UNIVERSIDADE DE SÃO PAULO

ESCOLA DE ENGENHARIA DE SÃO CARLOS

Programa de Pós-Graduação em Ciências da Engenharia Ambiental

ANDRÉ LUÍS SANCHEZ

Avaliação de risco ecológico em cenários de contaminação por pesticidas: respostas de indivíduos a ecossistemas

São Carlos
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Ecological risk assessment in pesticide contamination scenarios: from individuals to ecosystems responses

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Tese apresentada à Escola de Engenharia de São Carlos, da Universidade de São Paulo, como parte dos pré-requisitos à obtenção do título de Doutor em Ciências - Área Ciências da Engenharia Ambiental.

Orientador: Prof. Tit. Evaldo Luiz Gaeta Espíndola

São Carlos
Estado de São Paulo
2016

Sanchez, André Luís
S211a Avaliação de risco ecológico em cenários de contaminação por pesticidas: respostas de indivíduos a ecossistemas / André Luís Sanchez; orientador Evaldo Luiz Gaeta Espíndola. São Carlos, 2016.

Tese (Doutorado) - Programa de Pós-Graduação e Área de Concentração em Ciências da Engenharia Ambiental -- Escola de Engenharia de São Carlos da Universidade de São Paulo, 2016.

1. Pesticidas. 2. Ecotoxicologia. 3. Avaliação de risco ecológico . 4. Biodisponibilidade. 5. Interações ecológicas. 6. Respostas comportamentais. 7. Serviços ecossistêmicos. 8. Mesocosmos. I. Título.

FOLHA DE JULGAMENTO

Candidato: Licenciado e Bacharel **ANDRÉ LUIS SANCHEZ.**

Título da tese: "Avaliação de risco ecológico em cenários de contaminação por pesticidas: respostas de indivíduos a ecossistemas ".

Data da defesa: 28/06/2016

Comissão Julgadora:	**Resultado:**
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Presidente da Comissão de Pós-Graduação:
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ACKNOWLEDGEMENTS

My supervisors Dr. Evaldo Luiz Gaeta Espíndola and Dr. John Jensen for guidance, support, encouragement and trust. Thanks to John for accepting my stay in Aarhus University and giving me the opportunity to have worked with fantastic people.

The members of the Ecology and Applied Ecotoxicology Center (São Carlos School of Engineering - USP), especially Ana Vasconcelos, Bruna Vieira, Claudia Fileto, Edna Nunes, Juliana Resende, Haroldo Lobo, Lucas Mendes, Marina Reghini and Vanessa Menezes.

My world friends from Silkeborg/DK, especially Amaia Irizar from Basque Country, Jennifer Mariyadas from India, Jiayi Qin and Hu En from China, Luís Mendes from Portugal, Nicolás Vidal from Uruguay and Stine Slotsbo and Sissel Gently from Denmark.

The University of São Paulo and Aarhus University for the facilities, especially the staff of the Water Resources and Environmental Studies Center (São Carlos School of Engineering - USP, Itirapina, Brazil) and the National Environment Research Institute (Department of Bioscience - AU, Silkeborg, Denmark) for their great assistance respectively.

The Brazilian National Council for Scientific and Technological Development (CNPq) for the Ph.D. grant and the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for the doctorate sandwich grant.

The democratic Federal Government of Brazil to support my education in high academic level since 2005.

All my colleagues and friends.

My family to the incondicional support.

Thank you so much everybody!!!

:)

A seca avança em Minas, Rio, São Paulo
O Nordeste é aqui, agora
No tráfego parado onde me enjaulo
Vejo o tempo que evapora
Meu automóvel novo mal se move
Enquanto no duro barro
No chão rachado da represa onde não chove
Surgem carcaças de carro

Os rios voadores da Hiléia
Mal desaguam por aqui
E seca pouco a pouco em cada veia
O Aquífero Guarani
Assim do São Francisco a San Francisco
Um quadro aterra a Terra
Por água, por um córrego, um chovisco
Nações entrarão em guerra

Quede água? Quede água?
Quede água? Quede água?
Agora o clima muda tão depressa
Que cada ação é tardia
Que dá paralisia na cabeça
Que é mais do que se previa
Algo que parecia tão distante
Periga, agora tá perto
Flora que verdejava radiante
Desata a virar deserto

O lucro a curto prazo, o corte raso
O agrotóxico, o negócio
A grana a qualquer preço, petro-gaso
Carbo-combustível fóssil
O esgoto de carbono a céu aberto
Na atmosfera, no alto
O rio enterrado e encoberto
Por cimento e por aslfalto

Quede água? Quede água?
Quede água? Quede água?
Quando em razão de toda a ação humana
E de tanta desrazão
A selva não for salva, e se tornar savana
E o mangue, um lixão
Quando minguar o Pantanal e entrar em pane
A Mata Atlântica tão rara
E o mar tomar toda cidade litorânea
E o sertão virar Saara

Lenine (2015)
Quede água?

ABSTRACT

SANCHEZ, A. L. (2016). Ecological risk assessment in pesticide contamination scenarios: from individuals to ecosystems responses. Thesis (Ph.D. degree) - São Carlos School of Engineering, University of São Paulo, São Carlos, SP, Brazil.

Ecological risk assessment (ERA) studies are important to assess environmental changes that have been caused by anthropogenic activities. These integration models show the estimation of adverse risk effects across the levels of biological organization potentially exposed to perturbation, including a better understanding of the ecosystems complexity. It is well known that the pesticide have severe environment effects contributing to biodiversity loss and trophic levels changes. In this context, the aim of this study was to evaluate the ecological risk assessment in pesticide contamination scenarios for aquatic and terrestrial compartments. To attempt it direct and indirect effects on individual response for different biological organization and for multi trophic interactions responses with ecosystems models were evaluated. Thus the environmental impacts in relation to losses and changes of the ecosystems functions and services were analyzed. For this purpose, a risk scenario was designed to compare the Ivermectin contamination exposure routes, via dermal (soil) and oral (food) on *Eisenia fetida* reproduction tests. An experimental approach was constructed to characterise the effects of the fungicide Scala® (Pyrimethanil) in spraying application comparing to homogenous soil application on a constructed soil multi-species test system. n experiment was performed to reported the effects of the fungicide Mythos® (Pyrimethanil) with terrestrial plant test followed by elutriate test with non-targets freshwater organisms and avoidance test with soil invertebrates and quantify the ecosystems services framework. A holistic higher tier fungicide risk assessment was done with terrestrial and aquatic responses and trophic levels with multitrophic interactions in ecosystem models and supplementary with individuals' responses. The results obtained suggest that the analyzed reproduction parameters for earthworms were affected with the increase of ivermectin concentrations with statistical significant differences between the contamination exposure routes. The fungicide pyrimethanil has adverse effect on soil invertebrates' response for the application and spatial distribution with the habitat preferences and foraging abilities has affected directly or indirectly by the fungicide toxicity. The impacts by the runoff and leaching pesticides into adjacent water bodies and surrounding soil showed changes in the organism's structure with changes and loss in the provisioning, regulatory and supporting services. The integrated holistic four-tiered fungicide risk assessment showed the possible impacts and the adverse effects on the terrestrial and aquatic organisms, ecosystems and processes in the simulate scenarios. From the results, it is possible to conclude that the experiments performed crossed the multiple aspects of contaminations and show the individuals to ecosystems responses approaches using the exposure routes of contamination, multi trophic interactions of experimental ecosystems models, behavioral, individual and some comparatives responses with aquatic and terrestrial compartments in risk assessment. Furthermore, this study are an important register for the deleterious effects and responses to impacts of pesticides, prompting the possible environmental losses and changes of the ecosystems functions and services in disturbances areas.

Key-words: Pesticides, ecotoxicology, ecological interactions, ecosystem services, mesocosms

RESUMO

SANCHEZ, A. L. (2016). Avaliação de risco ecológico em cenários de contaminação por pesticidas: respostas de indivíduos a ecossistemas. Tese (Doutorado) - Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, SP, Brasil.

Os estudos de avaliação de risco ecológico consistem em avaliar os riscos ecológicos ocasionados pelas diversas atividades antropogênicas a um determinado sistema. Essa abordagem de integração reporta a estimativa dos efeitos de risco adverso através dos níveis de organização biológica potencialmente expostos a pertubação, incluindo assim uma melhor compreensão da complexidade dos ecossistemas. É bem conhecido que os pesticidas possuem efeitos nocivos ao meio ambiente, contribuindo para a perda de biodiversidade e mudanças nos níveis tróficos. A partir dessa análise, o objetivo geral desse estudo foi uma avaliação de risco ecológico em cenários de contaminação por pesticidas em relação aos compartimentos terrestres e aquáticos. Para tanto, foram avaliados os efeitos diretos e indiretos sobre as respostas individuais para diferentes níveis de organização biológica e para as interações multi tróficas através de modelos ecossitêmicos. Assim, foram analizados os impactos ambientais em relação as perdas e mudanças das funções e serviços dos ecossistemas. Para esse propósito, foram desenvolvidos cenários de risco em relação as rotas de exposição do antiparasitário Ivermectin para a minhoca *Eisenia fetida* em relação a testes de reprodução, através da via dermal (solo) e oral (comida). Foi construída uma abordagem experimental para caracterizar os efeitos do fungicida Scala® (Pyrimethanil), comparando a aplicação através de pulverização por spray com a aplicação homogênea no solo em um sistema terrestre multi espécies. Experimentos foram realizados para reportar os efeitos do fungicida Mythos® (Pyrimethanil) em plantas terrestres alvo, seguidos por teste com elutriato com organismos de água doce não-alvo e testes de fuga com invertebrados terrestres não-alvo e uma quantificação dos serviços ecossitêmicos. Foi realizada uma avaliação de risco holística do fungicida pyrimethanil com respostas dos organismos terrestres e aquáticos e das interações tróficas através dos modelos ecossitêmicos e complementados com respostas indivíduais. Os resultados obtidos sugerem que os parâmetros de reprodução para as minhocas foram afetados com o aumento das concentrações de ivermectina com diferenças estatísticas significativas entre as rotas de exposição a contaminação. O fungicida pyrimethanil mostrou efeitos adversos sobre os invertebrados terrestres para as aplicações do pesticida e para a distribuição espacial, sendo as preferências de habitat e habilidade de forageio direta ou indiretamente afetadas pela toxicidade do fungicida. Os possíveis impactos do runoff e lixiviação nos corpos de água e solos adjacentes mostram mudanças na estrutura da comunidade com mudanças e perdas nos serviços ecossistêmicos de provisão, regulação e suporte. A avaliação de risco holística mostrou os impactos e efeitos adversos sobre os organismos terrestres e aquáticos, ecossistemas e processos nos diferentes cenários de simulação. Ao analisar os dados obtidos é possivel concluir que os experimentos realizados permeiam os multíplos aspectos da contaminação por pesticidas, mostrando respostas de indivíduos a ecossistemas através das rotas de exposição da contaminação, interações multi tróficas a partir dos experimentos de modelos ecossistêmicos, respostas individuais, comportamentais e comparativas com os sistemas terrestres e aquáticos em avaliações de risco ecológico. Portanto, esse estudo se apresenta como um importante registro dos efeitos deletérios e das respostas dos impactos por pesticidas, levando a possíveis perdas e mudanças das funções e serviços ecossistêmicos em áreas com distúrbios.

Palavras chave: Pesticidas, ecotoxicologia, interações ecológicas, serviços ecossitêmicos, mesocosmos

SUMMARY

THESIS PRESENTATION

The present thesis contain four paper's draft in preparation for future publications, adding the judging committee contributions, and a general introduction where we included several theoretical aspects about pesticides, ecotoxicological studies, ecosystems services and risk assessment. The chronological Brazilian-Danish pesticide research levels is presented in the Figure 1. It is appropriate highlight that the general thesis structure is following the standards and recommendations of the São Carlos School of Engineering, University of São Paulo and that the thesis language was approved by the postgraduate committee (CCP-SEA, 29/04/2016).

The effect of exposure routes on the ecotoxicology of the antiparasitic Ivermectin to earthworms - An evaluation of the contamination exposure routes to earthworms, comparing the antiparasitic ivermectin contamination with exposure routes, via dermal (soil) and oral (food) on *Eisenia fetida* reproduction tests.

The impact of the fungicide Pyrimethanil on soil fauna measured in a soil multi-species (SMS) test system when exposure through surface and homogenous soil application - An experimental approach to characterise the effects of the fungicide Scala® (Pyrimethanil) on spraying application compare to homogenous soil application on a constructed soil multi-species (SMS) test system.

Scenarios of ecological risk assessment of the fungicide Pyrimethanil based on an ecosystem services approach - An experimental simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil), using laboratory-derived acute, chronic and avoidance toxicity data for plants and non-target freshwater and terrestrial organisms and a quantification of the ecosystems services.

Ecological risk assessment for ecosystem models in contamination scenarios of the fungicide Pyrimethanil - An ecological risk assessment study using a hypothetical risk scenarios experiments to integrate terrestrial and aquatic data responses to the fungicide Pyrimethanil, reporting a four-tiered pesticide risk assessment approach.

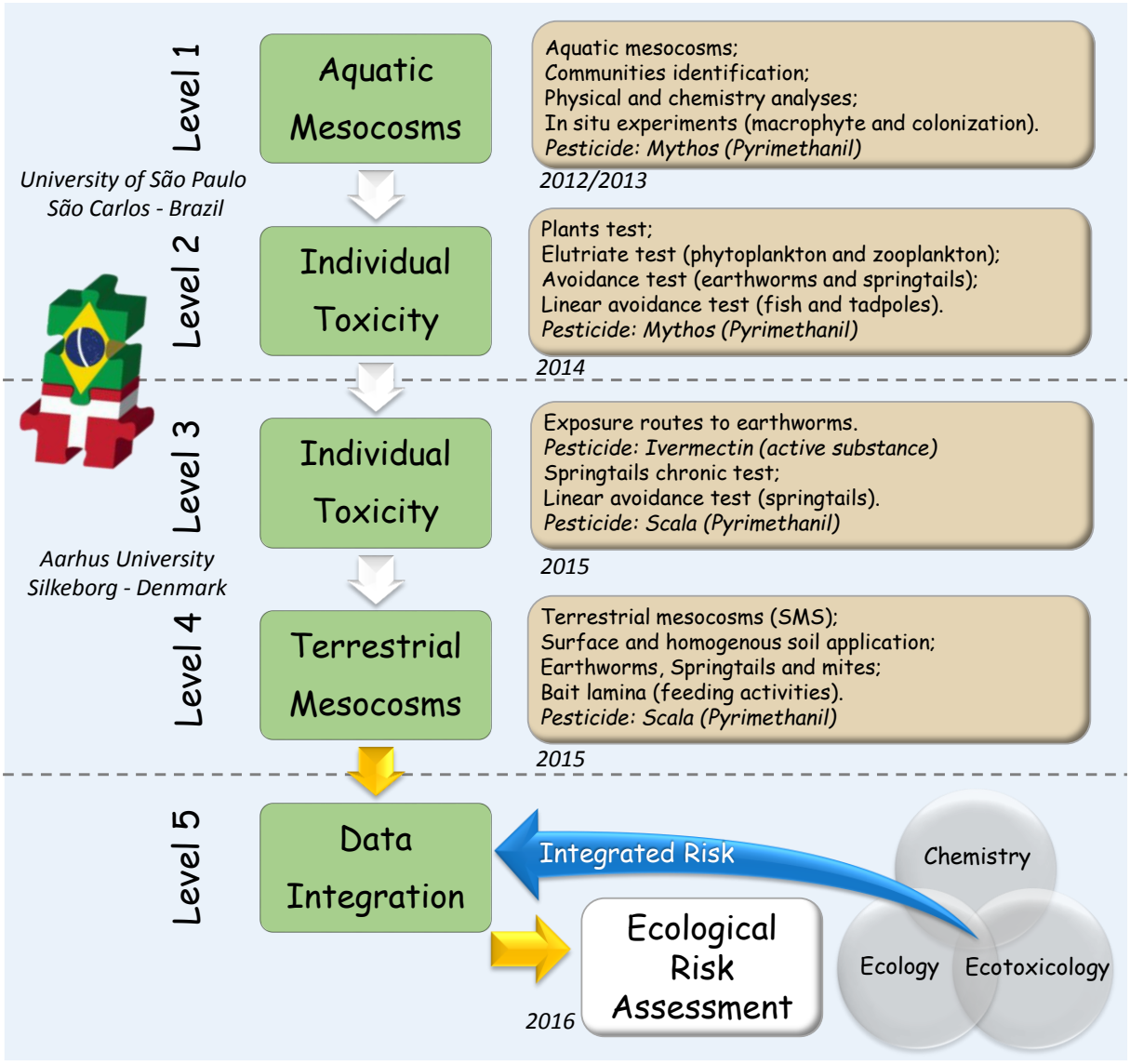

Figure 1. Chronological research level framework with the Brazilian and Danish experiments for the pesticide Pyrimethanil and Ivermectin. The levels represent the mesocosms tests (aquatic and terrestrial), individuals toxicity test with different organization levels and for the fungicide pyrimethanil a data integration in an ecological risk assessment.

Papers on related topics published during the Ph.D. study period

Araujo, C.V.M.; Shinn, C.; Mendes, L.B.; Delello-Schneider, D.; Sanchez, A.L.; Espindola, E.L.G. (2014). Avoidance response of Danio rerio to a fungicide in a linear contamination gradient. *Science of the Total Environment*, 484, 36-42.

Shinn, C.; Delello-Schneider, D.; Mendes, L.B.; Sanchez, A.L.; Muller, R.; Espindola, E.L.G.; Araujo, C.V.M. (2015). Immediate and mid-term effects of pyrimethanil toxicity on microalgae by simulating an episodic contamination. *Chemosphere*, 120, 407-413.

GOALS

GENERAL GOAL

The general goal for this research was evaluate the ecological risk assessment in pesticide contamination scenarios for aquatic and terrestrial compartments. To attempt it direct and indirect effects on individual response for different biological organization and for multi trophic interactions responses with ecosystems models were evaluated. Thus the environmental impacts in relation to losses and changes of the ecosystems functions and services were analyzed.

SPECIFIC GOALS

- Evaluate the relationship between exposure routes contamination and the effects of antiparasitic ivermectin concentrations on reproduction descriptors on earthworm *Eisenia fetida*;
- Evaluate the direct and indirect of two pesticide application methods (spraying and homogenous soil applications) on a soil multi-species (SMS) test system;
- Evaluate the fungicide Pyrimethanil effects on feeding activity of the soil invertebrates using the bait lamina test system;
- Evaluate the different effects on single-species reproduction tests for the springtails *Folsomia candida* with the commercial formulation and active ingredient of the fungicide Pyrimethanil;
- Evaluate the avoidance behaviour responses of the springtail *Heteromurus nitidus* to contaminated soils with the fungicide Pyrimethanil in a multi-compartmented static linear avoidance test system;
- Evaluate a simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil) considering plants seedling emergence and early growth;
- Evaluate the effects of fungicide Mythos® (Pyrimethanil) on freshwater organisms tests;
- Compare the avoidance behaviour responses of the earthworms *Eisenia andrei* and springtails *Folsomia candida* on the fungicide Mythos® (Pyrimethanil) contamination;

- Quantify the aquatic and terrestrial ecosystems services of the proposal scenarios;
- Evaluate the four-tiered ecological risk assessment for the fungicide pyrimethanil, integrating the responses of terrestrial and aquatic compartments.

HYPOTHESIS

- The antiparasitic ivermectin is a weakly metabolized substance and most of the dose given to the pasture animals is excreted relatively unaltered in the treated animals faeces. The exposure of *Eisenia fetida* to ivermectin via-food can cause stronger toxic effects on reproduction than via dermal exposure.
- A controlled terrestrial study (ecosystem model), simulating a crops situations pesticide applications by spray is an efficient tool to measure the pesticide effects on the soil processes and food web structure in a realistic scenario.
- The plant protect substances have the potential to affect the freshwater and terrestrial organisms by the runoff and leaching into adjacent water bodies and surrounding soils with deleterious impacts on the ecosystem functions and services.
- The pesticides have the intrinsic capacity to cause adverse effects over time and space on the individuals and mainly on multi trophic interactions with changes on the structure of the ecosystems functions and services.

CHAPTER I

General Introduction

Land use intensification and the Earth collapse

The Tree of Life is an apt metaphor for the diversity and relationships of organisms on Earth but the environmental changes are causing their pruning of whole twigs at a time (Dinnage et al. 2012). Some ecologists are considering that we are living in an age of extinction due to multiple anthropic drivers of biodiversity loss with potentially profound implications for the near future (Naeem et al. 2012). The biodiversity loss in the 21st century could rank among the major drivers of ecosystem change (Hooper et al. 2012; Thuiller et al. 2011). Large-scale habitat destruction and climate change result in the non-random loss of evolutionary lineages, reducing the amount of evolutionary history represented in ecological communities (Dinnage et al. 2012) (Figure 1). In a world that is being transformed by humans, ecology will have to respond relevant scientific knowledge on how ecosystems function and change, how they are linked to human well-being and how humankind can use and transform them in a sustainable way (Loreau, 2010).

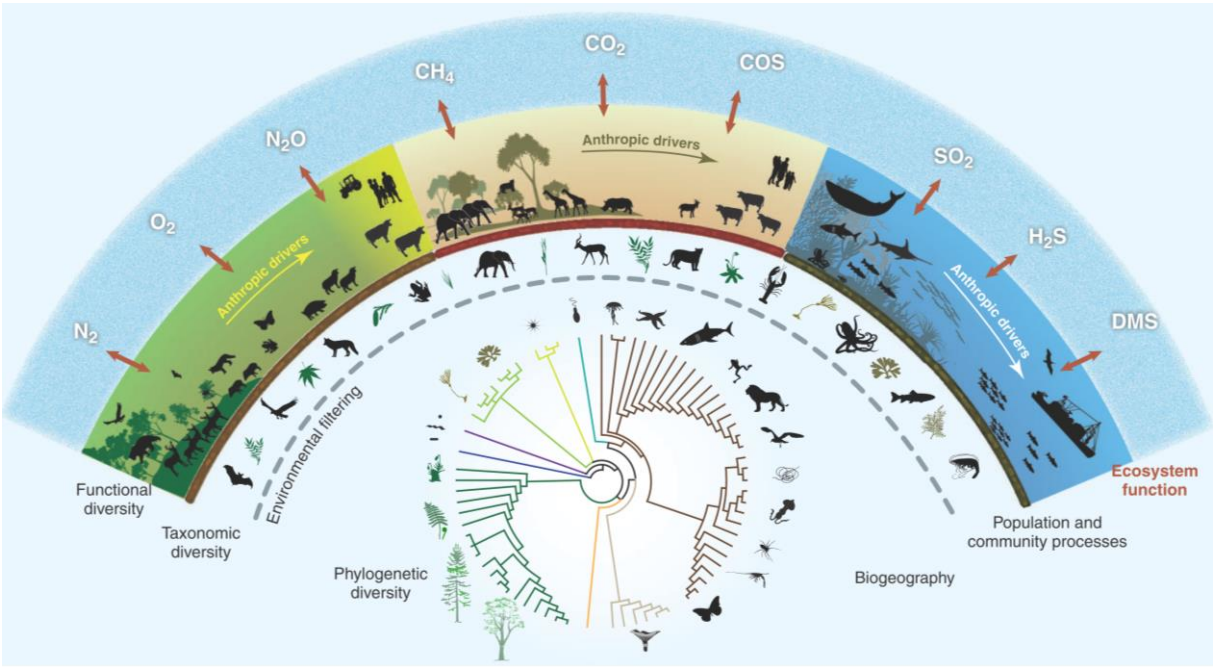

Figure 1. The phylogenetic tree of life of which only a few representative phyla and divisions are shown as icons at the tips of the branches. The species phylogenetic and taxonomic diversity from the global pool are found largely determined by environmental filters, represented as a barrier with pores (dashed arch). Three representative ecosystems are illustrated with the chemical exchanges between the atmosphere and biosphere shown in the outermost arch*:* Forested ecosystem (left arch) like Amazon and Atlantic forest in Brazil, temperate-zone bamboo forests in central China, Tamil Nadu forests in south India or the temperate forest in Denmark (emissions changes from Nitrogen gas (N_2) to Nitrous oxide (N_2O)), Savanna ecosystem (centre arch) like the Brazilian and African savanna (emissions changes from Methane (CH_4) to Carbonyl sulfide (COS)) and the Marine ecosystem (right arch) like the Basque Country coast besides the Pacific and Arctic fisheries areas (emissions changes from Sulfur dioxide (SO_2) to Dimethyl sulfide (DMS)). Human transformations of ecosystems going from left to right in each arch lead to biotic impoverishment and biotic homogenization. Source: Naeem et al. 2012.

Human activities have been and are continuing to change the environment on local and global scales, increasing the species invasions and species extinctions (Hooper et al. 2005). The conversion of land from complex natural systems to simplified agricultural monocultures is a major cause of the current unprecedented rates of global biodiversity loss with functional diversity changes with agricultural intensification (Flynn et al. 2009). The agricultural practices are essential for sustaining the human population, but also directly disrupt ecosystem functioning (Galic et al. 2012). The intensification of agriculture have shown a devastating effect on biodiversity (Krebs, et al. 1999). However, agro-ecosystems provide ecosystem services such as food and soil fertility but depend strongly on a suite of ecosystem services provided by natural, unmanaged ecosystems (Power, 2010). In this sense, the agricultural process also receives ecosystem disservices that reduce productivity or increase production costs, including herbivory, habitat loss for biodiversity conservation, nutrient runoff, sedimentation of waterways, and pesticide poisoning of humans and non-target species (Zhang et al. 2007).

The croplands and pastures have become one of the largest terrestrial biomes on the planet, rivalling to forest cover in extent and occupying approximately 40 % of the land surface (Foley, 2005). These authors report changing land-use practices have enabled world grain harvests to double in recent decades and most of these production gains resulted from "Green Revolution" technologies, including high-yielding cultivars, chemical fertilizers and pesticides, and mechanization and irrigation (Figure 2). A comparison of soil organic carbon stocks in Viking Age (700-1066 AD) and modern land use systems in Denmark showed a loss in the surrounding soils due to liming and drainage that increased the decomposition of organic matter in the soils (Breuning-Madsen et al. 2009). In Brazil, the land-use changes are currently common, altering soil concentrations, stocks and elemental ratios of carbon, nitrogen and phosphorus with possible impact on the subsequent vegetation, decreasing soil carbon and increasing nitrogen limitation but alleviating soil phosphorus deficiency (Groppo et al. 2015).

Notwithstanding, impoverished aquatic communities in agricultural landscapes have been associated with pesticide contamination and input of nutrients with evidence of adverse effects (Brock et al. 2006; Maltby & Hills, 2008; Schäfer et al. 2012). Agricultural activities can contribute with residues of applied chemicals to hydrological compartments such as surface and groundwater being one of the most fundamental problems affecting the management of river basins on a worldwide basis (Domagalski et al. 2008). These abiotic factors can influence the toxicity of aquatic organisms and an important factor in measuring the environmental

conditions to assess the impacts of contaminants (Clements et al. 2010; Seeland et al. 2012) with substantial changes in biodiversity, functions and ecosystem services.

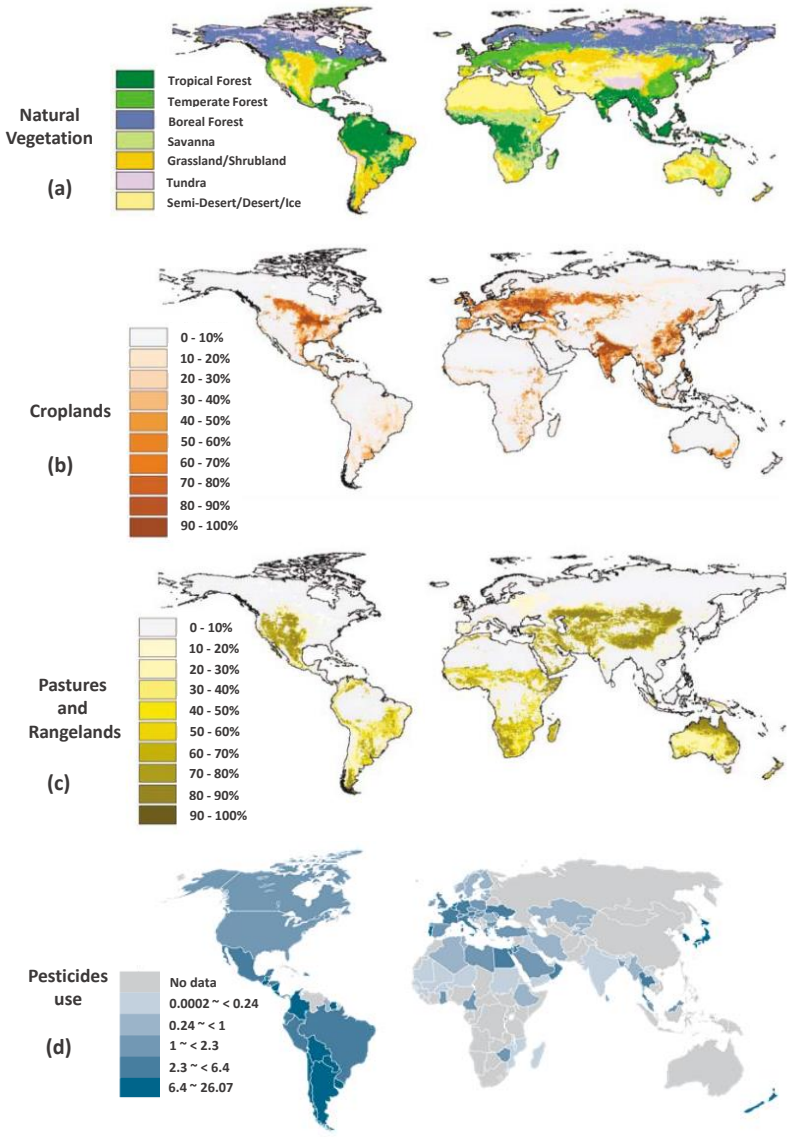

Figure 2. Worldwide extent of human land-use and land-cover change maps. These maps illustrate the geographic distribution of vegetation that would most likely exist in the absence of human land use (a), and the extent of agricultural land cover such as croplands (b) and pastures and rangelands (c) across the world during the 1990s. The pesticides use per ha of arable land (kg/ha, 2007 to 2012) (d). Source: Foley et al. 2005; FAO, 2015.

The ecosystem degradation results from increased input of nutrients, sediments, and toxic substances, which come from agricultural multi-use areas (Sánchez et al. 2006). The pesticides may enter in fresh waters directly via spray-drift or indirectly leaching, runoff, and/or accidental spills (Brock et al. 2006; Maltby & Hills, 2008), besides the discharges of domestic and industrial wastewater, urban runoff, seepage, storm water drainage, and agricultural effluents drainage systems (Whitehead et al. 2015) (Figure 3).

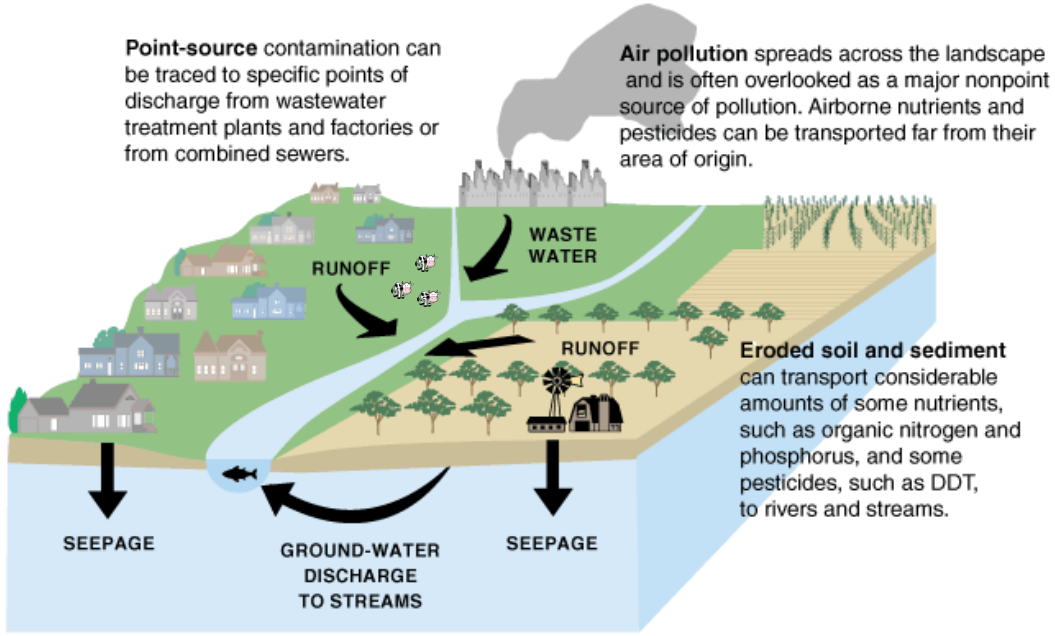

Figure 3. Sources of water and surrounding soil contamination and the movement of pollutants into different water reservoirs of the water cycle. The contamination start with spray-drift or indirectly leaching, seepage, runoff, and/or accidental spills in agricultural area, discharges of domestic and industrial wastewater and air pollution. *Source:* USA Geological Survey (USGS).

The hazards and risks of pesticide: from individuals to ecosystems

The wildlife ecotoxicology has its roots in acute poisoning events in the late 19th century with public concern over the undesirable environmental effects of chemicals arose in the early 1960s with the Rachel Carson (1907-1964) publication "*Silent Spring"*, which publicly broached the issue of the environmental risks of pesticide use for the first time (Köhler & Triebskorn, 2013). The highlights of the toxic side effects of organochlorine insecticides residues, such as DDT, that had fuelled the green revolution, were found to persist in the bird's food chain, reaching higher concentrations and hence having more severe effects, at successive trophic levels (Krebs et al. 1999). Some authors called the new losses in biodiversity, ecosystems functions and services as the "Second Silent Spring", although associated with the intensification and industrialization of agriculture, involving more subtle and indirect effects on wildlife by pesticide residues (Krebs et al. 1999).

Currently, pesticide residues constitute a potential toxicological hazard for the non-target organisms, possibly contributing to biodiversity loss and to side effects in higher trophic levels (Rico et al. 2016). Some of these pesticide effects at the sub-individual or individual levels and may linked to their consequences in populations and ecosystems (Köhler & Triebskorn, 2013) (Figure 4). Indeed, food-web approaches consider the species diversity and the fluxes of energy and materials between species through their interactions to provide

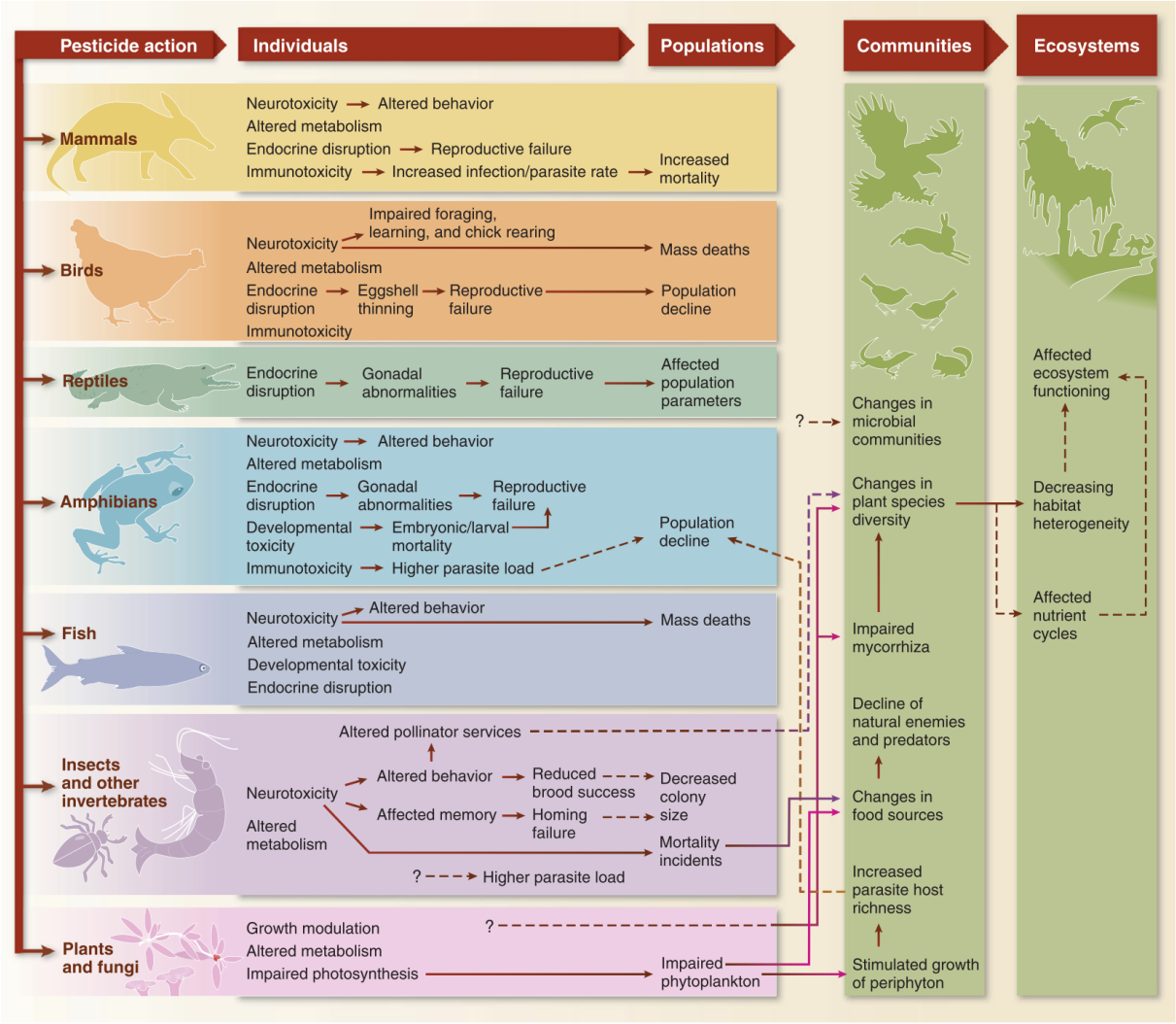

Figure 4. The pesticide effects on wildlife at different levels of biological organization such as individuals, populations, communities and ecosystems (solid arrows) or evidence supported, anticipated (dashed arrows) and the interrelations among them. Research remains to be conducted wherever plausibly interrelated effects are not connected by arrows. Most of the sub-individual data for mammals are derived from non-wildlife studies. Some of these pesticide effects at the sub-individual or individual levels have been causally or plausibly linked to their consequences in populations and ecosystems. Source: Köhler & Triebskorn, 2013.

a natural framework for understanding species' ecological roles and the mechanisms through which biodiversity influences the number and the distribution of functional groups in ecological communities (Montoya et al. 2015). However, species interactions will not be the major structuring force in ecosystems communities, where factors such as competition and predation are important, the relative strength of these interactions will likely influence how communities respond to anthropogenic disturbance (Clements & Rohr, 2009). For example, the plant diversity has strong bottom-up effects on multitrophic interaction networks, with particularly strong effects on lower trophic levels and effects on higher trophic levels are indirectly mediated through bottom-up trophic cascades (Scherber et al. 2010). With this insight, the plants support

a wide array of herbivore species in the ecosystems that feed upon them, as well as the predators that feed on herbivores, the loss of plant diversity should propagate up to consumers, influencing the structure and diversity of associated communities at higher trophic levels (Dinnage et al. 2012). However, the functional and phylogenetic diversity of natural zooplankton communities determines their ability to produce biomass, as well as suppress phytoplankton through top-down grazing (Thompson et al. 2015). These improved understanding of basic ecological concepts has enhanced the ability to predict effects of contaminants in aquatic and terrestrial ecosystems (Clements & Rohr, 2009).

