMANOVA analyses, and to quantify the contribution of each group of organisms to the differences in similarities observed.
Data gathered from the analysis of collembolan morphological traits of replicates per treatment were used to estimate the community trait weighted mean (mT) as described by Vanderwalle et al. (2010), Chelinho et al. (2010), and Rieff et al. (2020). The mT was estimated as an average for a given trait weighted by species abundance, according to the formula:

$$mT = \sum_{i=1}^{S} P_i X_i$$

Where P_i is the proportion of the i-th species and X_i the trait value of the i-th species in a sample. For each category, the relative abundance of a particular group is calculated by the index.

II.3 – Results

II.3.1 – Microarthropod community

The initial community (IC) of the test soil was composed of 11 groups of microarthropods dominated by collembolans (66.83 % of total individuals) followed by mites (30.73 % of total individuals) (Table II.1 – Annex II). Together, representing 97.56 % of the total organisms found. For mites, Oribatida and Astigmata dominated the initial community (35.47 % and 33.91 %, respectively). Prostigmata and Mesostigmata were less represented (17.44 % and 13.18 %, respectively). As for collembolans, epiedaphic organisms dominated the community (52.5 %) followed by hemiedaphic (29.95 %) and the euedaphic were the less represented (17.56 %).

Regarding the test with Coragen SC 20, mites and collembolans dominated in abundance both in controls and the treated samples with 70% and 27.6% representativeness, respectively (97.6% together). As for other microarthropod groups, members of Chilopoda, Coleoptera, Diplura, Formicidae, Hymenoptera, Diptera, Pauropoda, and also other groups such as Nematoda, Enchytraeidae, and Oligochaeta were also found in control/treatments but none of these groups had more than 10 individuals in total across all the samples.

For the test with Movento O-TEQ, members of Chilopoda, Coleoptera, Diptera, Hymenoptera, Enchytraeidae, Oligochaeta, and Nematoda were recovered from controls/treatments, but again, none of them had more than 10 individuals across all samples. Collembolans and mites were the groups most represented across all samples with 61,8% and 37,6% of representativeness, respectively, together making a total of 99,4% of the total abundance found of all organisms.
Thus, the effect of Coragen SC 20 and Movento O-TEQ was evaluated uniquely on the community of mites and collembolans.

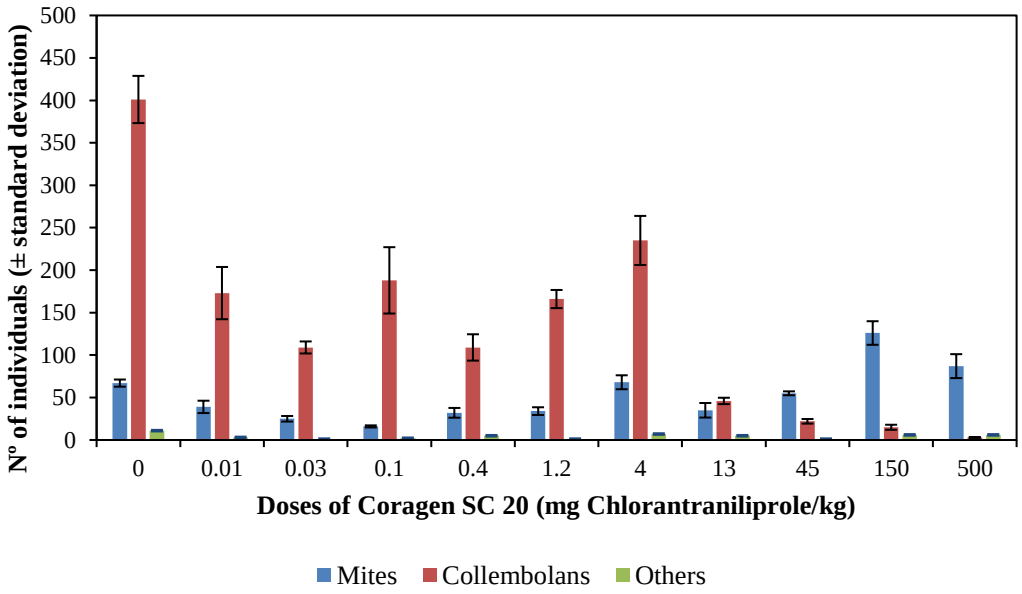

Figure 2: Total abundance (average ± standard deviation; *n* = 8 or 5) of mites, collembolans, and other soil fauna when exposed to a natural soil with increasing concentrations of Coragen SC 20 (a.i. Chlorantraniliprole) for 6 weeks.

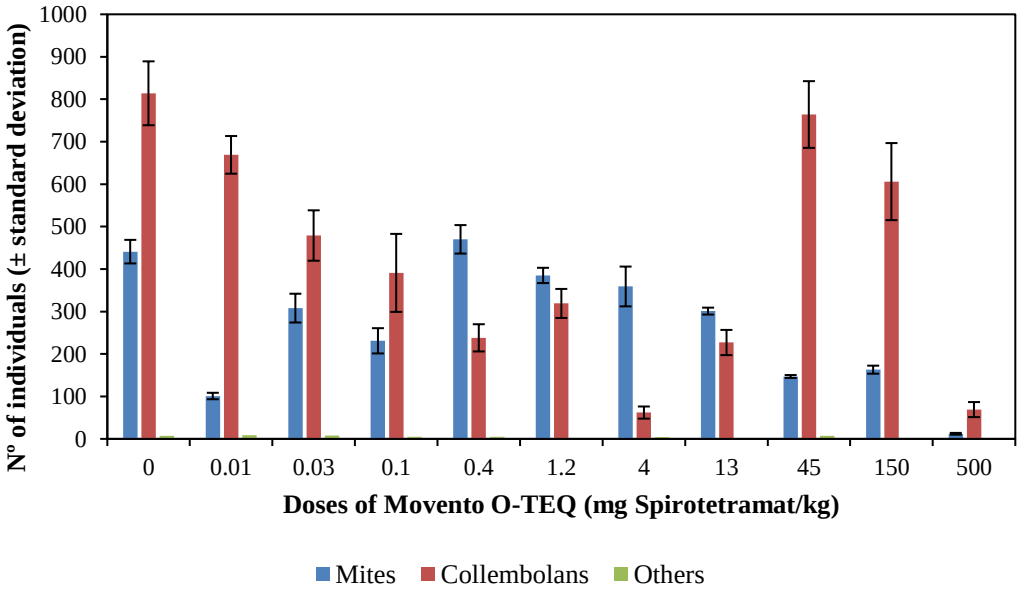

Figure 3: Total abundance (average ± standard deviation; *n* = 8 or 5) of mites, collembolans, and other soil fauna when exposed to a natural soil with increasing concentrations of Movento O-TEQ (a.i. Spirotetramat) for 6 weeks.

II.3.2 – Effects of Coragen SC 20 on mites

The total number of mites recovered in controls at the end of the test, and comparing to the IC, had a decrease of 12.98 %.

At the end of the test period, a total of 584 individuals were sampled and identified in all replicates from control and treatments. The most represented group was the suborder Oribatida with 451 organisms, which counts for around 77% of the total number of mites found. The other mite groups had a low abundance of organisms with Prostigmata having 74, Mesostigmata with 44, and Astigmata with 15 individuals. There was also a high variability between replicates and treatments in the total number of mites (Figure 4).

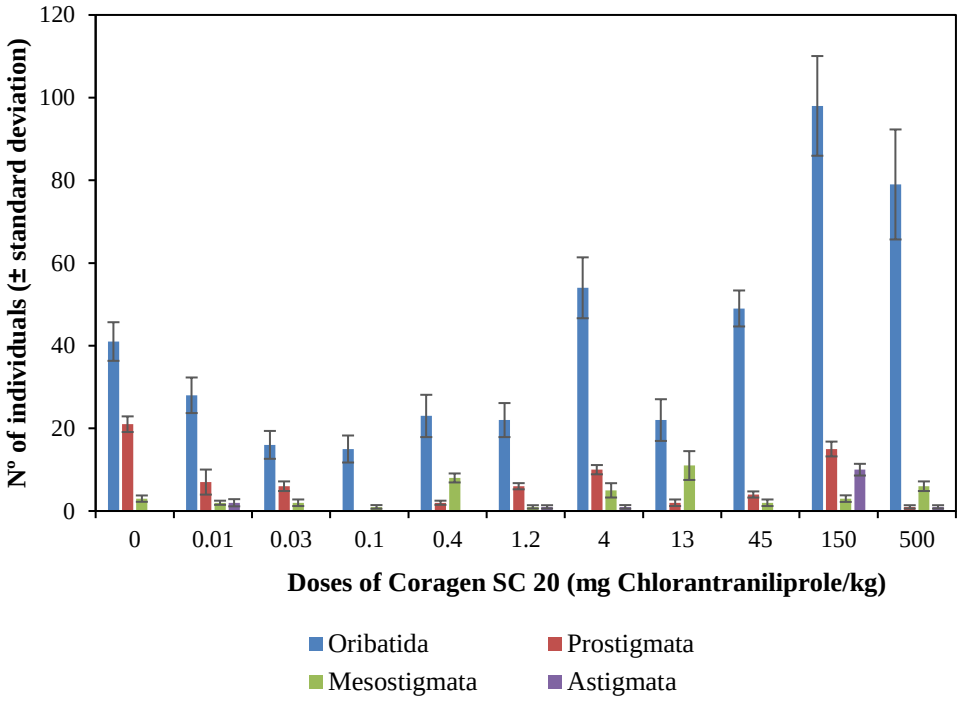

Figure 4: Total abundance (average ± standard deviation; *n* = 8 or 5) of different groups of mites (Oribatida, Prostigmata, Mesostigmata and Astigmata) when exposed to a natural soil with increasing concentrations of Coragen SC 20 (a.i. Chlorantraniliprole) for 6 weeks.

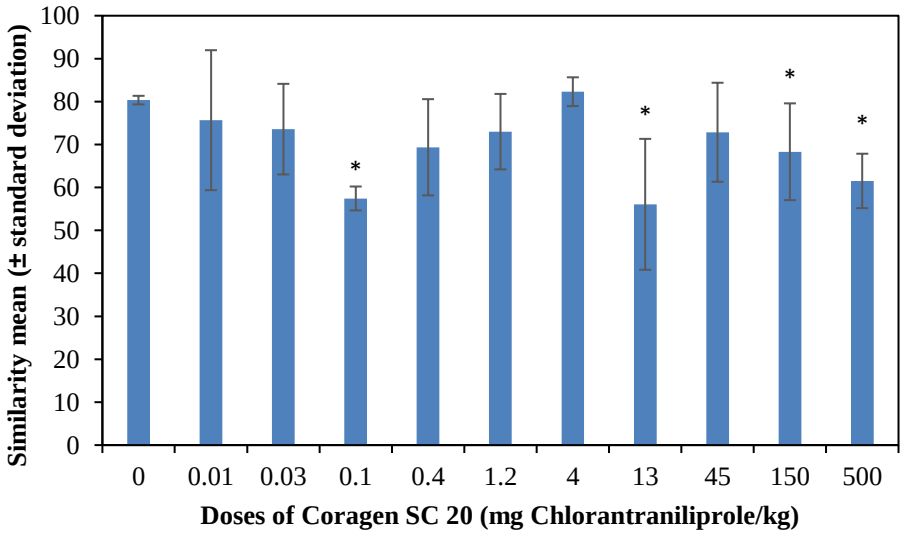

Figure 5: Similarity (through Bray-Curtis indices; average ± standard deviation; *n* = 8 or 5) of mite community between control and treatments when exposed to a natural soil with increasing

concentrations of Coragen SC 20 (a.i. chlorantraniliprole) for 6 weeks. * Significantly different from control.