The effect of pesticides may impair the individual's metabolic functions such as thermoregulation, water and/or food intake, and behaviour (activity, foraging time, learning ability) in vertebrates such as tadpoles and fishes, with consequences of weight loss, impaired development and reduced reproduction and hatching success (Köhler & Triebskorn, 2013). Furthermore, the contaminant concentrations, community structure and ecosystem processes vary naturally along environmental gradients, likely responses to contaminants will also differ over time and space (Clements et al. 2012).

One of the worldwide used pesticides are the veterinary medicinal products to treat diseases, protect animal health, enhance productivity and promote growth (Jensen et al. 2009). The antiparasitic ivermectin released to the environment may be degraded, transported and distributed between different compartments (Kolar et al. 2008). Thus, residues or their metabolites are excreted in dung and may have environmental impact on the development and survival on dung-dwelling fauna (Suarez, 2002; Iglesias et al. 2006; Fernandez et al. 2014) (Table 1). Notwithstanding, other authors state that ivermectin could do not affect earthworms (Kaneda et al. 2006; Svendsen et al. 2003; Torkhani et al. 2011). However, the risk posed by soil contaminants strongly depends on their bioavailability (Van Der Wal et al. 2004). The chemical Ivermectin (CAS: 70288-86-7) were obtained from Sigma-Aldrich Denmark ApS. The purity of the chemicals was 90 %. Due to the low solubility of ivermectin in water, a stock solution was prepared by diluting ivermectin in acetone. The structure of ivermectin is shown in Figure 5. The antiparasitic ivermectin is macrocyclic lactones isolated from a fermentation broth of the soil actinomycetes *Streptomyces avermitilis* belonging to the avermectin family (i.e. ivermectin, abamectin, doramectin) (Römbke et al. 2010). The chemical compound is a mixture of two chemically modified avermectins that contain at least 80 % of 22,23-dihydroavermectin-B 1a and > 20 % 22,23-dihydroavermectin-B 1b as a highly lipophilic substance that dissolves in most organic solvents, but is practically insoluble in water (0.0004 % m/v) (Lumaret et al. 2012).

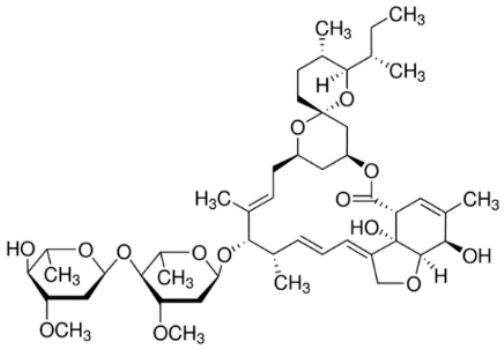

Figure 5. Chemical structure of Ivermectin.

Table 1. Framework of the literature review on the effects of Ivermectin ($mg.kg^{-1}$) on soil model organisms, ecotoxicological test and EC_{50} concentration.

Reference	Organism	Species	Test	EC_{50}
Jensen et al. 2003	Collembola	*Folsomia fimetaria*	Chronic 28 days, LC50	8,4
			Chronic 28 days, EC50	1,7
	Oligochaeta	*Enchytraeus crypticus*	Chronic 28 days	36
Rombke et al. 2010	Oligochaeta	*Eisenia fetida*	Chronic 28 days, LC50	10
			NOEC	2,5
	Collembola	*Folsomia candida*	Chronic 28 days, EC50	1,7
			NOEC	0,3
	Acari	*Hypoaspis aculeifer*	Chronic 28 days, LC50	31,6
			NOEC	3,2
Jensen et al. 2009	Collembola	*Folsomia fimetaria*	Chronic 28 days, LC50 single-specie	5,3
			Chronic 28 days, EC50 single-specie	0,93
			Chronic 28 days, LC50 two-specie	0,14
			Chronic 28 days, EC50 two-specie	0,11

The most frequently used fungicides in the European Union and worldwide are the compound of the anilinopyrimidine class called Pyrimethanil (Anfossi et al. 2007). It controls the grey mould with high affinity for the soil solid phase and not easily degraded (Sadlo, 2002). Pyrimethanil inhibits the secretion of fungal enzymes relevant for pathogenicity widely used for example in champagne's vineyards in France (Verdisson et al. 2001), grapevine's vineyards in Portugal (Gil et al. 2015), tomatoes and vegetables fields in Poland (Sadlo, 2002) and apples and outdoor strawberries fields in Denmark. Pyrimethanil (CAS: 53112-28-0) [IUPAC name N-(4,6-dimethylpyrimidin-2-yl)-aniline] is a colorless crystalline substance practically insoluble in water (121 mg/L), belonging to the anilinopyrimidine class (mode-of-action: methionine biosynthesis inhibition). In the form of concentrates, it is used as a contact fungicide with protective and curative properties (Sadlo, 2002). The structure of pyrimethanil is shown in Figure 1. The chemical compound has a moderate persistence in soil with half-life value of 55 days (Wightwick et al. 2010). The commercial formulations are found in a chemical composition of suspension concentrate with tradenames of Scala® (400 g/l Pyrimethanil, 37.4 % w/w) indicated for the control of leaf scab in apples and grey mould in outdoor strawberries

and the moderate control of grey mould in protected strawberries and tradenames of Mythos® (300 g/l Pyrimethanil, 30.0 % w/w) indicated for the control of treatment of various diseases on banana, potato, onion, carrot, apple, strawberries, tomato and grapes crops.

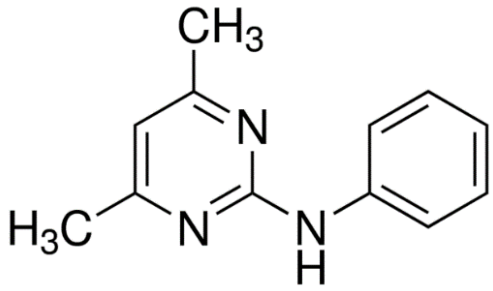

Figure 6. Chemical structure of Pyrimethanil.

In general, for the ecosystem this fungicide has low ecological risk when applied according to agricultural recommended practices, but the risk is likely to increase in case of accidental spills, inadequate application and environmental loading or disposal (European Commission, 2005; EFSA, 2010; Verdisson et al. 2001; Müller et al. 2012; Seeland et al. 2012; Gil et al. 2015; Bandow et al. 2016) (Table 2).

Table 2. Framework of the literature review on the effects of Pyrimethanil ($mg.L^{-1}$ or $mg.kg^{-1}$) on aquatic and soil model organisms, ecotoxicological test and EC_{50} concentration ($^{*}EC_{20}$).

Reference	Organism	Species	Test	EC_{50}
Wightwick et al. 2010	Oligochaeta	*Eisenia fetida*	Chronic 28 days	252
Seeland et al. 2012	Algae	*Desmodemus subspicatus*	Chronic 72 hours	13.7
		Scenedemus acutus	Chronic 48 hours	23.1
	Macrophyte	*Lemna minor*	Chronic 6 days	46.1
	Oligochaeta	*Lumbriculus variegatus*	Chronic 28 days	12.7
	Insecta	*Chironomus riparius*	Acute 48 hours	2.92
	Crustacea	*Daphnia magna*	Acute 48 hours	3.61
			Chronic 21 days	1.18
	Fish	*Oncorhynchus mykiss*	Chronic 21 days	10.6
Seeland et al. 2013	Mollusc	*Physella acuta*	Embryo test	33.8
			Juvenile growth test	65.6
			Chronic	39.7
Bandow et al. 2013	Oligochaeta	*Enchytraeus bigeminus*	Chronic 28 days, 50 % WHC	499
			Chronic 28 days, 70 % WHC	829
Bandow et al. 2014	Collembola	*Folsomia candida*	Chronic 28 days	55.6
		Sinella curviseta	Chronic 28 days	81.5
Araujo et al. 2015	Amphibian	*Lithobates catesbeianus*	Avoidance 12 hours	0.48
Gil et al. 2015	Bacteria	*Saccharomyces cerevisiae*	Chronic 6 hours	45
	Nematode	*Caenorhabditis elegans*	Chronic 72 hours	1.4^{*}
	Oligochaeta	*Enchytraeus crypticus*	Chronic 28 days	185
	Collembola	*Folsomia candida*	Chronic 28 days	19.9

WHC = water holding capacity

Relationship between biodiversity, ecosystem functions and services

In 1881, Charles Darwin (1809-1882) published his last scientific book entitled "*The formation of vegetable mould through the action of worms with observations on their habits*", as result of several decades of detailed observations and measurements on earthworms and the natural sciences (Feller et al. 2003). These authors' highlights about Darwin's clear demonstrated about the importance of earthworms on biological activities in ecosystems processes for the maintenance of the soil fertility and play the role to soil formation.

The potential for diversity to affect ecosystem functions and services was recognized by mid-twentieth-century researchers and originally dates back to Darwin (Hector & Bagchi, 2007). In this sense, it has become increasingly well known that ecosystems provide a wealth of benefits to human society, and the provision of such ecosystem services depends fundamentally on functions performed by organisms (Millennium Ecosystem Assessment, 2005). Biodiversity increases the ability of ecosystems to provide multiple functions with a positive relationship between species richness and the number of ecosystem functions (Montoya et al. 2015). For example a little declines in plant diversity have prompted concern over the consequences for the stability of ecosystem functioning and the reliable provisioning of ecological services (Hautier et al. 2014) (Figure 7).

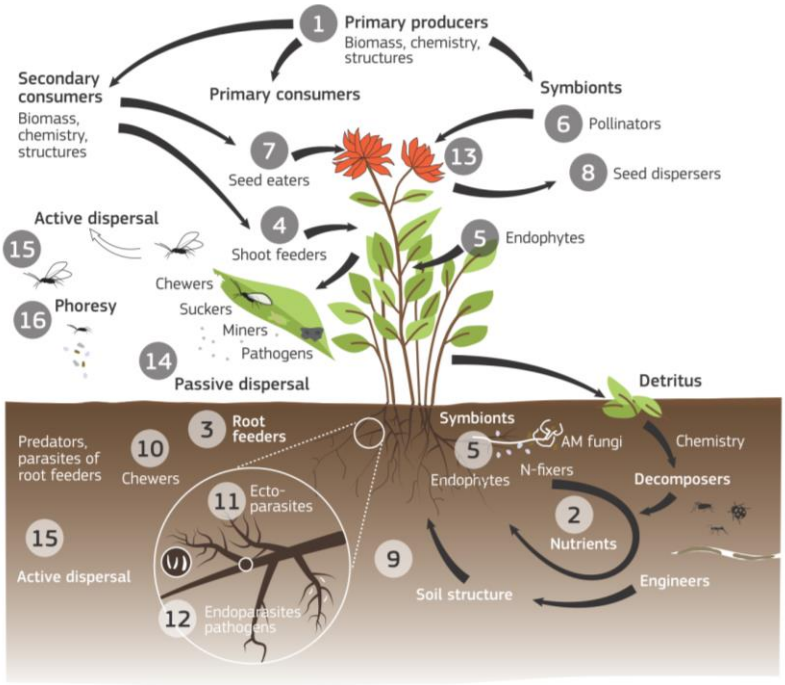

Figure 7. Plant community biomass, chemistry and structures with the plant-soil interactions (1), the dependence of plants characteristics and the activity of soil functional groups, such as decomposers, symbionts and engineers which make nutrients available (2), the belowground and aboveground herbivores and pathogens (3-4), endophytes living symbiotically in shoots, leaves or roots (5), pollinators (6), seed eaters (7), seed dispersers (8), soil organisms interacting with a single plant root system (9), mobile generalist feeders (10-11), specialised endoparasitic plant association (12), flowers, fruits or seeds (13), passive dispersal (flying, walking, crawling or burrowing) (14), an aboveground life phase enabling targeted active dispersal (15), dispersal by air, water or take a ride via phoresy (16). Source: Orgiazzi et al. 2016.

The Millennium Ecosystem Assessment (2005) established the scientific basis for actions needed to enhance the conservation and sustainable use of ecosystems and their contributions to meeting human needs. These authors proposed an inextricably linked between biodiversity and human well-being, changing human conditions drive, directly and indirectly, changes in biodiversity, changes in ecosystems and changes the services ecosystems provide (Figure 8).

The ecosystem services (ES) are quantifying in four categories: **provisioning services** (production of goods) such as food and water, **regulating services** (life support processes) such as the regulation of climate and water quality, **cultural services** (life fulfilling conditions) such as recreation and aesthetic values and **supporting services** (life support processes) such as soil formation, photosynthesis and nutrient cycling (Millennium Ecosystem Assessment, 2005). The ES are usually results from complex interactions between and within abiotic and biotic components of ecosystems articulated by human activities (Schäfer, 2012). Additionally, the ES concept can aid efficient communication between different stakeholder groups and with risk managers, in particular when defining specific protection goals (Nienstedt et al. 2012).

The fundamental challenge for sustainability is meet society's current needs by using Earth's natural resources without compromising the needs of future generations (Liu et al. 2015). Understanding the ecology–society links we can better manage, maintain, restore or evaluate ecosystem services such as the knowing that there are seasonal fluctuations in stream flows needed for irrigation we can prepare for this variability though water collection or better irrigation management (Fisher et al. 2009). Ecosystems worldwide are rapidly losing functional diversity as a result of human appropriation of natural resources with impacts of habitats loss and diversity (Krebs, et al. 1999; Solan et al. 2004; Hector & Bagchi, 2007; Isbell et al. 2011; Naeem, et al. 2012). For example, the increased use of pesticides has caused concern over sublethal effects on bees, such as impacts on reproduction or learning ability with crucial implications of their ability to deliver the pollination services to plants necessary for ecosystem functioning (Stanley & Raine, 2016).

Currently, efforts to conserve wild nature are expanding into realms well beyond reserves, charity and biodiversity the new fronts of conservation are much bigger and much more complex, including new places dominated by human activity, new revenue streams from public and private sectors, and new goals of ecosystem service provision (Daily & Matson, 2008). In the other hand, managing the agricultural landscapes to provide sufficient supporting and regulating ecosystem services and fewer dis-services (e.g., herbivory) will require research

that is policy-relevant, engaging at a minimum the fields of ecology, hydrology, economics and political science (Zhang et al. 2007).

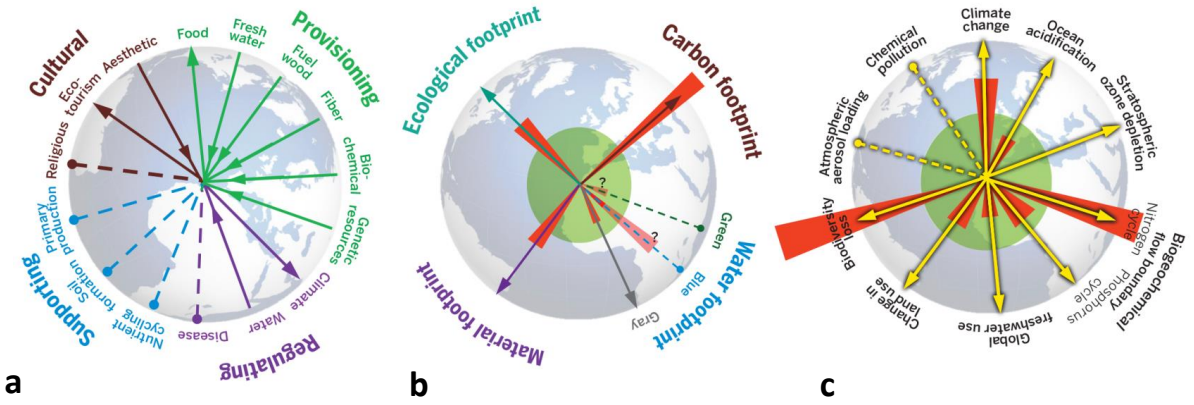

Figure 8. Conceptual framework of ecosystem services categories (cultural, provisioning, regulation and supporting) (a), environmental footprints (ecological, carbon, material and water) (b), environmental changes (c). Outward arrows indicate increases in the values, inward arrows indicate decreases and dashed lines indicate no data. In (b) and (c), the inner green shading represents maximum sustainable footprints and safe operating space for nine planetary system variables, respectively. Red wedges refer to the estimated current positions for the variables. Source: Liu et al. 2015.

The use of ES may lead to larger environmental footprints once these integrated frameworks of hazard substances have been studied largely in isolation, although they are interconnected through human activities (Liu et al. 2015). The integration will be facilitate the process to determine modifications or enhancements to traditional ecological risk assessment (ERA) measurement endpoints or to identify additional studies that would be required for injury determination and restoration scaling (Munns et al. 2009). Within the context of ERA, this new approaches are needed to facilitate the assessment of environmental health and the capacity of nature to provide the services (Faber & Van Wensem, 2012).

Environmental risk assessment: an environmental holistic systems integration

The 1992 Earth Summit in Rio de Janeiro/Brazil awakened the global interest in understanding how biodiversity loss might affect the dynamics and functioning of ecosystems, and thus affecting society by the supply of goods and services (Cardinale et al. 2012). The international researches increase the initiatives with hundreds of experiments performed in ecosystems and new ecological theories developed and tested against experimental results (Cardinale et al. 2012; Montoya et al. 2015). In this sense, the USA Environmental Protection Agency postulated the Ecological risk assessment (ERA) (EPA, 1992). Described as being the risk a subject to intrinsic capacity of the stressor to cause adverse effects and for the interactions

with biological components by time and with sufficient intensity to cause the identified adverse effects and may evaluate one or more elements stressors and ecological components.

ERA is an increasingly important part of the decision-making process for managing the global sustainability challenges (Weeks et al. 2004). These challenges include air pollution, biodiversity loss, climate change, energy and food security, disease spread, species invasion, water shortages and ecosystem pollution and they are interconnected across three dimensions (organizational levels, space and time) (Liu et al. 2015). The aim of ERA is the estimation of adverse risk effects across the levels of biological organization in locations that are potentially exposed to pollutants and other substances (Solomon & Sibley, 2002) (Figure 9). Thus, ERA is a process of collecting, organising and analysing environmental data to estimate the potential risk of the stressors for ecosystems (Jensen & Mesman, 2006).

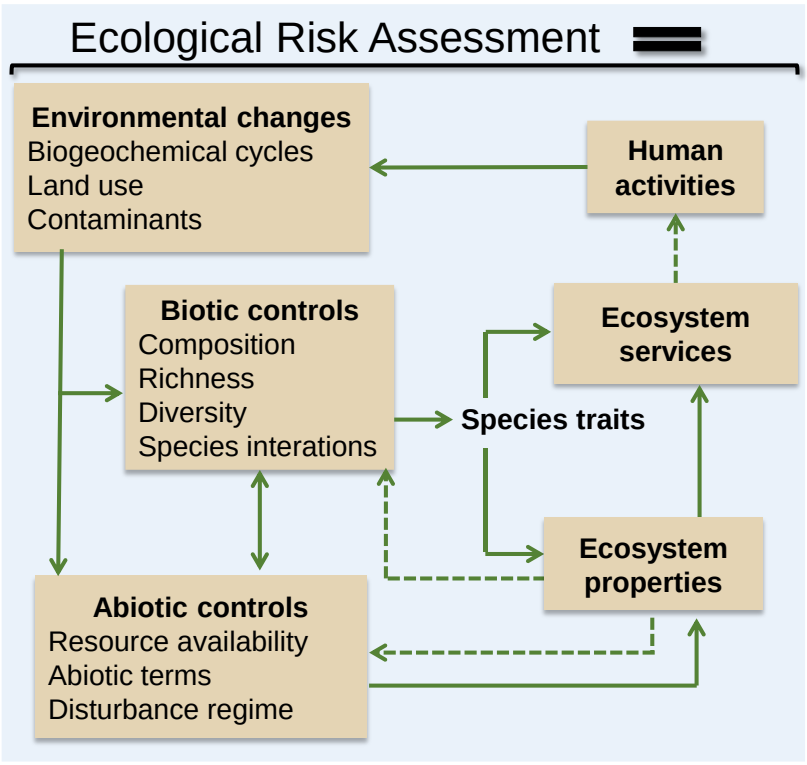

Figure 9. Ecological risk assessment framework between human activities, the environmental changes and the biotic and abiotic controls on the species traits and ecosystem properties and services. Changes in ecosystem properties can feed back and alter the biotic community either directly or via further alterations in abiotic controls (dashed arrows). The altered ecosystem services can lead to modification of human activities, as evidenced in a variety of responses to environmental problems (dashed arrows) (adapted from Hooper et al. 2005).

The regulatory risk assessments has a focus on risk at the level of individuals by means of hazard quotients or toxicity exposure ratios in which exposure is compared with toxicological endpoints measured on individuals in laboratory or semifield studies (Schmolke et al. 2010). However, another approach of the disturbances effects is the analysis of the triad (Chapman & Hollert, 2006), integrating the different lines of evidence to determine the hazard effects to ecosystems (Figure 10).

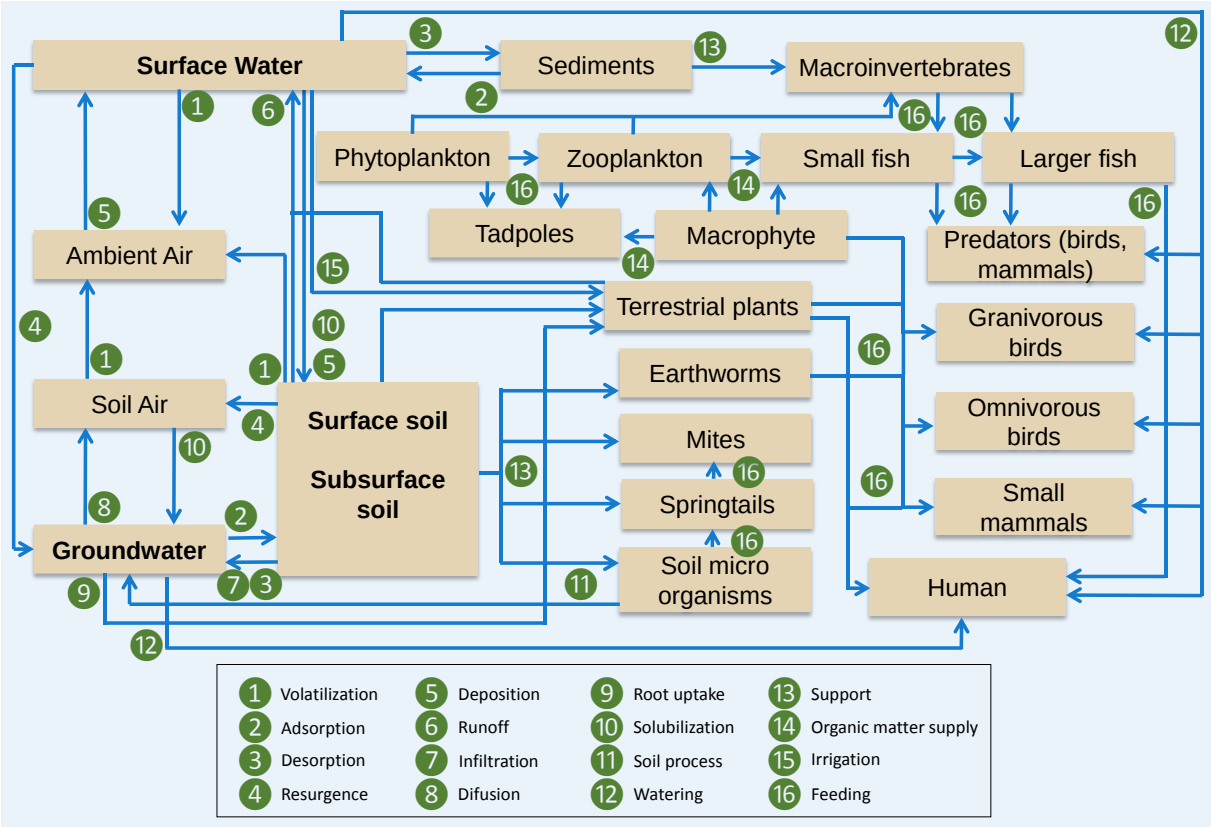

Figure 10. Conceptual model using the ecological interactions in aquatic and terrestrial ecosystems compartments (subsurface and surface soil, groundwater and surface water) for the ecological risk assessment in potential risk scenarios of the fungicide Pyrimethanil. The number represent the hypothetical environmental physical process and ecosystem functions and services.

Thus, integration a line of evidence of three different sources lead to a more detailed analysis than an approach based on only one aspect, as the site concentration of pollutants (Jensen & Mesman, 2006). Some report suggest the incorporation of lines of evidence to the triad analyses becomes tetrad, possibly a pentad, a hexad, or whatever formulation best addresses the problem and provides the necessary decision-making information for its solution (Chapman & Hollert, 2006). The ARE studies can help identify environmental problems, set priorities and provide a scientific basis for regulatory actions and report an identify or predict the risks of stressing elements not yet present in the environment (EPA, 1992). However, the understanding of how populations, communities, and ecosystems are affected by pesticides could be increased by integrating the fields of toxicology, chemistry, ecology, and bioinformatics at different levels of biological organization (Van den Brink, 2008).

These holistic approaches, integrating various components of coupled human and natural systems, it becomes critical to understand socioeconomic and environmental interconnections and create the present and future sustainability solutions (Liu et al. 2015). If we look at the recent backwards shall see great environmental and social disturbances such as

Chernobyl catastrophic nuclear accident in Ukraine (1986), the big tsunami in Southeast Asia (2004), the Gulf of Mexico oil spill in USA, the largest accidental spill in world history (2010), the Japanese earthquake, tsunami and Fukushima nuclear disaster in Japan (2011) and more recently the collapse of wastewater dam at an iron-ore mine on the Doce river in Brazil (2015), besides the increasing of worldwide impact of pesticides use. The questions that need to be answered and improved in ERA science are how to have effective risk management, how to have mitigation for nature and human societies and how to have a regulatory, public perception and effective risk communication to work in these past damage, fix the present and manage in a sustainable manner the future.

In the following pages, the present thesis will present tools to better evaluate the ERA of pesticides, informing hypothetical environmental impacts through the incorporation data that are more comprehensive, ecological modelling and ecosystem functions and services endpoints. Our experiments will cross the multiple aspects of contaminations and show the individuals to ecosystems responses approaches using the exposure routes of contamination, multi trophic interactions of experimental ecosystems models, behavioral, individual and some comparatives responses with aquatic and terrestrial compartments in risk assessment.

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CHAPTER II

The effect of exposure routes on the ecotoxicology of the antiparasitic Ivermectin to earthworms

SANCHEZ, A. L., ESPÍNDOLA, E. L. G. & JENSEN, J.

The effect of exposure routes on the ecotoxicology of the antiparasitic Ivermectin to earthworms

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Abstract

It is assumed that earthworms have a strong impact on improve structure and fertility of soil ecosystems. These organisms have a close contact with allochthonous materials becomes able to contact different contamination exposure routes. The studies emphasis of the antiparasitic ivermectin is placed on dung feeding invertebrates and the effects that faecal residues may have on invertebrates' ecology. In this context, the focus of this research was assess the contamination exposure routes for earthworms. To attempt it, the risk scenario was design to compare the ivermectin contamination exposure routes, via dermal (soil) and oral (food), on *Eisenia fetida* reproduction tests. The results obtained suggest that the analyzed reproduction parameters such as number of cocoons and juveniles and the hatchability were affected with the increase of ivermectin concentrations (0.5 to 12.5 mg soil or cow-dung dry weight) with statistical significant differences between the contamination exposure routes. We concluded that found differences between the antiparasitic ivermectin routes of exposure to earthworm. The environmental perturbation responses should be consider the contamination exposure routes to to assess and better understand soil risk assessment.

Keywords: Earthworms, veterinary products, bioavailability, soil risk assessment

INTRODUCTION

Earthworms are considered in the group of ecosystem engineers and have been identified as one of the most important soil engineers with the largest component of the soil animal biomass (Jones et al. 2008; Jouquet et al. 2006). Those organisms control directly or indirectly the availability of resources to other organisms causing physical state changes in the abiotic or biotic materials (Jones et al. 2008). It is assumed that they have a large impact on improving structure and fertility of soil ecosystems (Faber & Van Wensem, 2012).

The saprophagous species (e.g., earthworms, springtails, and some nematodes), can be considered part of the group of dung-feeding species, they are not reliant on dung, but they are common in dung at later stages of decomposition (Adler et al. 2016). Furthermore, their contact with the soil allochthonous materials provides different exposure routes of contamination to non-target organisms and the resources of terrestrial food web. Since earthworms can take up chemicals from outside soil and pore water both through the skin (dermally) as well as from ingestion (orally) (e.g. Jager et al. 2003; Lee et al. 2015; Sijm et al. 2000; Vijver et al. 2003; Wen et al. 2015). The earthworms in manure fields or the soil organisms that colonize dung pats may be impacted by the application of veterinary medical products to livestock and potentially retard the degradation of dung on pastures (Adler et al. 2016).

The processes and organisms involved in dung disappearance may be affected by the treatment of animals with antiparasitic drugs, particularly the avermectins (Svendsen et al. 2003). Into the avermectin family (i.e. ivermectin, abamectin, doramectin) the ivermectin is a macrocyclic lactones isolated from a fermentation broth of the soil actinomycetes *Streptomyces avermitilis* (Römbke et al. 2010). This substance is used worldwide to control the ecto and endoparasites (mites and nematodes) of livestock, protect animal health, enhance productivity and promote growth (Jensen et al. 2009; Taylor et al. 2009). Moreover, this antiparasitic is weakly metabolized and most of the dose given to the animals is excreted relatively unaltered in the faeces (Halley et al. 1989).

The ivermectin released to the environment may be degraded, transported and distributed between different compartments (Kolar et al. 2008). Thus, ivermectin residues or their metabolites are excreted in dung and may have environmental impact on the development and survival of the dung-dwelling fauna (e.g. Suarez, 2002; Iglesias et al. 2006; Fernandez et al. 2014). Notwithstanding, other authors state that ivermectin might not affect earthworms (e.g. Kaneda et al. 2006; Svendsen et al. 2003; Torkhani et al. 2011). However, the risk posed by soil contaminants strongly depends on their bioavailability (Van Der Wal et al. 2004).

In this context, considering the exposure routes to assess the risk of soil pollution to earthworms the aim of the present study was to analyse the interaction between exposure routes contamination, via dermal (soil) and oral (food), and the antiparasitic ivermectin concentrations on reproduction descriptors of non-target epigeic earthworm *Eisenia fetida*.

MATERIAL AND METHODS

Test soil

The soil test used was from Askov, a Danish agricultural soil (N 55° 28.34', E 9° 6.6'), belonging to the Danish Institute of Agricultural Sciences (Askov, Jutland, Denmark). The Askov soil is a sandy loam and has the following particle size distribution: coarse sand (200–2,000 mm) 38.4 %, fine sand (63–200 mm) 23.6 %, coarse silt (20–63 mm) 10.0 %, fine silt (2–20 mm) 12.3 % and clay (< 2 mm) 13.0 %. The humus content of the soil was 2.8 %, the total content of organic carbon 1.6 % and the soil retention capacity 18 % (Holmstrup et al. 2001). The soil, density was 1.135 g/cm^3 dry soil and the total cation exchange capacity were 8.14 meq/100 g (Sverdrup et al. 2001) and the pH-H_2O was 6.2. To defaunation test soil was dried at 80 °C for 24 h and sieved through a 2 mm mesh prior to use.

Test species

The specimens of the earthworm *Eisenia fetida* were obtained from a stock culture in the laboratory of NERI (National Environment Research Institute, Silkeborg, Denmark). The laboratory conditions were at 22 ± 1 °C with a 12:12 h light:dark cycle. The animals remained in cultures with adjusted soil pH and feeding regularly with cow-dung from non-medicated cows.

Test substance

Ivermectin (CAS: 70288-86-7) were obtained from Sigma-Aldrich Denmark ApS. The purity of the chemicals was 90 %. Due to the low solubility of ivermectin in water, a stock solution was prepared by diluting ivermectin in acetone. The structure of ivermectin is shown in Figure 1. The antiparasitic ivermectin (Sigma-Aldrich Co. LLC) the purity was ≥ 90 %. The chemical compound is a mixture of two chemically modified avermectins that contain at least 80 % of 22,23-dihydroavermectin-B 1a and > 20 % 22,23-dihydroavermectin-B 1b as a highly lipophilic substance that dissolves in most organic solvents, but is practically insoluble in water (0.0004 % m/v) (Lumaret et al. 2012). In dung from treated animals the level of ivermectin varies according to type of animal, administrated doses and application form (Jensen et al. 2003). The recommended dosage applied in cattle corresponding to 0.2 mg ivermectin per live weight animal (Suarez, 2002). Residues of this substance are secreted into urine and faeces and excreted onto the land or into the water (Montforts et al. 1999). The animal excreted is hardly in urine less than 2 % and approximately 90 % of a dosage is excreted via faeces in the 7-14 days following administration, depending on the route of administration (Halley et al. 1989). The excretion of the labelled ivermectin to cattle is described in two phases, phase 1 in two 2 days releasing 60 % of the label and phase 2 releasing 39 % in the following 4 days and the decrease of unchanged ivermectin is also a two-step process: from 100 to 93.5 % in 2 days and from 93.5 to 44 % in the following 4 days (Montforts et al. 1999). These authors reported the calculation of ivermectin exposure concentrations in ruminants dung requires a dosage, animal body weights and dung production data.

To soil contamination the ivermectin was dissolved in acetone and the entire soil and the cow-dung batch for a particular treatment concentration. For the acetone evaporation, before adding the animals, the soil and cow-dung with ivermectin were left under a fume head overnight. Next day, following the water holding capacity of the soil, water was add and the spiked soil divided among individual replicates. The soil and cow-dung contamination followed the concentrations of 0.5, 1.25, 2.5, 5.0 and 12.5 mg ivermectin.kg^{-1} soil or cow-dung dry

weight. The concentrations were chosen from literature information regarding the toxicity of ivermectin for earthworms (Rombke et al. 2010) and the limit values found in cow dung field situations.

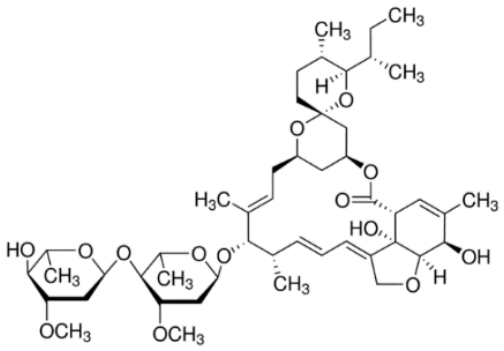

Figure 1. Chemical structure of Ivermectin.

Test experimental design

An adapted version of Organization for Economic Cooperation and Development (OECD, 2004) guideline 222 for chemical testing conducted our reproduction tests experiments. Three experimental designs were used with the exposure route of contamination with the earthworms: only the soil contamination (Soil), soil plus weekly food contamination (Soil+Food) and only the weekly food contamination (Food). The earthworms were fed with wet cow-dung (20 % nitrogen), pH of 8.5, from non-medicated cows. The dung was dried 24 hours at 100° C, ground to pass a 2 mm sieve and then rewetted prior to addition as food. For each 10 g dry cow-dung 30 ml glass-distilled water was added. For the Soil treatment and the Control treatment the food recourses were a wet cow-dung without ivermectin and the Soil+Food and Food treatment the food recourses were a wet cow-dung with ivermectin concentrations. The feeding procedure used 0.7 ± 0.05 g cow-dung wet weight for each vessels for all additions on the days 0, 7, 14 and 21 as food resource for earthworms.

The experiment was conducted in plastic containers (13.5 cm diameter and 12 cm height) containing 500 g wet soil. For the animals, adult earthworms were cleaned with distilled water and left on wet filter paper in Petri dishes for 24 hours to depurate the gut contents. The adults animals used in the experiment had clitellum and weight of 250-350 mg. Four replicates of each concentration and controls were prepared, each containing 10 earthworms. The test vessels were maintained for 56 days at 21° ± 1° C in 12:12 hours light:dark cycle.

After 28 days the adult survival was removed carefully, counted, cleaned and weighed. The cocoons were maintained in the soil for additional 28 days to allow hatching. At the end of

the experiment, after 56 days, the cocoons and juveniles were collected with water washing in 1.0 and 2.0 mm mesh and counted.

Statistical analysis

The logistic model was used to estimates the concentrations that caused 10 % and 50 % reduction in reproduction output (EC_{10} and EC_{50}).

EC_{50} calculations:

$$y = \frac{c}{1 + \left(\frac{Conc}{ECp}\right)^{b}}$$

EC_{10} calculations:

$$y = \frac{c}{1 + \left(\frac{0{,}10}{0{,}90}\right) * \left(\frac{Conc}{ECp}\right)^{b}}$$

, where c is the mean control value, b is the slope parameter.

For pairwise comparison of means with the control with 0.05 significance were used ANOVA and Dunnett's test for the determination of the no observed effect concentration (NOEC) and the low observed effect concentration (LOEC) values. The checking of the homogeneity of variances preceded the variance analysis. For compare the interaction between the three exposure routes and the concentrations for the reproduction descriptors was used pairwise multiple comparisons (Tukey test) after a multi-factor ANOVA for analysis of the differences between the categories with a confidence interval of 95 %. The data were analyzed using STATISTICA software (version 7.0, StatSoft, Inc.) and XLSTAT (version 2014.5.03).

RESULTS

The results for the reproductive outputs showed a decrease with the increase of the ivermectin concentrations (Figure 2). The average of number of cocoons and juveniles showed a particular distribution for each exposure route with a trend of distribution for Soil and Soil+Food treatment. In general, after 56 days the soil exposure route were similar compare to the only food exposure route resulted in lower EC_{10} and EC_{50} values of all three exposure route (Table 1). Food exposure route reported less toxicity for the reproduction outputs. The cocoons hatchability evidence this distribution difference for each exposure routes (Figure 3). When compare to the percentage of the control the Food exposure showed a complete hatching for all

the ivermectin concentrations while the others exposures decrease with the increase of the concentrations.