The Permanova main test indicated significant differences in the similarity between control and treatments (Pseudo-F = 2.555, $p < 0.05$). Going through with the analysis, the Permanova pair-wise test indicated significant differences in the community of mites at doses 0.1 mg/kg, 13 mg/kg, 150 mg/kg, and 500 mg/kg, compared with control ($p < 0.05$) (Fig. 4 and 5). This is corroborated by the dose-response curve performed with the similarity between controls and between controls and each treatment, based on the Bray-Curtis distance matrix, which revealed higher decreases in similarity comparing to control on these doses (Fig. 5). Prostigmatic mites were the group that contributed the most for the significant differences between control and the doses 0.1mg/kg and 13mg/kg (61,72% and 50,41% contribution, respectively) reflected by the decrease in their relative abundance (Fig. 4). At dose 150 mg/kg, mites belonging to the Oribatida group contributed more to the significant differences compared with control (Table III.3 – Annex III) with more than two times the abundance found in control (Fig. 4). Astigmata was the second group that contributed more to the dissimilarity (Table III.3 – Annex III) since 67% of the total individuals from this group were found at this dose. Prostigmata mites contributed with 19.57 % reflected by a decrease in its relative abundance. Lastly, Mesostigmata contributed with 15.21 % to the dissimilarity again with a decrease in the relative abundance. At dose 500 mg/kg, Prostigmata mites contributed more for the significant differences compared to control (40.46% contribution) reflected by a great decrease in abundance, with only 1 individual found. Oribatida was the second group that contributed most for the dissimilarity with 36.97 % reflected by a 92.7 % increase in abundance compared to control. Finally, Mesostigmata contributed with 18.50 % to the dissimilarity with 1 more individual compared to control. Also, it is worth mentioning the almost consistent increase in numbers of Oribatid mites, along the contamination gradient. The abundance of Oribatid mites was the highest at the two last doses (150 mg/kg and 500 mg/kg), compared with the other treatments and control (39.25% of the total numbers of Oribatid mites found in all treatments and control were at the two highest doses).

Regarding the toxic values for mites along the contamination gradient with Coragen SC 20, an EC_{10} = 497 mg a.i./kg and EC_{50} > 500 mg a.i./kg were derived and a NOEC = 45 mg a.i./kg and a LOEC = 150 mg a.i./kg were chosen (Table 6). Although significant differences, compared to control, were observed at doses 0.1 and 13 mg a.i./kg, we assume here that they might be related to the natural variability of microarthropod communities extracted in the field. Moreover, an increase in similarity occurred after 0.1 and 13 mg a.i./kg (Figure 5) and these are the reasons behind the decision of not considering these concentrations as LOEC's.

II.3.3 – Effects of Coragen SC 20 on collembolans

The total number of collembolans recovered in the controls, compared to IC, decreased to 35.74 % after the test period.

In total, 1467 collembolans were sampled in control and all treatments, distributed by 20 morphotypes. Ep4, Ep2 Ed3, Ep7, and He1 were the most represented morphotypes with 247, 199, 187, 138, and 137 individuals, respectively. Five morphotypes stand out for having less than 20 individuals: Ep6, He5, He6, He2, and Ed4. Epiedaphic collembolans (Ep) represented 67% (985 individuals) of the total number of collembolans found among control and treatments, while hemiedaphic (He) represented 15% (215 individuals) and euedaphic 18% (267 individuals).

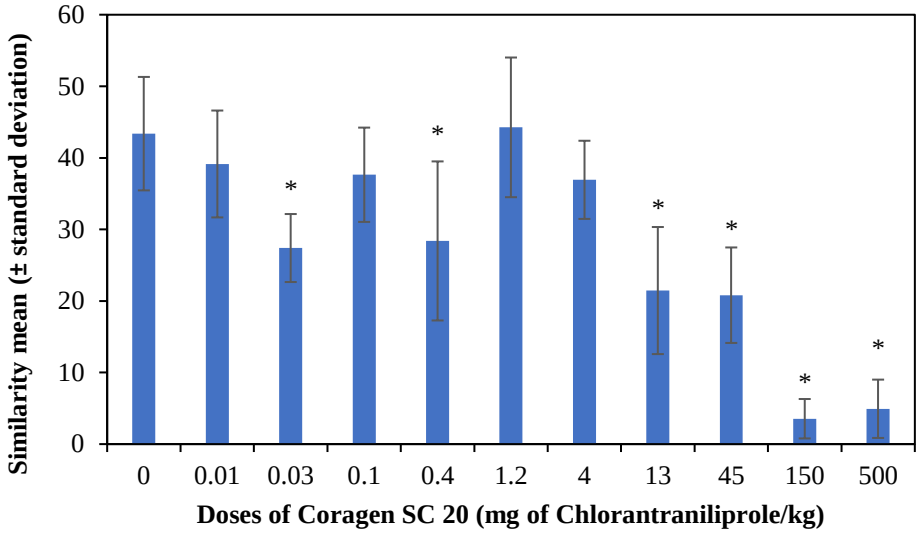

Figure 6: Similarity (through Bray-Curtis indices; average ± standard deviation; *n* = 8 or 5) of Collembola community between control and treatments when exposed to a natural soil with increasing concentrations of Coragen SC 20 (a.i. chlorantraniliprole) for 6 weeks. *Significantly different from control.

The Permanova main test analysis indicated significant differences in the similarity of the Collembola community between control and treatments (Pseudo-F = 3.4218, $p < 0.05$). From the observation of the dose-response curve, the major significant drops in the similarity between treatments and control occur at the last four doses (13 mg/kg, 45 mg/kg, 150 mg/kg, and 500 mg/kg). The Permanova pair-wise test supports this observation, indicating significant differences in similarity starting from 13 mg a.i./kg but also at doses 0.03 mg a.i./kg and 0.4 mg a.i./kg, compared to control ($p < 0.05$), which is in agreement with the similarity graphic of Fig. 4, where it is observable a decrease in similarity in these both doses. Interestingly, the same number of collembolans was found in these two doses (109 individuals in each), which was the lowest

abundance observed among all the treatments, except for the last four doses. Moreover, at dose 0,03 mg/kg, individuals characterized by morphotypes Ep7 and Ep10 were absent (but they were well represented in the control) and a high abundance of Ep2, compared to control, was observed (the contribution to the dissimilarity of these morphotypes was 9.78%, 8.28%, and 7.95%, respectively; Table III.5 – Annex III). As for the dose 0,4 mg/kg, Ep2, Ep1, and Ep7 were the morphotypes that most contributed to the different dissimilarities compared to control (10.16%, 9.16%, and 8.75% contribution, respectively; Table III.6 – Annex III). Ep2 had a high abundance at 0,4 mg/kg compared with control, Ep1 was well represented in control but had no individuals at 0,4 mg/kg and Ep7 was well represented in control but not at 0,4 mg/kg.

At dose 13 mg/kg morphotypes Ep7, Ep1, and Ep10 contributed the most for the dissimilarity comparing to control (11.28%, 11.05%, and 10% contribution, respectively,). Ep7 had a high abundance of individuals at control comparing to the dose 13 mg/kg, while Ep1 and Ep10 were well represented in control but no individuals were found for this dose.

Regarding the dose 45 mg/kg, the morphotypes Ep10, Ep1, and He1 contributed more for the dissimilarity when compared with control (10.18%, 10.15%, and 9.91%, respectively). Ep10 and He1 were well represented in control but no individuals were found at 45 mg/kg and Ep1 was also well represented in control but at dose 45 mg/kg only 1 individual was found.

At dose 150 mg/kg, Ep7, Ep1, and Ep10 contributed more for the dissimilarity comparing to control (13.12%, 11.79%, 9.40% contribution). All these morphotypes were well represented in control but had no individuals at 150 mg/kg.

Finally, at the highest dose (500 mg/kg) the same morphotypes Ep7, Ep1, and Ep10 contributed more to the dissimilarity observed (13.77%, 12.44%, and 9.74% contribution, respectively). All of them were well represented in control but had no individuals at the last dose.

The toxic values derived for collembolans along the concentration gradient with Coragen SC 20 were EC_{10} = 10.46 mg a.i./kg and EC_{50} = 35.59 mg a.i./kg and a NOEC = 4 mg a.i./kg and LOEC = 13 mg a.i./kg were chosen (Table 6). Again, significant differences in similarity were found at lower doses (0.03 and 0.4 mg a.i./kg) that might be due to the natural variability of field microarthropod communities, so they were not considered as LOEC's. Also, an increase in similarity is observed after these doses (Figure 6).

Regarding the mT values obtained along the contamination gradient, significant differences were obtained at the last 4 doses (13, 45, 150, and 500 mg a.i./kg) comparing to control (Table 7). This was reflected by a shift in community composition of collembolans at these doses where practically only epiedaphic collembolans were found (Table II.4 – Annex II).

II.3.4 – Effects of Movento O-TEQ on mites

The total numbers of mites recovered in the controls at the end of the test period, compared to IC, decreased to 85.47 %.

In contrast to the total abundance obtained in the community test with the insecticide Coragen SC 20, here, the total number of mites was higher, with 2918 mites sampled and identified between controls and all treatments. The most represented group was the cohort Astigmata with 1680 individuals, followed by the suborder Oribatida with 1122 individuals. The remaining groups had a low abundance of mites compared with these. Mesostigmata had a total of 78 individuals and Prostigmata was represented with 38 individuals. Again, a high variability occurred in the relative abundance of mites between replicates and treatments.

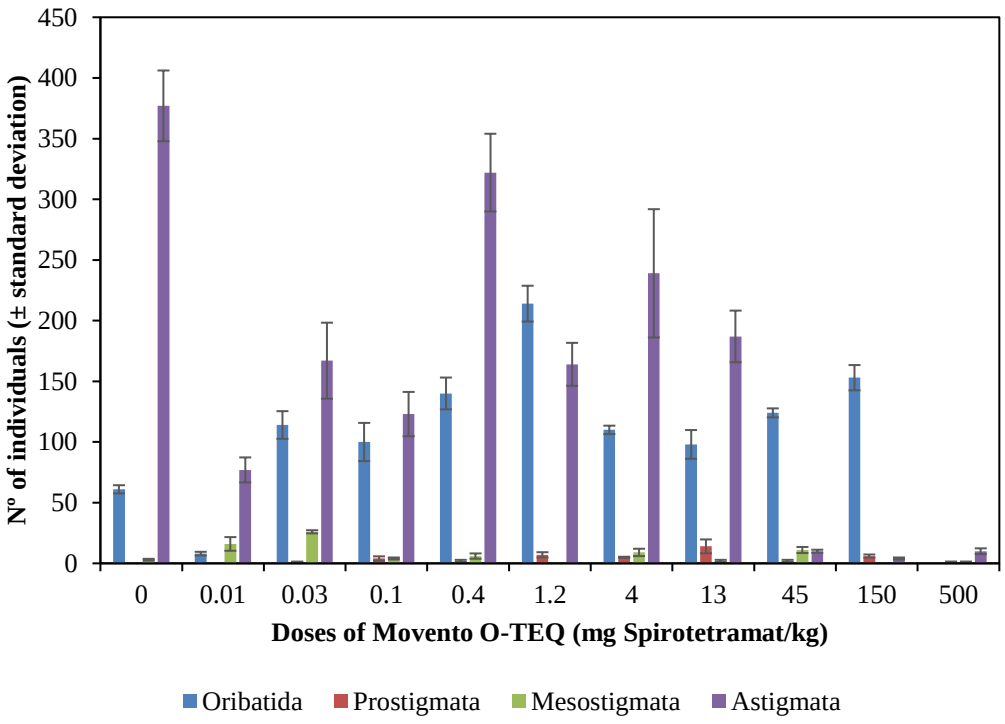

Figure 7: Total abundance (average ± standard deviation; *n* = 8 or 5) of different groups of mites (Oribatida, Prostigmata, Mesostigmata and Astigmata) when exposed to a natural soil with increasing concentraations of Movento O-TEQ (a.i. spirotetramat) for 6 weeks.

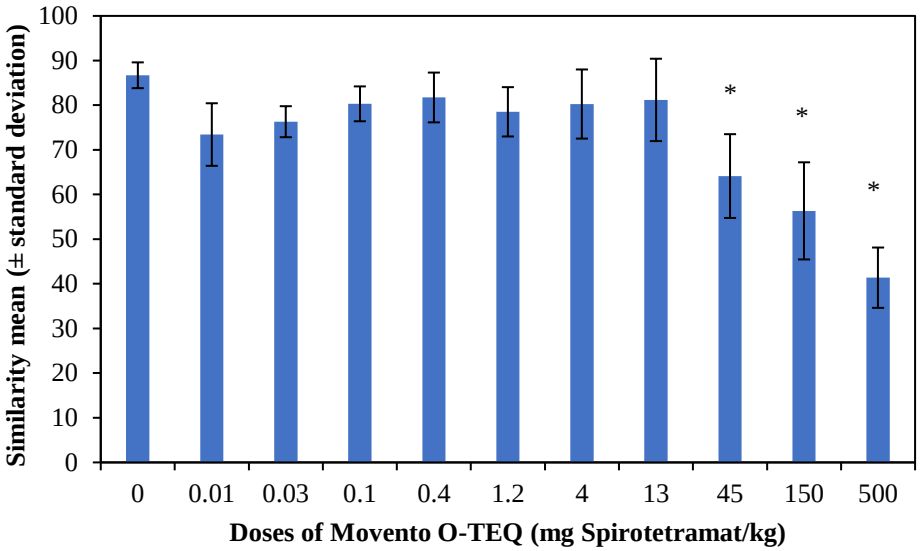

Figure 8: Similarity (through Bray-Curtis indices; average ± standard deviation; *n* = 8 or 5) of mite community between control and treatments when exposed to a natural soil with increasing concentrations of Movento O-TEQ (a.i. Spirotetramat) for 6 weeks. * Significantly different from control.

The Permanova main test indicated significant differences between control and treatments (Pseudo-F = 10.35, $p < 0.05$). The dose-response relationship (Fig. 8) shows a relatively consistent similarity until the last 3 highest doses (45, 150, and 500 mg/kg) tested, where a significant drop occurred and thus effects on mites became statistically detectable. At dose 45 mg/kg, Astigmata was the group that most contributed to the significant differences comparing to control (57.97 % contribution), followed by Oribatida (26.61 % contribution) and Mesostigmata (12.12 %). The same trend was observed at dose 150 mg/kg with Astigmata contributing more for the significant differences (59.50 % contribution) than Oribatida (29.40 % contribution) and Prostigmata mites (8.33 %). Indeed, at both doses, it was observed a great decrease in abundance of Astigmata mites (only 10 individuals at dose 45 mg/kg and 4 individuals at dose 150 mg/kg, which represented a decrease in 97.35 % and 98.94 %, respectively, compared to control which had 377 individuals) (Table II.3 – Annex II; Table III.11 and III.12 – Annex III). Contrarily, Oribatida mites had an increase in abundance at both 45 and 150 mg/kg doses. Control had 61 individuals and this number raised to 124 individuals, at dose 45 mg/kg, and 153 individuals, at dose 150 mg/kg (representing an increase of 103,28 % and 150.82 % in abundance, respectively) (Fig. 7).

At the maximum dose tested (500 mg/kg), once more, Astigmata mites were the ones that most contributed to the dissimilarity with control (58.74 % contribution), followed by Oribatida mites (33.86 % contribution). Here, only 10 individuals of Astigmata were found (representing a decreased of 97.35 % in abundance compared to control) whereas Oribatida were not represented at all (Fig. 7).

Along the contamination gradient of Movento O-TEQ an EC_{10} = 18.08 mg a.i./kg and EC_{50} > 500 mg a.i./kg were derived and a NOEC = 13 mg a.i./kg and a LOEC = 45 mg a.i./kg were chosen (Table 6). This is because significant differences in similarity comparing to control were observed starting from dose 45 mg a.i./kg.

II.3.5 – Effects of Movento O-TEQ on collembolans

The total number of collembolans recovered in the controls at the end of the test, comparing to IC, was of 72.55 %.

In total, 4638 individuals were sampled in control and all treatments, distributed by 17 morphotypes. Ep7 and He1 were the most represented morphotypes with 2825 and 1228 individuals, respectively, which represented more than 87% of the total Collembola abundance found. Seven morphotypes stand out for having less than 10 individuals: Ep1, Ep3, Ep5, Ep8, Ep10, He3, and Ed2. Epiedaphic collembolans (Ep) represented 69,6% (3263 individuals) of the total number of collembolans found among all the samples, hemiedaphic (He) 29,8% (1397 individuals), and euedaphic (Ed) 0,6% (30 individuals).

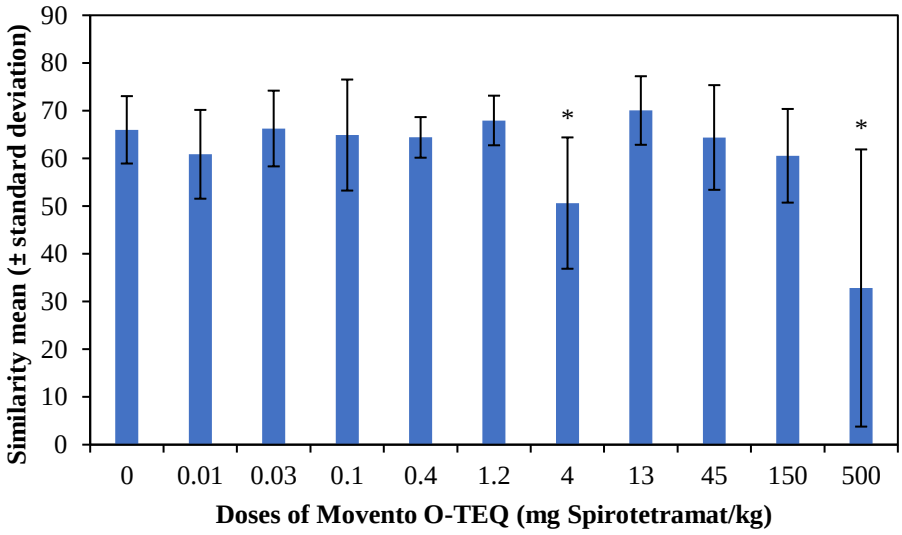

Figure 9: Similarity (through Bray-Curtis indices; average ± standard deviation; *n* = 8 or 5) of Collembola community between control and treatments when exposed to a natural soil with increasing concentrations of Movento O-TEQ (a.i. spirotetramat) for 6 weeks. *Significantly different from control.

Looking at the dose-response curve obtained, there were no significant changes in the similarity between controls and the rest of the doses, except for the dose 4 mg a.i./kg and the highest dose tested (500 mg a.i./kg). The Permanova main test indicated significant differences in the similarity between treatments and controls (Pseudo-F = 2.0187, $p < 0.05$), and the Permanova pair-wise test showed significant differences on doses 4 mg/kg and 500 mg/kg (the highest tested), compared to control ($p < 0.05$). At dose 4 mg/kg, the lowest abundance in collembolans (62 individuals) was found among all treatments and control, even when comparing with dose 500 mg/kg (which had 69 individuals). Morphotypes that most contributed to the dissimilarity between dose 4 mg/kg and control were He1, Ep7, Ep9, and Ep6 (with 28.15 %, 22.74 %, 19.10 %, and 14.13 % contribution, respectively). Regarding the two most represented morphotypes, Ep7 had a total number of 525 individuals in control but at dose 4 mg/kg only 23 individuals were found. For He1, control had 202 individuals whereas dose 4 mg/kg had only 18 individuals.

For the highest dose tested (500 mg/kg), morphotypes that most contributed to the dissimilarity comparing to control were Ep7, He1, and Ep6 (with 32.29 %, 24.98 %, and 23.97 % contribution, respectively). Morphotype Ep7 had 525 individuals in control but at 500 mg/kg dose only 53 individuals were recovered. He1 had 202 individuals in control and only 15 at the highest dose. For Ep6, control had 60 individuals but was not represented at 500 mg/kg.

Regarding the toxic values, an EC_{10} = 196.53 mg a.i./kg and EC_{50} > 500 mg a.i./kg were derived, and a NOEC = 150 mg a.i./kg and LOEC = 500 mg a.i./kg were chosen (Table 6). At dose 4 mg a.i./kg significant differences in similarity comparing to control were obtained (Figure 9). Again, it was not considered as a LOEC because we assume that the natural variability of microarthropod communities could explain the result, and, after this dose the similarity increases.

The mT values derived along the contamination gradient did not show significant differences in the community composition of collembolans comparing to the control (Table 7). Although significant differences in similarity were observed at doses 4 and 500 mg a.i./kg, most collembolan species in the community had similar traits (Table 7), so the mT value did not change significantly.

Table 6: No observed effect concentrations (NOEC), lowest observed effect concentrations (LOEC), and 10% (EC_{10}) and 50% (EC_{50}) effect concentrations (with 95% confidence intervals) for Chlorantraniliprole (sourced by Coragen SC 20) and Spirotetramat (sourced by Movento O-TEQ) to mites and collembolan communities. Values are expressed in mg of active ingredient/kg soil (dry weight equivalent).