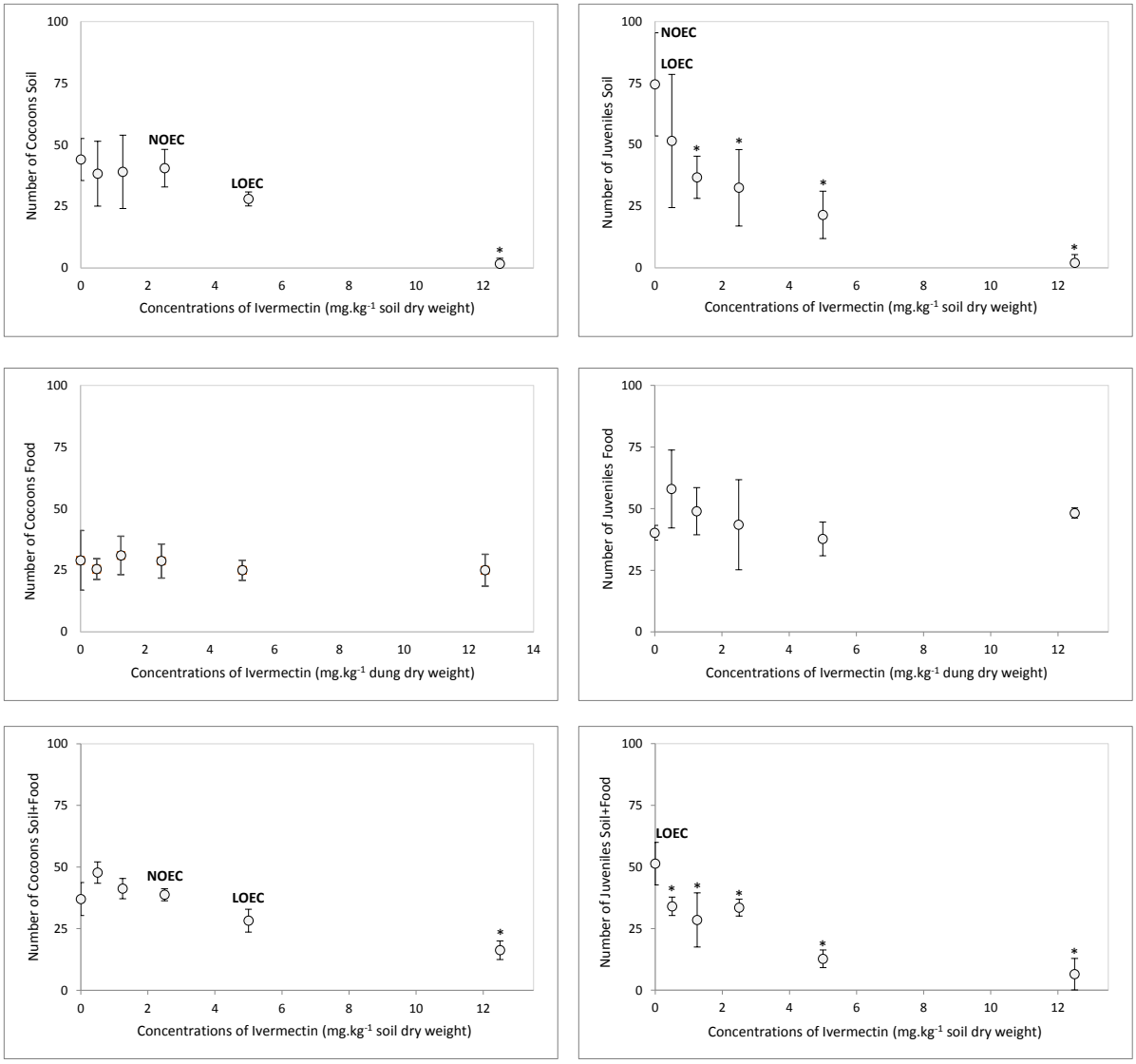

Figure 2. Average of the number of cocoons (a) and juveniles (b) production for the exposure routes (Soil, Food and Soil+Food) and multiple comparison using Dunnett's procedure at 5% significance level representing by (*) and the representative of the Low observed effect concentration (LOEC) and No observed effect concentration (NOEC).

The values of recommended dose to causing 10 and 50 % reduction in the number of individual (EC_{10} and EC_{50}) for the cocoons and juveniles production on *E. fetida* for the exposure routes (Soil, Food and Soil+Food) on the ivermectin concentrations after 56 days shown notably differences between Soil and Food exposures (Table 2). The Dunnett's test detected a statistically significant difference of the reproduction parameters between the control and the treatments (Table 1). Therefore, NOEC and LOEC can be estimated: for cocoons

production the Soil and Soil+Food LOEC value was 2.5 mg.kg^{-1} soil dry weight and NOEC value was 5.0 mg.kg^{-1} soil dry weight; for juveniles production the Soil NOEC value was 1.25 mg.kg^{-1} soil dry weight.

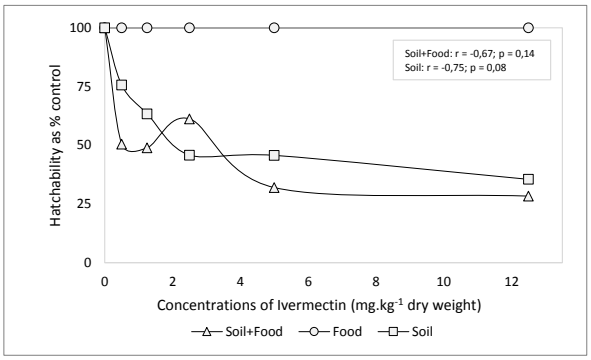

Figure 3. Hatching success of cocoons following 56 days reproduction test for the ivermectin concentrations (mg.kg^{-1} soil dry weight) as percentage of the control for the exposure routes (Soil, Food and Soil+Food) with *E. fetida*.

The results of the multi-factor ANOVA (Table 2) showed that exposure routes affect the reproductive outputs significantly (p-value < 0.05), the ivermectin concentrations and the statistical interaction between the exposure routes versus concentration affect the cocoons and juveniles production and for hatchability only the exposure route is significant. The Tukey's test evidence the statistical significant differences between the exposure routes. Food versus Soil had differeces with the juveniles production and hatchability, Food versus Soil+Food affect all the parameters and Soil versus Soil+Food to be closer distribuction to reproductive parameters had no significant differences.

Table 1. EC_{10} and EC_{50} values (mg.kg^{-1} soil dry weight) for the cocoons and juveniles production on *E. fetida* for the exposure routes (Soil, Food and Soil+Food) on the ivermectin concentrations after 56 days. Calculation used the logistic model with 95 % confidence level.

Parameter		Soil	Food	Soil+Food
Cocoons	NOEC	2.5	n.a.	2.5
	LOEC	5.0	n.a.	5.0
	EC_{10}	3.65 (1.45–5.86)	8.6 (-20–37.2)	2.23 (-0.05–4.52)
	EC_{50}	6.0 (3.96–8.03)	78.2 (-660–816)	8.68 (5.16–12.2)
Juveniles	NOEC	1.25	n.a.	n.a.
	LOEC	n.a.	n.a.	n.a.
	EC_{10}	0.12 (-0.12–0.36)	26.17 (-258.7–311.1)	0.14 (-0.11–0.4)
	EC_{50}	1.42 (0.29–2.56)	1004.4 (-66307–68316)	2.16 (0.6–3.72)

Table 2. Results of the multi-factor ANOVA and interaction between the exposure routes (Soil. Food and Soil+Food) and ivermectin concentration for the reproduction descriptors (Cocoons. Juveniles and Hatchability) and Tukey HSD test for analysis of the differences between the categories with a confidence interval of 95 %.

	Cocoons	Juveniles	Hatchability
R^2	0.732	0.711	0.665
F	8.782	7.904	6.394
p-value			
Exposure route	0.014*	< 0.0001*	< 0.0001*
Concentrations	< 0.0001*	< 0.0001*	0.068
Exposure routes x Concentrations	0.001*	0.044*	0.376
Tukey´s test			
Food vs Soil	0.580	< 0.0001*	< 0.0001*
Food vs Soil+Food	0.011*	< 0.0001*	< 0.0001*
Soil vs Soil+Food	0.119	0.274	0.170

DISCUSSION

In this study the *E. fetida* reproduction parameters had reduction with the increase of the antiparasitic ivermectin concentrations but shown different effects according to the way of the exposure route of the pesticide (Figure 2). Earthworms take up organic compounds in the soil through their skin as well as from their food, but the contribution of each route is unclear (Jager et al. 2003; Schmitt & Römbke, 2008). Tourinho et al. (2015) showed that relation between nanoparticles and body concentration reached 5 times higher in soil exposure than dietary exposure. In this sense, our study showed that the production of cocoons, juveniles and consequently the hatchability showed a particular distribution when the exposure route was related to the soil or only food with a trend of distribution for the soil contaminations treatments. (Figure 2 and 3).

The epigeic earthworms, living in the leaf litter near the surface can occur in high numbers below dung pats, feeding on dung particles and attached microbes (Adler et al. 2016). However, the two skin exposure (Soil and Soil+Food) with the contaminated soil showed similar distribution when compare to only food exposure resulted in lower EC_{10} and EC_{50} values (Table 1). The oral exposure with the cow-dung as food showed no toxicity for the earthworm's reproduction parameters. The lower bioavailability and toxicity upon dietary exposure was explained by the higher organic matter content of food, resulting in strong binding of contaminants in comparison to soil's exposure (Vijver et al. 2006). The bioavailability is defined as a complex process with all kinds of relationship between the concentration, portion and the way of uptake by the organisms in the environment (Sijm et al. 2000). For earthworms,

this process becomes more vulnerable than other soil invertebrates due the absence of protective cuticle (Jager et al. 2003).

The results reported here suggest, as well as the literature, that the principal ivermectin route of toxicity for earthworms is probably the dermal via. Ivermectin is used worldwide to control internal and external parasites of livestock and the literature emphasis is placed on dung feeding invertebrates and the effects that faecal residues may have on invertebrates ecology (Lumaret et al. 2012). Although some reports in dung beetles communities showed no observable effect of the administered drugs on dung (Kryger et al. 2005; Römbke et al. 2010). Besides that some reports for earthworm activity and the disappearance of dung pats showed no significance affected with the faecally-excreted pesticide residues (Kaneda et al. 2006; Svendsen et al. 2003; Svendsen et al. 2002). The oral sealing test performed by Vijver et al. (2006) with the oral uptake excluded using a medical glue in the earthworm's mouth, blocking the ingestion of soil particles and pore water, show a predominance of dermal uptake over oral uptake, demonstrating that dermal uptake is the most important uptake route.

Thus, our results corroborate this predominance and importance of dermal uptake over oral uptake for earthworm's soil pollution risk with the exposure routes affecting the reproductive parameters significantly (Table 2). In the soil exposure the cocoons production reported the no observed effect concentration (NOEC) and the low observed effect concentration (LOEC) of Soil and Soil+Food with the same values (2.5 $mg.kg^{-1}$ soil dry weight and 5.0 $mg.kg^{-1}$ soil dry weight respectively) and for juveniles production the NOEC for Soil was 1.25 $mg.kg^{-1}$ soil dry weight. The report of Gunn and Sadd (1994) suggested a decrease of *E. fetida* response at ivermectin soil exposure higher than 8 $mg.kg^{-1}$ soil dry weight. Svendsen et al. (2003) reported that ivermectin metabolites on cow dung have no adverse effects on *Lumbricus terrestris*. While Torkhani et al. (2011) showed more than 70 % of the earthworms preferred soil with ivermectin concentrations of 8, 64, and 256 $mg.kg^{-1}$ soil dry weight. On the other hand a report on two soil invertebrate species, the ivermectin had a toxic effect with EC_{50} values of 1.7 $mg.kg^{-1}$ soil dry weight and NOEC values of 0.3 $mg.kg^{-1}$ soil dry weight for the springtails and 36 and 3 soil $mg.kg^{-1}$ soil dry weight respectively for the enchytraeids (Jensen et al. 2003).

The statistical interactions Food versus Soil was significant for the juveniles production and hatchability, Food versus Soil+Food affected significantly all the parameters and Soil versus Soil+Food, to sharing the soil as exposure route and be closer distribuction to reproductive parameters, had no significant differences. This interchange and impact by the pesticides on biota is highly relevant to the growing interest in the role that species attributes

have in driving terrestrial ecosystem processes (Wardle et al. 2011). Agricultural practices are essential for sustaining the human population, but in the other hand they can directly disrupt the ecosystem functions (Galic et al. 2012). However, earthworms are typical ecosystem engineers as they have a large impact on architecture of soils structure and have thus been recognized as typical ecosystem engineers with an high potential partner for humans in managing ecosystem services (Lavelle et al. 2006; Blouin et al. 2013). Fonte & Six (2010) highlight the application of litter inputs and proper management of earthworm populations can have important implications for the provision of ecosystem services such as carbon sequestration, soil fertility and plant production.

The results obtained in this study characterized the differences between the antiparasitic ivermectin routes of exposure to the earthworm *E. fetida* as regards the dermal (soil) and oral (food) exposure. The results showed significant differences on the reproduction descriptors for the ivermectin concentrations and the both exposure routes and highlighted the probable route of toxicity to earthworms as the dermal via. This information points that the contamination exposure routes should be considered to assess an accurate veterinary pharmaceuticals, as well as in other chemical compounds, soil pollution risk assessment. In addition, we note that management and environmental remediation of sites with anthropogenic disturbances requires a better understand of the mechanisms and responses on the ecosystems functions and services to perturbations.

ACKNOWLEDGEMENTS

The authors would like to thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for the doctorate sandwich grant (process number 99999.008342/2014-08) and Aarhus University for the facilities. We thank the technicians at the Institute of Bioscience (Aarhus University, Silkeborg, Denmark) Tanja Quottrup Egholm, Elin Jørgensen, Zdenek Gavor and John Geert Hytter for technical assistance and guidance and finally, we thank Sissel Geyti for assistance during experiments.

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CHAPTER III

The impact of the fungicide Pyrimethanil on soil fauna measured in a soil multi-species (SMS) test system when exposure through surface and homogeneous soil application

SANCHEZ, A. L., ESPÍNDOLA, E. L. G. & JENSEN, J.

The impact of the fungicide Pyrimethanil on soil fauna measured in a soil multi-species (SMS) test system when exposure through surface and homogeneous soil application

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Abstract

The pesticides have severe environment effects contributing to biodiversity loss and trophic levels changes. To evaluate the chemical stressors effects on the terrestrial species interactions have been designed controlled studies simulating the real field situations. The present study tested an experimental approach to characterise the effects of the fungicide Scala® (Pyrimethanil) on soil invertebrates. To attempt it, a constructed soil multi-species (SMS) test system was compared with spray and homogeneous soil application. The study was supplemented with the measure of feeding activity, single-species tests with the fungicide commercial formulation and active ingredient and a multi-compartmented linear avoidance test system. The results obtained suggest that the fungicide has adverse effect on soil invertebrates' response for the application and spatial distribution. The organisms' habitat preferences and foraging abilities was affected directly or indirectly by the fungicide toxicity. We concluded that information set assess an accurate fungicide risk assessment as an efficient tools for ecosystem disturbance responses.

Keywords: Pesticide exposure, ecological interactions, behaviour responses, soil risk assessment

INTRODUCTION

The pesticides play a major role in modern worldwide agriculture, but it have also potential risks hazard to non-target organisms and ecosystems (Maltby & Hills, 2008). Their residues were found to persist in the food chain, reaching elevated concentrations and hence having more severe effects, contributing to biodiversity loss and to side effects in higher trophic levels (Krebs et al. 1999). This persistence occur in agricultural soils with chemicals that have the ability to accumulate and adsorb to soil particles like the fungicides, causing adverse effects to soil organisms and the ecosystem functions as the decomposition of organic matter and facilitating of nutrient cycling (Komárek et al. 2010; Wightwick et al. 2010).

One of the most frequently used fungicides in the European Union is the compound of the anilinopyrimidine class called Pyrimethanil (Anfossi et al. 2007). It controls the grey mould with high affinity for the soil solid phase and not easily degraded (Sadlo et al. 2002). Pyrimethanil inhibits the secretion of fungal enzymes relevant for pathogenicity as commercial formulation Scala®. This chemical is widely used for example in champagne's vineyards in France (Verdisson et al. 2001), grapevine's vineyards in Portugal (Gil et al. 2015), tomatoes and vegetables fields in Poland (Sadlo, 2002) and apples and outdoor strawberries fields in Denmark.

The regulatory risk assessment of fungicides in Europe uses information from ecotoxicological studies ranging in complexity from standardized single-species toxicity tests to semifield studies and also field studies (Brock et al. 2006). In the past 25 years, research interest has shifted from documenting incidents and exclusively quantifying chemical exposure, to effect studies to linking laboratory, mesocosms and field experiments (Ippolito et al. 2012; Köhler & Triebskorn, 2013).

A controlled study simulating the real field situations have been designed to evaluate the chemical stressors effects on the terrestrial species interactions (Jensen & Scott-Fordsmand, 2012). This semi-field system can be the terrestrial model ecosystems (TME) based on the sampling of field soil-cores containing an indigenous pool of organisms to which the toxicant is added (e.g. Knacker et al. 2004; Schaeffer et al. 2009; Bandow et al. 2016). The other tool are the soil multi-species systems (SMS) designed to contain standardized multispecies system, combining the advantages of laboratory conditions and monitoring with the ecology relevance of the soil ecosystems interactions as predation, competition and commensalism (e.g. Jensen & Scott-Fordsmand, 2012; Menezes-Oliveira et al. 2014; Schnug et al. 2014; Scott-Fordsmand et al. 2008; Sechi et al. 2014).

The soil ecosystems has been linked to biodiversity and especially the relative abundances of keystone species or functional groups that support the soil processes and food web structure (Chagnon et al. 2015; Nielsen et al. 2011). The anthropogenic stressors affect the abundance of the species on ecosystem functions in multi trophic level communities include top-down or bottom-up trophic cascades, keystone predation, exploitative competition, apparent competition and indirect facilitation (Mcmahon et al. 2012; Relyea & Hoverman, 2006). Furthermore, it is noteworthy that the pesticides affects cause not only modulations in the predator-prey dynamics, but also cause changes on interspecific behaviour (Köhler & Triebskorn, 2013). Notwithstanding, the behavioural responses are a relevant and sensitive endpoints in environmental risk assessment, with rapid cost-effective and ecologically relevant biological screening tools for initial assessment and habitat preference (Luz et al. 2004).

In pesticides risk assessment realistic simulation scenarios are essential to report accurately the ecosystems responses. Is is well known that the major pesticides applications take place often as liquids sprayed on the crop and/or the soil surface (Van Der Werf, 1996). Here we adopt an experimental approach to characterise the effects of pesticides using a lower and higher tier test and compared with the pesticide properties, such as applications and the chemical formulation. The principal focus is on a gradients of concentrations of the fungicide Scala® (Pyrimethanil) and the way of exposure (surface spraying and homogeneous soil

application) on a constructed food-web system, a soil multi-species (SMS) test system. The objective of the present study was to measuring the effects of the fungicide on soil invertebrates' communities and functions. The standardized test system was supplemented with a measure of feeding activity of the soil invertebrates using the bait lamina test system. In addition, the results of the SMS test system were compared with single-species tests on the effects of the fungicide in a commercial formulation and active ingredient on the standard springtail *Folsomia candida.* Finally, we wanted to assess the foraging behaviour of the springtail *Heteromurus nitidus* in a multi-compartmented static avoidance test system.

MATERIAL AND METHODS

Experimental design

A selected set of soil invertebrates was exposed to pesticide on soil multi species mesocosms (1 kg moist weight, i.e. 820 dry soil and 180 ml inoculation solution) in test containers (polyethylene tubes, 33 cm height and 9.3 cm width) during 28 days at 21 ± 1 ºC in a 12/12 hours light-dark interval in a surface application (Spray) and homogeneous application (Soil). Except for the way of pesticide application, the following procedure was identical for both experiments with four replicates each test concentration and six replicates for the control.

Test soil

The soil test used was from Askov, a Danish agricultural soil (N 55° 28.34', E 9° 6.6'), belonging to the Danish Institute of Agricultural Sciences (Askov, Jutland, Denmark). The Askov soil is a sandy loam and has the following particle size distribution: coarse sand (200–2,000 mm) 38.4 %, fine sand (63–200 mm) 23.6 %, coarse silt (20–63 mm) 10.0 %, fine silt (2–20 mm) 12.3 % and clay (< 2 mm) 13.0 %. The humus content of the soil was 2.8 %, the total content of organic carbon 1.6 % and the soil retention capacity 18 % (Holmstrup et al. 2001). The soil, density was 1.135 g/cm^3 dry soil and the total cation exchange capacity were 8.14 meq/100 g (Sverdrup et al. 2001) and the pH-H_2O was 6.2. To defaunation test soil was dried at 80 °C for 24 hours and sieved through a 2 mm mesh prior to use.

Test substance

Pyrimethanil (CAS: 53112-28-0) [IUPAC name N-(4,6-dimethylpyrimidin-2-yl)-aniline] is a colorless crystalline substance practically insoluble in water (121 mg/L), belonging to the anilinopyrimidine class. In the form of concentrates with tradenames of Scala® it is used as a contact fungicide with protective and curative properties (Sadlo, 2002). The structure of

pyrimethanil is shown in Figure 1. The chemical compound has a moderate persistence in soil with half-life value of 55 days (Wightwick et al. 2010). The commercial formulation Scala® (concentrated suspension; Bayer CropScience, BASF, Cheshire, United Kingtom) is a chemical composition of suspension concentrate containing 400 g/l Pyrimethanil (37.4 % w/w) indicated for the control of leaf scab in apples and grey mould in outdoor strawberries and the moderate control of grey mould in protected strawberries.

All concentrations in this study refer to the recommended dose for outdoor strawberries with the commercial fungicide Scala® and the active substance. The maximum single application rate for Scala® is 2 L/ha in strawberry cultures. The homogeneous and surface application followed the Scala® recommended dose for outdoor strawberries with values of 2.5, 10, 25, 50 and 75 times the doses (1.4, 5.4, 13.5, 27 and 40 mg pyrimethanil.kg^{-1} soil dry weight).

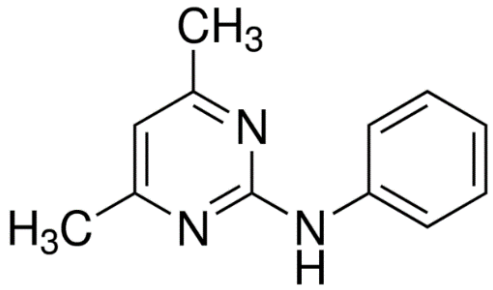


Figure 1. Chemical structure of Pyrimethanil.

For the springtails single specie reproduction test and for linear avoidance test the concentrations, using the commercial formulation, following the same values and procedures used at the soil Scala® mesocosms as recommended dose of 0, 2.5, 10, 25, 50 and 75 (0, 1.4, 5.4, 13.5, 27 and 40 mg pyrimethanil.kg^{-1} soil dry weight) times the doses. While for the active ingredient Pyrimethanil the values were 0, 10, 25, 50, 75 and 100 mg pyrimethanil.kg^{-1} soil dry weight. The active ingredient was dissolved in acetone and the entire soil and batch for a particular treatment concentration. For the acetone evaporation before adding the animals, the soil were left under a fume head overnight.

Test species

A laboratory invertebrate food-web was design based on the species composition found in a Danish agro-ecosystem, on habitat features and the species ecological interaction (Table 1) (Jensen and Scott-Fordsmand, 2012). The earthworms (Annelida: Lumbricidae) were

composed on the surface epigeic specie *Eisenia fetida* (Savigny, 1826). The animals were obtained from a stock culture in the laboratory of NERI (National Environment Research Institute, Silkeborg, Denmark). The laboratory conditions were at 22 ± 1 °C with a 12:12 hours light:dark cycle. The animals remained in cultures with adjusted soil pH and feeding regularly with cow-dung from non-medicated cows.

The springtails species (Arthropoda: Collembola) were taken from cultures routinely maintained at the same laboratory (NERI), where they were kept at 20 ± 1 ºC with a 12/12 hours light:dark interval. When in culture, the collembolans were all bred on Paris-charcoal plaster (8:1) in Petri dishes and fed with dried bakers's yeast. To reflect the natural variation in age, none of the test animals was synchronized in the soil multi species mesocosms. *Heteromurus nitidus* (Templeton, 1835) is a detritivore and epedaphic as a surface-active animal. *Hypogastrura assimilis* (Krausbauer, 1898) is a detritivore, pigmented, epi to hemiedaphic and have eyespots, with reproduces sexually. *Protaphorura fimata* (Gisin, 1952) is a detritivore/herbivore, relative large non-pigmented euedaphic springtail. *Proisotoma minuta* (Tullberg, 1871) is a detritivore, widespread small and greyish mycophagous and hemiedaphic collembolan, found in very large numbers in habitats with high organic matter. *Folsomia fimetaria* (Linné, 1758) is a detritivore, euedaphic, non-pigmented, eyeless springtail, which reproduces sexually.

Table 1. List of the species used and their relative group, functional groups, life-forms soil ecosystem, and the number of individuals added in each of the 46 experimental units (mesocosms) (J: juvenile, F: female, M: male) (Jensen and Scott-Fordsmand, 2012).

Group	Test species	Functional group	Life-form	Nº. indiv.
Bacteria	Microbial community	Decomposer/Prey	-	n.a.
Oligochaeta	*Eisenia fetida*	Detritivore	Epigeic	5 (J)
Collembola	*Heteromurus nitidus*	Detritivore	Epedaphic	30
	Hypogastrura assimilis	Detritivore	Epi-hemiedaphic	30
	Protaphorura fimata	Detritivore/Herbivore	Euedaphic	30
	Proisotoma minuta	Detritivore	Hemiedaphic	30
	Folsomia fimetaria	Detritivore	Euedaphic	15 (F) 15 (M)
Acari	*Hypoaspis aculeifer*	Predator	Hemi-euedaphic	10 (F) 5 (M)

The mite specie (Arthropoda: Acari) of *Hypoaspis aculeifer* (Canestrini, 1884) is a predatory mite and hemiedaphic/euedaphic. It has an arrhenotokous mode of reproduction, i.e. unfertilized females produces only male offspring. The animals were bought from Company CropBio - Denmark, extracted with light and maintained at the laboratory, where they were

kept at 21 ± 1 ºC with a 12/12 hours light:dark interval. The mites were bred on Paris-charcoal plaster (8:1) with a diet of juvenile springtails (*F. fimetaria*).

Procedures for the SMS test system

The experiments start with the microbial community, 3 days before the pesticide contamination (T-3). The microbial activity inoculation was obtained by shaking 1 kg of the freshly soil test soil with 2 liters of distilled water for approximately 3 hours and then sieved through a 50 mm mesh and maintained at 5 ºC. The inoculation solution following the water holding capacity of the soil for each treatment (T1).

One day before the contamination, the juvenile earthworms of 60 - 100 mg fresh weight were cleaned with distilled water and left on wet filter paper in Petri dishes for 24 hours to depurate the gut contents (T-1). The next day, five individuals were carefully cleaned, dry, weighed and later added to each container (T1).

In the contamination day (T1), for the soil homogeneous application, the pesticide solution and water was add and the spiked soil divided among individual replicates following the soil water holding capacity. For the surface application a pot sprayer was used that consist in a pesticide field applications simulator. The spraying is done with a moving boom equipped with two ordinary hydraulic nozzles. The spray liquid is driven to the nozzles by pressurized air. This is done by placing a beaker with the spray solution in a pressurized container. The spraying operation was calibrated for velocity of 6 km/h and solution as 2 liters pesticide to 150 liters water. The sprayer is located in a ventilated cabinet in order to avoid contamination of the outside environment.

After 2 days of the contamination, springtails were removed from the cultures with vacuum and added to the test containers (T2). Each species (*H. nitidus*, *H. assimilis*, *P. minuta*, *P. fimata* and *F. fimetaria*) were handled separately in order to avoid contamination of cultures with other species. All individuals were checked under the stereomicroscope to ensure that no legs or antennae were missing or injured. In total, 30 individuals of each springtail species were added to each test container. In the case of *F. fimetaria*, 15 males and 15 females were introduced to each replicate, while no sex differentiation was done in the case of the other species (Table 1). To avoid initial predation, the introduction of 15 individuals of *H. aculeifer* (10 female and 5 male) was postponed one week in relation to springtails (T10).

The bait-lamina test system was commercially obtained from Terra Protecta GmbH, Berlin (http://www.terra-protecta.de) (Von Törne, 1990; Förster et al. 2011). It consists of PVC-stripes (cm) perforated with 16 holes. The holes were filled with a standard substrate mixture

of 70 % cellulose powder, 25 % wheat bran and 5 % of active charcoal. For each replicates 3 sticks were inserted at the day 3, two hours after adding the springtails, day 14 and day 21 and removed after 1 week, in order to have the same period of feeding at all three sampling occasions. A score of 16 correspond to a situation where all holes were empty, and a score of 0 of the opposite situation. The bait lamina test is thought to be an easily applicable and low-effort screening method to assess the functional parameters of soil animals being a simple and fast evaluation tool to ARE (Gestel et al. 2003; Römbke et al. 2006).

The test containers were maintained at 21° ± 1 °C in 12:12 hours light:dark cycle. Furthermore, weekly the water and food resources for earthworms (0.7 ± 0.05 g wet cow-dung) were replaced in the containers. After the 28 days the soil from each container was divided up into three layers (top, middle and bottom) with the aid of a plunger with approximately 5 cm size each. Than the survival adults' earthworms were removed, cleaned, counted and weighted. From each of these layers four sample of approximately 80 g wet soil was taken by a soil corer (inner diameter of 5.8 cm and 5 cm high) for quantification the collembolan and mites. This soil were extracted in a high-gradient extractor over 7 days with benzoic acid beakers, than placed in a 50 ºC oven for 24 hours, sieved with 70 % ethanol and conserved in glycerol.

Springtails single specie reproduction test

An adapted version of Organization for Economic Cooperation and Development (OECD 2009) guideline 232 for chemical testing conducted the single reproduction tests experiments. The tests were performed with 30 g of moistened soil (24.6 g soil and 5.4 ml solution). Each container consisted of a transparent acrylic cylinder (height = 5.5 cm and diameter = 6 cm) with two meshes (1 mm) in the bottom and plastic lids at top and bottom to closed the system. At the start of the test, 10 individuals of synchronized *Folsomia candida* (Willem, 1902) (10-12 days) were added to each replicate (four replicates per concentration). The reproduction test was made with the commercial formulation (Scala®) and the active ingredient of the Pyrimethanil. Furthermore, the water and food resources (dried bakers's yeast) were replaced in the containers weekly. The test containers were maintained for 28 days at 21° ± 1° C in 12:12 hours light:dark cycle. After 28 days, the animals were extracted in a high-gradient extractor over 2 days with benzoic acid beakers, than placed in a 50º C oven for 24 hours, sieved with 70 % ethanol and conserved in glycerol.

Linear avoidance test

A multi-compartmented static test system was developed based on Araujo et al. (2014a). The system was composed by a transparent acrylic box with cover (15 cm height, 9.2 cm width and 6.3 cm depth) with six compartments (1 cm height, 2.5 cm width and 9.2 cm depth with 30 g wet soil) (Figure 2). Each compartment was constructed with transects using a polyethylene divider (9.1 cm x 6.5 cm) and removed for make a compacted and uniform soil surface. The foraging behavior was examined with adults' collembolan *H. nitidus* (1.5 mm length) to avoid Scala® contaminated soil. An avoidance assay for 48 hours in a multi-compartmented static test system with three replicates was used with a contamination gradient. After removed the divider and compacted the soil, five collembolan were added to each virtual compartment in the rectangular soil surface (total of 30 animals per system), through which the animals could move and choose the preferred compartments. The test containers were maintained at 21° ± 1° C in 12:12 hours light:dark cycle. Visual observations were made using light with the box closed to avoid the air movement influence on the individual's displacement.

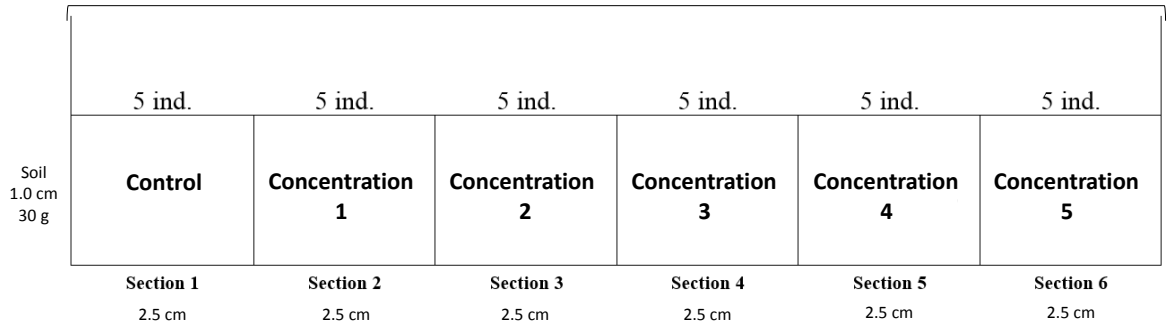


Figure 2. Schematic diagram of the multi-compartmented static avoidance test system with the six soil sections. The system was composed by a transparent acrylic box with cover (15 cm height, 9.2 cm width and 6.3 cm depth). The section 1 represent the control soil and the following sections represent the crescent concentrations gradient. Each section was formed by a rectangular cube with 2.5 cm height, 1.0 cm width and 9.2 cm depth with 30 g wet soil each section. Afterwards, the divider were removed and compacted the soil, five collembolan were added to each virtual compartment in the rectangular soil surface (total of 30 animals per system).

Statistical analysis

A correspondence analysis was run on the species with the layers and the concentrations to quantify all variation and the distribution in the data. To estimate the concentrations that caused 50 % reduction in the soil invertebrates (EC_{50}) was used best fit models.

Two-parametric Weibull models (for *F. fimetaria* and linear avoidance):

$$Y = c * \exp\left((\log(0{,}5)) * \left(\frac{\text{Conc}}{\text{ECp}}\right)^{b} \right)$$

Logistic model (for single-species test with *F. candida*):

$$Y = \frac{c}{1 + \left(\frac{Conc}{ECp}\right)^{b}}$$

Linear model (for *H. nitidus*, *H. assimilis*, *P. minuta*, *P. fimata*, *H. aculeifer* and *E. fetida* biomass):

$$Y = \left(\frac{-0{,}5 * c}{EC50}\right) * conc + c$$

where c is the mean control value, b is the slope parameter.

The linear avoidance tests calculations used the number of avoiders for each compartment following the equation Avoiders = NE - NO, where NE is the expected organisms and NO the number of observed organisms. The compartment with the highest concentration, NE is equal to the number of collembolan introduced in the compartment at the start of the test; for the remaining compartments, NE includes the organisms introduced initially in the compartment plus the organisms introduced in the adjacent compartments of higher concentration (Araujo et al. 2014a).

For correlation analysis between bait lamina scores and structural responses (composition and biomass of the soil invertebrates) the Pearson product e moment correlation coefficient r with a two-tailed p value and the coefficient of determination R^2 were calculated. The feeding activity measured by the bait lamina test system is a measure of function of the SMS system in the top and middle layers of the soil.

For pairwise comparison of means with the control with 0.05 significance were used ANOVA and Dunnett's test for the distribution of the soil invertebrates and for the linear avoidance test. The checking of the homogeneity of variances preceded the variance analysis. For compare the interaction between the pesticide application and the layers of the soil was used pairwise multiple comparisons (Tukey test) after a multi-factor ANOVA for analysis of the differences between the categories with a confidence interval of 95 %. The data were analyzed using STATISTICA software (version 7.0, StatSoft, Inc.) and XLSTAT (version 2014.5.03).

RESULTS

SMS test system

The results after the 28 days exposure of the fungicide Scala® (Pyrimethanil) concentrations on homogeneous soil and surface spray mesocosms applications showed adverse effect on the community structure. The relative abundance of collembolans, mites and earthworms exposed to Scala® (Pyrimethanil) concentrations on soil and spray mesocosms

applications compared to the control showed a decrease for both exposure (Figure 5b). The soil application was notably more affected, a decrease of 45 to 14 % relative abundance, compare to the spray application, a decrease of 58 to 26 % relative abundance.

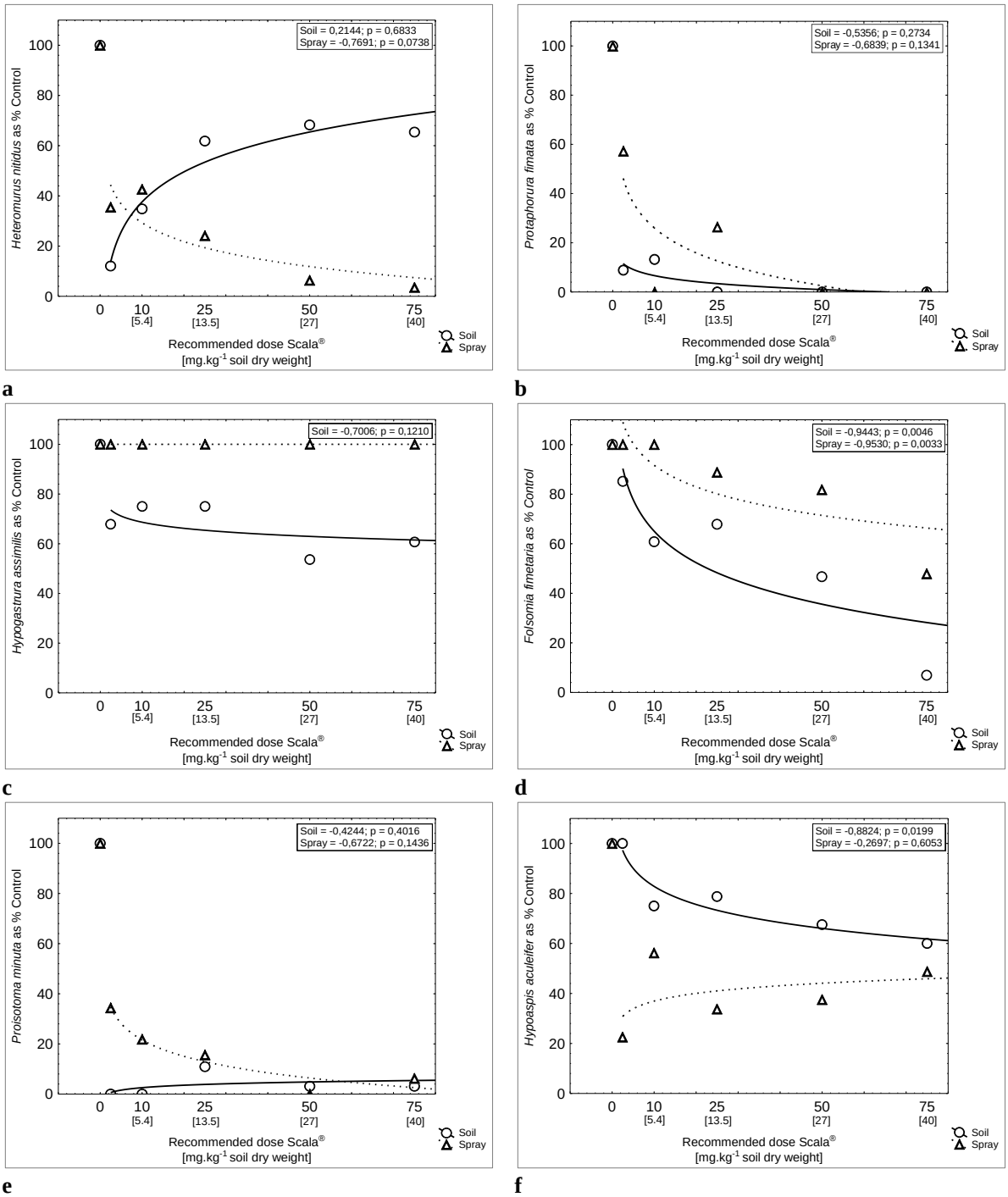
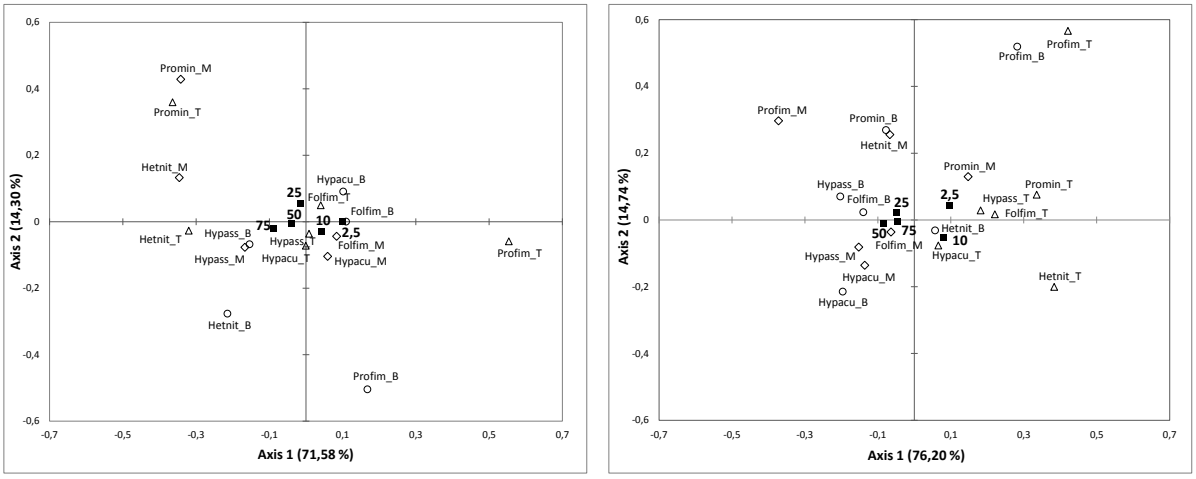


Figure 3. Average of the springtails *H. nitidus* (a), *P. fimata* (b), *H. assimilis* (c), *F. fimetaria* (d) and *P. minuta* (e) and mite *H. aculeifer* (f) abundance as percentage of the control after Scala® (Pyrimethanil) concentrations on Soil (solid line) and Spray (dashed line) mesocosms applications. The present concentration are the recommended dose for strawberries with the control simulator without fungicide (0). Values in brackets correspond to pyrimethanil concentrations (mg pyrimethanil.kg^{-1} soil dry weight).

The abundance of the springtails and mites were presenting as percentage of the control (Figure 3). For the *H. nitidus*, a surface active animal, showed an increase with increasing of the concentrations in soil applications. On the other hand for the spray applications the number of animals decrease with the increase of the concentrations. *H. assimilis* for all the concentrations showed similar results to the control in the spray applications and a little decrease with the concentrations in the soil applications. *P. minuta* showed decrease in spray and soil applications. The same pattern was registered for *P. fimata* showed decrease in spray and soil applications. For the *F. fimetaria*, the dominance specie in the system, showed a significance decrease in spray and soil applications. For the mite specie *H. aculeifer*, the soil applications showed a significance decrease and a little increase for the spray applications.

The correspondence analysis shows the distribution on the springtail species and the mites in relation to the concentrations and the preference habitat in the soil column mesocosm on the two exposure applications (Figure 4). The soil applications results shown the lower concentrations predominance of middle and bottom species and a highlight to *H. nitidus* individuals in the highest concentrations with an avoidance for the soil to surface and the middle and bottom individuals of *H. assimilis*.



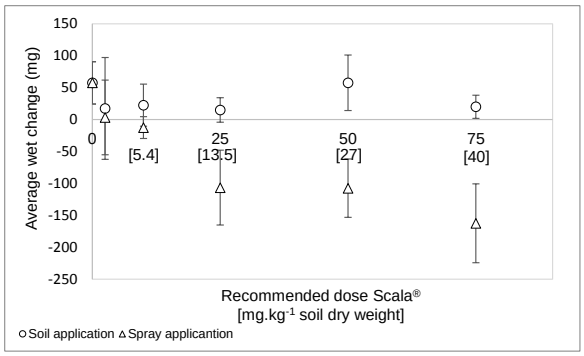
a **b**

Figure 4. Correspondence analysis of the replicates ordination containing the springtails (*H. nitidus* (Hetnit), *H. assimilis* (Hypass), *P. minuta* (Promin), *P. fimata* (Profim) and *F. fimetaria* (Folfim)) and mite (*H. aculeifer* (Hypacu)) after 28 days exposed to Scala® (Pyrimethanil) recommended doses concentrations on Soil (a) and Spray (b) mesocosms applications. The three layers of the mesocosm soil column were plotted with symbols (top: T - Δ; middle: M - ◊; bottom: B - ○) representing the spacial distribution of the species. For the soil applications the first axis explains 71.58 % and second axis represent 14.30 % variation and for the spray applications the first axis explains 76.20 % and second axis represent 14.74 % variation.

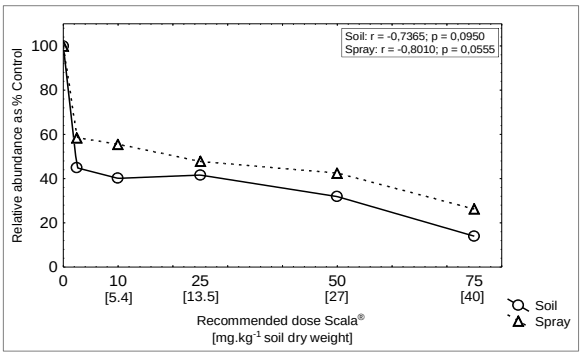
The spray applications shown the high abundance of top species in the lower concentrations and for the highest concentrations only the middle and bottom species. For the

soil applications, the first axis explains 71.58 % and second axis represent 14.30 % variation and for the spray applications the first axis explains 76.20 % and second axis represent 14.74 % variation.

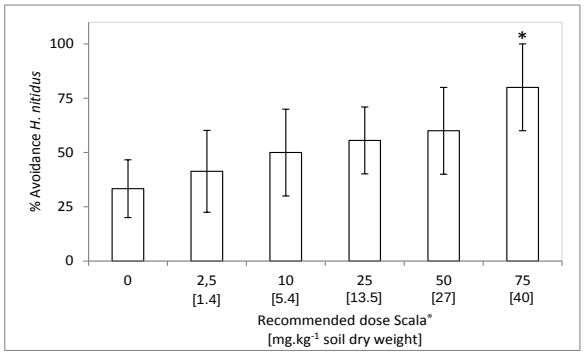
The average of the earthworms *E. fetida* biomass, representing as fresh body weight change, showed different pattern for the soil and spray applications during the 28 days exposure (Figure 5a). For the control the increase of biomass was 58 mg fresh weight and for the soil application the increase was 18 to 58 mg fresh weight. However the spray application shows a decrease to 13 to 163 mg fresh weight. It is appropriate to point out that the survival of earthworms considered was above 80 %.



a



b



c

Figure 5. Earthworm exposure to Scala® (Pyrimethanil) exposure concentrations on soil (circles) and spray (triangles) mesocosms applications after 28 days considering the average of fresh body weight biomass change (mg) of the earthworm *E. fetida* (a), the relative abundance of collembolans, mites and earthworms compared to the control (b) and the avoidance (%) of *H. nitidus* exposed to a gradient of the fungicide for 48 hours in a multi-compartmented static test system with the multiple comparison using Dunnett's procedure at 0.05 significance level representing by (*) (c).

The pearson correlation analysis with bait lamina score in soil and spray applications on the top and middle layers versus the soil mesocosm structural responses with fresh body weight change of the earthworms and the sun of the springtails abundance are summarized in Table 3. The bait lamina score was significantly for the springtails abundance ($r > 0.6$) but with a weak correlation with weight of earthworms ($r < 0.6$).

The results of the multi-factor ANOVA (Table 2) showed the significance between the categories application and soil layers (p-value < 0.05). The statistical interaction between the soil and spray applications showed significant differences for all the organisms (sprigtails, mite

and earthworms), except to the springtail *P. fimata.* The Tukey's test evidence the statistical significant differences between most of the invertebrates and the soil layers. However, the layer no had significant effect in the distribution of the springtails *H. assimilis, P. fimata* and *F. fimetaria.* Bottom vs Top had differences with the springtails *H. nitidus, P. minuta* and the mite *H. aculeifer*; Bottom vs Middle had differences with *P. minuta* and *H. aculeifer* and Middle vs Top had differences with *H. nitidus* and *H. aculeifer.*

The values of recommended dose to causing 50% reduction in the number of individual (EC_{50}) for the soil multi species mesocosms showed differences between soil and spray mesocosms applications (Table 2). For *H. nitidus* do not possess values for soil applications ($EC_{50spray}$ = 17.33 mg.kg^{-1} soil d.w.) and for *H. assimilis* because the homogeneous distribution of data do not possess the EC_{50} values for spray applications (EC_{50soil} = 50.95 mg.kg^{-1} soil d.w.). The individuals of *P. minuta* (EC_{50soil} = 16.04 mg.kg^{-1} soil d.w.; $EC_{50spray}$ = 16.58 mg.kg^{-1} soil d.w.), *P. fimata* (EC_{50soil} = 14.85 mg.kg^{-1} soil d.w.; $EC_{50spray}$ = 15.85 mg.kg^{-1} soil d.w.), *F. fimetaria* (EC_{50soil} = 21.32 mg.kg^{-1} soil d.w.; $EC_{50spray}$ = 39.63 mg.kg^{-1} soil d.w.), *H. aculeifer* (EC_{50soil} = 36.50 mg.kg^{-1} soil d.w.; $EC_{50spray}$ = 46.20 mg.kg^{-1} soil d.w.) present high values of EC_{50} for spray compare to soil applications. However for the biomass of *E. fetida* the values of were notably highest in the soil than in spray applications (EC_{50soil} = 159.0 mg.kg^{-1} soil d.w.; $EC_{50spray}$ = 3.29 mg.kg^{-1} soil d.w.).

Springtails single specie reproduction test

The single reproductions tests with *F. candida* exposed to Pyrimethanil reported different EC_{50} values for commercial formulation ($EC_{50c.f.}$ = 17.10 mg.kg^{-1} soil d.w.) and active ingredient ($EC_{50a.i.}$ = 48.16 mg.kg^{-1} soil d.w.). For the linear avoidance the values of causing 50% avoidance in the number of individual (AC_{50}) was 28.53 mg.kg^{-1} soil d.w. of Scala® for the springtail *H. nitidus* (Table 2).

Linear avoidance test

The avoidance test of *H. nitidus* exposed to a gradient of the commercial formulation Scala® in a multi-compartmented static test system is illustrated in Figure 5c. After 48 hours exposure, collembolan avoidance response in the concentrations highest than 10 recommended dose showed avoidance above 50 %. For the multiple comparison with the control using Dunnett's procedure the avoidance reported significative differences in the concentration of 75 recommended doses (80 % avoidance).

Table 2. Soil multi species mesocosms results of Tukey HSD test for analysis of the differences between the categories application with layers, values of recommended dose causing 50% reduction in the number of individuals EC_{50} (*H. nitidus, H. assimilis, P. minuta, P. fimata, F. fimetaria, H. aculeifer* and biomass of *E. fetida*) extracted from Scala® (Pyrimethanil) concentrations on Soil and Spray mesocosms applications, single reproductions tests with *F. candida* exposed to commercial formulation (c.f.) Scala® and active ingredient (a.i.) of Pyrimethanil and linear avoidance with *H. nitidus* exposed to Scala® concentrations ($mg.kg^{-1}$ soil dry weight). All the calculation used a confidence interval of 95 %, representing by (*).

	Soil multi species mesocosms							Single test c.f.	Single test a.i.	Avoidance[1]
	H. nitidus	*H. assimilis*	*P. minuta*	*P. fimata*	*F. fimetaria*	*H. aculeifer*	*E. fetida*	*F. candida*		*H. nitidus*
Spray vs Soil	0.001*	< 0.0001*	0.000*	0.070	< 0.0001*	< 0.0001*	< 0.0001*	-	-	-
Bottom vs Top	< 0.0001*	0.278	0.004*	0.324	0.502	< 0.0001*	-	-	-	-
Bottom vs Middle	0.644	0.615	0.017*	0.881	0.897	< 0.0001*	-	-	-	-
Middle vs Top	< 0.0001*	0.823	0.887	0.603	0.776	0.027*	-	-	-	-
EC_{50} Soil	-	50.95	16.04	14.85	21.32	36.50	159.0	17.10	48.16	28.53
Conf. limit		(4.84-97.02)	(7.59-24.50)	(9.41-20.30)	(13.16-29.48)	(12.57-60.44)	(-1611-1928)	(13.46-20.73)	(34.75-61.6)	(4.15-52.9)
EC_{50} Spray	17.33	-	16.58	15.83	39.63	46.20	3.29	-	-	-
Conf. limit	(10.64-24.0)		(9.91-23.24)	(9.98-21.68)	(29.49-49.78)	(-4.91-97.32)	(0.47-6.11)			

1 - AC_{50} for the avoidance test results.

Table 3. Pearson correlation analysis of bait lamina score in soil and spray applications on the top and middle layers versus the soil mesocosm structural responses with biomass of the earthworms (*E fetida*) and the sum of the springtails abundance (*H. nitidus, H. assimilis, P. minuta, P. fimata, F. fimetaria*). The Pearson correlation used a significance level of 0.05, representing by (*).

Bait lamina score versus:	Soil application				Spray application			
	Top layer		Middle layer		Top layer		Middle layer	
	Earthworm	Springtails	Earthworm	Springtails	Earthworm	Springtails	Earthworm	Springtails
Pearson	-0.062	0.634	0.105	0.652	0.142	0.637	0.130	0.695
p value	0.825	0.011*	0.680	0.003*	0.573	0.004*	0.608	0.001*
R^2	0.004	0.402	0.011	0.425	0.020	0.406	0.017	0.483

DISCUSSION

SMS test system

The results after the 28 days exposure of Scala® (Pyrimethanil) concentrations on homogeneous soil and surface spray mesocosms applications and soil layers showed different adverse effect on soil invertebrates' communities and functions. The short term ecological direct and indirect responses to disturbance on earthworms, micro-arthropods interactions and bait-lamina feeding activity were monitored and combined with the single and behaviour responses. These habitants of soil have traditionally portrayed by the ecologists as "decomposers" essentially, a single trophic level responsible by the recycling all aboveground material but in a subterranean world the ecology is on a smaller scale with a complex soil food web as the aboveground web (Sugden et al. 2004).

The results of the SMS species showed a particular habitat preference on the soil column, corroborating the initial design on life-forms species in soil ecosystem. For the epedaphic springtail *H. nitidus* the distribution showed an increase with increasing of the concentrations in soil applications as results of the avoidance of the contaminated soil and the dispersal for the outer surface of soil (Figure 3 and 4). The habitat preferences and dispersal abilities are still imperfectly known in springtails (Auclerc et al. 2009). Indeed, species with similar or compatible ecological requirements may disperse at varying rates, and thus may respond differently to environmental change (Ponge et al. 2006). In this sense, the distribution for this surface-active animal on spray applications showed a decrease with the increase of the concentrations as results of the habitat suppression in the soil surface by the accumulation of sprayed fungicide.

Song et al. (2016) highlight about the surface-active epedaphic collembolans can avoid part of the damages that happen in the soil while the euedaphic and hemiedaphic collembolan are more vulnerable to soil perturbation to dwell in soil in their lifetime and directly interact with the soil by feeding and some other activities. The hemi-euedaphic behaviour animals (*H. assimilis*, *P. minuta* and *P. fimata*) registered a few extracted individuals in the system showed a decrease with the concentrations gradient. However, the euedaphic springtail *F. fimetaria*, the dominant specie on the system, showed a significance decrease of distribution in spray and soil applications. According to Schnug et al. (2014) this specie to be omnivorous behaviour is less dependent on fungi as a food resource, may be less affected by the indirect effects of fungicide, and suggested a low direct toxicity of fungicides to this specie. It is known that the fungal communities are included as available resource not regulated by top-down factors, such as predation, but rather by bottom-up factors (Crowther et al. 2011). However, the anthropogenic

levels of habitat modification can benefit species which have invested in traits associated with colonization ability survive as fugitives, colonizing and recolonizing habitat patches in which the competitor has either gone extinct or failed to colonize as yet (Marshall et al. 2000). In this scenario, can occur the reduction of the intensity of interspecific interactions, whether they be predation or competition.

The distribution of the predator mite *H. aculeifer* in the gradient of concentrations showed decrease for soil applications and a little increase for the spray applications with high values of EC_{50} for spray compare to soil applications with possible decrease of the predation interaction. A possible explanation could be the preference and availability of hemi-euedaphic prey as observed by Scott-Fordsmand et al. (2008), in this case mainly *F. fimetaria.* Jensen et al. (2009) reported the predatory-prey interactions between these two species and reported the influence response of test organisms to toxic substances. Although, the predatory mites were affected by all the exposure concentrations with a few extracted animals.

The correspondence analysis (Figure 4) showed the distribution of the springtail species and the mites in relation to the concentrations and the preference habitat in the soil column mesocosm on the two exposure applications. The pesticides applications may change the dominance structure of a springtail community in SMS test systems (Jensen and Scott-Fordsmand, 2012; Schnug et al. 2014). In this sense, the statistical interaction between the soil and spray applications showed significant differences for all the organisms in the system (sprigtails, mites and earthworms), except to the springtail *P. fimata* (Table 2). The habitat preference contributed to the distribution of hemi-euedaphic springtails species with no significance difference for the layer for *H. assimilis, P. fimata* and *F. fimetaria.*

The habitat preference and dispersal ability of species play a prominent role in the building of springtails (Auclerc et al. 2009). In this sense, the system layers shown different patterns as bottom versus top layer with differences of the springtails *H. nitidus, P. minuta* and the mite *H. aculeifer,* bottom versus middle layer with differences of the *P. minuta* and *H. aculeifer* and middle versus top layer with differences of *H. nitidus* and *H. aculeifer.* The soil applications results showed in the lower concentrations predominance of hemi-euedaphic species. The spray applications showed the top species near the lower concentrations and for the highest concentrations only the middle and bottom species as a result of the direct toxicity. The reduced of individuals number may be caused by the fungicide indirect toxicity by killing their hyphal food (Hopkin, 1997) as a bottom-up effect. Furthermore, the effects of habitat structure on the shape of the functional response determine energetic efficiencies of the predator-prey interactions (Vucic-Pestic et al. 2010).

For all the invertebrates species were registered an increased at low and medium exposure concentrations before they decreased at higher concentrations. This distribution was present in both exposure but more evident in soil exposure, that indicate a hormesis effect. The hormesis effect, under low stress, organisms not only repair any damage, but also overcompensate and reduce background damage more effectively (Chapman, 2001, 2002). This author report as a response to stress may not be just a generalized toxicological phenomenon, but also a generalized ecological phenomenon, for instance the intermediate disturbance hypothesis in ecology. Thus the effect, similar to acclimation, may be a physiological/structural mechanism of gaining increased tolerance with metabolic cost. In presence of fungicides Schnug et al. (2014) showed the hormesis distribution for *F. fimetaria* and *H. nitidus* and Krogh (1995) for the mite *H. aculeifer*. In other words, the ecological resilience, capacity of a system to absorb disturbances and retain the same level of fundamental functions, changes in ecological systems and determine the capacity for reorganization while undergoing change (Mori, 2013). The natural ecosystems can differ greatly in how they respond to disturbance, both in terms of their resistance and resilience being the differences in how they are impacted by disturbance are driven by both abiotic and biotic factors (Wardle & Jonsson, 2014).

In our case, the relative abundance of collembolans, mites and earthworms exposed to Scala® (Pyrimethanil) concentrations on soil and spray mesocosms applications compared to the control showed a decrease for both exposure (Figure 4b). When compare the habitat structure, spray application was mainly affected in the top layer, with a pesticide crust on the soil surface while in the soil application was committed in the entire soil column with indirect toxicity causing elimination of the base food resources (fungal structures) by the fungicide. Nevertheless, competition is a major regulatory factor in population and community dynamics with the effects on organisms can be either direct in intraspecific competition or indirect by exploitative intraspecific and interspecific competition (Bourlot et al. 2014).

The fresh body weight change of the earthworm *E. fetida* shown notably different pattern for the soil and spray applications (Figure 4a). While in the soil applications the food (cow-dung) in the surface was available, the spray application blocked the surface with the fungicide affecting the feeding zone for epigeic soil organism. *E. fetida* is an epigeic specie that feeds mainly on plant litter or manure in soil surface (Khorram et al. 2016). Furthermore, the earthworms are located near the bottom of the terrestrial trophic level, use the sensitive receptors on their body surfaces to sense, and avoid chemicals in the soil (Bouché, 1992). A study with the fungicide carbendazim combining the *E. fetida* highly sensitive chemoreceptors reported a high avoidance response and correspondence between the observed mortality effects

(Rico et al. 2016). Notwithstanding the earthworms are considered as soil engineers because of their effects on soil properties, structure (increased macroporosity) and their influence on the availability of resources and habitat (Jouquet et al. 2006). The earthworms probably create a favorable environment for micro arthropods as the distribution of the springtail *H. nitidus* reported in some studies, at least partly, controlled by earthworm density and particularly by the earthworms' mucus excretion, faeces and urine in a commensalism relation (Salmon & Ponge, 1999, 2001; Salmon, 2004).

For the soil mesocosm structural responses, the system were compared with the pearson correlation analysis. The bait lamina score in soil and spray applications, on the top and middle layers, showed significantly for the springtails abundance, but a weak correlation with fresh body weight change of the earthworms (Table 3). Schnug et al. (2014) showed weak effects of the fungicide triclosan in a SMS test systems on earthworm performance. These authors highlight the correlation of bait lamina scores with springtail abundances is partly a result of the interrelationship between earthworms and springtails. In a SMS test systems without earthworms species the springtail densities were not correlated with bait lamina scores (Jensen and Scott-Fordsmand, 2012). Gestel et al. (2003) reported a bait-lamina as a good tool to reflect the biological activity of soil animals showed a consumption rate highest in mesocosms containing earthworms and increased with increasing earthworm density. These conclusions are corroborate by the studies shown above about the distribution of the springtail *H. nitidus* versus the earthworms.

Springtails single specie reproduction test

The pyrimethanil chemical formulation for single reproductions tests with springtail *F. candida* reported different EC_{50} values for active ingredient ($EC_{50a.i.}$ = 48.16 $mg.Kg^{-1}$ soil d.w.) and commercial formulation ($EC_{50c.f.}$ = 17.10 $mg.kg^{-1}$ soil d.w.). The similar patterns were reported in others *F. candida* standard tests studies. The EC_{50} for pyrimethanil as active ingredient on the climate change and chemical stress study ($EC_{50a.i.}$ = 55.6 $mg.Kg^{-1}$ soil d.w.) (Bandow et al. 2014) was higher than the EC_{50} found for the commercial formulation Scala® on a toxicity of pyrimethanil sprayed soils via surface runoff ($EC_{50c.f.}$ = 19.9 $mg.Kg^{-1}$ soil d.w.) (Gil et al. 2015). The inert ingredients present in commercial formulations are not supposed to be toxic and their identification and percentages are rarely disclosed (Pereira et al. 2009). Although these substances can contribute to the overall toxicity of the commercial formulation, exerting toxic activity or interacting with the active ingredient and often exhibit higher toxicity to non-target organisms (Krogh et al. 2003; Pereira et al. 2009). The EC_{50} for commercial formulation

Scala® is within the range of EC_{50} confidence limit found for the SMS hemi-euedaphic species (*P. minuta, P. fimata and F. fimetaria*) tested in the present study.

Linear avoidance test

The avoidance test of *H. nitidus* exposed to a gradient of the commercial formulation Scala® in a multi-compartmented static test system shown avoid to the perturbation (Figure 5c). After 48 hours exposure, collembolan avoidance response showed avoidance above 50 % in the concentrations highest than 10 recommended dose with significative differences in the concentration of 75 recommended doses. The linear avoidance shown values to causing 50 % avoidance in the number of individual (AC_{50} = 28.53 mg.kg^{-1} soil d.w.) (Table 2). Additionally when we compare the AC_{50} with the EC_{50} found in the SMS test systems for the springtails, we found values within the range of confidence limit. In the SMS test systems the springtail *H. nitidus* showed a foraging behavior away from the contaminated soil. As result of the direct toxicity, this behavior reported an increase with the concentrations increase on soil application, but not supported the fungicide sprayed on spray application. In collembolan the olfactory cues are an important sense for foraging behavior orientating the movement away from high toxicity substances (Staaden et al. 2011). Furthermore, they are able to avoid on different sensitivities for the contaminated soils (Luz et al. 2004) and the choice behavior may indeed be affected by intraspecific interactions (Filser et al. 2014). This ability to detect and avoid to contaminated sites has been studied in a linear gradient exposure as efficient tools for aquatic (Araujo et al. 2014a; Araujo et al. 2014b; Vasconcelos et al. 2016) and soil organisms (Chauvat et al. 2014). The introduction of foraging behavioral aspects of soil animals in ecological risk assessment would help to better assess the habitats disturbance responses (Boitaud et al. 2006).

The ecological resilience showed changes on interaction patterns such as the exploitative competition, apparent competition and indirect facilitation and interspecific interactions with collembolan and the mites, the predation process of the mites on *F. fimetaria* and the commensalism relation between the earthworms' *E. fetida* and the springtail *H. nitidus.*

Conclusions

In summary, the fungicide showed deleterious effects for the constructed food-web system interactions on SMS test systems. The pesticides have direct effects on individual organisms but also has effects on the interactions on the food web in terrestrial ecosystems with few studies on the effects of chemical on the ecosystem structure and functioning (Sechi et al. 2014). Our results reported changes on exploitative competition, apparent competition and

indirect facilitation, interspecific interactions with collembolan and mites, the predation process of the mites on *F. fimetaria* and the commensalism relation between the earthworms' *E. fetida* and the springtail *H. nitidus.* However, we reported changes on feeding activity with the fungicide concentrations gradient.

Nevertheless, on spray application the soil surface was cover with the fungicide liquid application, blocking the resources supply zone for the earthworms. This point of view corroborates the ecological characteristics of the earthworm *E. fetida* being an ultra epigeic species, living almost entirely in organic matter and feeds almost entirely on the soil surface (Langdon et al. 2005) and the availability of the contaminant may influence their susceptibility to perturbation. However, the soil invertebrate community has different response for the applications (soil homogeneous and surface spray) and for the spatial distribution (column layers) with effects of diversity loss and environmental changes. The food-web approaches provide a natural framework for understanding the ecological roles and the mechanisms through which biodiversity influences the number and the distribution of functional groups in ecological communities (Hector & Bagchi, 2007; Montoya et al. 2015). The indirect pesticide effects act on food webs and species competition through the removal of prey or competing species besides effects on interspecific behavior, may change predator-prey interactions (Köhler & Triebskorn, 2013). The food-web interactions improve the ecological representativeness in pesticides ERA. The risk assessment for agricultural procedure involves the use of the available information on exposure risk and toxicity to specific non-target sensitive organisms to provide an adequate environmental protection from the agricultural chemicals being applied (Hewitt, 2000). For this procedure, the spray application are more realist scenario through the simulate on agricultural field situations, but the soil applications are an important tool to assess the chemical toxicity and regulatory. The EPA (1999) highlights the worldwide concern of plant protection products spray from the target site to any non- or off-target site can affect the wildlife and its habitat and human health.

In conclusions, the SMS test system showed that the exposure of Scala® (Pyrimethanil) concentrations has adverse effect on soil invertebrates' response for the fungicide application and spatial distribution. The results showed the springtails community and predator mite had habitat preferences and foraging abilities affected by direct and indirect toxicity. The epigeic earthworms was influence by the availability of resources and habitats with indirect toxicity on spray application. However, the soil mesocosm structural responses was correlated with the springtails abundance and earthworms in a **commensalism** intraspecific interaction**.** Finally, the results reported the commercial formulation had more toxicity than the active ingredient

and the linear gradient exposure response represent efficient tools for habitats disturbance responses. Therefore, this set of fungicide risk assessment information combined with sprayed applications provides more realistic simulation scenarios approaching the ecotoxicology science with the agricultural field pesticides situations, reporting accurately the ecosystems responses.

ACKNOWLEDGEMENTS

The authors would like to thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for the doctorate sandwich grant (process number 99999.008342/2014-08) and Aarhus University for the facilities. We thank the technicians at the Institute of Bioscience (Aarhus University, Silkeborg, Denmark) Tanja Quottrup Egholm. Elin Jørgensen, Zdenek Gavor, Lise Lauridsen and John Geert Hytter for technical assistance and guidance.

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CHAPTER IV

Scenarios of ecological risk assessment of the fungicide Pyrimethanil based on an ecosystem services approach

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Scenarios of ecological risk assessment of the fungicide Pyrimethanil based on an ecosystem services approach

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Abstract

Ecological risk assessment (ARE) studies are important to assess environmental changes caused by anthropogenic activities on ecosystems function and services. New frameworks have been proposed to integrated more level of ecology into the risk results linking the ecosystems to human well-being. Our study adopt an experimental simulate scenarios from the recommended dose of tomatoes, carrots and onions of the fungicide Mythos® (Pyrimethanil). To attempt it, the experiment consists in a terrestrial plant test followed by elutriate test with non-targets freshwater organisms and avoidance test with soil invertebrates with quantify an ecosystems services framework. The results obtained suggest that the possible impacts by the runoff and leaching into adjacent water bodies and surrounding soil showed changes in the organism's structure with changes and loss in the provisioning, regulatory and supporting services. We concluded that the original ecological risk assessment were more relevant when included new analyzes factors such as the measurement of the effects of contaminants on the functions and ecosystem services in disturbances areas.

Keywords: Pesticides, phytotoxicity, elutriate assays, avoidance behavior

INTRODUCTION

The focus of the ecological risk assessment (ERA) is determine the risk to ecological receptors posed by chemical and physical stressors against the environmental conditions and inform accurately for decision making (Munns et al. 2009). To counteract the ecological deficiency in ERA, new frameworks have been proposed to integrated more level of ecology into the risk results (Van den Brink, 2008). The anthropogenic threats may lead to detrimental ecosystem degradation, providing strong motivation to evaluate the response of ecological communities to various anthropogenic pressures (Mori et al. 2013). The ecosystem services (ES) were developed to focus on the linkages between ecosystems and human well-being and the benefits people obtain from ecosystem structures and processes (Millennium Ecosystem Assessment, 2005). These authors quantifying the ES in four categories: **provisioning services** such as food and water; **regulating services** such as the regulation of climate and water quality; **cultural services** such as recreation and aesthetic values and **supporting services** such as soil formation, photosynthesis and nutrient cycling. The ES usually are results from complex interactions between and within abiotic and biotic components of ecosystems articulated by human activities (Schäfer, 2012). Additionally, the ES concept can aid efficient communication between different stakeholder groups and risk managers, in particular when defining specific protection goals (Nienstedt et al. 2012).

In general, the reduction in species diversity is associated with a decrease of ES and consequently with the services performed (Hooper et al. 2005), emphasizing the importance of the biodiversity conservation. These authors elucidate the changes in biodiversity influence onecosystem properties requires an understanding of the functional traits of the species involved, based on the influence of ecosystem properties or the responses of species against the environmental conditions. Thus, nature conservationists and environmental scientists hope to communicate the importance of ecosystems and the worthiness of maintaining their present condition and function by translating their value into ES (e.g. Groot et al. 2010; Frank et al. 2012; Maltby, 2013; Cavender-Bares et al. 2015).

Agricultural ecosystems provide and rely upon important ES such primarily managed to optimize the provisioning food and bioenergy and in the other hand a variety of supporting and regulating services such as soil structure and fertility and pollination that determine the underlying biophysical capacity of agricultural ecosystems (Zhang et al. 2007). The plant protection products and their residues are highly persistent, reaching elevated concentrations, and hence having more severe effects, contributing to biodiversity loss and changes on trophic levels (Krebs et al. 1999) and repeated application can lead to build up of environmental concentrations in soils (Chagnon et al. 2015). This persistence occur in agricultural soils with chemicals that have the ability to accumulate and adsorb to soil particles like the fungicides, causing adverse effects to soil organisms and the ecosystem functions as the decomposition of organic matter and facilitating of nutrient cycling (Komárek et al. 2010; Wightwick et al. 2010). However, they have high runoff and leaching potential into adjacent water surface and groundwater (Bonmatin et al. 2015). There are evidence that they have direct and indirect impacts at field realistic environmental concentrations on a wide range of non-target species, mainly aquatic and terrestrial organisms (Pisa et al. 2014).

The use of ES may lead to larger environmental footprints once these integrated frameworks of hazard substances have been studied largely in isolation, although they are interconnected through human activities (Liu et al. 2015). Our study adopt an experimental approach to characterise the ecological risk assessment based on an ecosystem services approach. The focus is on a simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil) architected based on fungicide application. The goal of the present study was to measuring the fungicide effects estimated using laboratory-derived acute, chronic and avoidance toxicity data and quantify the ecosystems services. The experiment includes seedling emergence of plants followed by the possible effect of the runoff and leaching potential to surrounding soil, surface and groundwater for freshwater and terrestrial organisms. In

addition, we wanted to assess the foraging behaviour of the non-targets soil invertebrates in an avoidance test system.

MATERIAL AND METHODS

Soil test

The soil test used was from Itirapina (22°10'03,4"S 47°54'05,5"W), São Paulo State/Brazil, in area belonging to the Water Resources and Environmental Studies Center (Itirapina, Brazil) with no history of contamination (Nunes et al. 2016) (Table 1). The soil in this area is sandy with fertile patches due to the presence of basalt (Tundisi & Matsumura-Tundisi, 2014). Given that, vegetation is associated with soil type, Brazilian savanna formations (Cerrado) are dominant, being replaced with mesophilic forest in upland sites where soils are more fertile, and riparian forests in nutrient-rich soils near watercourses. To defaunation, the test soil was dried at 80 °C for 24 h and sieved through a 2 mm mesh prior to use.

Table 1. Physicochemical description (mean, n = 3) of the Itirapina, São Paulo, Brazil soil.

Total organic nitrogen (%)	1.05
Total organic phosphorus (µg.g^{-1})	0.50
pH	6.15
Conductivity (µs)	48.6
Water capacity retention (%)	49.0
Organic matter (%)	14.0
Soil particle size (%)	
Sandy	72.0
Silt	18.0
Clay	10.0
Metals (mg.L^{-1})	
Iron	5.96
Manganese	3.73
Zinc	0.44
Lead	0.29
Copper	0.21

Test substance

Pyrimethanil (CAS: 53112-28-0) [IUPAC name N-(4,6-dimethylpyrimidin-2-yl)-aniline] is a colorless crystalline substance practically insoluble in water (121 mg/L), belonging to the anilinopyrimidine class. In the form of concentrates is used as a contact fungicide with protective and curative properties (Sadlo, 2002). The structure of pyrimethanil is shown in Figure 1. The chemical compound has a moderate persistence in soil with half-life value of 55 days (Wightwick et al. 2010). The commercial formulation Mythos® (Bayer CropScience Ltda) is a chemical composition of suspension concentrate containing 300 g/l Pyrimethanil (30.0 %

w/w) indicated for the control of treatment of various diseases on banana, potato, onion, carrot, apple, strawberries, tomato and grapes crops.

The concentrations followed the fungicide Mythos® recommended dose values for the agriculture cultures plants of onion, carrots and tomatoes with the ratio of 0.5, 1, 2, 4, 8 and 16 times the doses. The tests used two scenarios based on recommendation dose for the tomatoes and carrots and onions crops. The recommended dose for tomato followed 2.5 liters commercial formulation to 100 liters water per hectare (0.8, 1.6, 3.2, 13, 103 and 1658 µg pyrimethanil.kg $^{-1}$ soil dry weight). The carrot and onion crops followed recommended dose of 200 ml commercial formulation to 100 liters water per hectare (0.05, 0.11, 0.22, 0.86, 6.9 and 110 µg pyrimethanil.kg^{-1} soil dry weight).

Terrestrial plant test

An adapted version of Organization for Economic Cooperation and Development (OECD, 2006) guideline 208 for the testing of chemical testing a terrestrial plant test: seedling emergence and early seedling growth test conducted our experiments with plants of the fungicide pyrimethanil. Three experiments with commercial seeds plants of the fungicide pyrimethanil were used: *Solanum lycopersicon* (tomato), *Daucus carota* (carrot) and *Allium cepa* (onion). The soil contamination was realized with the pesticide solution and water and spiked soil divided among individual replicates following the soil water holding capacity. The plants was exposed to fungicide on seedling emergence and early growth test (fresh plants biomass, length seedling (cm), roots elongation (cm) and number of leaves). The experiments contained four replicates per treatment with 30 g soil and ten seeds each (30 g moist weight, i.e. 15.3 g dry soil and 14.7 ml inoculation solution). All the seeds were select in stereomicroscope prior to use.

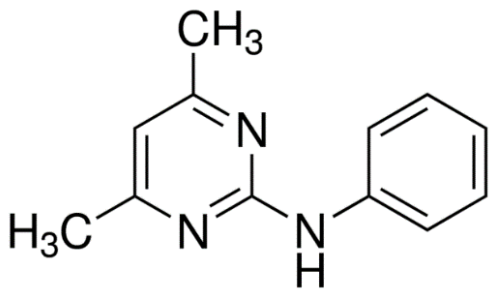


Figure 1. Chemical structure of Pyrimethanil.

The test containers (250 ml transparent plastic cups, height = 8.5 cm, width = 7.5 cm) were maintained at 22° ± 1° C in 16:8 hours light:dark cycle with the amount of light intensity followed 350 ± 50 µE/m^2/s in 21 days exposure. The terrestrial plant tests were analyzed with visual counted of the seedling emergence number in three different germination periods (7, 14 and 21 days). The early seedling growth parameters were analyze after 21 days exposure through the fresh plants biomass (g), the length seedling (cm), length roots (cm) and the number of leaves.

The earthworms avoidance concentrations followed the fungicide Mythos® recommended dose values for the target plants carrot and onion (200 ml commercial formulation to 100 liters water per hectare) with the ratio of 0.5, 1, 2, 4, 8 and 16 times the doses (0.005, 0.01, 0.02, 0.04, 0.08 and 0.16 µg pyrimethanil.kg^{-1} soil dry weight) calculated by the containers superficial area. The springtails avoidance concentrations followed the fungicide Mythos® recommended dose values for the target plants carrot and onion (200ml commercial formulation to 100 liters water per hectare) with the ratio of 0.5, 1, 2, 4, 8 and 16 times the doses (0.04, 0.08, 0.17, 0.33, 0.67 and 1.3 µg pyrimethanil.kg^{-1} soil dry weight) calculated by the containers superficial area.

Elutriate preparation

The elutriate solutions were prepared with the remaining soil of each plants test (tomato and onion) and used in non-targets freshwater organisms tests. For both plants the remaining soil of each concentrations replicates were summed. The elutriate solution followed an adapted version of USA Environmental Protection Agency (EPA, 1998) evaluation of dredged material proposed for discharge in waters of the US testing manual. The elutriate solutions were prepared used the remaining soil of the plant test and unfiltered distilled water. Then combined in a sediment-to-water ratio of 1:4 on a volume basis and mixture on volumetric displacement in a stirred vigorously for 1 hour with a mechanical mixture. After the stirred, the solution was kept in the refrigerator at 5 ºC for 24 hours to particles sedimentation.