Active ingredient	**Community**	**NOEC (mg/kg)**	**LOEC (mg/kg)**	**EC_{10} (mg/kg)**	**EC_{50} (mg/kg)**
Chlorantraniliprole	Mites	45	150	497 [a]	>500 [a]
	Collembolans	4	13	10.46 (0.71-20.21)	35.59 (11.86-59.32)
Spirotetramat	Mites	13	45	18.08 [a]	>500
	Collembolans	150	500	196.53 [a]	>500

[a] – Data obtained did not allow the estimation of the 95% confidence intervals.

Table 7: Community trait weighted mean (mT; average ± standard deviation; *n* = 8 or 5) estimated for collembolan communities and the respective number of morphospecies (n) considered when exposed to a natural soil with increasing concentrations of the commercial formulations Coragen SC 20 (a.i. Chlorantraniliprole) and Movento O-TEQ (a.i. Spirotetramat). For more details about the estimation of mT see in the text.

Test dose (mg a.i./kg)	0	0.01	0.03	0.1	0.4	1.2	4	13	45	150	500
Coragen SC 20											
mT	7.70 ± 2.68	9.31 ± 1.43	9.39 ± 3.87	8.95 ± 2.54	8 ± 1.86	7.22 ± 1.07	8.2 ± 2.68	5.55±1.44*	5.2± 2.55*	3.6 ± 3.29*	4 ± 4.9*
n	19	13	11	15	12	15	17	6	5	1	2
Movento O-TEQ											
mT	6.97 ± 0.67	8 ± 0.97	7.23 ± 0.65	6.56 ± 0.53	5.94 ± 3.39	7.17 ± 0.75	5.68 ± 3.22	7.02 ± 0.42	7.21 ± 0.66	7.39 ± 0.45	4.08 ± 3.73
n	7	9	10	7	8	5	5	8	6	9	3

* Significantly different from control.

II.4 - Discussion

II.4.1 – Effects of Coragen SC 20 and Movento O-TEQ on the community of mites and collembolans

The community of mites and collembolans was affected along the contamination gradient with Coragen SC 20, with shifts in the taxonomic groups of mites and changes in the morphological traits of collembolans. On the other hand, the effects of Movento O-TEQ on the community of mites and collembolans were less evident compared with the effects of Coragen SC 20. Indeed, shifts in the taxonomic groups of mites were observable along the contamination gradient but changes in the morphological traits of collembolans were not significant (Table 7).

Overall, in the test with Coragen SC 20, Prostigmata, Mesostigmata, and Astigmata mites had a very low abundance when comparing to Oribatids (Table II.2 – Annex II) and this fact made difficult the statistical analysis of data and its interpretation on the first three groups mentioned above. On the other hand, in the test with Movento O-TEQ, Astigmata mites that were practically absent in the test with Coragen SC 20 (only 15 individuals among all samples) were the most represented group (1680 individuals found in controls plus treatments). These differences in soil community are related to the natural seasonal variance occurring in the field since the soil collections for community extractions occurred at different times of the year. For Coragen SC 20, the soil cores containing the community were collected on the 28th of November, while for Movento O-TEQ they were collected on the 6th of January. Both mites and collembolans have peak populations in spring and autumn in temperature climates (Edwards and Thompson, 1973). The same method was used, in the same site and by the same persons for both tests with Coragen SC 20 and Movento O-TEQ, so these aspects did not contribute to the differences in the soil community.

Mites were not very affected by Coragen SC 20 based on the derived EC_{10} = 497 mg a.i./kg and LOEC = 150 mg a.i./kg (Table 6). This is in agreement with previous studies that also reported low toxicity of Coragen SC 20 to *Typhlodromus pyri* with an LR_{50} and ER_{50} > 750 g a.i./ha (APVMA, 2008; EFSA, 2013a; NFSA, 2010; USEPA, 2008a).

Movento O-TEQ was more toxic to mites compared with Coragen SC 20 based on the EC_{10} of 18.08 mg a.i./kg and LOEC of 45 mg a.i./kg estimated (Table 6). However, the EC_{50} values derived for mites in both tests with Coragen SC 20 and Movento O-TEQ were higher than the highest dose

tested (> 500 mg a.i./kg). The toxicity of Movento O-TEQ towards mites was higher at the beginning of the dose-response curve but then dissipated. Data from tier 1 laboratory studies have shown very high toxicity of Movento O-TEQ to the predatory mite *Typhlodromus pyri* based on LR_{50} = 0.33 g a.i./ha and NOEC = 0.15 g a.i./ha derived for acute contact toxicity, assessing mortality (APVMA, 2009; EFSA, 2013b; Maus et al., 2008; USEPA, 2008b). On the other hand, the toxicity of Spirotetramat-enol, which is the primary metabolite of the active substance Spirotetramat, showed lower toxicity towards *Hypoaspis aculeifer* based on a NOEC = 316 mg metabolite/kg derived in mortality and reproduction tests (APVMA 2009; EFSA, 2013b; Maus et al., 2008; USEPA, 2008b). The higher toxicity found for Movento O-TEQ compared to Coragen SC 20 on mites might be associated with the fact that Spirotetramat belongs to the chemical class of cyclic ketoenols (Lummen et al., 2014) that have been used both as insecticides and acaricides (Marcic et al., 2011).

These two species (*T. pyri* and *H. aculeifer*) are predatory mites and standard indicators used in the first tier risk assessment step to non-target arthropods exposed to PPPs (Candolfi et al., 2001; European Comission, 2013; OECD, 2008) but direct comparisons with these data must be made cautiously. EFSA (2017) reported that the representativeness of *H. aculeifer* to other groups of soil mites could not be drawn because of the low toxicity data available.

In the test with Coragen SC 20, Oribatids had their highest abundance in the highest two doses and were the main group of mites to contribute to the significant differences between dose 150 mg/kg and control (Table III.3 – Annex III). As for the last dose (500 mg/kg), Oribatida was the second group that contributed the most to the dissimilarity comparing to control (Table III.4 – Annex III). This observation of increased abundance along the contamination gradient is in agreement with a study performed by Chelinho et al. (2014) where Oribatid mites from a brazilian soil consistently increased their abundance over increasing Carbofuran doses and, the authors argued that one possible explanation could be the reduction in the number of competitors (such as collembolans) for the available food (supplied during the test in the form of yeast). In our study with Coragen SC 20, a great reduction in the number of collembolans and a great increase in the number of Oribatid mites at the highest three doses, compared to control (Table II.2 – Annex II; Fig. 4), corroborates this hypothesis reflecting a potential indirect effect via competition of Coragen SC 20 on these groups of microarthropods. Regarding the test with Movento O-TEQ, potential effects of competition were also observed along the contamination gradient. Astigmata, generally, were dominant in control and at most treatments until dose 13 mg/kg compared to Oribatid mites (Table II.3 – Annex II; Fig. 5). Astigmata are known to be specialists in exploiting spatially and/or temporally restricted to microhabitats (Krantz and Walter, 2009) which might give them an advantage in terms of avoiding contact with contaminants in soil. Moreover, they have a short-generation time contrarily to Oribatida (Behan-Pelletier, 2000; Krantz and Walter, 2009) so they can reproduce more rapidly, reaching higher abundances faster. At control and lower doses (until 13 mg/kg), these characteristics might have favoured them in terms of competing with Oribatida for the available food. Also, Collembola had a high abundance (except at 4 mg/kg and 500 mg/kg)

(Table II.5 – Annex II) and might as well competed with Oribatida for food (provided during the tests in the form of yeast). Collembolans, Astigmata, and Oribatida mites are mainly saprophagous (feeding on decaying vegetation or animal detritus) and/or mycophagous (feeding on fungi) (Krantz and Walter, 2009; Rusek, 1998). Considering doses that showed significant decreases compared to control in the test with Movento O-TEQ, at 45 mg/kg and 150 mg/kg, Oribatida were more abundant than Astigmata (Table II.3 – Annex II; Fig. 5). Indeed, at 45 mg/kg and 150 mg/kg, Astigmata had a higher contribution for the significant differences compared to control (Table III.11 and III.12, respectively – Annex III) due to its great decrease in abundance, that was followed by Oribatida which were still well represented at these doses (Table II.3 – Annex II; Fig. 7). This might be related to the fact that feeding stages of Astigmata have low body sclerotization compared with Oribatida (Krantz and Walter, 2009) thus making them more susceptible to be affected by these higher pesticide doses. Another observation made at these doses was the higher abundance of collembolans compared with lower doses (Table II.5 – Annex II) which also could be explained by the general decrease in the abundance of competitors (especially Astigmata) for the available food. At 500 mg/kg, both Astigmata and Oribatida were the groups that contributed more to the significant differences compared to control (Table III.13 - Annex III.). Astigmata had a very low abundance (10 individuals) and Oribatida was completely absent which reflects direct toxic effects of the insecticide on these organisms (Koehler, 1997).

Mites have a chitinous exoskeleton (Krantz and Walter, 2009) and, in particular, Oribatid mites have a high level of sclerotization (Krantz and Walter, 2009; Norton and Behan-Pelletier, 2009), making their body tissues very hard, which might work as a biological barrier against contaminants, giving them higher resistance to pesticides such as Coragen SC 20 or Movento O-TEQ, at least in terms of direct contact toxicity. Moreover, Oribatids have long-life cycles and slow metabolic rates, when comparing with other mite groups (Norton and Behan-Pelletier, 2009), which could improve their resistance or delay the effects of Coragen SC 20 and Movento O-TEQ on them. Regarding delayed effects, Natal da Luz et al. (2019) reported that effects of chemicals depend upon the fate and behaviour of the active substance in the soil as well as the application timing, the life stage of the exposed organisms, and both toxicokinetics and toxicodynamics which could only be detectable later in time. Indeed, Pelosi et al. (2015) conducted a study over 15 years comparing the effects of different cropping systems on earthworms. A reduction in the abundance of earthworms was observable in conventional farming systems (where pesticides were used) compared to organic farming only after 9 years. The duration of our tests was 6 weeks, and more long-term effects could not be assessed.

Regarding Mesostigmata and Prostigmata mites, species of the first group are mainly free-living predators (Krantz and Walter, 2009) and the families of the second group are mostly predators as well (Kethley, 1990), although they sometimes can feed on fungi (Walter and Proctor, 2013). Edwards and Thompson (1973) reported increased numbers of Oribatid mites following exposure to some organochlorine and organophosphorus pesticides and this was related to the decrease of predatory mites that normally preyed upon Oribatids, reflecting predator/prey indirect effects of

these pesticides. In our study with Coragen SC 20, the number of predatory mites found in all treatments was relatively low compared to the abundance of Oribatids (Fig. 4). Indeed predatory mites from Mesostigmata were represented in control and all treatments but the total abundance was only of 44 individuals across all samples. Because of this, the effect of predatory/prey relationships was probably neglected. This is enforced by the low contribution of Mesostigmata mites to the dissimilarity between control in the two higher doses (Table III.3 and III.4 – Annex III).