Zooplankton elutriate tests

The animals were obtained from a stock culture in the laboratory of Water Resources and Environmental Studies Center (São Carlos School of Engineering, Itirapina, Brazil). The laboratory conditions were at 24 ± 2º C with a 16:8 hours light/dark. Neonates of cladocerans (Cladocera, Crustacea) *Daphnia similis* (Claus, 1876), *Ceriodaphnia silvestrii* (Daday, 1902) and *Ceriodaphnia dubia* (Richard, 1894) were cultivated in reconstituted water with 7.0–7.6

pH, conductivity of 160 µS cm^{-1} and hardness between 40 and 48 mg L^{-1} for $CaCO_3$. The organisms were fed daily with *P. subcapitata* chlorophycean algae (10^6 cells.mL^{-1}).

The acute toxicity tests were performed with four replicates with five organisms (< 24 hours old) each, exposed in nontoxic plastic cups containing 10 mL of the *S. lycopersicon* or *A. cepa* elutriate solution. The containers were kept under controlled temperature (20 ± 2º C for *D. similis* and 25 ± 2º C for *Ceriodaphnia* sp.) and photoperiod (16:8 h light/dark) for 48 hours. The results were analyzed with visual counted of the individuals' survival. All procedures followed an adapted version of the Brazilian standard ABNT (ABNT, 2009) guideline ABNT NBR12713 for aquatic ecotoxicology with acute toxicity test with *Daphnia* spp (Crustacea, Cladocera).

Phytoplankton elutriate test

A strain of the freshwater green microalga (Chlorophyta, Chlorophyceae) *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák 1990 (formerly known as *Selenastrum capricornutum*) was cultured in a 500 mL Erlenmeyer flask containing 250 mL of LC Oligo medium. The cultures were maintained in continuous white light (4000 lux), at 22º C and agitation (100–175 rpm; Shaker Table, Ética). The algal cells used in the assay were three days old (exponential growth phase). The assays were performed with three replicates per treatment in 125 mL glass Erlenmeyer flasks.

The flasks containing 100 mL of *S. lycopersicon* or *A. cepa* elutriate solution inoculated with an initial algae cell concentration of 10^4 cells.mL^{-1}. The test flasks were incubated under the same conditions as the cultures. After 96 hours the algae cells were sampled from each flask in 5 ml aliquots, fixed with lugol acetic and counted in a Neubauer chamber using a microscope. The algae growth inhibition percentages were calculated by comparison of the area under the curve at 96 hours of the control. All procedures followed an adapted version of the Brazilian standard ABNT (ABNT, 2011) guideline ABNT NBR12648 for aquatic ecotoxicology method of chronic toxicity test with green algae (Chlorophyceae).

Earthworms avoidance test

The earthworm (Annelida, Lumbricidae) was composed by the specie *Eisenia andrei* (Bouché, 1972). The earthworms were obtained from the company Minhobox (Juiz de Fora, Brazil) and were maintained in the laboratory conditions with adjusted soil pH (6.0 ± 0.5) and feeding regularly with oatmeal pre-cooked in distilled water. The laboratory conditions were at 22 ± 1 °C with a 12:12 hours light:dark cycle.

The avoidance procedures followed an adapted version of International Organization for Standardization (ISO, 2008) guideline 238 for the soil quality avoidance test for testing the quality of soils and effects of chemicals on behavior. Tests used plastic rectangular containers with a perforated lid (18 cm length, 13 cm width, and 5 cm height). The containers were divided into two equal sections with a plastic card, and approximately 250 g dry weight of a particular soil type were placed into each of the two sections; one section was filled with the control soil and the other one with the contamineted soil. After placing the soils into the containers, the card divider was removed and ten individuals of *E. andrei*, previously washed and dried were placed on the middle line of each test container. The assays were performed with four replicates kept under controlled temperature (22 ± 2 ºC) and photoperiod (16:8 hours light/dark) during 48 hours. The results were obtained dividing again the container with the plastic divider and registered the number of animals in each test soil section. Is appropriate to point out in the case where a individual was found under the midline, the recorded as being in the section that contained the anterior segments.

Springtail avoidance test

The springtail specie (Arthropoda, Collembola) *Folsomia candida* (Willem, 1902) was taken from cultures routinely maintained at the laboratory, where they were kept at 22 ± 1 ºC with a 12/12 hours light:dark interval. When in culture, the collembolans were all bred on Paris-charcoal plaster (8:1) in plastic containers and fed with dried bakers's yeast. At the start of the test, the individuals used were synchronized with 10-12 days age.

The avoidance test was performed with an adapted version of International Organization for Standardization (ISO, 2007) guideline 264 for the avoidance test collembolans. Test used a cylindrical plastic containers (height = 8.5 cm, width = 7.5 cm) cover with a perforated lid of parafilm. The containers were divided into two equal sections with a plastic card, and approximately 30 g dry weight of a particular soil type were placed into each of the two sections; one section was filled with the control soil and the other one with the contamineted soil with five replicates. After removal the plastic divider, 20 individuals of *F. candida* were placed into the soil in the midline of each test container. All individuals were checked under the stereomicroscope to ensure that no legs or antennae were missing or injured. The assays were performed with four replicates kept under controlled temperature (20 ± 2º C) and photoperiod (16:8 hours light/dark) during 48 hours. The results were obtained dividing again the container with the plastic divider and adding water until the middle of the container enabling the animals float and the recorded the sections individuls number.

Conceptual framework for ecosystem services assessment

The experimental test scenarios results were translated in an ecosystem service framework based on specific protection goals considering the legal requirements derived to a European legislation (EFSA, 2010). The possibly impacted were described by the agricultural use of the fungicide Mythos® in field situations. The analysis followed the key drivers of ecosystem services and specified in terms of ecological entity and the impact scale (i.e. attribute, magnitude and spatio temporal) for the target and non-target terrestrial and aquatic organims.

Statistical analysis

For the estimates of the concentrations that caused 50 % reduction in organisms output (EC_{50}) was used the logistic model calculations:

$$y = \frac{c}{1 + \left(\frac{Conc}{ECp}\right)^{b}}$$

where c is the mean control value, b is the slope parameter.

The earthworms and springtails avoidance percentage was calculation according to the equation:

$$\%\, Avoidance\ = \left[\frac{(nc - nt)}{N}\right] * 100$$

where, n_c = number of earthworms in the control soil, n_t = number of earthworms in the test soil, N = total number of earthworms. Results obtained in the dual tests from both test organisms were analyzed by the Fisher exact test.

For the comparison of distributions to fit a species sensitivity distribution (SSD) curve were estimated the chemical concentration (proportion x (%) of the species would be affected (the x% hazardous concentration [HCx]), using the lognormal distribution). This analysis reported a hazardous concentration, predicting the risk of ecological structure using the information from a subset of the species (Frampton et al. 2006).

For pairwise comparison the means of survive, avoidance and plants' germination and growth were compared with the control with 0.05 significance were used ANOVA and Dunnett's test values. The checking of the homogeneity of variances preceded the variance analysis. The data were analyzed using STATISTICA software (version 7.0, StatSoft, Inc.) and XLSTAT (version 2014.5.03).

RESULTS

Terrestrial plants test

The terrestrial plants test results in the three different germination periods (7, 14 and 21 days) for the plants *S. lycopersicon* (tomato), *D. carota* (carrot) and *A. cepa* (onion) of the fungicide pyrimethanil as the commercial formulation Mythos® showed different effects on germination and early plant development (Figure 2). The seedling emergence showed different patterns variation according to the time of the specie specific seed development. The initial analyze period (day 7) showed significative difference distribution for the germination of dicots plants *S. lycopersicon* and *D. carota* and few germination for the monocots plants *A. cepa*. The germination of *S. lycopersicon* showed a decrease with increase of concentration ($F_{6,21}$ = 115.74; $p < 0.0001$; Dunnet multiple comparisons test) but the other species showed a little decrease (*D. carota*: $F_{6,21} = 2.14$; $p = 0.09$ and *A. cepa*: $F_{6,21} = 1.64$; $p = 0.183$; Dunnet multiple comparisons test). The biomass for the control treatment registered more weight for the *S. lycopersicon* (0.62 ± 0.06 g) than the *D. carota* (0.17 ± 0.015 g) and *A. cepa* (0.28 ± 0.063 g).

For the values causing 50% reduction in the number of individual (EC_{50}) for the toxicity endpoints of germination and plant development showed specie specific values (Table 2). For the dicot plants, S. lycopersicon showed increase emergence according to the three periods and a similar pattern to the seedling growth parameters. The carrot specie *D. carota* showed a crescent seed emergence with a similar pattern to the plants development, but a different value for the number of leaves. The monocot *A. cepa* had a few emergence for the initial period of day 7 with similar values for the plant growth and a high value for the number of leaves.

Elutriate test

The non-target freshwater algae *P. subcapitata* cell growth distribution showed significative differences for all concentrations compare to the control (*S. lycopersicon*: $F_{6,14}$ = 58.04; $p < 0.0001$ and *A. cepa*: $F_{6,14} = 10.72$; $p = 0.000$; Dunnet multiple comparisons test) (Figure 3). The *S. lycopersicon* elutriate report a decrease of algae cells until a complete absence of the algae in concentrations above eight recommended dose of Mythos® while the *A. cepa* showed a decrease but with cell in all the concentrations. The results were calculated the values of pyrimethanil to causing 50% reduction in the number of cells (EC_{50}) for the chronic algae toxicity test (Table 2). The *S. lycopersicon* showed high value compare to the *A. cepa* elutriate ($EC_{50S.lycopersicon}$ = 1.25 µg.Kg^{-1} soil d.w., $EC_{50A.cepa}$ = 0.57 µg.Kg^{-1} soil d.w.).

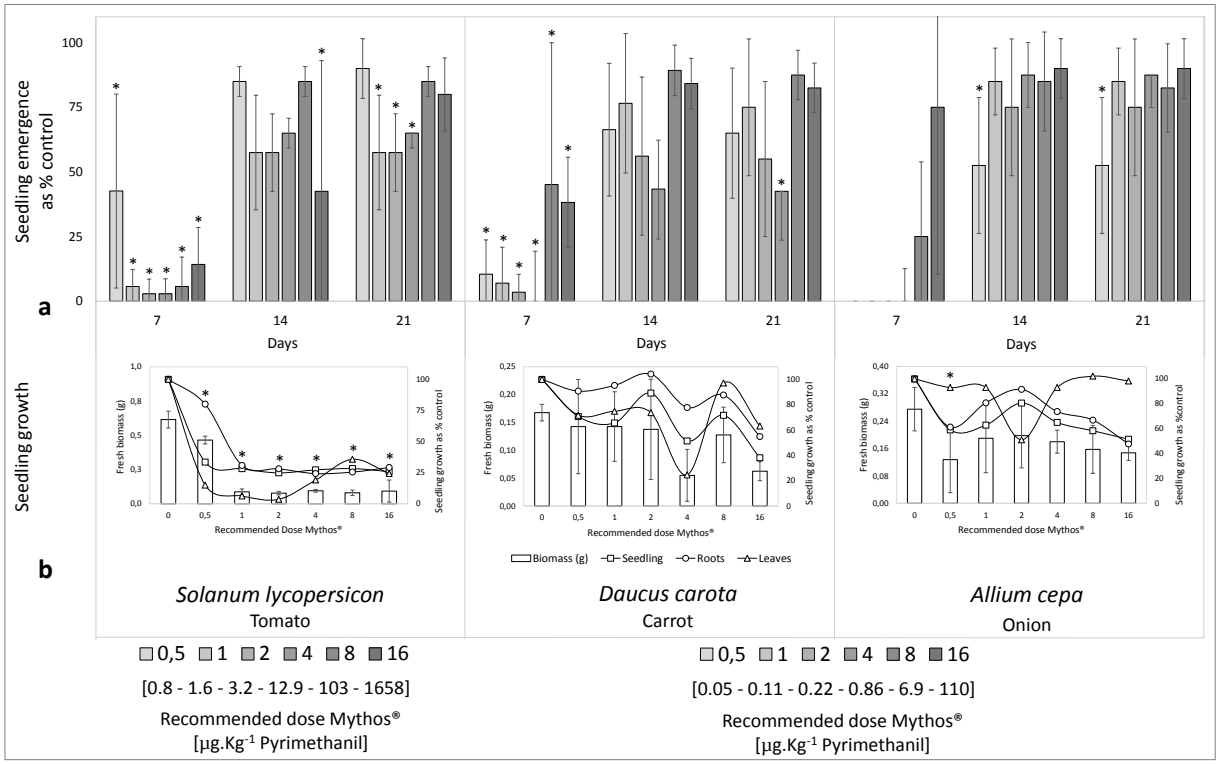


Figure 2. Terrestrial plant test results for *S. lycopersicon* (tomato), *D. carota* (carrot) and *A. cepa* (onion) of the fungicide pyrimethanil as the commercial formulation Mythos® (recommended dose) and pyrimethanil concentrations (µg.L^{-1}). Seedling emergence are represent as percentage of control and analyze in three different germination periods (7, 14 and 21 days) (a). The early seedling growth parameters (b) are analyze after 21 days exposure and represent the fresh plants biomass (g) with bar graphs and the complementary biomass contribution with a secondary line graphs and symbols represent the length seedling (cm) (□) roots elongation (cm) (○) and number of leaves (Δ) as percentage of control. The multiple comparison using Dunnett's procedure at 5% significance level is representing by (*) for the seedling emergence and plants fresh biomass.

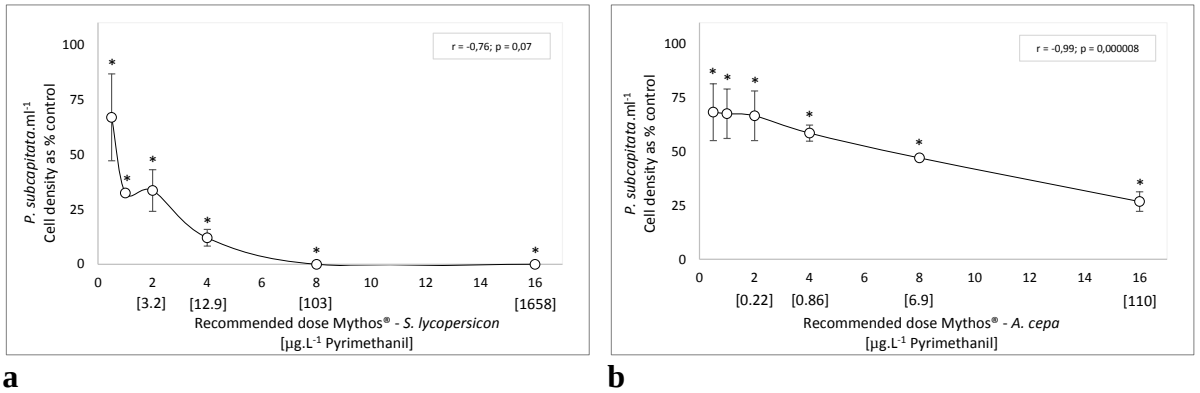


Figure 3. Chronic toxicity test for the non-target freshwater algae *P. subcapitata* on an elutriate exposure at 96 hours with terrestrial plants test *S. lycopersicon* (tomato) (a) and *A. cepa* (onion) (b) of the fungicide pyrimethanil. The multiple comparison using Dunnett's procedure at 5% significance level is representing by (*).

The results of the non-targets freshwater cladoceran acute toxicity test on the elutriate test with remaining plants soil test are shown in the Figure 4 and Table 2 with the multiple comparison using Dunnett's procedure test. For the *S. lycopersicon* elutriate the survival

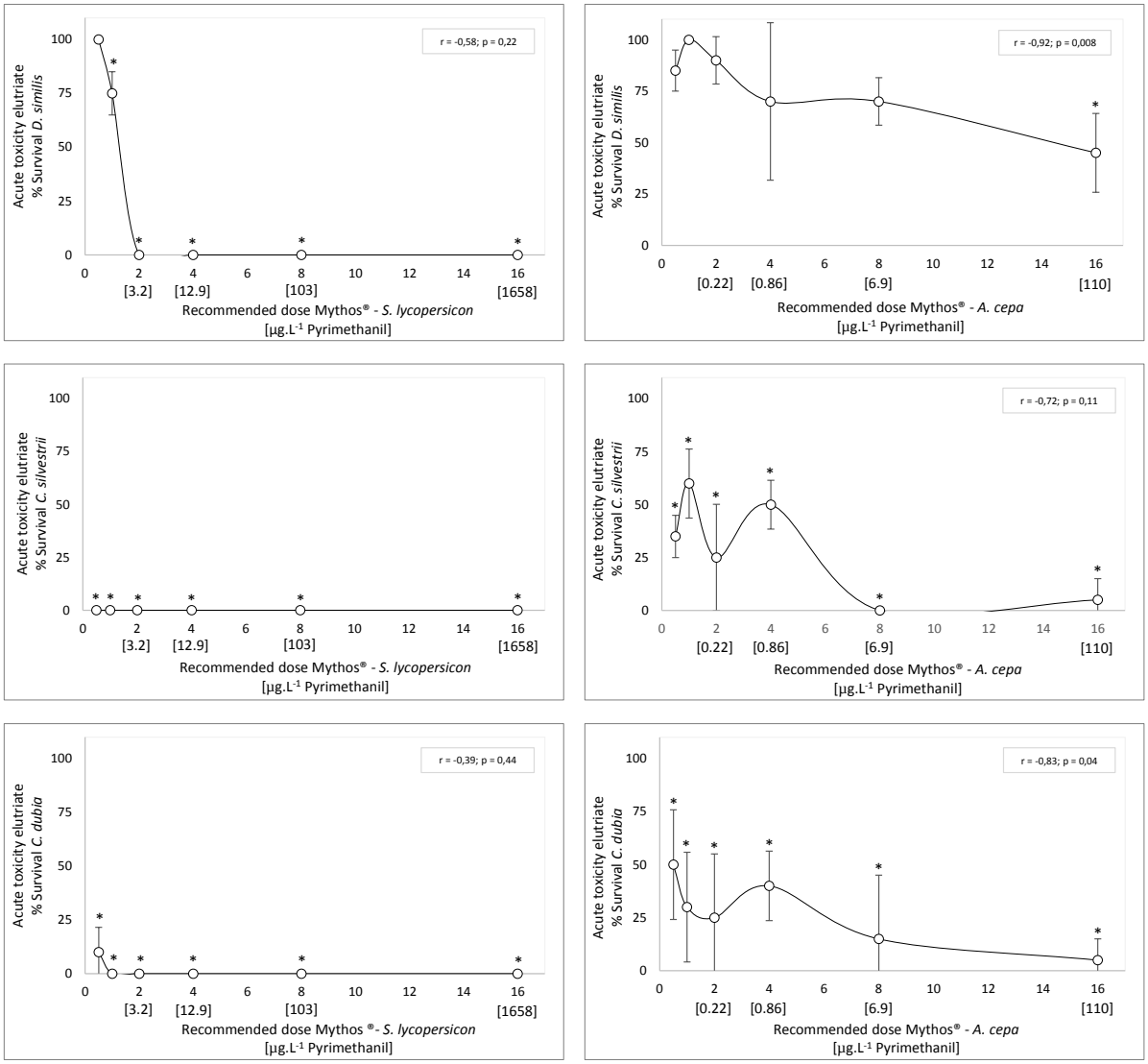
distribution showed a notably sensitive effects with drastic mortality for *D. similis* ($F_{6,21}$ = 644.37; $p < 0.0001$; Dunnet multiple comparisons test) and *C. dubia* ($F_{6,21}$ = 293.00; $p < 0.0001$; Dunnet multiple comparisons test) on the initial concentrations and a complete death for *C. silvestrii* in all the concentrations. The *A. cepa* elutriate showed a hormesis distribution of the survival with decrease with the increase of the concentrations (*D. similis*: $F_{6,21}$ = 4.984; p = 0.0026; *C. dubia*: $F_{6,21}$ = 7.79; p = 0.0002; *C. silvestrii*: $F_{6,21}$ = 26.89; $p < 0.0001$; Dunnet multiple comparisons test). The values of pyrimethanil to causing 50% reduction in the individuals number (EC_{50}) were calculated the for the acute cladoceran toxicity test (Table 2). The *S. lycopersicon* showed high toxicity ($EC_{50D.similis}$ = 1.7 µg.Kg^{-1} soil d.w.) for the organisms compare to the *A. cepa* elutriate ($EC_{50D.similis}$ = 1.6 µg.Kg^{-1} soil d.w., $EC_{50C.dubia}$ = 0.041 µg.Kg^{-1} soil d.w., and $EC_{50C.silvestrii}$ = 0.061 µg.Kg^{-1} soil d.w.).

Terrestrial avoidance test

In avoidance tests, was observed mortality for few tested soils invertebrates. For the earthworms, the results reported a clear avoidance response to highly contaminated soils, with significant differences for the high concentrations combinations tested in Fisher exact test (Figure 5a). The results for pyrimethanil to causing 50 % avoidance (AC_{50}) showed value 0.0036 µg.Kg^{-1} soil d.w. (Table 2). For the springtail *F. candida* showed a more variance avoidance with significant differences in all the concentrations combinations tested in Fisher exact test (Figure 5b). The results for pyrimethanil to causing 50 % avoidance (AC_{50}) showed value 0.0018 µg.Kg^{-1} soil d.w. The results report in a pyrimethanil exposure the earthworms more sensitive to the collembolan specie in contamination soils.

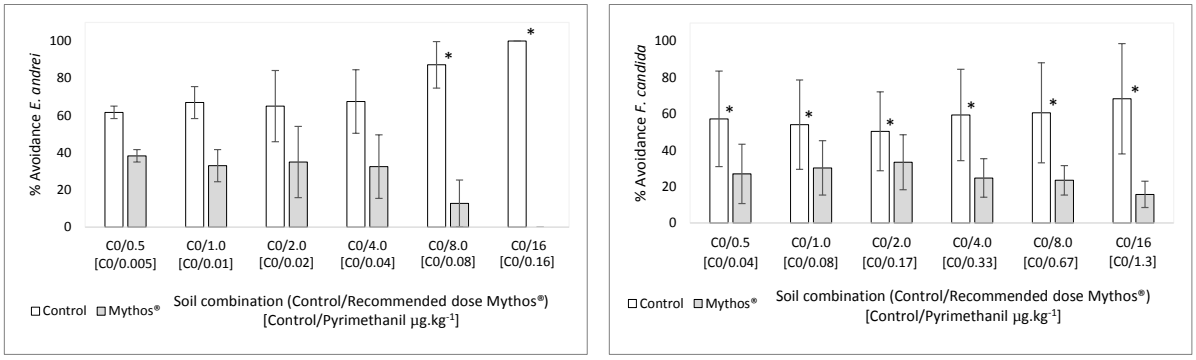
Cumulative risk probability

The cumulative risk probability was calculated by the SSD using all the available data and showed the terrestrial and aquatic compartments organisms' distribution for the simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil) (Figure 6). The plot show for the aquatic compartment the tomato elutriate as a high risk for the aquatic organism and for the terrestrial compartment the risk followed the plants, earthworms and springtails.



a **b**

Figure 4. Acute toxicity test for the non-targets freshwater cladoceran *D. similis, C. silvestrii* and *C. dubia* on an elutriate exposure at 48 hours with terrestrial plants test *S. lycopersicon* (tomato) (a) and *A. cepa* (onion) (b) of the fungicide pyrimethanil. The multiple comparison using Dunnett's procedure at 5% significance level is representing by (*).



a **b**

Figure 5. Avoidance test for the non-targets soil organism *E. andrei* (a) and *F. candida* (b). The results are represent after for 48 hours exposure. An * indicates statistical differences ($p < 0.05$).

Table 2. Results for the values causing 50% reduction in the number of individuals EC_{50} of all the experiments; the terrestrial plant test about the seedling emergence (7, 14 and 21 days) and growth test (fresh biomass, seedling, roots and leaves) for the plants *S. lycopersicon* (tomato), *D. carota* (carrot) and *A. cepa* (onion) exposed to Mythos® concentrations ($\mu g.kg^{-1}$ soil dry weight). The elutriate of the soil test (*S. lycopersicon* (tomato) and *A. cepa* (onion)) with the freshwater zooplankton 48 hours acute toxicity (*D. similis*, *C. dubia* and *C. silvestrii*) and freshwater phytoplankton 96 hours chronic toxicity (*P. subcapitata*). The avoidance test is represent with the values causing 50% individuals avoidance AC_{50} with soil organisms (*E. andrei* and *F. candida*).

Terrestrial plant test: seedling emergence and growth test

Plants	*S. lycopersicon*							*D. carota*							*A. cepa*						
	Emergence (days)			Biomass	Seedling	Roots	Leaves	Emergence (days)			Biomass	Seedling	Roots	Leaves	Emergence (days)			Biomass	Seedling	Roots	Leaves
	7	14	21					7	14	21					7	14	21				
EC_{50}	10	30	$1.3E^{+6}$	12	20	17	30	$1.6E^{+4}$	$2.42E^{+5}$	$2.46E^{+5}$	1.5	1.7	2.1	6.4	-	$2.6E^{+5}$	$2.65E^{+5}$	2.9	2.3	2.0	$2.8E^{+5}$

Elutriate test: freshwater organisms

S. lycopersicon				*A. cepa*		
Zooplankton	*D. similis*	*C. dubia*	*C. silvestrii*	*D. similis*	*C. dubia*	*C. silvestrii*
EC_{50}	1.7	-	-	1.6	0.041	0.061
Phytoplankton	*P. subcapitata*			*P. subcapitata*		
EC_{50}	1.25			0.57		
	-			**Avoidance test: soil organisms**		
Soil invertebrates	-		-	*E. andrei*		*F. candida*
AC_{50}	-		-	0.0036		0.0018

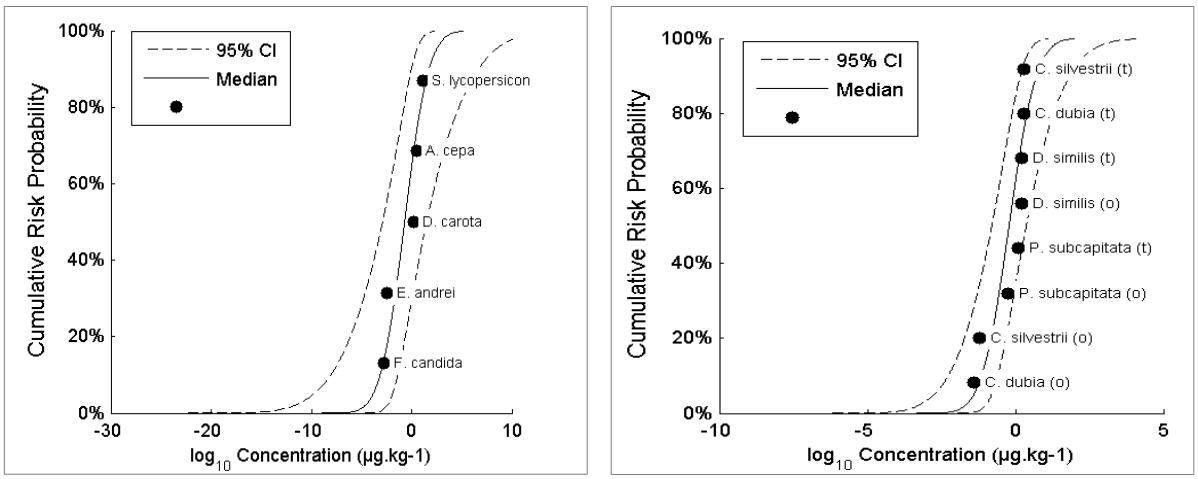


Figure 6. Cumulative risk probability calculated by the species sensitivity distribution. The calculation used the simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil) representing the effect concentrations on non-target organisms. The terrestrial compartment (a) are represent by the early growth fresh biomass of plants (*S. lycopersicon*, *D. carota* and *A. cepa*) and the foraging behavior of soil invertebrates (*E. andrei* and *F. candida*). The aquatic compartment (b) are represent by the elutriate tests with tomato (t) and onion (o) solution for the non-target organisms such as the cell growth of freshwater microalgae (*P. subcapitata*) and the survival of cladoceran (*D. similis*, *C. dubia* and *C. silvestrii*).

Ecosystem services framework

The ecosystem services framework approach were derived adopting the European scientific opinion on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the guidance documents on aquatic and terrestrial ecotoxicology (EFSA, 2010). The categories and the specific protection goals it was proposed for the experimental test scenarios. The possible impacts by the agricultural use of the fungicide Mythos® in field situations were categorized through the key drivers of ecosystem services and ecological parameters on non-targets terrestrial and aquatic organisms (Table 3).

DISCUSSION

This study has shown changes in the structure of the laboratory-derived acute, chronic and avoidance toxicity data for plants and non-target freshwater and terrestrial organisms in the simulated experimental scenarios impacted by the recommended dose of the fungicide Mythos® with changes in the ecosystem services framework. Due of their properties and use, it is expected that the primary effect of pesticides is on the mortality of sensitive non-target organisms and from these direct toxic effects and resulting shifts in species interactions on community structure and followed by indirect effects in ecosystem processes (Van den Brink et al. 2005).

Table 3. Ecosystems services framework based on specific protection goals for the experimental test scenarios possibly impacted by the agricultural use of the fungicide Mythos® (Pyriemthanil) in field situations analyzed through the key drivers of ecosystem services non-target terrestrial and aquatic organims considering the legal requirements derived to a European legislation (EFSA, 2010) and specified in terms of ecological entity, attribute, magnitude of impact and the spatio temporal scale of the impact**.**

Compartment	Experimental test	Category	Key driver	Ecosystem services	Legal requirement	Specific protection goal	Ecological entity	Attribute	Impact scale		
									Magnitude	Spatial	Temporal
Terrestrial	Seedling emergence	Provisioning	Plants	Food/Fibre	No decrease of production	In a short term no effect on biomass of functional groups and keystone species	Population to functional groups	Biomass as affected by survival/growth	No negligible	Field	Days to weeks in edge of field
	Avoidance	Regulatory	Earthworms	Soil formation and retention Nutrient cycling	No lethal and sublethal effects, no effects on ongoing behaviour	No temporary impacts on density of functional groups	Functional groups	Abundance/ biomass and foraging behaviour	Small to medium effect in agro-ecosystems negligible effects in other off-crop areas	Field to landscape	Weeks in field and edge of field and no to days in other off-crop areas
			Springtail	Biodiversity	No decrease of biodiversity	No decrease of biodiversity in the landscape, temporary impact on local populations			Negligible effect	Field to landscape	
Aquatic	Elutriate	Supporting	Zooplankton	Biodiversity Nutrient cycling, Pest and disease regulation	No lethal and sublethal effects, no effects on ongoing behaviour	In a short-term no effects on densities/biomass of functional groups	Functional groups	Abundance and biomass	Negligible effect	Edge of field to watershed	Days to weeks in edge of field to days in protected areas and watershed
			Phytoplankton	Primary produce Photosynthesis Nutrient cycling Water purification	No lethal and sublethal effects, no effects on ongoing growth	In a short-term no effects on densities/biomass of functional groups and communities	Functional groups and communities	Function and biomass	Negligible effects to small effect	Edge of field to watershed	

Terrestrial plants test

The results for the terrestrial plants showed different effects on germination and early plants development and variation according to the time of the specie specific seed development. The *S. lycopersicon*, because the high-recommended dose, showed a notably decrease of germination with increase of concentration and the other species (*D. carota* and *A. cepa*) showed a little decrease for this toxicity endpoints. Toxicity tests using plants are used to monitoring environmental contaminant concentrations, to assess biotransformation, phytotoxicity and bioaccumulation (Lytle & Lytle, 2001). The seed germination and root elongation is a rapid and widely used acute phytotoxicity test with several advantages of sensitivity, low cost and suitability for unstable chemicals or samples (Lin & Xing, 2007). These authors reported the seed coat plays a very important role in protecting the embryo from harmful external hazards factors with a selective permeability. Thus the pollutants, though having obviously inhibitory effect on root growth, may not affect germination if they cannot pass through seed coats. This idea may explain that seed germination in this study was not greatly altered by the fungicide concentrations (Figure 2) with high values causing 50% reduction in seedling emergence number (EC_{50}) of all the experiments.

Higher-level pesticide effects, such as changes in plant communities, will probably interfere with the effects of global change on biodiversity and thus affect ecosystem function (Köhler & Triebskorn, 2013). These changes when extrapolation for the categories for ES showed the importance and relevance of loss this attributes for the human societies. The category provisioning include the seedling emergence of the terrestrial plants as an ecosystem service to providing the food and bioenergy to human wellbeing. Due to such importance the legal requirement of this service would be the no decrease of crop production with specific protection goal such no to short-term effect on biomass of functional groups and keystone species. In turn, Power (2010) reported that the value of these ecosystem services to agriculture is enormous and often underappreciated becoming depend strongly on a suite of ecosystem services provided by natural ecosystems.

The ecological entity related to population and functional groups reporting the attributes relate to production and productivity as affect by plants survival and/or growth. Furthermore, the tolerable impact scale showed a no negligible magnitude in days to weeks in edge of field being with constant management. In this sense, the agricultural process also receives ecosystem disservices that reduce productivity or increase production costs, including herbivory, habitat loss for biodiversity conservation, nutrient runoff, sedimentation of waterways, and pesticide poisoning of humans and non-target species (Zhang et al. 2007).

Elutriate test

The plant protection products and their residues may enter freshwaters indirectly via surface runoff or drainflow induced by rainfall or irrigation but the biological and ecological consequences of such contamination are less clear (Maltby & Hills, 2008). Our experimental scenario, based on a elutriate test simulating the runoff and leaching potential into adjacent water surface and groundwater for freshwater plankton organisms. The results showed the effects on the mortality and with possible impacts on species loss and changes in composition by the fungicide concentrations.

For the non-target freshwater microalgae *P. subcapitata* in a chronic toxicity test showed decrease with increase of the concentrations of the fungicide pyrimethanil (Figure 3). The cell growth distribution showed significative differences for all concentrations compare to the control. The results report a decrease until a complete absence of the algae in high concentrations with deadly effect in the elutriate solutions with high recommended dose (*S. lycopersicon*). This microalgae constitute an important group of photosynthetic organisms that present high sensitivity and are widely used for the assessment the impacts on aquatic ecosystems (e.g. Giloni-Lima et al. 2010; Braun et al. 2012; Rodgher et al. 2012; Shinn et al. 2015). Environmental effects caused by pesticides can potentially the impact of microalgae community and modulate the primary productivity system and photosynthesis (Ferraz et al. 2009).

Freshwater ecosystems provide essential goods and services for human societies such as clean water, food, purification of wastes, recreation and spiritual values (Schäfer et al. 2012). The aquatic elutriate to non-target phytoplankton are categorized in supporting service such as primary produce, photosynthesis, nutrient cycling and water purification. The ES of agricultural landscapes are potentially affected by pesticides with adversely effects on the key taxa or functional groups responsible for providing them (Maltby, 2013). The legal requirement for the algae would be no unacceptable lethal and sublethal effects, no effects on ongoing growth and such specific protection goal no to short-term effects on densities and/or biomass of functional groups and phytoplankton communities.

Ecosystem degradation results from increased input of nutrients, sediments, and toxic substances, which come from agricultural multi-use areas (Sánchez et al. 2006). Tundisi et al (2015) reported a eutrophication by the cyanobacteria blooms of a regional freshwater ecosystems as results of global changes. These authors reported this phytoplankton growth with immediate impacts on human wellbeing mainly on recreation, sport fisheries, water quality, aquatic sport activities beyond the danger of toxicity for aquatic ecosystem. Therefore, the

ecological entity for this ES affect the functional groups and communities as attribute the function and biomass. The tolerable impact scale showed a negligible magnitude effects to small effect from edge of field to watershed. The temporal scale for both aquatic organism shoed days to weeks in edge of field to days in protected areas and watershed.

The non-targets freshwater cladoceran *D. similis, C. dubia* and *C. silvestrii* in an acute toxicity test showed notably sensitive dose effects for the high concentrations. For the *S. lycopersicon* elutriate the survival distribution showed a drastic mortality for *D. similis* and *C. dubia* on the initial concentrations and a complete death for *C. silvestrii* in all the fungicide concentrations (Figure 4a and Table 2). The *A. cepa* elutriate showed a gradual decrease of the survival with the increase of the recommended fungicide dose concentrations (Figure 4b and Table 2). Zooplankton is widely used in ecotoxicological bioassays because this is one of the most sensitive organisms to toxic chemicals and this organism occupies a central position in the aquatic food chain (Czech et al. 2014). The recommended dose concentration that may be expected in runoff water after pesticides application at crop sites were reported highly toxic effects on *D. similis* (Novelli et al. 2012) such as *C. dubia* (Braun et al. 2012) and *C. silvestrii* (Casali-Pereira et al. 2015).

The functional characteristics of species influence the ecosystem properties, including effects of dominant species, keystone species, ecological engineers, and ecological interactions among species (Hooper et al. 2005). The aquatic elutriate to non-target freshwater zooplankton are categorized in supporting services representing important well-being such as biodiversity, nutrient cycling and pest and disease regulation. The legal requirement of these services would be no unacceptable lethal and sublethal effect with no unacceptable effect on ongoing behaviour and such specific protection goal no to short-term effects on densities and/or biomass of functional groups (EFSA, 2010). The freshwater biota are severely threatened by anthropogenic stressors such as organic pollution, heavy metals and pesticides with unclear structural changes on ecosystem functions (Schäfer et al. 2012). The tolerable impact scale for these organisms show a negligible effect, being dependent upon impact on keystone species such as fish mortality.

Terrestrial avoidance test

Notwithstanding, followed the second part of the simulated experimental scenarios design thought the possible impacts by the runoff and leaching potential into surrounding soil of the recommended dose of the fungicide Mythos® we focus in the structure of the terrestrial ecosystem functions with species foraging behaviour. The behavioural responses are a relevant

and sensitive endpoints in environmental risk assessment, with rapid cost-effective and ecologically relevant biological screening tools for initial assessment and habitat preference for soil invertebrates (Luz et al. 2004). The earthworms avoidance tests reported a clear behavior response to highly contaminated soils with significant differences for the high concentrations combinations tested in Fisher exact test (Figure 5a). For the springtail *F. candida* showed a more variance avoidance with significant differences in all the concentrations combinations tested in Fisher exact test (Figure 5b).

The results report that the collembolan species were more sensitive than the earthworms in the dual control contamination soils. The values to causing 50 % avoidance (AC_{50}) shown values for the collembolan (0.0018 $\mu g.Kg^{-1}$ soil d.w.) high than earthworms (0.0036 $\mu g.Kg^{-1}$ soil d.w.) (Table 2). In Collembola the olfactory cues are an important sense for foraging behavior orientating the movement away from high toxicity substances (Staaden et al. 2011). However, the earthworms use the sensitive receptors on their body surfaces to sense and avoid chemicals in the soil with correspondence between the avoidance response and the observed mortality effects when exposure to hazard substances (Rico et al. 2016).

The soil animals can stimulate nutrient mobilization and plant nutrient uptake, they also have the potential to indirectly affect aboveground consumers such as the plant-sucking aphids were found to perform better when host plants were grown in the presence of microbial-feeding collembolan or earthworms than when these organisms were absent (Wardle et al. 2004). Soil invertebrates are key mediators of soil function for the diversity of ecosystem engineering processes in which they share (Lavelle et al. 2006). These authors reported many effects they have on other organisms through their activities such as the incorporation of litter into soil, the building and maintenance of structural porosity and aggregation in soils through burrowing, casting and nesting activities, the control of microbial communities and activities, plant protection against some pests and diseases and the acceleration of plant successions. The terrestrial avoidance with non-target soil invertebrates was categorized in regulatory service, regulating the soil formation and retention, nutrient cycling and biodiversity. The agricultural systems has reduced the multiples terrestrial ecosystem services and has led to the degradation of soils and the capacity to support life (Fonte & Six, 2010). The legal requirement of this service would be no unacceptable lethal and sublethal effects, no effects on ongoing behaviour and no decrease of biodiversity with specific protection goal such no to temporary impacts on density of functional groups. The ecological parameters related to functional groups such entity and abundance and/or biomass and foraging behaviour. The tolerable impact scale showed small to medium magnitude effect in agro-ecosystems negligible effects in other off-crop areas,

such field to landscape and the temporal scale understood to weeks in field and edge of field and no to days in other off-crop areas. In the context of ERA this new point of view for the perturbations are needed to facilitate the assessment of soil health and the capacity of soils to provide ecosystem services forward decision-making (Faber & Van Wensem, 2012).

Cumulative risk probability

To predict effects of pollutants on ecosystem the information must be extrapolated from a small subset of key species used to estimate the hazardous concentration and predicts the risk to ecological structure and functional attributes (Maltby et al. 2005; Frampton et al. 2006). The cumulative risk probability calculated by the terrestrial and aquatic organisms' SSD for the simulate scenarios from the recommended dose of the fungicide Mythos® (Pyrimethanil) showed a dynamic risk front the different levels of biological organization tested. The risk curves showed for the terrestrial compartment a high risk for the plants early growth followed by the earthworms and springtails foraging behaviour and for the aquatic compartment the tomato elutriate solutions showed a high risk for the aquatic organism with potential to change the ecosystem functions and services.

Conclusions

Human and natural systems interact in a multiple ways and quantifying the services that ecosystems provide for societal needs helps assign value to natural components for humans (Liu et al. 2015). The plants, besides the importance of the human supply, has the importance along trophic cascades that the species richness effects are passed from one trophic level (Scherber et al. 2010). The earthworms are typical ecosystem engineers as they have a large impact on architecture of soils structure and have thus been recognized as typical ecosystem engineers with an high potential partner for humans in managing ecosystem services such as carbon sequestration, soil fertility and plant production (Lavelle et al. 2006; Blouin et al. 2013). Furthermore the earthworms are located near the bottom of the terrestrial trophic level and use the sensitive receptors on their body surfaces to sense and avoid chemicals in the soil (Bouché, 1992). For collembolan, behavioral avoidance are an important strategy for their relatively high tolerance to several substances (Staaden et al. 2011). Furthermore, it are able to avoid on different sensitivities for the contaminated soils (Luz et al. 2004). The local extinction of certain species from an ecosystem can have a greater impact on ecosystem functioning than the extinction of other species such as the functional and phylogenetic diversity of zooplankton

determines their ability to produce biomass and suppress phytoplankton through top-down grazing (Thompson et al. 2015).

Indeed, the agricultural practices are essential for sustaining the human population, but in the other hand, they can directly disrupt the ecosystem functions (Galic et al. 2012). The agricultural production is highly dependent on the services provided by neighbouring natural ecosystems (Power, 2010). It is to be predict that the global changes the world ecosystems are going to experience during the coming decades pose larger questions regarding pesticide impact on biodiversity and thus affect ecosystem function (Köhler & Triebskorn, 2013). The sustainability challenges from maintaining biodiversity provides and human society needs faced by direct and indirect pesticide effects across levels of biological complexity. This dualism requires of human societies think about how to management the resources in a fair form, looking for solutions to mitigate the damages.

Our simulated experimental scenarios, using the recommended dose for tomatoes, carrots and onions of the fungicide Mythos® (Pyrimethanil) diagnose the possible impacts by the runoff and leaching into adjacent water bodies and surround soils. The results showed notably changes in the structure of the laboratory-derived acute, chronic and avoidance toxicity data for plants and freshwater and terrestrial invertebrates with changes and loss in the ecosystem services framework. This holistic measurement showed more accuracy in the environmental impacts on the structure and functioning of natural ecosystems making it an important tool for pesticides risk management programs. We concluded that the original ecological risk trends and potential projections of the studies for use in diagnostic of environmental impacts were more relevant when included new analyzes factors such as the holistic measurement of the effects of contaminants on the functions and ecosystem services in disturbances areas.

ACKNOWLEDGEMENTS

The authors would like to thank the Brazilian National Council for Scientific and Technological Development (CNPq) for the Ph.D. grant (process number 140415/2013-0) and University of São Paulo for the facilities. We thank the technicians at the Water Resources and Environmental Studies Center (São Carlos School of Engineering, Itirapina, Brazil) Amandio Nogueira and Marcelo Nogueira for technical assistance and guidance and finally, we thank Vanessa M. Oliveira and Bruna H. Vieira for assistance during experiments.

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CHAPTER V

Ecological risk assessment for ecosystem models in contamination scenarios of the fungicide Pyrimethanil

SANCHEZ, A. L., MENDES, L. B., JENSEN, J. & ESPÍNDOLA, E. L. G.

Ecological risk assessment for ecosystem models in contamination scenarios of the fungicide Pyrimethanil

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Abstract

Ecological risk assessment (ERA) studies are important to assess environmental changes that have been caused by anthropogenic activities. This integration models show the estimation of adverse risk effects across the biological organization levels potentially exposed to perturbation, including a better understanding of the ecosystems complexity. Our study adopts a two-tiered risk scenarios experiments to integrate terrestrial and aquatic responses to the fungicide Pyrimethanil. To attempt it, the experiments evaluated the exposure effects of the fungicide on different compartments and trophic levels with multitrophic interactions in ecosystem models and supplementary with individuals' responses, integrating and reporting a holistic four-tiered fungicide risk assessment approach. The results obtained suggest the possible impacts and the adverse effects on the terrestrial and aquatic organisms, ecosystems and processes in the simulate scenarios. We concluded that the integrated ERA for the fungicide pyrimethanil is an important register for the deleterious effects and responses to impacts of pesticides, prompting the possible environmental losses and changes of the ecosystems functions and services.

Keywords: Pesticides, ecotoxicology, mesocosms, ecosystem risk assessment

INTRODUCTION

Ecological risk assessment (ERA) is an increasingly important part of the decision-making process for managing the global sustainability challenges (Weeks et al. 2004). These challenges include air pollution, biodiversity loss, climate change, energy and food security, disease spread, species invasion, water shortages and ecosystem pollution and they are interconnected across three dimensions (organizational levels, space and time) (Liu et al. 2015). ERA is a process of collecting, organising and analysing environmental data to estimate the potential risk of the stressors for ecosystems (Jensen & Mesman, 2006). The aim of ERA is the estimation of adverse risk effects across the biological organization levels in locations that are potentially exposed to pollutants and other substances (Solomon & Sibley, 2002).

An approach for the analyses the disturbances effects is the analysis of the triad (Chapman & Hollert, 2006), integrating the different lines of evidence to determine the hazard effects to ecosystems. However, for the definitions of ecotoxicological hazards and risk, the risk is assessed through a combination of dangerousness due to exposure to a stressor and the effects on organisms, while the hazard is define only as to their ecotoxicological potential (EPA, 1992; De Lange, 2010; c). As a result, the hazard can be defined as the potential for production risks (De Lange, 2010) it is estimated from integrated analyses of environmental exposure factors. ERA also encompasses the relationship between stressors and the vulnerability of

species to environmental degradation, describing the processes that determine the level of exposure to individuals and the expression of this exposure into direct and indirect effects to show how the response on individuals to ecosystem levels (Van den Brink et al. 2011).

The pesticides risk assessment in Europe uses information from toxicity studies performed on vertebrates, invertebrates and primary producers (Maltby et al. 2009). In Netherlands the environmental risk evaluation procedure followed the predicted environmental concentration and compared to a reference effect concentration for the substances (Wipfler et al. 2015). The United Kingdom has published a set of detailed scientific reports for toxicants relies on comparing some exposure estimate for each chemical of interest with a corresponding toxicity threshold for a series of biological tests and a proposed decision-making framework (Weeks, 2004). However, USA Environmental Protection Agency provide an overview and guidelines for examination the ecological effects or toxicity, chemical fate and transport and the environmental exposure characterization for the evaluation the pesticides potential risk (EPA, 2005). The ERA conceptual framework in Canada is the application to multiple receptor groups via multiple exposure pathways considering various lines of evidence and often applied a weight of evidence approach (FCSAP, 2010). In addition, in Brazil a special committee on solid residues has discussed methodologies for ERA implementation of a guideline to be applied on contaminated sites (Niva et al. 2016).

Ecosystems worldwide are rapidly losing functional diversity as a result of human appropriation of natural resources with impacts on habitats loss and diversity (Solan et al., 2004; Hector & Bagchi, 2007; Naeem et al. 2012) and the intensification of agriculture have contributed to a devastating effect on biodiversity (Krebs et al. 1999). Nevertheless, the importance of the biodiversity for the integrated functioning of ecosystems remains unclear because most information focus only on the effect on individual functions and taxonomic groups (Lefcheck et al. 2015). Currently, one of the greatest challenges is how to use the fundamental ecological processes that link biodiversity, ecosystem functions and services, to meet the attempts to forecast the societal consequences of diversity loss and to meet an objective conservationist policies (Hooper et al. 2012).

A holistic systems integration approaches with various components of coupled human and natural systems across all dimensions is necessary to address complex interconnections and identify effective solutions to environmental degradation (Liu et al. 2015). Our study adopt an experimental approach to characterise the fungicide episodes hazards by a simulate scenarios of runoff and leaching on terrestrial and aquatic compartments on four-tiered ERA approach. The focus is on ecological effects of a pesticide to several non-target terrestrial and aquatic

organisms, the chemical fate, transport and the environmental exposure characterization in a three dimension risk scenarios (organizational levels, spacial and temporal exposure). The goal of the present study was to integrating the terrestrial and aquatic compartment responses to the fungicide pyrimethanil in an ERA framework. The risk scenarios experiments was designed to cross the immediate, middle and short term exposure effects of the fungicide on different compartments and trophic levels. In this sense it was analyzed by the responses of terrestrial and aquatic multitrophic interactions with ecosystem models and supplementary with individuals responses of terrestrial plants, soil invertebrates, cladoceran, algae, fish and tadpoles, integrating the data and reporting a holistic two tiers fungicide risk assessment.

MATERIAL AND METHODS

Experimental design

The fungicide pyrimethanil risk assessment was architected in a simulate scenarios with a terrestrial and aquatic compartments responses (Table 1). Our study considered as a risk modulate a three dimension risk assessment scenarios related to the organizational levels, temporal and spacial scale of impact. The simulate exposure considered three gradient of exposure scenarios appointed as immediate term (near from the contamination source), middle (intermediate from the contamination source) and short (far from the contamination source) term fungicide exposure. The hypothetical contamination source will be considered the distance in space, such as the gradient of concentration to ecotoxicological data, and time, such as the exposure for the ecological data.

The terrestrial compartment, based on a fungicide application on plants and an ecosystems models with the fungicide spray application, showed the effects on plants and soil invertebrates as well as the foraging responses of the perturbations. The aquatic compartment was based on a simulated runoff and leaching potential for aquatic non-target invertebrates and algae. Moreover an aquatic ecosystem models (mesocosms) was analysed with different trophic levels, simulating the fungicide effects on a shallow lakes. In addition, for the aquatic foraging behaviour was performed a linear avoidance tests with fishes and tadpoles and a fish acute test. Finally, integrating scaled data and calculating the contribution of each line of evidence for the compartments an accurately and holistic ecosystem approach was performed for the fungicide environmental risk assessment.

Test substance

Pyrimethanil (CAS: 53112-28-0) [IUPAC name N-(4,6-dimethylpyrimidin-2-yl)-aniline] is a colorless crystalline substance practically insoluble in water (121 mg/L), belonging to the anilinopyrimidine class (mode-of-action: methionine biosynthesis inhibition). In the form of concentrates, it is used as a contact fungicide with protective and curative properties (Sadlo, 2002). The structure of pyrimethanil is shown in Figure 1. The chemical compound has a moderate persistence in soil with half-life value of 55 days (Wightwick, et al. 2010). The commercial formulation are found in a chemical composition of suspension concentrate with tradenames of Scala® (400 g/l Pyrimethanil, 37.4 % w/w) indicated for the control of leaf scab in apples and grey mould in outdoor strawberries and the moderate control of grey mould in protected strawberries. The tradenames of Mythos® (300 g/l Pyrimethanil, 30.0 % w/w) indicated for the control of treatment of various diseases on banana, potato, onion, carrot, apple, strawberries, tomato and grapes crops. In general, for the ecosystem this fungicide has low ecological risk when applied according to agricultural recommended practices, but the risk is likely increase of accidental spills, inadequate application and environmental loading or disposal (EFSA, 2010; Verdisson et al. 2001; Müller et al. 2012; Seeland et al. 2012; Gil et al. 2015; Bandow et al. 2016)

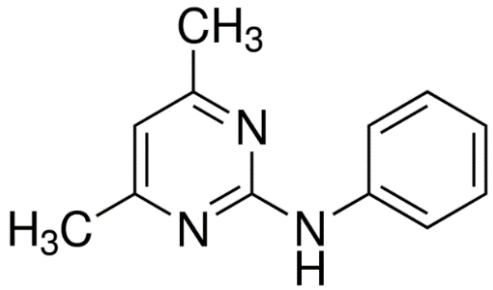

Figure 1. Chemical structure of Pyrimethanil.

Terrestrial plant test

An adapted version of Organization for Economic Cooperation and Development (OECD, 2006) guideline 208 for the testing of chemical testing a terrestrial plant species was used: seedling emergence and early seedling growth test conducted our experiments with terrestrial plants of the fungicide pyrimethanil. The experiments with commercial seeds plants (Plantae) of the fungicide pyrimethanil used *Solanum lycopersicon* (tomato) and *Allium cepa* (onion) seeds. The concentrations followed the fungicide Mythos® recommended dose values for these target agriculture cultures plants with the ratio of 1, 8 and 16 times the doses. The tests

used two scenarios based on tomato crops recommendation dose with 2.5 liters commercial formulation to 100 liters water per hectare ($1.6E^{-3}$, 0.11 and 1.6 mg pyrimethanil.kg $^{-1}$ soil dry weight) and onion crops with 200 ml commercial formulation to 100 liters water per hectare (1 E^{-4}, $6.1E^{-3}$ and 0.11 mg pyrimethanil.kg^{-1} soil dry weight). The terrestrial plants test was analyzed in three different germination periods (7, 14 and 21 days). The seedling growth parameters were analyzed after 21 days exposure through the fresh plants biomass (g), the length seedling (cm), length roots (cm) and the number of leaves (For more information see the Chapter IV).

Elutriate test

The elutriate solution was prepared with the remaining soil of the tomato and onion seedling emergence and growth test and unfiltered water using non-target freshwater organisms tests. The elutriate solution followed an adapted version of United State of America Environmental Protection Agency (EPA, 1998) evaluation of dredged material proposed for discharge in waters of the US testing manual.

The acute toxicity tests were performed with cladoceran neonates (Cladocera, Crustacea) representing the zooplankton such as *Daphnia similis* (Claus, 1876), *Ceriodaphnia silvestrii* (Daday, 1902) and *Ceriodaphnia dubia* (Richard, 1894), exposed of the *S. lycopersicon* or *A. cepa* elutriate solution. All procedures followed an adapted version of the Brazilian standard ABNT (ABNT, 2009) guideline ABNT NBR12713 for aquatic ecotoxicology with acute toxicity test with *Daphnia* spp (Crustacea, Cladocera). For the algae (Chlorophyta, Selenastraceae), representing the phytoplankton, the flasks containing 100 mL of *S. lycopersicon* or *A. cepa* elutriate solution were inoculated with an initial algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák 1990 (formerly known as *Selenastrum capricornutum*) cell concentration of 10^4 cells.mL^{-1}. After 96 hours the algae cells were sampled from each flask in 5 ml aliquots, fixed with lugol acetic and counted in a Neubauer chamber using a microscope. All procedures followed an adapted version of the Brazilian standard ABNT (ABNT, 2011) guideline ABNT NBR12648 for aquatic ecotoxicology method of chronic toxicity test with green algae (Chlorophyceae) (For more information see the Chapter IV).

Avoidance test

The earthworm (Annelida: Lumbricidae) was represented by the specie *Eisenia andrei* (Bouché, 1972). An adapted version of International Organization for Standardization (ISO,

2008) guideline 238 for the soil quality avoidance test for testing the quality of soils and effects of chemicals on behavior was used. The concentrations followed the fungicide Mythos® recommended dose values for the terrestrial plants carrot and onion (200 ml commercial formulation to 100 liters water per hectare) with the ratio of 1, 8 and 16 times the doses ($1E^{-5}$, $8E^{-5}$ and $1.6E^{-4}$ mg pyrimethanil.kg^{-1} soil dry weight) (For more information see the Chapter IV).

The springtail species (Arthropoda: Collembola) was *Folsomia candida* (Willem, 1902) followed the same procedures of the earthworms with 20 individuals of *F. candida* per container during 48 hours. The avoidance test was performed with an adapted version of International Organization for Standardization (ISO, 2007) guideline 264 for the avoidance test collembolans. The concentrations followed the fungicide Mythos® recommended dose values for the terrestrial plants carrot and onion (200ml commercial formulation to 100 liters water per hectare) with the ratio of 1, 8 and 16 times the doses ($8E^{-5}$, $6.7E^{-4}$ and $1.3E^{-3}$ mg pyrimethanil.kg^{-1} soil dry weight) (For more information see the Chapter IV).

The linear avoidance test in a multi-compartmented static test system was developed based on the foraging behavior of aquatic and terrestrial organisms. For the aquatic compartment was used frogs tadpoles (Amphibians: Anura) and the zebrafish (Pisces: Cypriniformes). The tadpoles was used two amphibian species, the tropical anuran *Leptodactylus latrans* (Steffen, 1815) and the North American bullfrog *Lithobates catesbeianus* (Shaw, 1802) with a 12 hours avoidance test (for more information see Araujo et al., 2014a). The zebrafish *Danio rerio* (Hamilton, 1822) followed the 4 hours and 12 hours avoidance (for more information see Araujo et al. 2014b). For the terrestrial compartment the foraging behavior was examined with adults' springtails specie (Arthropoda: Collembola) *Heteromurus nitidus* (Templeton, 1835) a detritivore and epedaphic as a surface-active animal. The soil system was composed by a transparent acrylic box with cover and six compartments. An avoidance assay for 48 hours with concentrations following the same values and procedures used at the soil application Scala® mesocosms as recommended dose of 2.5, 25 and 75 (1.4, 13.5 and 40 mg pyrimethanil.kg^{-1} soil dry weight) times the doses (more information see the Chapter III).

Table 1. Pyrimethanil environmental risk assessment framework based on a triad scenarios (chemical, ecotoxicological and ecological) for aquatic and terrestrial exposure. The risk calculation using different trophic levels potential risk organisms such as aquatic (zooplankton, phytoplankton, fish, anura, macroinvertebrates and macrophyte) and terrestrial (plantae, oligochaetas, collembolan and acari), integrating the simulate risk scenarios of the fungicide exposure.

RISK SCENARIOS		AQUATIC				TERRESTRIAL			
Triad		Taxon		Data-set	Endpoint	Taxon		Data-set	Endpoint
Chemical	Fungicide Pyrimethanil	Mesocosm concentrations (HPLC)		mg.L^{-1}	Decrease concentrations	Gil et al. 2015 (HPLC)		mg.Kg^{-1}	Decrease concentrations
Ecotoxicological	Individual toxicity	Crustacea	*D. similis* *C. silvestrii* *C. dubia*	Acute (48h)	Survival	Plantae	*S. lycopersicon*	Emergence (7, 14 and 21d)	Germination
		Algae	*P. subcapitata*	Chronic (96h)	Cell growth		*A. cepa*	Seedling (21d) Roots (21d) Leaves (21d) Biomass (21d)	Growth
		Fishes	*D. rerio*	Acute (48h) Acute (96h)	Survival				
				Avoidance (4h) Avoidance (12h)	Avoidance	Oligochaeta	*E. andrei*	Avoidance (48h)	Avoidance
		Amphibians	*L. latrans* *L. catesbeianus*	Avoidance (12h)	Avoidance	Collembola	*H. nitidus* *F. candida*	Avoidance (48h)	Avoidance
								Chronic (28d)	Reproduction
Ecological	Ecosystems models (Mesocosms)	Phytoplankton	Community	Diversity	Diversity	Oligochaeta Collembola Acari	Bait lamina	Functional responses (7, 14 and 28d)	Feeding activities
		Zooplankton	Community						
		Macroinvertebrates	Community	Colonization					
		Macrophyte	*P. stratiotes*	Decomposition	Decomposition				

Individual toxicity test

The fish acute toxicity test with the zebra fish *D. rerio* followed an adapted version of the Brazilian standard ABNT (ABNT, 2011) guideline ABNT NBR15088 for aquatic ecotoxicology with acute toxicity test with fish. The test was analyzed with fish mortality and immobility in 48 and 96 hours of exposure with concentrations of Mythos® (1, 30 and 45 $mg.L^{-1}$ pyrimethanil).

The springtail single specie reproduction test followed an adapted version of Organization for Economic Cooperation and Development (OECD 2009) guideline 232 for chemical testing conducted the single reproduction tests experiments. At the start of the test, 10 individuals of synchronized *Folsomia candida* (Willem, 1902) (3 weeks age) were added to each replicate (four replicates per concentration). The 28 days reproduction test was made with the commercial formulation (Scala®) and the active ingredient of the Pyrimethanil as recommended dose of 2.5, 25 and 75 (1.4, 13.5 and 40 mg $pyrimethanil.kg^{-1}$ soil dry weight) times the doses.

Ecosystems models

A constructed soil multi-species (SMS) test system was designed with a selected set of soil invertebrates exposed to pesticide in test containers during 28 days at 21 ± 1ºC in a 12/12 hours light-dark interval in a surface application (Spray) with a pesticide field applications simulator. The surface application followed the Scala® recommended dose values of 2.5, 25 and 75 (1.4, 13.5 and 40 mg $pyrimethanil.kg^{-1}$ soil dry weight) times the doses. To evaluate the fungicide effects on the terrestrial species interactions were added springtails species (Arthropoda: Collembola) *Heteromurus nitidus* (Templeton, 1835), *Hypogastrura assimilis* (Krausbauer, 1898), *Protaphorura fimata* (Gisin, 1952), *Proisotoma minuta* (Tullberg, 1871) and *Folsomia fimetaria* (Linné, 1758), earthworm (Annelida: Lumbricidae) with the surface epigeic specie *Eisenia fetida* (Savigny, 1826) and finally a mite specie (Arthropoda: Acari) *Hypoaspis aculeifer* (Canestrini, 1884). After 28 days the SMS organims was extracted. The SMS test system was supplemented with a measure of feeding activity using the bait-lamina test system. It consists of PVC-stripes perforated with 16 holes. The holes were filled with a organic substrate and sampling at three occasions (7, 14 and 28 days) (more information see Chapter III).

For the aquatic compartment an aquatic mesocosms system were designed with polypropylene water tanks (1500 liters) filled with natural water and sediment (Lobo reservoir,

Itirapina, Brazil). The tanks were constructed and started monitored six months before the experiments start. The zooplankton community was analyzed with integrated samplings on the water column with a PVC tube and sieved in a plankton mesh (68 µm) while the phytoplankton community were collected in the water sub-surface. For this risk assess were considered the initial samplings periods of daily in the first week and during 27 days after the fungicide applications, the interval of the sampling was 3 days. Moreover, it should be emphasized that all biological material was identified with the lowest possible taxonomic resolution with the aid of specific keys for each taxon and experts help. The decomposition rate experiments were constructed with dry aquatic plant *Pistia stratiotes* (Plantae: Araceae). The litter bags were design with polypropylene mesh (1 mm) with 15 x 10 cm and approximately 10 g of dry macrophyte. The rectangular bags were arranged in the middle of the water tanks mesocosms with help of a float and weight bearing. The bags were remove, dry the excess of water and reinstalled again in the tanks during the time 5, 10 and 30 days after the fungicide application (Pompeo & Moschini-Carlos, 2003). To measure the macroinvertebrates community of the system were design a colonization experiments. The artificial substrates were constructed with a mesh (1.5 cm) bags filled with different sizes rocks and left in the tank bottom. The removal of the structures followed the time -5, 0, 15 and 45 days after the fungicide exposure (Kikuchi & Uieda, 2005).

The mesocosms were contaminated with surface pyrimethanil application by the commercial formulation Mythos® followed the size of the water tank with dose values of 1.4 $mg.L^{-1}$ pyrimethanil. The chemical qualification and quantization of pyrimethanil in the mesocosms followed the methodologies of Müller et al. (2012) conducting by high pressure liquid chromatography (HPLC Shimadzu LC-20A, UV-VIS detector SPD-20A) using a C18 column (precolumn 4.3 × 10 mm, main column 4.3 × 150 mm, 5-µm particle size, 120-Å pore size). The applied isocratic method operated with 40 % methanol (A level) / 60 % pure water (B level) as a mobile phase (1 $mL \times min^{-1}$). After injection of 100 µl of sample, the mobile phase was gradually increased to 94 % A/6 % B within 15 min (25ºC).

Risk calculations

Overview of the framework

The environmental risk assessment followed a four-tiered approach (Jensen and Mesman 2006; Adler et al. 2016). These authors rescribed tier 0 assessing the development of a conceptual model, identifying potential contaminants, pathways and receptors. The Tier I

assesses the simple screening of the risk relevance based on data on the use, environmental release, and predicted exposure of the specific pesticide. In this case, including the initial organisms responses, such as the acute and avoidance responses. The Tier II normally is required only if risks assessed during Tier I exceed an exposure threshold value or action limit. However, if the pesticide product is being authorized as a plants protection, non-target effects must be specifically addressed and Tier II tests are mandatory. This phase decribed a refined screening, including the chronic organisms responses, such as the colembolan reproduction tests and the algae chronic tests. While Tier II are performed to reduce uncertainties about the actual risk, Tier I is essentially a screening phase, aiming to produce a first representation of the risk and to determine whether an experiment can be excluded from higher tiers of testing or it needs to be further evaluated (Weeks et al. 2004). If risks assessed exceed an exposure threshold value or action limit the Tier III is required. This phase described a detailed screening, including the ecosystem responses, representing by the terrestrial and aquatic experimental ecosystem models (mesocosms). The Tier IV described a final assessment, defining acceptable results and perform the data integration (Jensen and Mesman 2006).

Triad approach

The first model to pyrimethanil risk assessment calculation followed the principles of the triad approach. The risk characterization was based on the triad of the chemical, ecotoxicological and ecological line of evidences (Loe). The exposure scenarios (immediate, middle and short exposure) represented the aquatic, terrestrial and ecosystem risk with the integration of the information from all the Loe. It is appropriate to point out that the ecosystem and the integrated risk are not a sum of the compartments but a mathematical integrated. The chemical line of evidence was calculated based on the toxic pressure for the measure extractable fraction of the fungicide pyrimethanil from the contaminated water and soil samples. The HC50 (hazardous concentration for 50% of the species) was used to calculate the toxic pressure of one contaminant. When the $HC50_{EC50}$ value was not available in the literature the safety factor of 10 to the $HC50_{NOEC}$ (Rutgers et al. 2008) was applied. The aquatic HC50 followed the EC_{50} for *D. magna* (Seeland et al., 2012) and the terrestrial followed the EC_{50} for *F. candida* (Gil et al., 2015). For the terrestrial toxic pressure was used the measure extractable fraction of the fungicide pyrimethanil in soil samples by Gil et al. (2015). The ecotoxicological line of evidence used as default the results from the terrestrial and aquatic toxicity test expressed as percentages. The ecological line of evidence considered ecological observations of the terrestrial and aquatic ecosystem model such as results of the aquatic organisms' diversity and

the soil invertebrates feeding activities. The risk calculations followed the approach proposed by Jensen and Mesman (2006) where the lines of evidence were integrated with the reference in a risk score expressed from zero (no risk) to one (high risk). The formulas and steps calculations are shown in the Box 1.

Box 1. Environmental risk assessment framework based on the triad of the line of evidence chemical, ecotoxicological and ecological and the steps calculations for the integrated risk and the risk indicators based on Jensen & Mesman (2006) risk assessment model.

1 - CHEMICAL LOE	2 - ECOTOXICOLOGICAL LOE	3 - ECOLOGICAL LOE
Step 1. Measured concentration (mg.Kg $^{-1}$) *R1 Samples* **Step 2.** Generic screening values (mg.Kg $^{-1}$) *R2 = HC50* **Step 3.** For each contaminant, calculate toxic pressure *R3=1/(1+exp^((logR2-logR1)/0,4)* **Step 4.** Correct for background concentrations *R4=(R3-R3ref) / (1-R3ref)* **Step 5.** Calculate the combined risk for n chemicals *TPMM = 1-(1-R5(1)) * (1-R5(2))*…* (1-R5(n))* **Step 6.** Scale the potentially affected fraction of species values *R6= (Sample-REF) / (1-REF)* **Obs.** When no $HC50_{EC50}$ available in literature are applied the safety factor of 10 to the $HC50_{NOEC}$ $HC50_{EC50} = 10 * HC50_{NOEC}$	**Step 1.** Divide data test result by 100 *R1=(100-X) / 100* **Step 2.** Scale difference between X and reference (Negative values are converted in zero) *R2= (X-Ref) / (1-Ref)* **Step 3.** Integrated the ecotoxicological data *R1= log (1-X)* **Step 4.** Average of the test *R2 = Average (X1… Xn)* **Step 5.** Retro-transform values *R3= 1-(10 ^ X)*	**Step 1.** Ratio between site x and reference *R1=X / 100* **Step 2.** Positive values are converted to zero *Calculate absolute values of log (R1)* **Step 3.** Calculate sum of all values and multiply with -1 *R3 = -1 * Σ (R2)* **Step 4.** Calculate number of endpoints. *R4 = N* **Step 5.** Apply formula *1-10 ^ (R3 / R4)*
	4 - INTEGRATED RISK	**RISK INDICATORS (IR = Integrated Risk)**
	Step 1. Risk values for lines of evidence **Step 2.** Calculate log to (1-scaled result). *R1= log (1-X)* **Step 3.** Average all log-values to one integrated log value *R2 = Average (X1… Xn)* **Step 4.** Transform log-values into integrated risk (IR) values *R3= 1-(10 ^ X)*	0 < IR < 0.25 **No risk** 0.26 < IR < 0.50 **Low risk** 0.51 < IR < 0.75 **Moderate risk** 0.76 < IR < 1.00 **High risk**

Deterministic approach

The second model to pyrimethanil risk assessment calculation is the most widely used method. In this system, the environmental concentrations of a stressor are compared to an effect concentration with the simple ratios of single exposure and effects values and may be used to express hazard or relative safety (Solomon & Sibley, 2002). The approach based on the technical overview of ecological risk assessment proposed by the United States Environmental Protection Agency (EPA, 2016). That the risk estimation compares exposure and effects data, considers integrated exposure and effects data and states the potential for risk. The risk

description interprets risks based on assessment endpoints and the risk assessor evaluates the lines of evidence supporting or refuting the risk estimates. This methodologie uses a deterministic approach to compare toxicity to environmental exposure. In the deterministic approach, a risk quotient (RQ) is calculated by dividing a point estimate of exposure by a point estimate of effects (Box 2).

Box 2. Environmental risk assessment framework based deterministic approach proposed by the United States Environmental Protection Agency (EPA, 2016).

INTEGRATED RISK	RISK QUOTIENT (RD)
$Risk\ Quotient\ (RQ) = \frac{Exposure}{Toxicity}$	$0.00 < RQ \leq 0.05$ **acute endangered species** $0.05 < RQ \leq 0.1$ **acute restricted use** $0.05 < RQ \leq 0.1$ **acute restricted use** $RQ \geq 1.0$: **chronic risk**

This ratio is a simple, screening-level estimate that identifies high or low risk situations. In this study, the estimated environmental concentration for the terrestrial and aquatic environmental is compared to pyrimethanil effect level used the EC_{50} (the concentration of a pesticide where caused 50 % reduction of the organisms) of the toxicity tests.

Data analysis

For the estimates of the concentrations that caused 50 % reduction in organisms output (EC_{50}) was used the logistic model calculations:

$$y = \frac{c}{1 + \left(\frac{Conc}{ECp}\right)^{b}}$$

where c is the mean control value, b is the slope parameter.

All toxicity tests were pairwise comparison of means with the control with 0.05 significance were used ANOVA and Dunnett's test values. The checking of the homogeneity of variances preceded the variance analysis.

The linear avoidance tests (fishes, frogs and collembolan) calculations used the number of avoiders for each compartment following the equation *Avoiders = ne - no*, where *ne* is the expected organisms and *no* the number of observed organisms. The compartment with the highest concentration, *ne* is equal to the number of organisms introduced in the compartment at the start of the test; for the remaining compartments, *ne* includes the organisms introduced initially in the compartment plus the organisms introduced in the adjacent compartments of

higher concentration (Araujo et al. 2014a). The earthworms and springtails avoidance test was calculation according to the equation.

$$\%\ Avoidance = \frac{(nc - nt)}{N} * 100$$

where nc = number of individuals in the control, nt = number of individuals in the test soil, N = total number of individuals.

Results obtained in the dual tests from both test organisms were analyzed by the Fisher exact test.

For the ecology parameters were calculated the diversity index for the aquatic ecosystem models. The Shannon-Wiener's diversity index is commonly used to characterize species diversity in a community accounts for both abundance and evenness of the species present.

$$H' = -\sum_{i=1}^{S} pi \ln pi$$

where $pi = ni/n$; ni = total number of individuals per taxon; n= total number of individuals.

Another way to represent the diversity was the Margalef's richness index represent the total number of species in the sample unit.

$$d = \frac{S - 1}{log2\ N}$$

where S = species number; N individuals number.

The principal response curves (PRC) was used to represent the effects of the fungicide pyrimethanil on the macroinvertebrates community colonization experiment. The treatment explained a significant part of the total variance, of which also a significant part is displayed in the first and second PRC ($p < 0.05$, Monte Carlo permutation test with permuting whole time series only) (Van den Brink et al. 2003). The data were analyzed using the statistical programs STATISTICA (version 7.0, StatSoft, Inc.), XLSTAT (version 2014.5.03), CANOCO (version 5.0) and the PAST (version 2.17).

RESULTS

Tier I - Simple screening

The integrated of the multiple terrestrial and aquatic results after nearly 30 days exposure of the fungicide pyrimethanil and the data transformation to environmental risk assessment showed adverse effects on the ecosystems. The fungicide concentrations affect all the organisms and the simulate scenarios of immediate, middle and short-term exposure showed

accurately the vision about the time, exposure and degradation of the contaminant to the ecosystem.

Tier II - Refined screening

The ecotoxicological Loe showed high risk for the immediate exposure. The ecological Loe reported low risk for the aquatic and ecosystem compartment but no risk for the terrestrial compartment. The chemical Loe showed high and moderate risk for the terrestrial and ecosystem compartment but without risk for compartment aquatic (Figure 3). However, for the trophic levels the response was variable according to the specific characteristics of the exposure and toxicity (Figure 2). For the plants of tomatoes the risk was strongest in the first days of seed emergence but for onions the values was not the same, varying the specie-specific time of seed emergence. The soil invertebrates showed, with some exceptions, moderate and high risk for the chronic and avoidance assays.

In relation to the aquatic compartment the cladoceran followed the exposure gradient with a high risk on the elutriate tests, except to *D. similis* with moderate risk in onion elutriate. The algae had the same pattern followed the exposure gradient and more moderate on onion elutriate. The fish showed high risk for the immediate term exposure and an opposite risk for the avoidance assays with a decrease of 4 hours exposure and increase of 12 hours exposure. Although the tadpoles showed a moderate to low risk on the 12 hours linear avoidance tests.

Tier III - Detailed screening

To exemplify and better understand the simulated scenarios considered in the risk assessment some results of the Tier 2 are plotted such as the single reproduction test of the soil invertebrates *F. candida* after 28 days exposure showed a decrease with the increase of the concentrations in reproductive outputs (Figure 2a). The decomposition of the macrophyte *P. stratiotes* in aquatic mesocosms experiments on 4 periods (0, 5, 10 and 30 days exposure) showed a decrease of the plants weight during the physical aquatic process (Figure 2b). In the terrestrial ecosystem models the invertebrates responses to the chemical was measured to the feeding activities representing a functional response by the bait lamina score in 3 periods (7, 14 and 28 days exposure).The terrestrial mesocosms experiments showed an increase with the time exposure (Figure 2c).

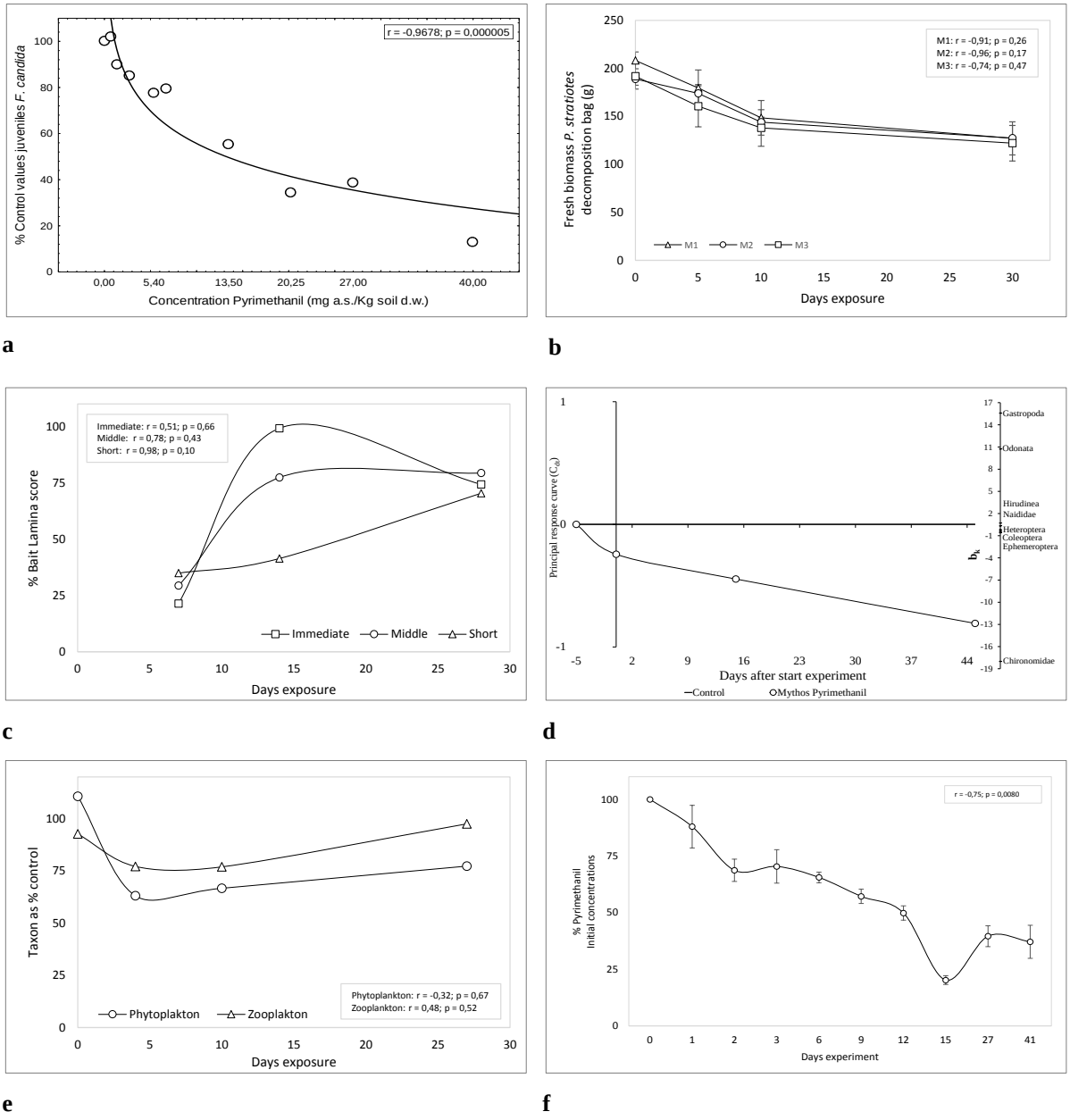

Figure 2. Pyrimethanil Tier 2 results such as the reproduction test of the soil invertebrates *F. candida* after 28 days exposure (a), macrophyte *P. stratiotes* decomposition results of the exposure in the aquatic mesocosms (M) experiments on 4 periods (0, 5, 10 and 30 days exposure) (b), feeding activities representing by the bait lamina score in terrestrial mesocosms experiments on 3 periods (7, 14 and 28 days exposure) (c), principal response curve (PRC) with species weights (bk) for macroinvertebrates colonization in the aquatic ecosystem models on 4 periods (-5, 0, 15 and 45 days exposure) (d), the taxon number average of phytoplankton and zooplankton as percentage of the control in the aquatic ecosystem models on 4 periods (0, 4, 10 and 27 days exposure) (e), and the Pyrimethanil HPLC analyze with the degradation of the fungicide in the water mesocosms (f).