Moreover, Prostigmata mites can predate upon collembolans or compete with Oribatids for the available food (Krantz and Walter, 2009) thus reducing their abundance. However, the total abundance of this group of mites, found in control and all treatments, was low (only 74 individuals) compared with Oribatida, and probably these effects were neglected. Prostigmata, at dose 150 mg/kg was the third group with the highest contribution to the dissimilarity compared to control (Table III.3 – Annex III) with a low decrease in relative abundance. However, at the last dose (500 mg/kg) they were the group that mostly contributed to the significant differences (Table III.4 – Annex III) with only 1 individual found at this dose. They are more susceptible to chemicals than Oribatids because their body sclerotization is softer (Krantz and Walter, 2009). Mesostigmata and Prostigmata, similarly to the test with Coragen SC 20, had a very low abundance in the test with Movento O-TEQ (78 and 38 individuals, respectively, across all samples) compared to Oribatida and Astigmata. Thus, the interpretation of Movento O-TEQ effects on these two groups was difficult, however, we can assume that again, predator-prey effects were neglected.

At dose 45 mg/kg Mesostigmata contributed with 12.12 % to the dissimilarity with control whereas Prostigmata did not contribute at all (Table III.11 – Annex III). At dose 150 mg/kg Prostigmata contributed with 8.33 % and Mesostigmata had no relevant contribution (Table III.12 – Annex III). In dose 500 mg/kg, none of these two groups contributed to the dissimilarity with control (Table III. 13 – Annex III).

The very low abundance of Astigmata with only 15 individuals found across all samples did not allow any conclusions regarding the effects of Coragen SC 20 on this group of mites. Despite that, at dose 150 mg/kg Astigmata was the second group that mostly influenced the dissimilarity with control (Table III.3 – Annex III). This is related to the fact that 67 % of the total abundance was found in this dose, with 10 individuals present in it (Table II.2 – Annex II; Fig 4).

Regarding collembolans, they were more sensitive to Coragen SC 20 in comparison to mites, as evidenced by the toxic values estimated for this group (LOEC = 13 mg/kg; EC_{50} = 35.59 mg/kg). This can be related to the mode of action of the pesticide on the organisms and the different affinity of ryanodine receptors to Chlorantraniliprole on mites and collembolans. The mode of action of Chlorantraniliprole consists of binding to the ryanodine receptors causing an unregulated activation of these receptor channels which ultimately impairs regulation of muscle contraction, leading to paralysis and death (Lahm et al., 2007). Earlier studies showed a low impact of Chlorantraniliprole on non-target organisms such as pollinators, beneficial insects, predatory mites, earthworms, and many other soil organisms (Bassi et al., 2009; Dinter et al., 2009; USEPA, 2008a). These findings

might be explained by the low affinity of Chlorantraniliprole to the Ryodine Receptors (RyR) in some insects (Lahm et al., 2007; Latvizar et al., 2016)). Notwithstanding, the high toxicity of Chlorantraniliprole to collembolans observed in this study was somehow expected since several other studies, conducted on the collembolan *Folsomia candida*, have shown very high toxicity of this substance to collembolans. Indeed, the effects of Chlorantraniliprole on the reproduction of *F. Candida* reported until date generated an EC_{50} of 0.48 mg/kg and a NOEC of 0.39 mg/kg (EFSA, 2013a; NFSA, 2010; USEPA, 2008a). Also, Latvizar et al. (2016) tested the toxicity of Chlorantraniliprole on the survival and reproduction of *F. candida* in four soils with different properties (Coimbra soil, Lufa 2.2 soil, Dutch grassland soil, and North Wales soil). In all soils, very high toxicity was reported. The highest toxicity was observed in the Lufa 2.2 soil (EC_{50} = 0.14 mg CAP/kg) and the lowest in the Dutch grassland soil (EC_{50} = 0.76 mg CAP/kg).

Earlier studies concluded that pore water is the main route of exposure for either mites and collembolans (Van Gestel, 1997; Smit and Van Gestel, 1998; Didden and Römbke, 2001). As mentioned before, the physical characteristics of some mites may protect them from chemicals present in the pore water. Also, the body of collembolans is revested by a permeable cuticle that permits them to actively take up pore water to restore their water balance (Hopkin, 1997). This cuticle can contribute as a biological barrier against the toxicity of water-soluble pesticides (Gillott, 1995), a similar function provided by the chitinous exoskeleton of mites. Another observation made by Latvizar et al. (2016) in tests with collembolans *F. candida* was that Chlorantraniliprole was less toxic in soils with higher organic matter content (10.6 % - 14.7 %). In fact, according to the literature, Chlorantraniliprole strongly binds to organic matter (Koc = 329 L/kg) (APVMA, 2008; NFSA, 2010). This explains the lower toxicity in soils with higher amounts of organic matter content. The natural soil used in this study had 15.73 % organic matter content (Table 5) and comparing to the study made by Latvizar et al. (2016) the toxicity towards collembolans was lower. The toxic values derived for the community of collembolans in our test with Coragen SC 20 were EC_{10} = 10.46 mg a.i./kg and EC_{50} = 35.59 mg a.i./kg and, in the study performed by Latvizar et al. (2016), the values obtained in the soil containing the most percentage of organic matter (14.7 %) were EC_{10} = 0.17 mg a.i./kg and EC_{50} = 0.62 mg a.i./kg. However, the tests performed by Latvizar et al. (2016) were single-species tests assessing effects on reproduction and did not cover the possible interactions occurring among different species and/or groups of organisms. It is known that different species of soil organisms have different sensitivity to pesticides (Edwards and Thompson, 1973; Haegerbaeumer et al., 2019), based on their intrinsic characteristics. This happens with any active substance, such as Chlorantraniliprole (Latvizar et al., 2016), present in Coragen SC 20, or Spirotetramat (Maus et al., 2008) present in Movento O-TEQ. Notwithstanding, different combinations of species and their interactions may also change the level of toxicity of pesticides (Sechi et al., 2014). The fact that the toxic values found in the literature for the collembolan *Folsomia candida* were lower compared with the values derived in our study on a community of collembolans are related to the type of tests performed. As referred before, the results described from the literature were assessed through single-species tests evaluating mortality and/or

reproduction. Contrarily, the tests performed in this study assessed the effects on communities. They were more ecological relevant because interactions, such as competition for food, could be evaluated, reflecting until a certain point what happens in Nature.

Regarding the assessment of the trait-based effects, in the test with Coragen SC 20, shifts in the community structure of collembolans were observed mostly at the four highest doses tested (13 mg/kg, 45 mg/kg, 150 mg/kg, and 500 mg/kg). At these doses, a significant decrease in taxon diversity (expressed as different combinations of trait scores) occurred (Table 7). Also, euedaphic species (high mT) were completely lost at these doses. Contrastingly, epiedaphic species (low mT) were still represented at these doses, but with very low abundance, compared to other treatments (0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.4 mg/kg, 1.2 mg/kg, 4 mg/kg) and control. A similar observation was made by Chelinho et al. (2014), which reported lower sensitivity of epiedaphic species than euedaphic species along the contamination gradient of Carbofuran. The authors defended that the different cuticular permeability, lower in epiedaphic species and higher in euedaphic species, could be the reason behind the differences observed. The lower permeability in the cuticle of epiedaphic collembolans is a natural defence of these species against dissection. Because they live in the upper soil layers they are more susceptible to the loss of water through evaporation so their cuticle is more resistant to this perturbation comparing to euedaphic species (Kaersgaard et al., 2004). Thus, epiedaphic collembolans which also are less in contact with soil pore water (Hopkin, 1997) and have higher mobility than euedaphic ones (Fountain and Hopkin, 2004) are less exposed to chemicals such as pesticides and, less affected. Moreover, euedaphic collembolans have a relatively low metabolism and nutritional needs (Hopkin, 1997) which can be an adaptation to the habitat in deeper soil layers where, generally, the amounts of food available are lower (Larsen et al., 2009). On the other hand, since this test was performed in laboratory conditions and the only available food was the yeast provided, it is possible that euedaphic collembolans lacked the means to feed, explaining their low abundance (Sechi et al., 2014).

Toxic effects of Movento O-TEQ on collembolans were much lower compared to the toxicity of Coragen SC 20 to the same group of organisms (EC_{10} = 196.53 mg a.i./kg, EC_{50} > 500 mg a.i./kg and EC_{10} = 10.46 mg a.i./kg, EC_{50} = 35.59 mg a.i./kg, respectively). Specific data on the toxicity of this insecticide or its active substance Spitotetramat on collembolans (e.g. *Folsomia candida*, as standard test species) was never reported in the literature. This is probably due to the fact that the use of Spirotetramat has been permitted in 2013 (EFSA, 2013) before the current legislation Nº 284/2013 (European Commission, 2013) laying down the data requirements for non-target arthropods exposed to PPP's was introduced. Before this legislation, the effects on non-target soil organisms exposed to PPP's were assessed based on the toxicity towards earthworms. Spirotetramat has shown very low toxicity to earthworms for acute toxicity in earlier studies that reported an LC_{50} of 1000 mg/kg and a NOEC of 1000 mg/kg (APVMA, 2009; EFSA, 2013b; Maus et al., 2008; USEPA, 2008b). Maus et al. (2008) reported that Spirotetramat has a very low persistence in soil (DT_{50} = 0.33 days). Moreover, they concluded that there was not a chronic risk to soil organisms

based on very low soil concentrations of Spirotetramat even after repeated applications (Maus et., 2008). Also, EFSA (2013b) reported that testing on Collembola was not required.

For the assessment of the trait-based effects, no significant shifts in the community structure of collembolans were observed (Table 7). The community had only 0.6 % of euedaphic collembolans (30 individuals) among control and all treatments (Table II.5 – Annex II). This means that the majority of collembolan species in the community test with Movento O-TEQ had similar traits. Even in doses where significant differences in similarity with control were obtained (4 mg a.i./kg and 500 mg a.i./kg) the mT value did not change significantly, so we can say that the ecological groups were affected in the same level of magnitude. Notwithstanding, at dose 500 mg/kg the mT value was the lowest (Table 7), but this value was not significantly different from the control. As referred before, euedaphic collembolans are more susceptible to chemicals comparing to epiedaphic ones because they have less mobility in soil and their cuticle has a higher permeability (Fountain and Hopkin, 2004; Hopkin, 1997).

II.4.2 – Testing method

An inevitability of the two community tests performed in this study is the fact that they are a closed system. This explains the decreased abundance of soil community extracted in controls at the end of the test compared with the abundances of the initial community. This was higher for the test with Coragen SC 20 comparing to the test with Movento O-TEQ. The same issue was reported by Chelinho et al. (2014) which used a very similar approach. The authors argued that this could be related to the fact that organisms were confined to a small area where they could not escape from potential predators such as arachnids (Marc et al., 1999) or unfavourable environmental conditions such as temperature, humidity, or light.

Another limitation is the natural variability intrinsic to the sample soil cores used to extract the natural community which ended in high variability between replicates and, consequently, reduced statistical power and repeatability of the test (Sechi et al., 2014; Scott-Fordsmand et al., 2008). A way to overcome this issue could be to increase the number of replicates to give more robustness to the data obtained. Also, the use of a more homogeneous area in the extraction of soil cores could reduce the variability.