The principal response curve (PRC) indicating the effects of the fungicide pyrimethanil on the macroinvertebrates colonization (Figure 2d). Of all variance, 36.9 % could be attributed to sampling date; this is displayed on the horizontal axis. The lines represent the course of the treatment levels in time, representing no direct effect with the fungicide to the colonization process. The species weight (b_k) can be interpreted as the affinity of the taxon with the Principal

Response Curves (C_{dt}). The species with positive b_k had a decrease during the time while the species with negative b_k had an increase during the time exposure. The aquatic ecosystem models showed phytoplankton and zooplankton community as on 4 periods (0, 4, 10 and 27 days exposure) represented as percentage of the control (Figure 2e). However, the degradation of the fungicide measure with HPLC analyze on the mesocosms' water during 30 days showed decrease of the concentration followed the half-life of the pesticide in water (Figure 2f).

For ecological responses in aquatic ecosystem models the phytoplankton diversity showed a decrease risk values from immediate to short term exposure, corroborating the distribution increase during the time in the mesocosms tanks. While the zooplankton diversity had an increase risk values from immediate to short term exposure, explain with the distribution decrease during the time in the mesocosms tanks. In the other hand, there was an increase of the macroinvertebrates risk followed the time exposure, but with the abundance showing an increase of colonization. The similar pattern was reported for the macrophyte decomposition rate. For the terrestrial ecosystems models the feeding activities represented by the bait lamina score showed an almost homogeneous low risk during the time exposure.

Tier IV - Final assessment

The environmental risk assessment for the fungicide pyrimethanil considered the triad with data of chemical (ChLoe), ecotoxicological (EcLoe), and ecological (ELoe) lines of evidence. The individual contribution and the combined calculated risk values for each Loe are shown in the Table 2. These values followed the risk score based on Jensen & Mesman (2006) risk assessment model.

The risk indicators score between zero (no risk) to one (high risk) indicate limits of accepted risk values for different soil uses (nature, agricultural, residential and industrial land-use), according to the risk indicators ($0.00 < IR < 0.25$ no risk; $0.26 < IR < 0.50$ low risk; $0.51 < IR < 0.75$ moderate risk; $0.76 < IR < 1.00$ high risk). The ternary graphs represent the contribution of each Loe for the integrated risk value being an indicator of the weight of evidence (Figure 3). However the risk was presented in terrestrial risk, aquatic risk and the integrated of ecosystem risk showed a decrease of the risk from immediate to short term exposure effects.

Table 2. Triad approach with the individual risk values for each exposure scenarios (immediate, middle and short exposure), representing the aquatic, terrestrial and ecosystem risk with the combination of the information from the chemical (ChLoe), ecotoxicological (EcLoe), and ecological (ELoe) lines of evidence and the integrated risk. The risk indicators (0.00 < IR < 0.25 no risk; 0.26 < IR < 0.50 low risk; 0.51 < IR < 0.75 moderate risk; 0.76 < IR < 1.00 high risk) were based on Jensen & Mesman (2006) risk assessment model.

ECOTOXICOLOGICAL Loe	Immediate	Middle	Short
- Terrestrial organisms			
Germination(Tomato-7d)	0.99	1.00	1.00
Germination(Tomato-14d)	0.96	0.92	0.94
Germination(Tomato -21d)	0.18	0.13	0.41
Seedling(Tomato-21d)	0.74	0.72	0.72
Roots(Tomato-21d)	0.71	0.75	0.69
Leaves(Tomato-21d)	0.76	0.64	0.94
Biomass(Tomato-21d)	0.85	0.87	0.86
Germination(Onion-7d)	0.00	0.33	0.99
Germination(Onion-14d)	0.03	0.08	0.08
Germination(Onion-21d)	0.11	0.08	0.19
Seedling(Onion-21d)	0.49	0.42	0.37
Roots(Onion-21d)	0.52	0.33	0.19
Leaves(Onion-21d)	0.02	0.00	0.07
Biomass(Onion-21d)	0.46	0.43	0.31
F. candida(28d)	0.87	0.00	0.66
E. andrei(Avoidance-48h)	0.99	0.67	0.87
F. candida(Avoidance-48h)	0.68	0.54	0.61
H. nitidus(Avoidance-48h)	0.80	0.41	0.55
-Aquatic organisms			
D. similis(Tomato-48h)	0.99	0.99	0.25
C. silvestrii(Tomato-48h)	0.99	0.99	0.99
C. dubia(Tomato-48h)	0.99	0.99	0.90
D. similis(Onion-48h)	0.55	0.30	0.00
C. silvestrii(Onion-48h)	0.95	0.99	0.40
C. dubia(Onion-48h)	0.95	0.85	0.70
P. subcapitata (Tomato-96h)	0.99	0.99	0.68
P. subcapitata (Onion-96h)	0.73	0.53	0.32
D. rerio(48h)	0.99	0.00	0.00
D. rerio(96h)	0.99	0.20	0.00
D. rerio(Avoidance-4h)	0.70	0.05	0.05
D. rerio(Avoidance-12h)	0.45	0.99	0.99
L. latrans(Avoidance-12h)	0.48	0.25	0.18
L. catesbeianus(Avoidance-12h)	0.16	0.18	0.18
Aquatic EcLoe	0.93	0.89	0.67
Terrestrial EcLoe	0.75	0.68	0.74
Ecosystem EcLoe	0.86	0.80	0.71

ECOLOGICAL Loe	Immediate	Middle	Short
- Aquatic mesocosms			
Phytoplankton			
Taxon	0.18	0.29	0.26
Abundance	0.03	0.07	0.01
Shannon	0.24	0.08	0.04
Margalef	0.21	0.32	0.30
Zooplankton			
Taxon	0.14	0.10	0.04
Abundance	0.48	0.35	0.12
Shannon	0.03	0.07	0.12
Margalef	0.12	0.09	0.06
Macroinvertebrates			
Taxon	0.04	0.07	0.03
Abundance	0.05	0.06	0.15
Shannon	0.00	0.06	0.04
Margalef	0.05	0.11	0.04
Macrophyte			
Decomposition rate	0.06	0.13	0.19
-Terrestrial mesocosms			
Bait Lamina	0.27	0.25	0.33
Aquatic ELoe	0.31	0.34	0.27
Terrestrial ELoe	0.06	0.05	0.07
Ecosystem ELoe	0.36	0.38	0.33

CHEMICAL Loe	Immediate	Middle	Short
Pyrimethanil water	0.15	0.12	0.08
Pyrimethanil soil	0.82	0.78	0.69
Aquatic ChLoe	0.15	0.12	0.08
Terrestrial ChLoe	0.82	0.78	0.69
Ecosystem ChLoe	0.84	0.80	0.72

INTEGRATED RISK	Immediate	Middle	Short
Integrated Risk$_{Aquatic}$	0.66	0.60	0.39
Integrated Risk$_{Terrestrial}$	0.65	0.59	0.58
Integrated Risk$_{Ecosystem}$	0.76	0.71	0.62

RISK INDICATORS (IR = Integrated Risk)
0.00 < IR < 0.25 No risk
0.26 < IR < 0.50 Low risk
0.51 < IR < 0.75 Moderate risk
0.76 < IR < 1.00 High risk

The triad approach, combination of the three lines of evidence into an integrated fungicide pyrimethanil ERA, showed the three dimensions of the risk along the risk scenarios. For the aquatic integrated risk had low risk to short term exposure to moderate risk for the others exposures with more weight for the ecotoxicological Loe (moderate to high risk) followed by the ecological Loe (low risk) and chemical Loe (no risk). The terrestrial integrated risk showed moderate risk increase followed the gradient terms of exposure with more weight for the chemical Loe (moderate to high risk) followed by the ecotoxicological Loe (moderate risk) and ecological Loe (no risk). Finally, the ecosystem integrated risk showed moderate risk for the short and middle-term exposure and high risk for the immediate-term exposure.

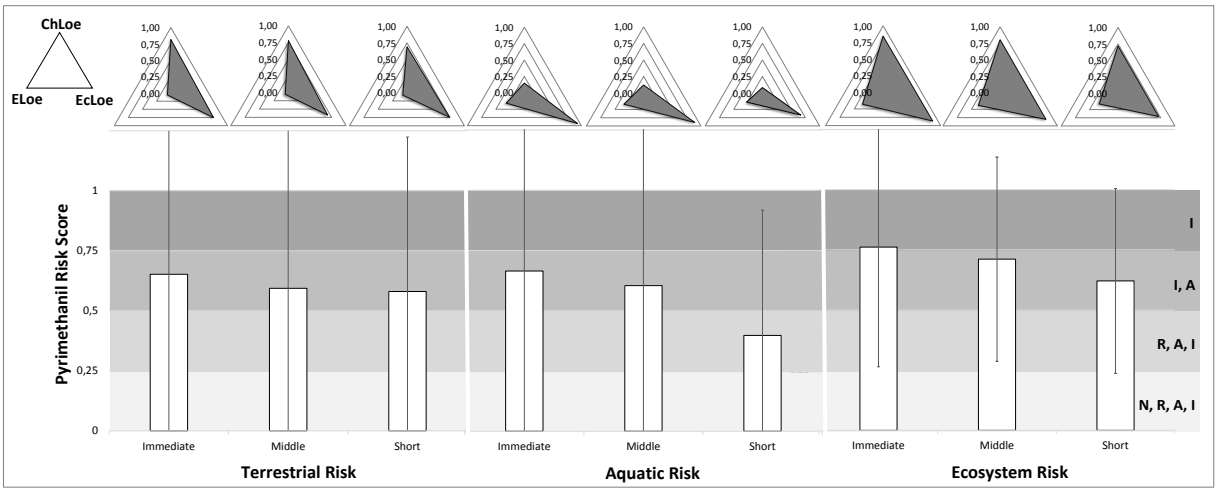

Figure 3. Pyrimethanil integrated environmental risk assessment values based on the triad approach for each exposure scenarios (immediate, middle and short exposure), representing the aquatic, terrestrial and ecosystem risk with the combination of the information from the chemical (ChLoe), ecotoxicological (EcLoe), and ecological (ELoe) lines of evidence (Loe). The gray bands indicate limits of accepted risk values for different soil uses (N = nature, A = agricultural, R = residential and I = industrial land-use), according to the risk indicators (0.00 < IR < 0.25 no risk; 0.26 < IR < 0.50 low risk; 0.51 < IR < 0.75 moderate risk; 0.76 < IR < 1.00 high risk). The ternary graphs on top of each bar represent the contribution of each Loe for the integrated risk value being an indicator of the weight of evidence (on the top left the example the length of each axis of the triangle represent maximum risk (1) from each Loe). The calculations were based on Jensen & Mesman (2006) risk assessment model.

The integrated risk values followed the exposure gradient from immediate, middle and short-term exposure. For the aquatic integrated risk showed low risk (0.39) to short-term exposure to moderate risk (0.60 and 0.66) for the others exposures. The terrestrial integrated risk showed moderate risk increase followed the exposure gradient (0.58, 0.59 and 0.65 respectively). Finally, the ecosystem integrated risk showed moderate risk for the short (0.62) and middle-term (0.71) exposure and high risk for the immediate-term exposure (0.76).

The deterministic approach, following the technical overview of ecological risk assessment proposed by the USA Environmental Protection Agency (EPA, 2016), showed a

general similar pattern to the previous model. This approach compares the different toxicity endpoint (EC_{50}) to the terrestrial and aquatic organsims to environmental exposure (Table 3). Moreover, the risk was presented in terrestrial risk, aquatic risk and the integrated of ecosystem risk showed a decrease of the risk from immediate, middle and short-term exposure (Figure 4). The fungicide toxicity had complexity responses from single species to a mesocosms exposure. For the terrestrial compartment, the plants and the soil invertebrates' avoidance had low risk quotient ranked as acute endangered species (Table 3). However, in the terrestrial mesocosms the invertebrates had high risk for immediate and middle-term exposure ranked as chronic risk. The aquatic compartment reported crescent high risk for the avoidance tests, cladoceran and algae exposure for short to immediate term exposure ranked as chronic risk.

DISCUSSION

Tier I - Simple screening

This study has shown the integrated holistic fungicide pyrimethanil ERA considered a three dimension risk scenarios (organizational levels, spacial and temporal exposure) of the terrestrial and aquatic compartment multiple data. The study, after nearly 30 days, crossing the immediate, middle and short term exposure effects, showing adverse effects on the ecosystems simulate scenarios. In this sense, following the basic principes of ERA, that is a process to evaluate the probability of adverse ecological effects from exposure to one or more stressors (EPA, 1998), a two-tiered approach was analyzed to proposed a two-tiered fungicide risk assessment.

The risk scenarios was designed according to plants protection products and their residues and the hypotethical entry on freshwaters ecosystems as a shallow lakes indirectly via surface runoff or drainflow induced by rainfall or irrigation and surronding soils with biological and ecological responses. The effects of species loss and the changes in composition by the chemical concentrations as consequences of this type of contamination are less clear (Maltby & Hills, 2008). The data compiled in the pyrimethanil ERA showed decrease of species such as the springtails survival in a chronic test, the maintained of the physical process of macrophyte decomposition rate on water mesocosms experiments and the increase of the species responses such as the feeding activities on the terrestrial ecosystem models (Figure 2).

Table 3. Deterministic approach with the individual risk quotient values for each endpoint of the compartments terrestrial ($mg.Kg^{-1}$) an aquatic ($mg.L^{-1}$) on mesocosms, acute, chronic and avoidance exposure. The calculated followed the exposure scenarios (immediate, middle and short exposure). The limits of accepted risk values are based on the risk quotient according to the risk quotient ($(0.00 < RQ \leq 0.05$ acute endangered species; $0.05 < RQ \leq 0.1$ acute restricted use; $0.1 < RQ < 1$ acute high risk; $RQ \geq 1.0$: chronic risk). The calculations were based on USA Environmental Protection Agency (EPA) risk assessment model.

Compartment		Endpoint	Exposure	Exposure scenarios				Risk Quotient		
				EC_{50}	Immediate	Middle	Short	RQ Immediate	RQ Middle	RQ Short
Terrestrial ($mg.Kg^{-1}$)	Plants	Germination(Tomato21d)	Acute	1300	1.6	0.11	$1.6E^{-3}$	$< 1E^{-3}$	$< 1E^{-3}$	$< 1E^{-3}$
		Seedling(Tomato)		0.02	1.6	0.11	$1.6E^{-3}$	0.08	$5E^{-3}$	$< 1E^{-3}$
		Biomass(Tomato)		0.012	1.6	0.11	$1.6E^{-3}$	0.13	$8.9E^{-3}$	$< 1E^{-3}$
		Germination(Onion21d)		265	0.11	$6.1E^{-3}$	$1E^{-4}$	$< 1E^{-3}$	$< 1E^{-3}$	$< 1E^{-3}$
		Seedling(Onion)		$2\ E^{-3}$	0.11	$6.1E^{-3}$	$1E^{-4}$	0.05	$2.7E^{-3}$	$< 1E^{-3}$
		Biomass(Onion)		$3\ E^{-3}$	0.11	$6.1E^{-3}$	$1E^{-4}$	0.04	$2.1E^{-3}$	$< 1E^{-3}$
	Terrestrial mesocosm	*E. fetida*(28d)	Mesocosm	3.3	40	27	1.4	12.2	8.2	0.43
		H. nitidus(28d)		17.3	40	27	1.4	2.3	1.6	0.08
		P. fimata(28d)		15.8	40	27	1.4	2.5	1.7	0.09
		P. minuta(28d)		16.6	40	27	1.4	2.4	1.6	0.08
		F. fimetaria(28d)		39.6	40	27	1.4	1.0	0.7	0.04
		H. aculeifer(28d)		46.2	40	27	1.4	0.9	0.6	0.03
	Terrestrial avoidance	*H. nitidus*(48h)	Avoidance	28.5	40	27	1.4	1.4	0.9	0.05
		E. andrei(48h)		$3.6E^{-6}$	$1.6E^{-4}$	$8E^{-5}$	$1E^{-5}$	$4.4E^{-2}$	$2.2E^{-2}$	$2.8E^{-3}$
		F candida(48h)		$1.8E^{-6}$	$1.3E^{-3}$	$7E^{-4}$	$8E^{-5}$	0.72	0.37	$4.4E^{-2}$
	Terrestrial	*F. candida*(28d)	Chronic	17.1	40	27	1.4	2.3	1.6	0.08
Aquatic ($mg.L^{-1}$)	Elutriate	*D similis*(Tomato)	Acute	$1.7E^{-3}$	1.6	0.11	$1.6E^{-3}$	0.97	0.06	$< 1E^{-3}$
		D similis(Onion)		$1.6E^{-3}$	0.11	$6.1E^{-3}$	$1E^{-4}$	0.07	$4E^{-3}$	$< 1E^{-3}$
		C. silvestrii(Onion)		$4.1E^{-5}$	0.11	$6.1E^{-3}$	$1E^{-4}$	2.68	0.15	$2E^{-3}$
		C. dubia(Onion)		$6.1E^{-5}$	0.11	$6.1E^{-3}$	$1E^{-4}$	1.80	0.10	$< 1E^{-3}$
		P. subcapitata (Tomato)	Chronic	$1.2E^{-3}$	1.6	0.11	$1.6E^{-3}$	1.32	0.08	$< 1E^{-3}$
		P. subcapitata (Onion)		$5.7E^{-4}$	0.11	$6.1E^{-3}$	$1E^{-4}$	0.19	0.01	$< 1E^{-3}$
	Aquatic	*D. rerio*(48h)	Acute	32.2	45	30	1	1.4	0.9	0.03
		D. rerio(96h)		27.4	45	30	1	1.6	1.1	0.04
	Aquatic avoidance	*D. rerio*(4h)	Avoidance	1.1	1.4	0.7	0.2	1.3	0.6	0.2
		L. latrans(12h)		0.41	1.4	0.7	0.2	3.4	1.7	0.49
		L. catesbeianus(12h)		0.48	1.4	0.7	0.2	2.9	1.5	0.42

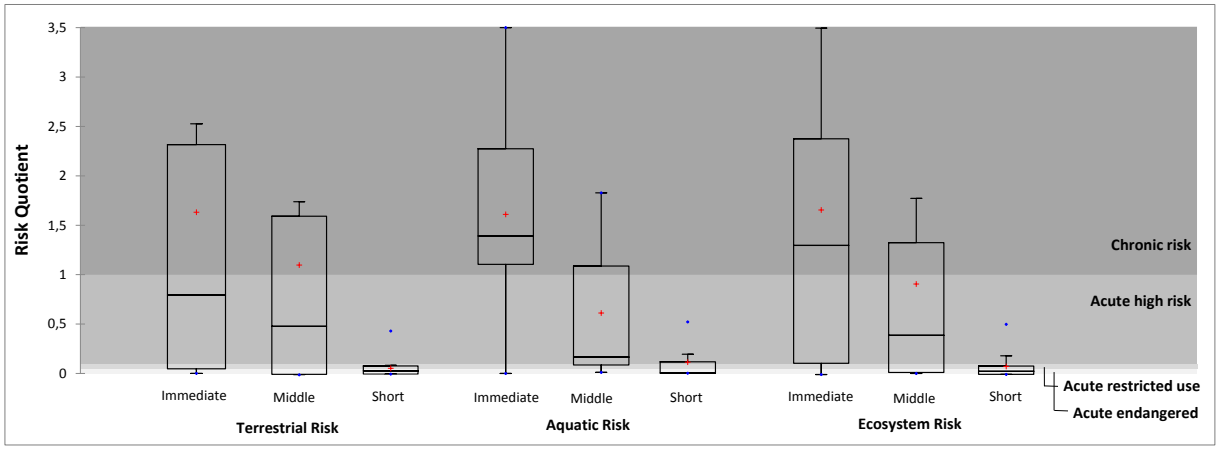

Figure 4. Boxplots of the pyrimethanil potencial environmental risk assessment values based on the deterministic approach with the risk quotient (RQ) for each exposure scenarios (immediate, middle and short term exposure), representing the aquatic, terrestrial and ecosystem risk. The gray bands indicate limits of accepted risk presumptions values, according to the risk quotient ($0.00 < RQ \leq 0.05$ acute endangered species; $0.05 < RQ \leq 0.1$ acute restricted use; $0.1 < RQ < 1$ acute high risk; $RQ \geq 1.0$: chronic risk). The calculations were based on USA Environmental Protection Agency (EPA) risk assessment model.

Ecosystems worldwide are losing some species and gain others, resulting in interchanges of species, traits and interactions and alteration of ecosystem functioning and services (Wardle et al. 2011). Besides the increase of complexity to communities of species and the function of ecosystems the trace and side of the chemicals effects are the major challenges in ecotoxicology (Köhler & Triebskorn, 2013). Here, we provided an integrated view of fungicide effects using the measure extractable fraction of the fungicide pyrimethanil analyze on the terrestrial and aquatic experiments, showing a decrease of the concentration and allowing the cross an immediate, middle and short term exposure effects on the different compartments and trophic levels.

Tier II - Refined screening

The chemical Loe showed high to moderate risk for the terrestrial and ecosystem compartment but without risk for the aquatic compartment. One plausible explanation is relative to the fungicide application on the water surface (1.4 $mg.L^{-1}$) on the aquatic ecosystem model, based in the EC_{50} for *D. magna* (Seeland et al., 2012), and it showed low effects for the ecological interactions and physical process changes. The assessment of pesticide risks to non-target organisms of the community structure is related to the abundance and biomass of all populations and their spatial, taxonomic, and trophic organization, where the function relates to the processes and the changes in time and flows (Van den Brink et al. 2005).

The ecotoxicological Loe showed moderate to high risk for the fungicide exposure. The trophic levels showed variable responses according to the specific characteristics of the exposure and toxicity. For the terrestrial compartment, tomatoes plants reported strongest risks in the first days of seed emergence but for onions the values were not the same, varying the specie-specific time of seeds emergence. The seeds germination and roots elongation is a rapid and widely used acute phytotoxicity test with several advantages of sensitivity, low cost and suitability for unstable chemicals or samples (Lin & Xing, 2007). These authors reported the seeds coat plays a very important role in protecting the embryo from harmful external hazards factors with a selective seed thickness and permeability. Thus the pollutants, though having obviously inhibitory effect on roots growth, may not affect germination if they cannot pass through seed coats. The plants support a wide array of herbivore species in the ecosystems that feed upon them, as well as the predators that feed on herbivores, the loss of plant diversity should propagate up to consumers, influencing the structure and diversity of associated communities at higher trophic levels (Dinnage et al. 2012).

The soil invertebrates showed, with some exceptions, moderate and high risk for the chronic and avoidance assays. The linear avoidance for the springtail *H. nitidus* showed high risk for immediate term exposure. In springtails the olfactory cues are an important sense for foraging behavior orientating the movement away from high toxicity substances (Staaden et al. 2011). Furthermore, it are able to avoid on different sensitivities for the contaminated soils (Luz et al. 2004) and the choice behavior may indeed be affected by intraspecific interactions (Filser et al. 2014). The introduction of foraging behavioral aspects of soil animals in ERA would help to better assess the habitats disturbance responses (Boitaud et al. 2006). This ability to detect and avoid to contaminated sites has been studied in a linear gradient exposure as efficient tools for soil (Chauvat et al. 2014) and aquatic organisms (e.g. Araujo et al. 2014a; Araujo et al. 2014b; Vasconcelos et al. 2016).

The risk for the aquatic linear avoidance with fishes and tadpoles registered a more variable values, decrease with the time exposure, relation to the mixed of the chemical in the water and the organisms foraging ability. The experiments assess the preferred spatial distribution along habitat gradients and the extent contamination with the swimming ability (Araujo et al. 2014b). For fishes and tadpoles living in environments receiving stressor substances, moving away seems an obvious way to avoid harm (Tierney, 2016). The cladoceran and algae followed the exposure gradient with a high risk on the elutriate tests. The recommended dose concentration that may be expected in runoff water after pesticides

application at crop sites were reported highly toxic effects on *D. similis* (Novelli et al. 2012), such as *C. dubia* (Braun et al. 2012) and *C. silvestrii* (Casali-Pereira et al. 2015). Furthermore the effects of the presence of fungicide pyrimethanil in aquatic system can cause immediate impact in cell growth of *S. capricornutum* (Shinn et al. 2015).

The ecological Loe reported low risk for the aquatic and ecosystem compartment but no risk for the terrestrial compartment. In higher tier risk assessments, endpoints often focus on the dynamics of populations and the structure and functioning of communities and ecosystems (Van den Brink et al. 2005). The local extinction of certain species from an ecosystem can have a greater impact on ecosystem functioning than the extinction of other species (Thompson et al. 2015; Winfree et al. 2015). For ecological responses in aquatic ecosystems models the phytoplankton diversity showed a decrease risk values from immediate to short term exposure while in the zooplankton diversity had an increase risk values from immediate to short exposure during the time in the mesocosms tanks (Figure 2e). Sensitive species may be impaired by sublethal effects or eliminated by lethality and this ecological alteration may initiate a trophic cascade or a release from competition that secondarily leads to responses in tolerant species (Fleeger et al. 2003). The ecological factors and threshold responses that determine community resistance and resilience should improve our ability to predict how and when communities will respond to environmental hazard and risks (Clements & Rohr, 2009). These authors reported the communities such as a midpoint between populations and ecosystems in the hierarchy of biological organization, offer an important insights regarding mechanisms of contaminant effects at lower levels and are intimately connected to ecosystem services at higher levels.

Tier III - Detailed screening

Indeed, the protection of communities-levels is critical for maintaining ecological integrity and are intimately linked to ecosystem function and services (Clements & Rohr, 2009). The ecosystem functions was direct analyzed in the pyrimethanil ERA on the aquatic compartment by the colonization process of macroinvertebrates and the physical process of macrophyte decomposition rate. In the aquatic ecosystems models these parameters showed a risk increase during the time exposure because of the increase of the resilience and ecosystem function in the system. The macroinvertebrates are potentially highly relevant indicators for contaminated sites being the mainly responsible for the ecosystem function of breakdown of allochthonous and autochthonous organic matter in aquatic ecosystems (Schäfer et al. 2012). The pyrimethanil ERA considered a colonization experiment with increase the organisms' abundance in the colonization structures and the risk during the time exposure (Figure 2d). The

community response was positive for Chironomidae (Diptera) species, often dominated and tolerant species for sediment-dwelling macroinvertebrates (Rasmussen et al. 2015), and response negative for Odonata and Gastropoda species, especially sensitive to elevated levels of contamination (Luek et al. 2015).

Furthermore, the ecosystem functions for the terrestrial compartment were direct analyzed by the soil invertebrates feeding activities on the ecosystems models measured by the bait lamina score showed an almost homogeneous low risk during the time exposure (Figure 2c). These results corroborated and improve our understanding of the relationship between biodiversity and ecosystem function, promoting ecosystem functionality and stability and thus contributes significantly to various ecosystem services (e.g. Connolly et al. 2013; Mori et al. 2013; Montoya et al. 2015). This evidences highlight the risk that loss of biodiversity may result in a decline or loss of crucial ecosystem services (Cardinale et al. 2012; Hooper et al. 2012).

Tier IV - Final assessment

The first ERA model proposed, the triad approach, followed the risk score based on Jensen & Mesman (2006) risk assessment model, considering the data of chemical, ecotoxicological and ecological lines of evidence (Table 2 and Figure 2). Semenzin et al. (2008) defined an ERA used a weight of evidence approach, integrating effect indexes to estimate the effects on terrestrial ecosystems caused by the stressors and proposed a tool for supporting the decision making process and monitoring a contaminated site in north of Italy. Dagnino et al. (2008) developed a new expert decision support system that can integrate triad data for assessing environmental risk and biological vulnerability at contaminated sites. However, Niemeyer et al. (2010) reported a screening phase of a site-specific ERA of a metal-contaminated area in northeast of Brazil with triad evaluation showed a low risk outside the smelter area and high risk levels inside the smelter area and in some possible residue deposits points.

The integrated holistic two-tier pyrimethanil ERA for the aquatic, terrestrial and ecosystem compartments with the combination of the three lines of evidence showed the three dimensions integrated risk along the hypothetical risk scenarios followed the gradient terms of exposure. However moderate and high levels of risk were found in the hypothetical risk scenarios, particularly in the immediate term exposure of the fungicide. Our analyses suggest that effects of the risk scenarios are within the land use restrictions limits, restricts to use to agricultural and industrial land use (Jensen & Mesman, 2006). The ecotoxicological Loe with the effects and response on the potential risk organisms showed strong weight of evidence on

the integrated analyses. The integration of the systems has led to fundamental discoveries, sustainability actions and environmental responsibilities that are not possible by using conventional compartmentalized approaches, including understanding the interconnectivity and complexity of ecosystems (Liu et al. 2015).

Notwithstanding to this train of thought, the second model to pyrimethanil ERA calculation, deterministic approach, following the technical overview of ecological risk assessment proposed by the USA Environmental Protection Agency (EPA, 2016) showed a general similar pattern results to the previous model (Table 3 and Figure 4). This regulation reported the risk characterization as the final phase of the ERA with two major components the risk estimation and risk description. The risk estimation, in essence, compares exposure and effects data, considers integrated exposure and effects data in context of levels of concern, and states the potential for risk. The risk description interprets the risks values based on assessment endpoints with interpretation by the risk assessor, evaluating the lines of evidence supporting or refuting risk estimates. The pyrimethanil ERA approach showed the impacts for different toxicity endpoint, from single species to a mesocosms exposure and to terrestrial and aquatic environmental risk scenarios exposure. This deterministic approach, is worldwide used and have been useful tools in predicting environmental effects based on laboratory data with some good and consistent experiences in Canada, USA, Netherlands and United Kingdom (Jensen et al. 2001).

The deterministic results showed the impact scale and magnitude for the organisms in the ecosystems, reporting for the short-term exposure scenarios as an acute endangered and restricted use for species while for the immediate and middle-term exposure scenarios as an acute high risk and chronic risk for species. Shinn et al. (2015) demonstrated that pyrimethanil had impact in aquatic system and the scale of impact may be attenuated by the term effect exposure with immediate effects on phytoplankton species observed due to the initial high availability of the fungicide and attenuated because of the degradation of the fungicide in the aquatic compartment after a short period. The decreased of the local occurrence or extinction of almost any species under different environmental change scenarios is expected to decrease ecosystem multi- functionality and services in some contexts at large spatial temporal scales in a changing world (Isbell et al. 2011). For example, declines in plants species richness may cause losses to neighbouring trophic levels interactions and may also alter mutualistic interactions such as pollination or mycorrhizal association (Scherber et al. 2010).

Conclusions

Biodiversity and ecosystem functioning can support efforts to safeguard the intrinsic capacity of ecosystems for self-renewal, adaptive dynamics and supporting humanity now and in the future (Naeem et al. 2012). In a world that is being transformed by humans, ecology will have to respond relevant scientific knowledge on how ecosystems function and changes, how they are linked to human well-being and how humankind can use and transform them in a sustainable way, demanding that ecology transform itself into a more integrated and predictive science (Loreau, 2010).

The fungicide pyrimethanil risk assessment, an integration approaches in two-tier, was design and performed, reporting the intrisec integrated effects on ecosystems by the trophic levels in the risk scenarios proposed. The three dimension risk scenarios tried to cross the major organizational levels and different ecological functions and process showed accurately the vision about the time, exposure and degradation of the contaminant to adverse effects on the ecosystems. The triad approach and the deterministic model showed an efficient tool to measured the pesticide risk assessment. We concluded that the integrated ecological risk assessment for the fungicide pyrimethanil is an important register for the deleterious effects and responses for potential risk organisms to environmental impacts of pesticides mainly relation to losses and changes of the ecosystems functions and services.

ACKNOWLEDGEMENTS

The authors would like to thank the Brazilian National Council for Scientific and Technological Development (CNPq) for the Ph.D. grant (process number 140415/2013-0) and the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) for the doctorate sandwich grant (process number 99999.008342/2014-08). We thank the University of São Paulo and Aarhus University for the facilities.

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CHAPTER VI

Summary and Outlook

Background

The pesticides have consistently demonstrated their worth by increasing global agricultural productivity, reducing insect-borne, endemic diseases and with potential of protection and restoration (Ecobichon, 2001). Brazil is the world's largest consumer of pesticides, accounting for approximately 20 % of the total world use (Albuquerque et al. 2016). These authors' highlights that Brazilian crops has been the world's top pesticide market consumer since 2008, with 381 approved pesticides. Is estimated over 90% of farmers rely on pesticide use (IBGE, 2006).

In Brazil, the basis for pesticide regulation was set by Federal Law No. 7802, enacted in 1989 and later by Acts 4074/2002 and 5981/2006 (ANVISA, 2011). These legal standards regulate all aspects related to pesticides, including registration, use, production, storage, transport and disposal.

Pesticide consumption and import has increased considerably in Brazil (Porto et al. 2010). These authors' highlights that between 1998 and 2007, pesticide companies' net revenue increased 107%; simultaneously, Brazilian pesticide imports between 1997 and 2006 increased 150%. However, between 1998 and 2007, the Brazilian crop area only increased 29%. The unexceptional exportation of hazardous substance or the transfer of production from USA and European Union countries to regions like Latin America, Africa, and Southeast Asia can further increase the environmental and health risks associated with hazardous materials in importing countries (Porto et al. 2010).

This intensive use of pesticides in agriculture of Brazil and others Latin America countries may cause contamination of ground water resources due to their leaching through the soils into aquifer regions (Laabs et al. 2002). The knowledge on pesticide impact in the tropical environment, however, is still lacking almost completely compared to temperate systems (Niva et al. 2016).

This gap in basic information about the effects of pesticides on the environment makes the studies increasingly challenging. The compiled set of information present in this thesis contributes substantially to explore and report the effects of these chemicals using various experimental forms and analysis. With this information, it would be possible to update and review the pesticide regulation and their standards. New information for monitoring and decision make would be requested such as the routes of exposure, the effects on the trophic food webs, ecosystem services changes, experimental ecosystem models and an integrated pesticides ecological risk assessment.

The researches of pesticides effect proves a crucial information nowadays in which this chemical are one of the major global challenges to balance agricultural production with environmental and human health. Indeed, the agricultural practices are essential for sustaining the human population, but in the other hand, they can directly disrupt the ecosystem functions. It is to be predict that the global changes the world ecosystems are going to experience during the coming decades pose larger questions regarding pesticide impact on biodiversity and thus affect ecosystem function and services.

The latest biodiversity conservation studies have shown an interest in the other side of conservation: the goods and services that ecological systems can provide to human societies. These ecosystem services are the result of complex interactions between abiotic and biotic components and the evaluation structures are intended to include the role of human beings in these interactions. The sustainability challenges from maintaining biodiversity provides and human society needs faced by direct and indirect pesticides effects across the levels of biological complexity. In this sense, the ecological risk assessment it is shown more accuracy when included new analyzes factors such as the holistic measurement of the effects of contaminants on the structure and functioning of natural ecosystems in episodic perturbation making it an important tool for pesticides risk management programs. Furthermore, this environmental impact tool making it possible assess the potential ecosystems risks and work in the past damage, fix the present and manage in a sustainable manner the future.

Technical recommendations

In general, the proposal ecological risk assessment in aquatic and terrestrial pesticides contamination scenarios proved to be useful to measure the possible environmental impacts. The inclusion of a mixture of pesticides should be considered, to improve the ecological relevance of the analysis. The study design in terrestrial ecosystems models is suitable to evaluate more ecological factors, such as organic matter decomposition rate, seed germination, dung degradation and microbial activity. The inclusion of functional endpoints, such as avoidance tests, should be required to compare the others laboratory results, including the animal behaviour into the toxicity. Sampling in real situations of agricultural fields in both aquatic and terrestrial ecosystems are necessary for an ecological representativity. Is appropriate to emphasize that the data analysis and integration methods are validated for field studies. The ecosystem services approach should be considered and improved. When the human beings are considering as part of ecological systems, an efficient tool is formed for the decision

making. Thus, this data interpretation makes it stronger and with some political weight for the environmental management.