Notwithstanding, the community tests performed in this study provided higher ecological relevance since the effects of both Coragen SC 20 and Movento O-TEQ on natural communities of

microarthropods were identified. The test methodology was done in the laboratory and although it could not resemble the real situation in the field, it permitted to assess the indirect effects of both pesticides, contrarily to standard single-species tests which mainly assess mortality and/or reproduction endpoints. Indeed, factors that affected the community dynamics might be related to toxic stress and competition for food. The competition was mostly observed for the test with Coragen SC 20 where Oribatid mites had an increased abundance in the last two doses after collembolans (their direct competitors) practically disappeared. For the test with Movento O-TEQ, the increase in abundance of collembolans at doses 45 mg/kg and 150 mg/kg might be related to the great decrease in abundance of Astigmata mites, which compete with them for the available food. Regarding predator-prey relationships, in both tests, they were probably neglected because a general low abundance of predatory mites was found.

Thus, community tests which have the advantage of a gain in ecological relevance compared to standard laboratory single-species tests and, are less expensive and time-consuming compared with fields tests, represent a very promising tool to use in risk assessment of soil organisms exposed to PPP's as intermediate tiers (EFSA, 2017).

II.5 - Conclusion

This study revealed the usefulness of community tests in assessing the effects of pesticides on communities of soil organisms (namely microarthropods) under laboratory conditions. Indirect effects of Coragen SC 20 and Movento O-TEQ were observed in the communities of microarthropods tested. For Coragen SC 20, Oribatida mites had their numbers increased along the contamination gradient which was associated with the decrease in abundance of their direct competitors (collembolans). Moreover, the trait-based assessment showed significant differences in mT values at higher doses, where euedaphic collembolans practically disappeared from the community. Regarding Movento O-TEQ, the increase in abundance of collembolans at doses 45 mg a.i./kg and 150 mg a.i./kg was associated with the great decrease of Astigmata mites which may have competed for food at lower doses. The mT values did not show a shift in the community composition of collembolans and this was due to the fact species with similar traits composed the community.

Moreover, community tests performed were sensitive enough to detect the effects of the mode of action of the two insecticides on the community level. Based on the results obtained, the recommended doses both for Coragen SC 20 and Movento O-TEQ (0.067-0.1 and 0.02-0,12 mg a.i./kg, respectively) are safe to these soil communities.

Comparing to single-species tests that mainly assess effects of pesticides on mortality and/or reproduction, community tests permitted to go beyond in terms of effect data on PPP's because they were sensitive enough to detect indirect effects on communities of microarthropods. They represent a more realistic exposure scenario under laboratory conditions and, together with data from single-species tests, they may help to extrapolate better the results to field situations.

Chapter III – General discussion

III – General discussion

The general aim of this study was to assess the toxicity of two recently developed pesticides (Coragen SC 20 and Movento O-TEQ) on natural microarthropod communities through community tests. Mites and collembolans were the only groups that could be evaluated due to the low number of organisms from other groups (Table II.1 – Annex II). For the test with Coragen SC 20, the community was affected along the contamination gradient with shifts in the abundance of taxonomic groups of mites and changes in the community composition of collembolans. Based on toxic values derived (Table 6) collembolans were more sensitive than mites to this pesticide with significant differences compared to control at doses 13, 45,150, and 500 mg a.i./kg (Fig. 4). At these doses, collembolans decreased strongly in abundance which probably is due to the direct toxicity effects of the pesticide. On the other hand, mites that were less represented than collembolans at lower doses had their numbers increased at the two highest doses (Table II.1 – Annex II; Fig. 4). This is probably associated with the indirect effects of the pesticide on the community. Indeed, the increase in Oribatida (Table II.2 – Annex II; Fig. 4) is most likely because their direct competitors for the available food (collembolans) decreased their abundance, which agrees with the observation made by Chelinho et al. (2004) on the impact of Carbofuran contamination on microarthropods from a brazilian soil following a very similar testing approach. Moreover, the trait-based assessment of collembolans showed a shift in the community composition of collembolans with significant shifts in the last 4 doses (Table 7). Here, the decrease in mT values was related to the disappearance of euedaphic collembolans that were more susceptible to the contamination gradient. Again, the same observation was made by Chelinho et al. (2014).

Regarding the test with Movento O-TEQ, effects were less evident for collembolans. They were not very affected by direct toxicity (Table 6) and the trait-based assessment did not show significant shifts in community composition based on the mT values derived (Table 7). As for mites, direct

toxic effects occurred (Table 6) as well as shifts in the taxonomic groups. Astigmata mites that were dominant at lower doses, practically were absent from the last 3 doses where significant differences compared to control were detected (Fig. 6). Thus, the significant increase of collembolans abundance at 45 and 150 mg a.i./kg might be associated with this fact, since Astigmata could compete directly with them for food. Collembola are known to rapidly increase when conditions are favourable (Edwards and Thompson., 1973). Movento O-TEQ which has properties of acaricides (EFSA, 2013b) might have affected Astigmata in such a way that they were practically absent from these doses, reflecting an indirect effect of the pesticide on the community of microarthropods.

These observations show that the community tests performed were sensitive enough to detect the effects of the mode of action of the two insecticides on the community level. Based on the results obtained it seems that the recommended doses both for Coragen SC 20 and Movento O-TEQ (0.067-0.1 and 0.02-0,12 mg a.i./kg, respectively) are safe to these soil communities. However, the natural soil used in this test had high organic matter content (15.73%) and it is known that soils with high percentages can lower the toxicity of certain chemicals (Latvizar et al., 2016). This may occur with Chlorantraniliprole which binds strongly to the organic matter (Koc = 329 L/kg) and also with Spirotetramat (Koc= 289 L/kg) (APVMA, 2009). Thus, in soils with lower organic matter content, the toxicity of Chlorantraniliprole towards collembolans and the toxicity of Spirotetramat to mites could be even higher than those obtained in these tests and may cause a risk to microarthropod communities.

Finally, this type of test allowed to assess indirect relationships that standard single-species tests do not allow, even though predator-prey relationships were probably neglected due to the low abundance of predatory mites found. Community tests constitute a promising tool to use in risk assessment to soil organisms exposed to PPPs where they can fill the gap between lower and higher tiers. Moreover, data from community tests together with data from single-species tests might help to extrapolate to what happens in real field situations.

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Annex 1- Collembola morphotypes

Table I.1: Morphotypes and corresponding EMI punctuations considered, as well as the EMI partial punctuations for each characteristic calculated for communities extracted from test with soil spiked with Coragen SC 20.

	Ocelli	Antennae lenght	Furca	Hairs/scales	Pigmentation	EMI punctuation
Ep1	0	0	0	0	2	2
Ep2	0	0	2	0	2	4
Ep3	0	2	0	0	2	4
Ep4	0	4	0	0	0	4
Ep5	0	2	2	0	2	6
Ep6	0	0	2	0	4	6
Ep7	0	4	2	0	0	6
Ep8	0	2	2	0	4	8
Ep9	0	4	0	0	4	8
Ep10	0	4	4	0	0	8
He1	0	4	2	0	4	10
He2	0	4	4	0	2	10
He3	0	4	4	0	4	12
He4	4	2	2	0	4	12
He5	4	4	0	0	4	12
He6	4	4	4	0	0	12
Ed1	0	4	2	4	4	14
Ed2	4	4	2	0	4	14
Ed3	4	4	4	0	4	16
Ed4	4	4	4	4	4	20

Table I.2: Morphotypes and corresponding EMI punctuations considered, as well as the EMI partial punctuations for each characteristic calculated for communities extracted from test with soil spiked with Movento O-TEQ.

	Ocelli	Antennae lenght	Furca	Hairs/scales	Pigmentation	EMI punctuation
Ep1	0	2	0	0	0	2
Ep2	0	4	0	0	0	4
Ep3	0	0	2	0	2	4
Ep4	0	2	2	0	0	4
Ep5	0	4	0	0	2	6
Ep6	0	2	2	0	2	6
Ep7	0	4	2	0	0	6
Ep8	0	4	2	0	2	8
Ep9	0	4	4	0	0	8
Ep10	0	4	4	0	0	8
He1	0	4	2	0	4	10
He2	0	4	4	0	2	10
He3	4	2	0	0	4	10
He4	0	2	2	4	4	12
He5	0	4	4	0	4	12
Ed1	4	4	2	0	4	14
Ed2	4	4	4	0	4	16

Annex II – Raw data gathered for both community tests

Table II.1: Composition of soil community from Freixo-do-meio, Portugal, exposed to soils contaminated with Coragen SC 20 and Movento O-TEQ (assessed sepaately). Values are expressed as total abundance of major taxonomic groups per treatment. IC, initial community; Aca, Acari; Coll, Collembola; Chil, Chilopoda; Coleo, Coleoptera; Dipl, Diplura; Form, Formicidae; Hym, Hymenoptera; Dipt, Diptera; Paurop, Pauropoda; Nem, Nematoda; Enchy, Enchytraeidae; Olig, Oligochaeta; Lar, Larvae.

Doses (mg/kg)	Aca	Coll	Chil	Coleo	Dipl	Form	Hym	Dipt	Paurop	Nem	Enchy	Olig	Lar
Coragen SC 20													
IC	237	693	1	3	-	-	-	3	1	4	2	5	7
0	67	401	-	1	4	-	1	-	1	2	-	1	1
0.01	39	173	-	1	-	-	-	-	-	1	-	-	1
0.03	25	109	-	-	-	-	-	-	-	-	-	-	1
0.1	16	188	-	-	-	-	-	-	-	1	-	1	-
0.4	32	109	-	1	-	-	-	-	-	1	-	-	3
1.2	34	166	1	-	-	-	-	-	-	-	-	-	-
4	68	235	-	4	-	-	-	-	-	1	1	-	1
13	35	46	1	-	-	-	-	-	-	-	-	1	3
45	55	22	1	-	-	-	-	-	-	-	-	-	-
150	126	15	2	1	-	-	1	-	-	2	-	-	-
500	87	3	1	1	-	-	1	1	-	1	-	-	1
Movento O-TEQ													
IC	516	1122	3	4	2	1	-	2	-	7	3	3	16
0	441	814	-	-	-	-	-	1	-	-	2	-	4
0.01	101	669	-	3	-	-	-	1	-	-	-	1	4
0.03	308	479	1	-	-	-	-	2	-	-	-	-	5
0.1	231	391	-	1	-	-	-	3	-	-	-	-	1
0.4	470	238	-	-	-	-	-	-	-	-	-	-	5
1.2	385	319	-	1	-	-	-	-	-	-	-	-	1
4	359	62	1	-	-	-	-	-	-	-	-	-	3
13	301	227	1	-	-	-	-	-	-	-	-	-	-

45	147	764	2	-	-	-	1	-	-	-	-	-	4
150	163	606	-	-	-	-	-	-	-	1	-	-	-
500	12	69	-	-	-	-	-	-	-	-	-	-	-

Table II.2: Total abundance of the different groups of mites identified for the test with Coragen SC 20.

Doses (mg/kg)	Oribatida	Prostigmata	Mesostigmata	Astigmata
0				
Rep 1	8	2	0	0
Rep 2	10	5	0	0
Rep 3	3	1	1	0
Rep 4	3	6	2	0
*Rep 5**	0	0	0	0
*Rep 6**	0	0	0	0
Rep 7	15	2	0	0
Rep 8	2	5	2	0
0.01				
Rep 1	5	2	0	0
Rep 2	6	2	1	0
Rep 3	9	3	1	0
Rep 4	8	0	0	2
*Rep 5**	0	0	0	0
0.03				
Rep 1	5	2	0	0
*Rep 2**	0	1	2	0
Rep 3	4	1	2	1
Rep 4	5	0	0	0
Rep 5	2	3	0	0
0.1				
Rep 1	6	0	0	0
Rep 2	3	0	0	0
*Rep 3**	0	0	0	0
Rep 4	3	0	0	0
Rep 5	3	0	1	0
0.4				
Rep 1	2	1	0	0
Rep 2	6	0	3	0
*Rep 3**	0	0	1	0
Rep 4	14	1	2	0

Rep 5	1	0	2	0
1.2				
Rep 1	5	0	0	0
Rep 2	1	2	0	0
Rep 3	1	1	0	0
Rep 4	7	1	1	1
Rep 5	12	2	0	0
4				
*Rep 1**	0	0	1	1
Rep 2	26	1	4	0
Rep 3	10	4	0	0
Rep 4	11	2	0	0
Rep 5	7	3	0	0
13				
Rep 1	5	2	2	0
Rep 2	14	0	9	0
Rep 3	1	0	0	0
Rep 4	1	0	0	0
Rep 5	1	0	0	0
45				
Rep 1	10	1	0	0
Rep 2	11	0	0	0
Rep 3	10	0	0	0
Rep 4	14	1	0	0
Rep 5	4	2	2	0
150				
Rep 1	18	5	1	4
Rep 2	15	4	2	2
Rep 3	13	2	0	0
Rep 4	9	0	0	1
Rep 5	43	4	0	3
500				
Rep 1	13	1	1	1
Rep 2	15	0	0	0
Rep 3	7	0	2	0
Rep 4	41	0	3	0
Rep 5	3	0	0	0

Table II.3: Total abundance of the different groups of mites identified for the test with Movento O-TEQ.

Doses (mg/kg)	Oribatida	Prostigmata	Mesostigmata	Astigmata
0				
Rep 1	9	0	0	94
Rep 2	6	0	0	71
Rep 3	6	0	0	10
Rep 4	15	0	2	46
Rep 5	5	0	1	68
Rep 6	6	0	0	22
Rep 7	9	0	0	20
Rep 8	5	0	0	46
0.01				
Rep 1	4	0	13	3
Rep 2	1	0	0	9
Rep 3	2	0	0	14
Rep 4	0	0	0	22
Rep 5	1	0	3	29
0.03				
Rep 1	35	1	4	88
Rep 2	15	0	5	29
Rep 3	11	0	7	24
Rep 4	35	0	6	14
Rep 5	18	0	4	12
0.1				
Rep 1	17	0	2	12
Rep 2	13	4	0	24
Rep 3	47	0	1	54
Rep 4	17	0	0	26
Rep 5	6	0	1	7
0.4				
Rep 1	25	0	0	26
Rep 2	38	0	1	47
Rep 3	15	2	5	94
Rep 4	45	0	0	101
Rep 5	17	0	0	54
1.2				
Rep 1	57	2	0	16
Rep 2	33	0	0	31
Rep 3	53	0	0	22
Rep 4	49	0	0	62
Rep 5	22	5	0	33

4				
Rep 1	22	0	7	29
Rep 2	24	1	2	6
Rep 3	16	0	0	38
Rep 4	24	0	0	26
Rep 5	24	0	0	140
13				
Rep 1	17	0	0	49
Rep 2	7	1	2	48
Rep 3	27	0	0	20
Rep 4	11	0	0	60
Rep 5	36	13	0	10
45				
Rep 1	24	0	6	3
Rep 2	27	0	0	3
Rep 3	30	0	3	0
Rep 4	21	0	2	2
Rep 5	22	2	0	2
150				
Rep 1	40	3	0	1
Rep 2	28	0	0	0
Rep 3	21	2	0	1
Rep 4	21	0	0	2
Rep 5	43	1	0	0
500				
Rep 1	0	0	0	6
Rep 2	0	0	0	0
Rep 3	0	0	0	1
Rep 4	0	1	1	2
Rep 5	0	0	0	1

Table II.4: Total abundance of the different collembolan morphotypes identified for the test with Coragen SC 20.

Doses (mg/kg)	Ep1	Ep2	Ep3	Ep4	Ep5	Ep6	Ep7	Ep8	Ep9	Ep10	He1	He2	He3	He4	He5	He6	Ed1	Ed2	Ed3	Ed4
0																				
Rep 1	1	3	0	20	0	0	3	0	0	25	4	1	2	9	0	1	3	0	14	0
Rep 2	11	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 3	2	3	0	0	0	0	7	0	0	11	1	0	8	0	0	0	0	0	0	0
Rep 4	0	0	0	15	1	0	7	0	1	10	9	0	0	0	0	0	5	0	12	0
Rep 5	1	0	2	18	1	0	11	0	1	2	31	0	0	0	0	2	0	0	12	0
*Rep 6**	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Rep 7	0	0	2	4	0	1	0	0	0	3	2	6	0	0	0	0	13	0	5	0
Rep 8	15	4	16	11	27	5	1	3	3	0	1	0	2	0	1	0	1	1	0	0
0.01																				
Rep 1	0	1	0	13	0	0	6	0	1	1	6	0	0	0	0	0	10	0	0	0
Rep 2	0	1	0	5	0	2	0	0	0	0	3	0	0	0	0	0	5	0	0	0
Rep 3	0	0	0	27	0	0	0	0	35	1	0	0	2	4	2	0	0	0	22	0
Rep 4	0	0	0	0	0	2	2	0	1	0	0	0	0	0	0	0	12	0	0	0

Rep 5	0	3	1	0	0	0	0	0	0	1	0	0	0	0	0	0	3	0	1	0
0.03																				
Rep 1	0	12	0	0	0	2	0	6	0	0	0	0	0	0	1	0	0	0	4	0
Rep 2	0	3	0	0	4	0	0	0	0	0	1	0	0	0	0	0	0	0	9	0
Rep 3	4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	5	0	0	24	2
*Rep 4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Rep 5	0	13	0	11	0	0	0	6	0	0	0	0	0	0	0	0	0	0	1	0
0.1																				
Rep 1	0	4	0	5	0	0	1	0	4	0	0	0	0	0	0	0	0	0	6	0
Rep 2	0	0	0	4	0	0	1	0	0	0	6	0	0	0	0	0	0	0	4	0
Rep 3	0	12	0	0	8	0	0	1	0	1	0	0	0	0	0	0	0	0	5	0
Rep 4	0	2	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	7	0
Rep 5	27	1	2	0	13	3	23	11	0	3	26	0	3	0	1	1	0	0	1	0
0.4																				
Rep 1	0	7	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	3	0
Rep 2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0

Rep 3	0	16	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Rep 4	0	26	0	7	0	0	1	5	3	1	0	0	0	0	0	0	0	0	6	0
Rep 5	0	4	0	1	0	0	3	0	0	0	2	0	1	0	0	0	0	2	4	0
1.2																				
Rep 1	0	1	0	4	0	0	5	0	5	2	5	0	0	0	0	0	0	0	5	0
Rep 2	0	12	0	5	0	0	0	0	0	9	0	0	1	0	2	0	0	0	1	0
Rep 3	0	6	0	21	0	0	11	0	1	5	8	0	1	0	0	0	0	0	1	0
Rep 4	13	0	0	0	1	2	4	0	0	1	1	0	0	3	0	0	0	0	6	0
Rep 5	0	11	0	0	0	0	2	5	0	0	0	0	0	0	0	0	0	2	4	0
4																				
Rep 1	0	0	0	2	2	0	2	0	0	0	0	0	0	0	0	0	0	5	0	0
Rep 2	0	23	0	11	4	0	0	3	17	0	0	0	0	0	3	0	0	3	15	0
Rep 3	0	1	0	2	0	0	5	0	0	0	3	0	0	2	0	1	0	13	9	0
Rep 4	0	27	0	0	0	0	30	0	0	1	21	2	2	0	0	0	0	0	0	0
Rep 5	2	0	8	12	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0
13																				

Rep 1	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 2	0	1	0	1	0	0	6	0	0	0	2	0	0	0	0	0	0	0	0	0
Rep 3	0	0	0	7	1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
Rep 4	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Rep 5	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45																				
Rep 1	1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 2	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 3	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0
Rep 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 5	0	0	1	8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
150																				
Rep 1	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 2	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Rep 5	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
500																				
Rep 1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Rep 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table II.5: Total abundance of the different collembolan morphotypes identified for the test with Movento O-TEQ.

Doses (mg/kg)	Ep1	Ep2	Ep3	Ep4	Ep5	Ep6	Ep7	Ep8	Ep9	Ep10	He1	He2	He3	He4	He5	Ed1	Ed2
0																	
Rep 1	0	0	0	0	0	2	7	0	0	0	0	0	0	0	0	0	0
Rep 2	0	0	0	0	0	1	44	0	0	0	25	0	0	0	0	1	0
Rep 3	0	0	0	0	0	14	45	0	0	0	10	0	0	0	0	0	0
Rep 4	0	0	0	3	0	9	137	0	1	0	91	0	0	0	0	0	0

Rep 5	0	0	0	0	0	3	136	0	0	0	46	0	0	0	0	3	0
Rep 6	0	0	0	3	0	7	16	0	0	0	11	0	0	9	0	0	0
Rep 7	0	0	0	0	0	0	39	0	0	0	8	0	0	0	0	0	0
Rep 8	0	0	0	0	0	24	101	0	7	0	11	0	0	0	0	0	0
0.01																	
Rep 1	0	0	0	0	0	0	81	0	0	0	8	43	0	41	0	1	1
*Rep 2**	0	0	0	0	0	8	0	0	0	0	0	18	0	0	6	0	0
Rep 3	0	0	0	0	0	9	138	0	0	0	54	0	0	0	6	0	0
Rep 4	0	0	0	0	0	0	59	0	0	0	31	1	0	0	2	0	0
Rep 5	0	0	0	0	0	13	121	0	0	0	58	0	1	0	0	1	0
0.03																	
*Rep 1**	0	0	0	0	0	7	0	0	1	0	0	0	0	0	0	2	0
Rep 2	0	0	0	0	0	0	61	0	0	0	21	0	0	0	0	0	0
Rep 3	2	0	0	0	0	2	99	0	1	0	10	0	0	0	0	0	0
Rep 4	1	15	1	0	1	26	105	1	2	0	65	0	0	0	0	1	0
Rep 5	0	0	0	0	0	3	30	0	2	0	30	0	0	0	0	0	0

0.1																	
Rep 1	0	0	0	0	0	9	197	0	1	0	37	0	0	0	2	0	1
Rep 2	0	0	0	0	0	1	70	0	0	0	22	0	0	0	0	0	0
*Rep 3**	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Rep 4	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0
Rep 5	0	0	0	0	0	3	31	0	0	1	13	0	0	0	0	0	0
0.4																	
Rep 1	0	0	0	0	0	0	39	0	0	0	20	0	0	0	0	0	0
Rep 2	0	0	0	0	0	5	6	0	3	0	4	0	0	0	0	2	0
Rep 3	0	0	0	0	0	5	57	0	0	0	23	16	0	0	3	0	5
*Rep 4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 5	0	0	0	0	0	0	45	0	0	0	5	0	0	0	0	0	0
1.2																	
Rep 1	0	0	0	0	0	1	70	0	0	0	36	1	0	0	0	0	0
Rep 2	0	0	0	0	0	13	1	0	0	0	1	0	0	0	0	0	0
Rep 3	0	0	0	0	0	5	19	0	0	0	16	9	0	0	0	0	0

Rep 4	0	0	0	0	0	7	36	0	1	0	6	0	0	0	0	0	0
Rep 5	0	0	0	0	0	0	57	0	0	0	40	0	0	0	0	0	0
4																	
*Rep 1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 2	0	0	0	0	0	5	1	0	2	0	0	0	0	0	0	1	0
Rep 3	0	0	0	0	0	4	16	0	2	0	18	0	0	0	0	0	0
Rep 4	0	0	0	0	0	0	3	0	1	0	0	0	0	0	0	0	0
Rep 5	0	0	0	0	0	3	3	0	3	0	0	0	0	0	0	0	0
13																	
Rep 1	0	0	0	0	0	3	29	0	0	0	11	0	0	0	0	0	0
*Rep 2**	0	0	0	0	0	6	0	0	3	0	0	0	0	0	0	0	0
Rep 3	0	0	0	0	0	19	11	0	9	0	1	0	0	0	0	0	0
Rep 4	0	0	0	4	0	6	15	0	0	0	8	0	0	1	0	1	1
Rep 5	0	0	0	0	0	21	46	0	0	0	41	0	0	0	0	0	0
45																	
Rep 1	0	1	0	0	0	46	4	0	12	0	0	0	0	0	0	0	0

Rep 2	0	0	0	0	0	0	47	0	2	0	10	0	0	0	0	1	0
Rep 3	0	0	0	0	0	2	130	0	0	0	50	0	0	0	0	1	0
Rep 4	0	0	0	0	0	18	99	0	0	0	83	0	0	0	0	0	0
Rep 5	0	0	0	0	0	0	128	0	0	0	130	0	0	0	0	0	0
150																	
Rep 1	0	0	0	0	0	8	3	0	5	0	0	0	0	0	2	0	0
Rep 2	0	0	0	0	0	2	156	0	0	0	35	5	0	0	3	1	0
Rep 3	0	0	0	0	0	0	16	0	0	0	2	0	0	0	0	5	0
Rep 4	0	0	0	0	0	6	139	0	7	1	87	0	0	0	0	0	1
Rep 5	0	0	0	0	0	8	78	0	1	0	35	0	0	0	0	0	0
500																	
Rep 1	0	0	0	0	0	0	16	0	0	0	4	0	0	0	0	0	0
Rep 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rep 3	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0
Rep 4	0	0	0	0	0	0	35	0	0	0	11	0	0	0	0	0	0
Rep 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Annex III – Simper analyses (% contribution for significant differences in similarity compared to control) performed in both community tests

Table III.1: Significant differences between control and dose 0.1 mg/kg (Mites – Coragen SC 20).

Groups 0 & 0.1 Average dissimilarity = 45.85				
Species	Group 0 Av. Abund	Group 0.1 Av. Abund	Contrib%	Cum.%
Prostigmata	1.79	0.00	54.79	54.79
Oribatida	2.46	1.91	25.33	80.12
Mesostigmata	0.64	0.25	19.88	100.00

Table III.2: Significant differences between control and dose 13 mg/kg (Mites – Coragen SC 20).

Groups 0 & 13 Average dissimilarity = 52.99				
Species	Group 0 Av. Abund	Group 13 Av. Abund	Contrib%	Cum.%
Prostigmata	1.79	0.28	41.54	41.54
Oribatida	2.46	1.80	35.37	76.91
Mesostigmata	0.64	0.88	23.09	100.00

Table III.3: Significant differences between control and dose 150 mg/kg (Mites – Coragen SC 20).

Groups 0 & 150 Average dissimilarity = 36.82				
Species	Group 0 Av. Abund	Group 150 Av. Abund	Contrib%	Cum.%
Oribatida	2.46	4.26	39.60	39.60
Astigmata	0.00	1.23	25.61	65.21
Prostigmata	1.79	1.53	19.57	84.79
Mesostigmata	0.64	0.48	15.21	100.00

Table III.4: Significant differences between control and dose 500 mg/kg (Mites – Coragen SC 20).

Groups 0 & 500 Average dissimilarity = 42.93				
Species	Group 0 Av. Abund	Group 500 Av. Abund	Contrib%	Cum.%
Prostigmata	1.79	0.20	40.46	40.46
Oribatida	2.46	3.65	36.97	77.43
Mesostigmata	0.64	0.83	18.50	95.93

Table III.5: Significant differences between control and dose 0.03 mg/kg (Collembolans – Coragen SC 20).

Groups 0 % 0.03 Average dissimilarity = 75.63				
Species	Group 0 Av. Abund	Group 0.03 Av. Abund	Contrib%	Cum.%
Ep7	1.26	0.00	9.78	9.78
Ep10	1.19	0.00	8.28	18.06
Ep2	0.58	1.27	7.95	26.01
Ep4	1.34	0.46	7.93	33.94
Ed3	1.02	1.59	7.82	41.77
Ep1	1.00	0.35	7.42	49.19
He1	1.24	0,25	7.22	56.41

Table III.6: Significant differences between control and dose 0.4 mg/kg (Collembolans – Coragen SC 20).

Groups 0 % 0.4 Average dissimilarity = 73.28				
Species	Group 0 Av. Abund	Group 0.4 Av. Abund	Contrib%	Cum.%
Ep2	0.58	1.70	10.26	10.26
Ep1	1.00	0.00	9.16	19.42
Ep7	1.26	0.46	8.75	28.16
Ep10	1.19	0.20	8.54	36.71
Ed4	1.34	0.53	8.25	44.96
He1	1.24	0.24	8.11	53.07

Table III.7: Significant differences between control and dose 13 mg/kg (Collembolans – Coragen SC 20).

Groups 0 % 13 Average dissimilarity = 78.54				
Species	Group 0 Av. Abund	Group 13 Av. Abund	Contrib%	Cum.%
Ep7	1.26	0.31	11.28	11.28
Ep1	1.00	0.00	11.05	22.33
Ep10	1.19	0.00	10.00	32.33
Ep4	1.34	1.46	9.56	41.89
Ed1	1.24	0.44	8.26	50.15
Ed3	1.02	0.	7.85	58.00

Table III.8: Significant differences between control and dose 45 mg/kg (Collembolans – Coragen SC 20)

Groups 0 % 45 Average dissimilarity = 79.19				
Species	Group 0 Av. Abund	Group 45 Av. Abund	Contrib%	Cum.%
Ep10	1.19	0.00	10.18	10.18
Ep1	1.00	0.25	11.15	20.33
He1	1.24	0.00	9.91	30.24
Ep4	1.34	1.10	9.49	39.74
Ep7	1.26	0.55	9.35	49.09
Ed3	1.02	0.00	7.98	57.07

Table III.9: Significant differences between control and dose 150 mg/kg (Collembolans – Coragen SC 20).

Groups 0 % 150 Average dissimilarity = 96.45				
Species	Group 0 Av. Abund	Group 150 Av. Abund	Contrib%	Cum.%
Ep7	1.26	0.00	13.12	13.12
Ep1	1.00	0.00	11.79	24.90
Ep10	1.19	0.00	9.40	34.30
He1	1.24	0.00	9.07	43.37
Ep4	1.34	0.01	8.93	52.30
Ep5	0.61	0.88	8.24	60.54

Table III.10: Significant differences between control and dose 500 mg/kg (Collembolans – Coragen SC 20).

Groups 0 % 500 Average dissimilarity = 95.06				
Species	Group 0 Av. Abund	Group 500 Av. Abund	Contrib%	Cum.%
Ep7	1.26	0.00	13.77	13.77
Ep1	1.00	0.00	12.44	26.21
Ep10	1.19	0.00	9.74	35.95
He1	1.24	0.00	9.39	45.33
Ep4	1.34	0.41	9.32	54.65
Ed3	1.02	0.00	7.50	62.16

Table III.11: Significant differences between control and dose 45 mg/kg (Mites – Movento O-TEQ).

Groups 0 & 45 Average dissimilarity = 51.27				
Species	Group 0 Av. Abund	Group 45 Av. Abund	Contrib%	Cum.%
Astigmata	6.53	1.26	57.97	57.97
Oribatida	2.71	4.97	26.61	84.58
Mesostigmata	0.30	1.12	12.12	96.70

Table III.12: Significant differences between control and dose 150 mg/kg (Mites – Movento O-TEQ).

Groups 0 & 150 Average dissimilarity = 58.14				
Species	Group 0 Av. Abund	Group 150 Av. Abund	Contrib%	Cum.%
Astigmata	6.53	0.68	59.50	59.50
Oribatida	2.71	5.47	29.40	88.90
Prostigmata	0.00	0.83	8.33	97.23

Table III.13: Significant differences between control and dose 500 mg/kg (Mites – Movento O-TEQ).

Groups 0 & 500 Average dissimilarity = 72.75				
Species	Group 0 Av. Abund	Group 500 Av. Abund	Contrib%	Cum.%
Astigmata	6.53	1.47	58.74	58.74
Oribatida	2.71	0.00	33.86	92.59

Table III.14: Significant differences between control and dose 4 mg/kg (Collembolans – Movento O-TEQ).

Groups 0 % 4 Average dissimilarity = 49.37				
	Group 0	Group 4		
Species	Av. Abund	Av. Abund	Contrib%	Cum.%
He1	1.88	0.51	28.15	28.15
Ep7	2.66	1.41	22.74	50.89
Ep9	0.33	1.17	19.10	69.98
Ep6	1.38	1.06	14.33	84.31
Ed1	0.29	0.25	7.36	91.68

Table III.15: Significant differences between control and dose 500 mg/kg (Collembolans – Movento O-TEQ).

Groups 0 % 500 Average dissimilarity = 95.06				
	Group 0	Group 500		
Species	Av. Abund	Av. Abund	Contrib%	Cum.%
Ep7	2.66	1.13	32.29	32.29
He1	1.88	0.65	24.98	57.27
Ep6	1.38	0.00	23.97	81.24
Ep9	0.33	0.20	6.63	87.87
Ed1	0.29	0.00	4.57	92.44