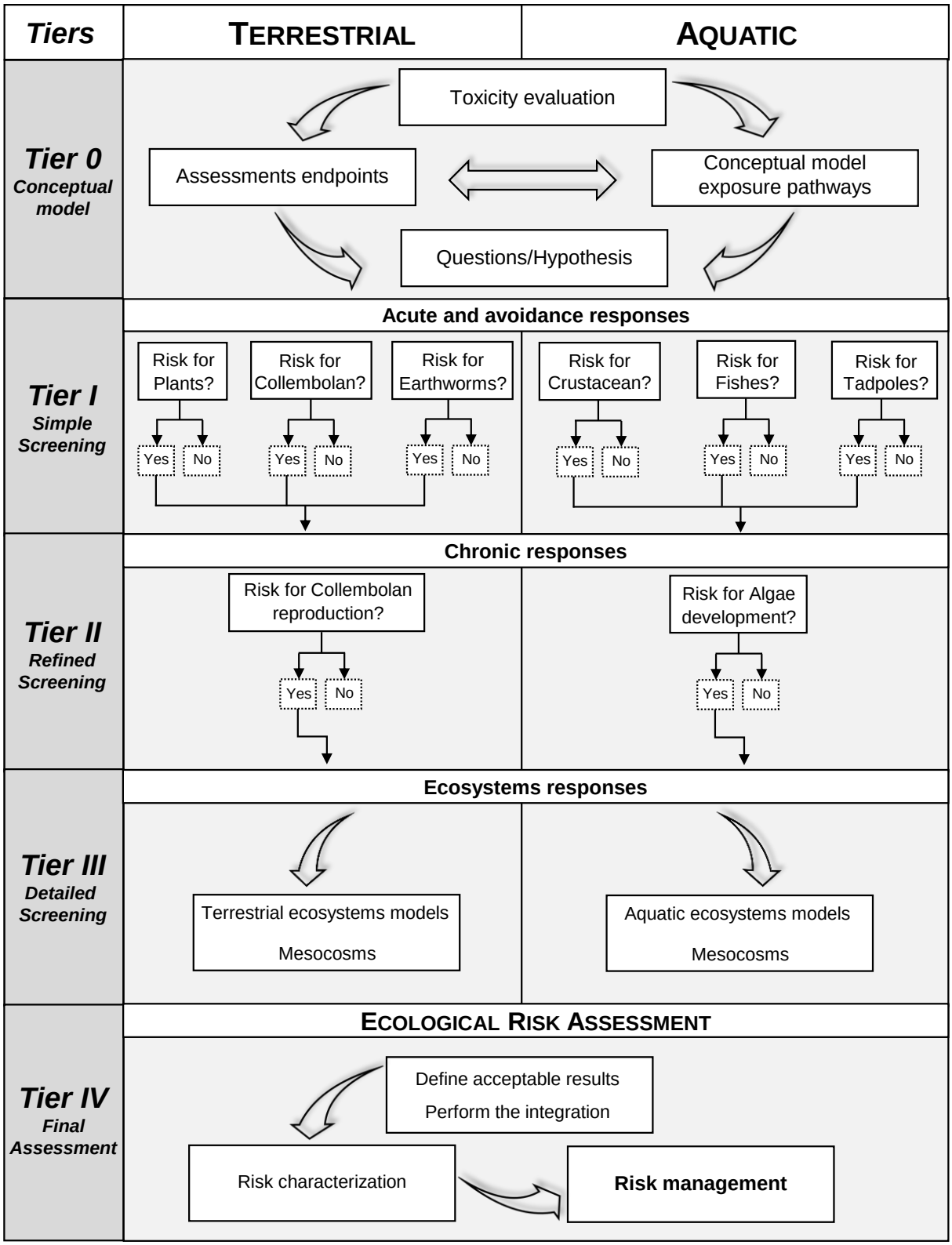
Data assessment and test strategy

In general, the ecological risk assessment (ERA) is a diagnosis process to get a prognosis. Jensen & Mesman (2006) proposes two major types of ERA. The first is a predictive model, involving an association with the regulation and disposal of hazardous substances, it's required before the chemical released for use. The second type is description, estimating the ecological adverse effects in populations or ecosystems under certain degraded areas. The conditions of ERA for realization are described in several review papers and books (e.g. EPA, 1992; EPA 1998; Weeks et al. 2004; Jensen and Mesman 2006; Niemeyer et al. 2010; Van den Brink et al. 2011; Adler et al. 2016; EPA, 2016).

The ERA are often performed in phases or tiers (Jensen and Mesman 2006). The environmental risk assessment can be followed a four-tiered approach (Jensen and Mesman 2006; Adler et al. 2016). The figure 1 are shown a model for ERA process. The process can be start with the Tier 0 assessing the development of a conceptual model and problem formulation, identifying potential contaminants, pathways and receptors. The Tier I assesses the simple screening of the risk relevance based on data on the use, environmental release, and predicted exposure of the specific pesticide. It is essentially a screening phase, aiming to produce a first representation of the risk and to determine whether an experiment can be excluded from higher tiers of testing or it needs to be further evaluated. This phase include the initial organisms responses, such as the acute and avoidance responses. The Tier II normally is required only if risks assessed during tier I exceed an exposure threshold value or action limit. However, if the pesticide product is being authorized as a plants protection, non-target effects must be specifically addressed and Tier II tests are mandatory. This phase described a refined screening, including the chronic organisms responses.

If risks assessed exceed an exposure threshold value or action limit the Tier III is required. This phase described a detailed screening, including the ecosystem responses, may be represent by the terrestrial and aquatic experimental ecosystem models (mesocosms) or field studies. The Tier IV described a final assessment, defining acceptable results and perform the data integration (Jensen and Mesman 2006). These authors highlights that it is always possible to stop further investigations after each tier and either re-define the land-use or if needed take necessary actions to remediate or prevent the area or dispersion of contaminants.

Figure 1. Suggested decision flowchart for assessing the risk of pesticide using a tiers approach for terrestrial and aquatic compartments. The tiers are presented with the thesis example.



The ecological risk assessment calculation can follow the model of the triad approach. This principles e risk characterization was based on the triad of the chemical, ecotoxicological

and ecological line of evidences (Loe). The chemical line of evidence was calculated based on the toxic pressure for the measure extractable fraction of the fungicide pyrimethanil from the contaminated water and soil samples. The ecotoxicological line of evidence used as default the results from the terrestrial and aquatic toxicity test expressed as percentages. The ecological line of evidence considered ecological observations of the terrestrial and aquatic ecosystem model such as results of the aquatic organisms' diversity and the soil invertebrates feeding activities. The risk calculations followed the approach proposed by Jensen and Mesman (2006) where the lines of evidence were integrated with the reference in a risk score expressed from zero (no risk) to one (high risk). The formulas and steps calculations are shown in the Box 1.

Box 1. Environmental risk assessment framework based on the triad of the line of evidence chemical, ecotoxicological and ecological and the steps calculations for the integrated risk and the risk indicators based on Jensen & Mesman (2006) risk assessment model.

1 - CHEMICAL LOE	**2 - ECOTOXICOLOGICAL LOE**	**3 - ECOLOGICAL LOE**
Step 1. Measured concentration (mg.Kg $^{-1}$) *R1 Samples* **Step 2.** Generic screening values (mg.Kg $^{-1}$) *R2 = HC50* **Step 3.** For each contaminant, calculate toxic pressure *R3=1/(1+exp^((logR2-logR1)/0,4)* **Step 4.** Correct for background concentrations *R4=(R3-R3ref) / (1-R3ref)* **Step 5.** Calculate the combined risk for n chemicals *TPMM = 1-(1-R5(1)) * (1-R5(2))*...* (1-R5(n))* **Step 6.** Scale the potentially affected fraction of species values *R6= (Sample-REF) / (1-REF)* **Obs.** When no $HC50_{EC50}$ available in literature are applied the safety factor of 10 to the $HC50_{NOEC}$ $HC50_{EC50} = 10 * HC50_{NOEC}$	**Step 1.** Divide data test result by 100 *R1=(100-X) / 100* **Step 2.** Scale difference between X and reference (Negative values are converted in zero) *R2= (X-Ref) / (1-Ref)* **Step 3.** Integrated the ecotoxicological data *R1= log (1-X)* **Step 4.** Average of the test *R2 = Average (X1... Xn)* **Step 5.** Retro-transform values *R3= 1-(10 ^ X)*	**Step 1.** Ratio between site x and reference *R1=X / 100* **Step 2.** Positive values are converted to zero *Calculate absolute values of log (R1)* **Step 3.** Calculate sum of all values and multiply with -1 *R3 = -1 * Σ (R2)* **Step 4.** Calculate number of endpoints. *R4 = N* **Step 5.** Apply formula *1-10 ^ (R3 / R4)*
	4 - INTEGRATED RISK	**RISK INDICATORS (IR = Integrated Risk)**
	Step 1. Risk values for lines of evidence **Step 2.** Calculate log to (1-scaled result). *R1= log (1-X)* **Step 3.** Average all log-values to one integrated log value *R2 = Average (X1... Xn)* **Step 4.** Transform log-values into integrated risk (IR) values *R3= 1-(10 ^ X)*	0 < IR < 0.25 **No risk** 0.26 < IR < 0.50 **Low risk** 0.51 < IR < 0.75 **Moderate risk** 0.76 < IR < 1.00 **High risk**

Another assessment model are represent by the deterministic approach, the most widely used method. In this system, the environmental concentrations of a stressor are compared to an

effect concentration with the simple ratios of single exposure and effects values and may be used to express hazard or relative safety (Solomon & Sibley, 2002).

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Box 2. Environmental risk assessment framework based deterministic approach proposed by the United States Environmental Protection Agency (EPA, 2016).

INTEGRATED RISK	RISK QUOTIENT (RD)
$Risk\ Quotient\ (RQ) = \frac{Exposure}{Toxicity}$	0.00 < RQ ≤ 0.05 **acute endangered species** 0.05 < RQ ≤ 0.1 **acute restricted use** 0.05 < RQ ≤ 0.1 **acute restricted use** RQ ≥ 1.0: **chronic risk**

Gaps and uncertainties

The bioassay testing has therefore the ability to account inherently for the complete mixture of contaminants, including degradation products and metabolites, in the sample (Jensen and Mesman, 2006). These authors highlights that a number of uncertainties or limitations may be associated with the use of bioassays and the interpretation of the results obtained and a precise information about the efficiency of a specific solvent to extract a pollutant from freshly samples.

In regulatory context of chemicals, the ERA use model species as surrogates for ecologically similar taxa in the environment are often extrapolated to assess the impact on ecosystems (Adler et al. 2016). This uncertainties involved with the predictions about effects

on one species/endpoint using data from another species/endpoint may be dealt with through extrapolation and the use of safety factors (e.g. species sensitivity distribution models) (Weeds, 2004).

The reduction of any outstanding uncertainties are the first steps in order to come to a decision making. However, the uncertainties associated with the contradictory information given by certain lines of evidence for certain sampling points show the need to confirm potential risks in a tiers analysis (Weeds, 2004). This author reported that upper tiers are performed to reduce uncertainties about the actual risk, while lower tiers is essentially a screening phase, producing a first spatial representation of the risk and to determine whether a site can be excluded from higher tiers of testing.

Thus, the reduction of uncertainties is done through sublethal bioassays data, determination of the available fraction of contaminants and inclusion of additional representative ecological data (Niemeyer et al. 2010). Knowledge about the life history and ecology are needed to improve modeling results, quantifying the effects of uncertainties in life-history traits and toxicant sensitivities of listed species (Forbes et al. 2016). Therefore, a multidisciplinary approach will help to minimise the uncertainties of the conclusions in ERA, taking into account the fact that ecosystems are too complex to analyse in one-factorial approaches (Jensen and Mesman, 2006).

Thesis summary

In this study, information and experience to better evaluate the pesticides ecological risk assessment, informing hypothetical environmental impacts through the incorporation data that are more comprehensive, ecological modelling and ecosystem functions and services endpoints, has been compiled. To attempt it, direct and indirect effects on individual response for different biological organization levels and for multi trophic interactions responses with ecosystems models were evaluated. Thus, the environmental impacts in relation to losses and changes of the ecosystems functions and services were analyzed.

Taking as reference the tested hypotheses in this study, we accept the entire hypothesis proposed. For the antiparasitic ivermectin, as a weakly metabolized substance and most of the dose given to the pasture animals is excreted relatively unaltered in the treated animals faeces, with direct and indirect effects on the development and survival on dung-dwelling fauna. Our experiments elucidates this contamination process with the differences between the antiparasitic ivermectin routes of exposure to the earthworm *E. fetida* as regards the dermal (soil) and oral (food) exposure. The hypothesis was accepted based on the results, showing

significant differences on the reproduction descriptors for the ivermectin concentrations and both exposure routes. These results highlighted the probable route of toxicity to earthworms as the dermal via, representing danger to the species when association mainly with livestock production.

The hypothesis that the soil multi-species systems (SMS), simulating a crops situations pesticides applications, is an efficient tool to measure the pesticides effects on the soil processes and food web structure in a realistic scenario was accepted. Our results showed the springtails community and predator mites had habitat preferences and foraging abilities affected by direct and indirect toxicity. Furthermore, the epigeic earthworms was influenced by the availability of resources and habitats with indirect toxicity on spray application. Thus, this more realistic simulation scenarios approaching the ecotoxicology science with the agricultural field pesticides situations, reporting with more accuracy the ecosystems responses.

The third hypothesis, about the plant protect substances, with the potential to affect the freshwater and terrestrial organisms by the runoff and leaching into adjacent water bodies and surrounding soils with deleterious impacts on the ecosystem functions and services was accepted also. Our simulated experimental scenarios, showed notably changes in the structure of the laboratory-derived acute, chronic and avoidance toxicity data for plants and freshwater and terrestrial organisms with changes and loss in the ecosystem services framework. Furthermore, this holistic measurement showed more accuracy in the environmental impacts on the structure and functioning of natural ecosystems making it an important tool for pesticides risk management programs.

The last hypothesis tested, cross the intrinsic capacity of pesticides to cause adverse effects over time and space on the individuals and on multi trophic interactions with changes on the structure of the ecosystems functions and services, was accepted also. The risk scenarios integration approaches cross the major organizational levels and different ecological functions and process showed accurately the vision about the time, exposure and degradation of the fungicide pyrimethanil to adverse effects on the ecosystems functions and services, confirming the initial hypothesis.

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APPENDIX I

Experimental overall view and photographic register

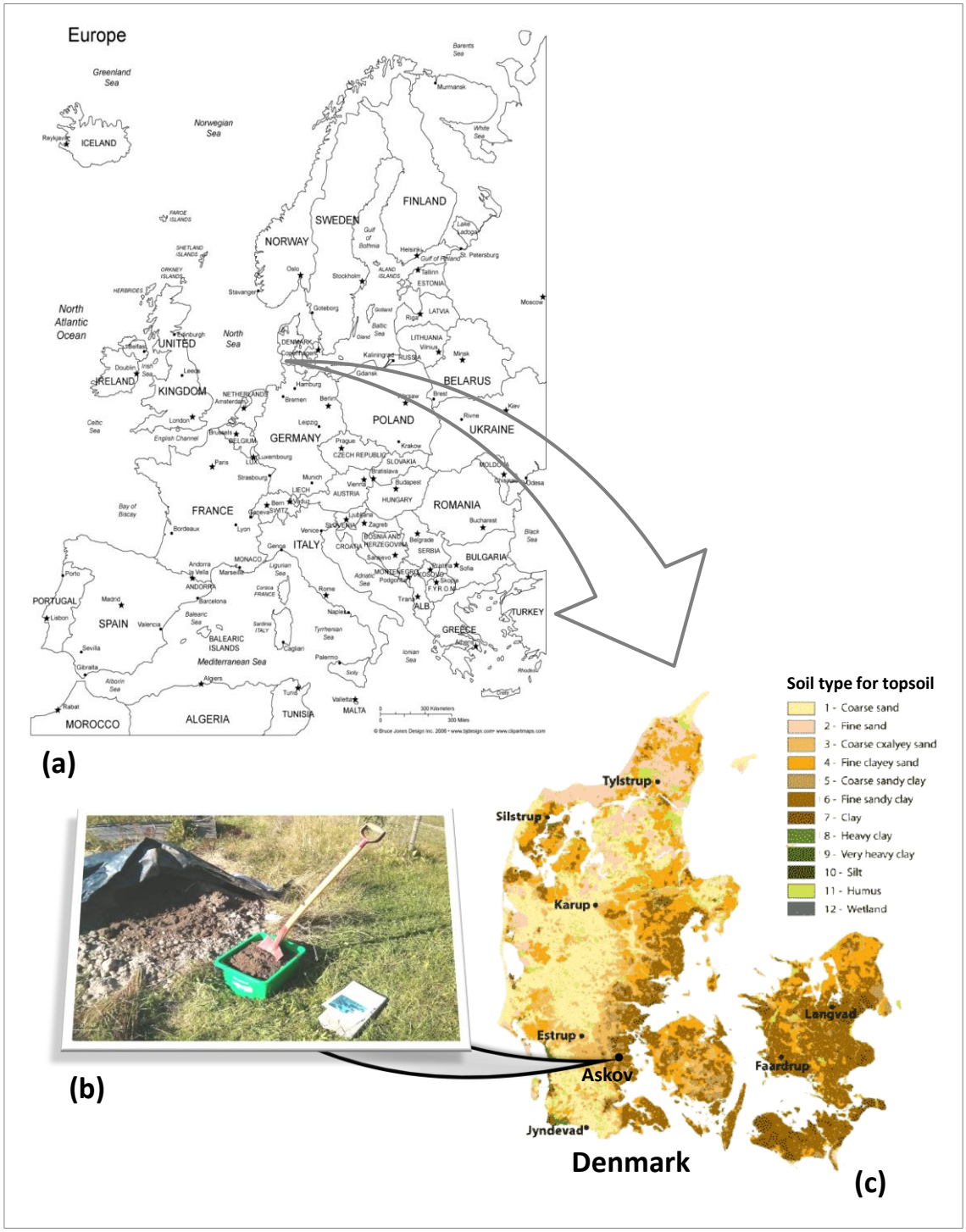

Figure 1. Study area map in Denmark, Northern Europe (a), the experimental soil sampling site on a Danish agricultural soil (Askov, Jutland) (b) and the *Danish Institute of Agricultural Sciences' nationwide topsoil property map of Denmark based on the texture classes of the Danish soil classification with the Askov sampling location* (Miljøstyrelsen Danish Environmental Protection Agency, 2007) (c) (Picture: André Luís Sanchez).

Figure 2. Experimental photographic register of the individual tests with the soil contamination with a mixed (1), the acetone evaporation (2), the earthworms on wet filter paper in Petri dishes for 24 hours to depurate the gut contents (3), the test containers (4), the containers in a acclimated room (5), the removed of organisms adults after 28 days (6 and 7), and after more 28 days the removal of juveniles and cocoons with water (8), the springtails single test containers (9), single test microcosms (10), inserted the animals in the containers (11), containers maintained in an acclimated room (12), added food weekly (13), the extraction in different temperatures in a high-gradient extractor (14) and sieved with 70 % ethanol and conserved in glycerol (15) (Pictures: André Luís Sanchez).

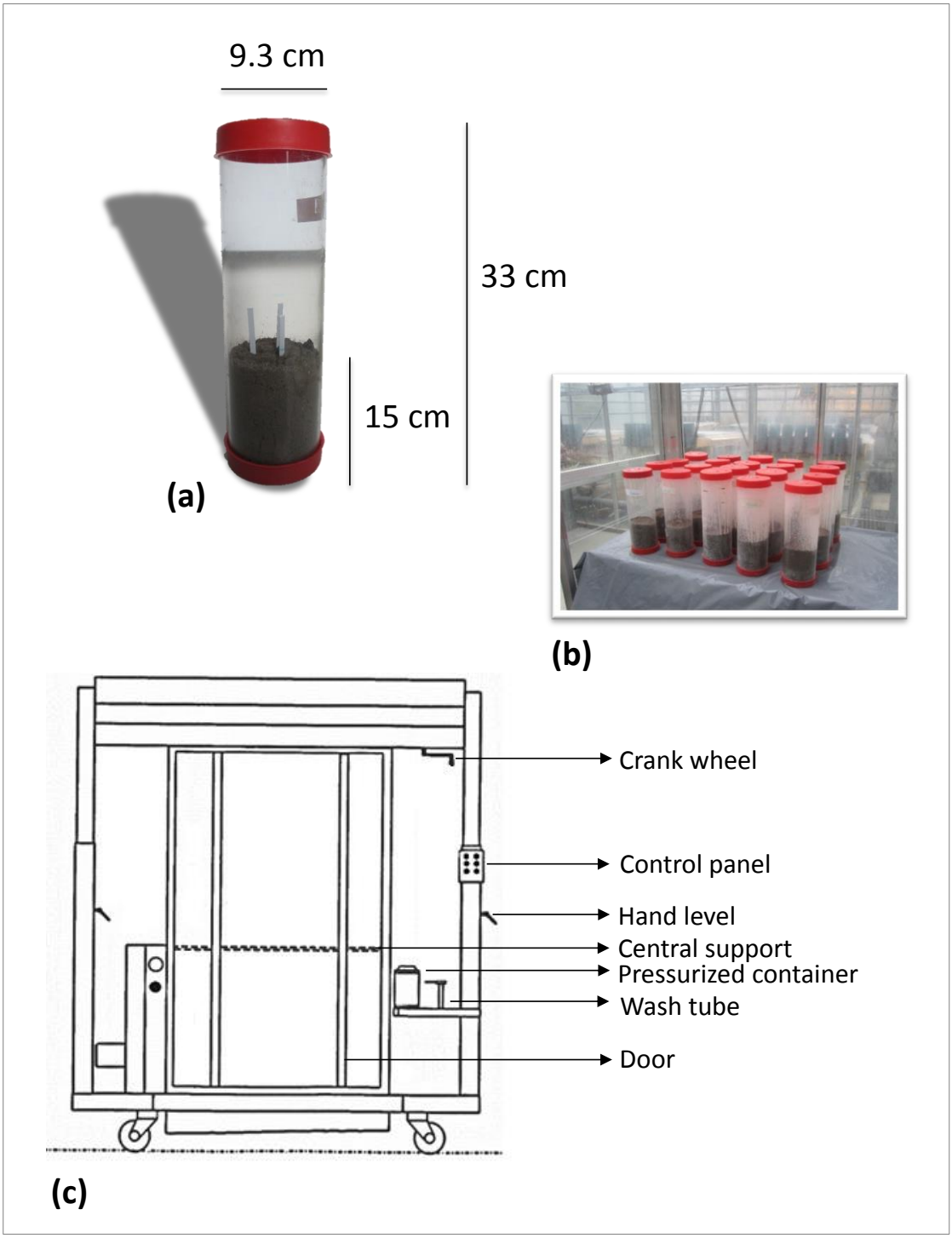

Figure 3. Overall view of the terrestrial mesocosms with the highlights the experimental acrylic tube dimensions of 9.3 cm diameter and 33 cm height and 15 cm soil column with the bait lamina sticks (a), the mesocosms after the fungicide drift sprayer (b) and the schematic design of the pot sprayer as seen from the front with the crank wheel, control panel, hand level, central support to mesocosms, pressurized container with the fungicide, wash tube and the door of the cabin (c) (Pictures: André Luís Sanchez).

Figure 4. Experimental photographic register of the terrestrial mesocosms with the microbial community extraction (1), the acrylic tubes for the mesocosms construction (2), mesocosms construction (3 and 4), preparation of the substrate mixture for bait lamina with cellulose powder, wheat bran and active charcoal (5), mesocosms with the bait lamina (6), pot sprayer (7), drift spray on the soil mesocosms (8 and 9), mesocosms external cleaning (10), mesocosms after the fungicide surface spray (11), bait lamina removed (12), extract mesocosms soil (13), three layers extraction (14) and the tunnels in the soil made by the earthworms (15) (Pictures: André Luís Sanchez).

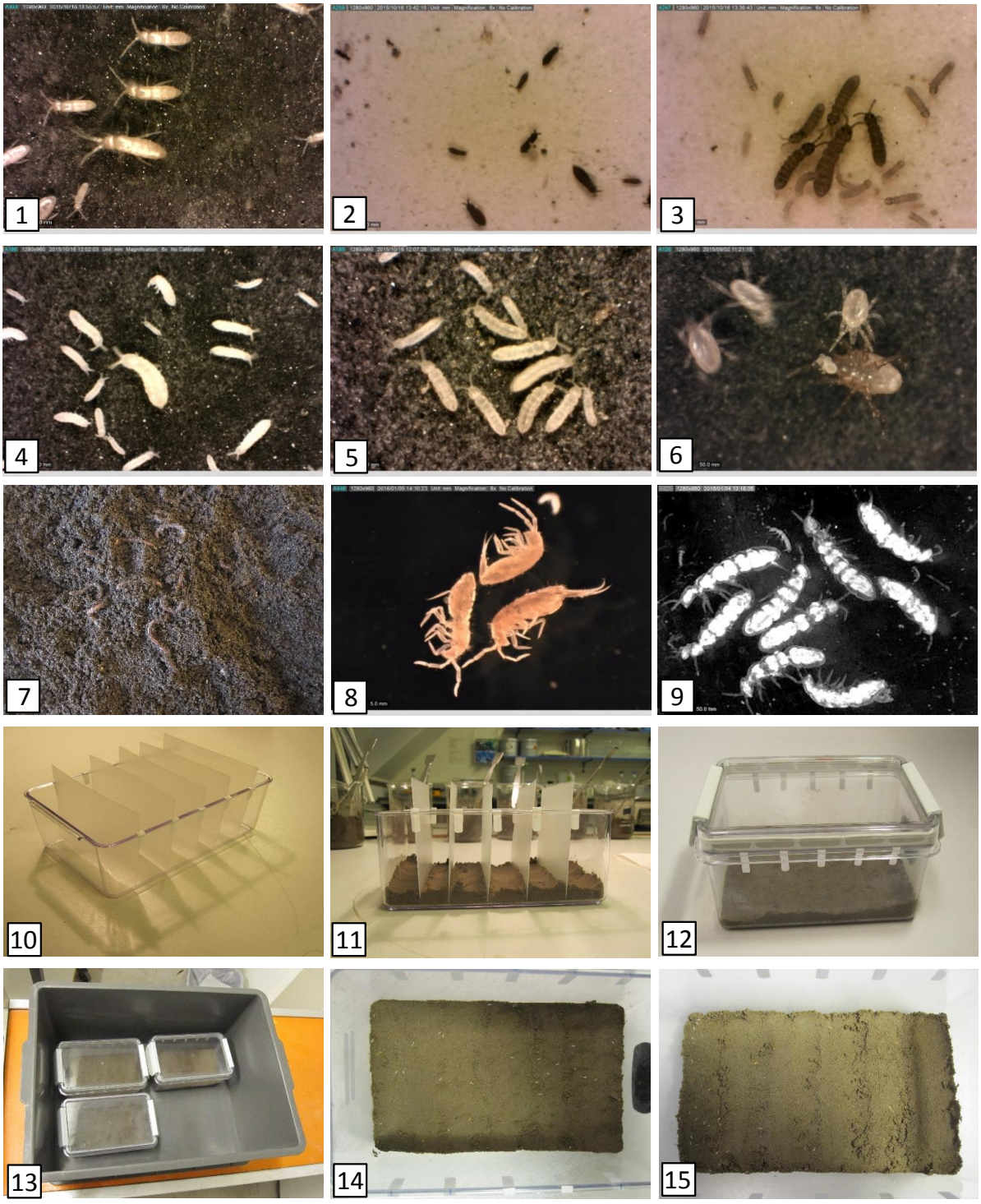

Figure 5. Experimental photographic register of the terrestrial mesocosms with the mesocosms springtails *Heteromurus nitidus* (1), *Hypogastrura assimilis* (2), *Proisotoma minuta* (3), *Protaphorura fimata* (4), *Folsomia fimetaria* (5), the mite *Hypoaspis aculeifer* (6), the juveniles earthworms *Eisenia fetida* (7), the avoidance test with the springtails *Heteromurus nitidus* (8), the single test with the springtails *Folsomia candida* (9), linear avoidance test containers with the dividers (10), the soil contaminants gradient container (11, 12 and 13), the soil surface immediate after the insert animals in each section (14), the soil surface after 48 hours test with the animals near the control section (15) (Pictures: André Luís Sanchez).

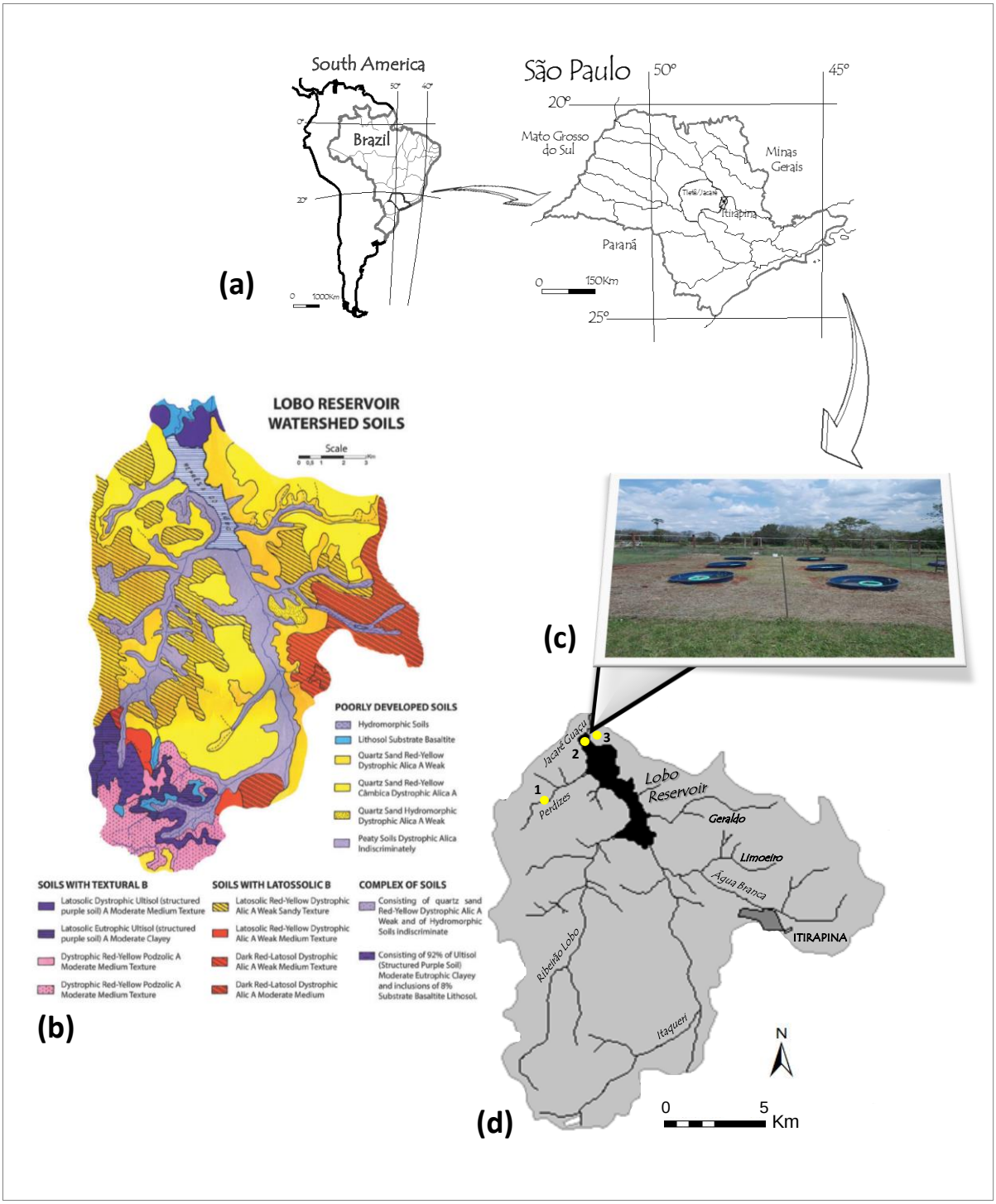

Figure 6. Study area map in Brazil in the Tietê/Jacaré Water Resources Management, central region of São Paulo State with emphasis on the Lobo watershed (a), the Lobo Reservoir watershed soils (Source: Tundisi & Matsumura-Tundisi, 2014) (b), the aquatic mesocosms arrangement of the outdoor tanks (c) and the Lobo Reservoir watershed with the lotic zones of streams and tributaries and the limnetic zone of the Lobo Reservoir with the sampling sites of sediment (1) and water (2) for the mesocosms construction and the soil for the terrestrial experiments (3) (d) (UTM datum *Córrego Alegre***,** *23 S* Zone) (Picture: André Luís Sanchez).

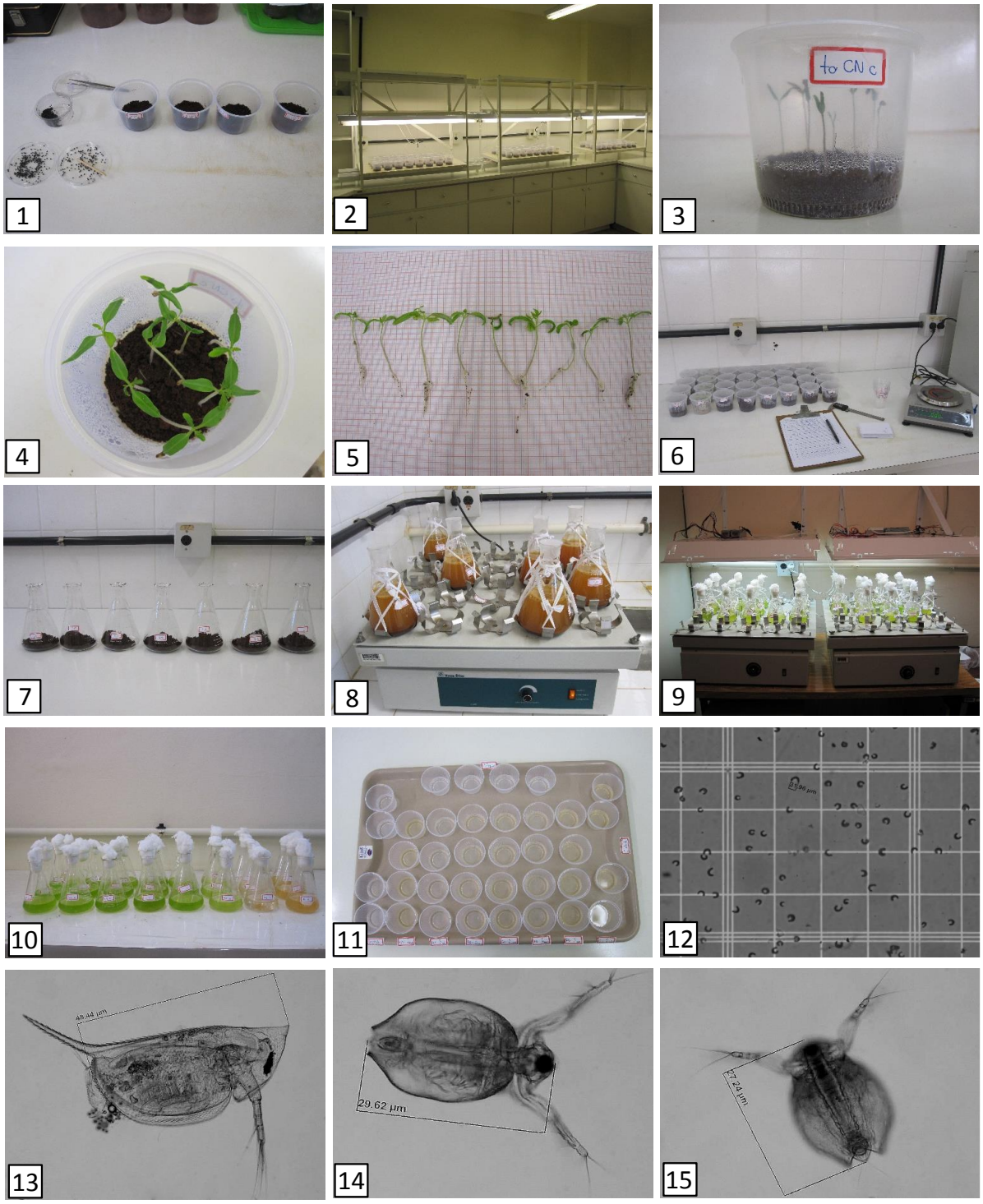

Figure 7. Experimental photographic register of the ecosystem services experiments with the germination and early growth test with place the seeds in the soil (1), the controlled room with light and temperature adjusted (2), the germination and seedling growth (3 and 4), seedling measures (5 and 6), the elutriate preparation with the remaining soil and mixed with water (7 and 8), the algae test with the elutriate (9 and 10) the cladoceran test with the elutriate (11), the algae *Pseudokirchneriella subcapitata* results after 96 hours (12), the cladoceran *Daphnia similis* (13), *Ceriodaphnia dubia* (14) *Ceriodaphnia silvestrii* (15) (Pictures: André Luís Sanchez).

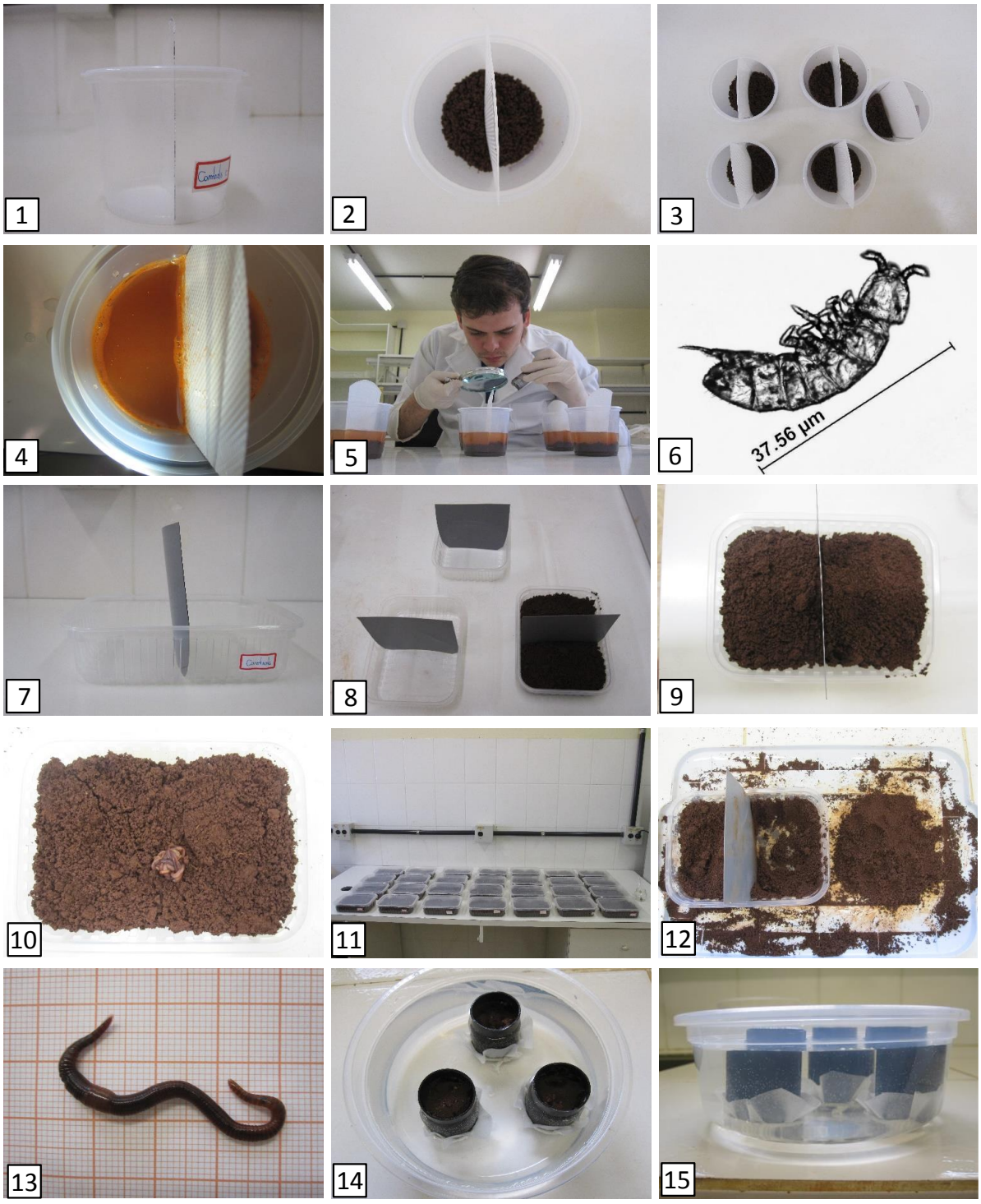

Figure 8. Experimental photographic register of the soil invertebrates avoidance tests with the Collembola dual control soil test containers (1, 2 and 3), the animals extract with water (4 and 5), the springtail *Folsomia candida* (6), the earthworms dual control soil test containers (7, 8, and 9), the test start with the animals in the middle of the container after the remove of the card divider (10), the test containers during the 48 hours test (11), the animals extraction in both sections (12), the earthworm *Eisenia andrei* (13) and the water holding capacity test (14 and 15) (Pictures: André Luís Sanchez).

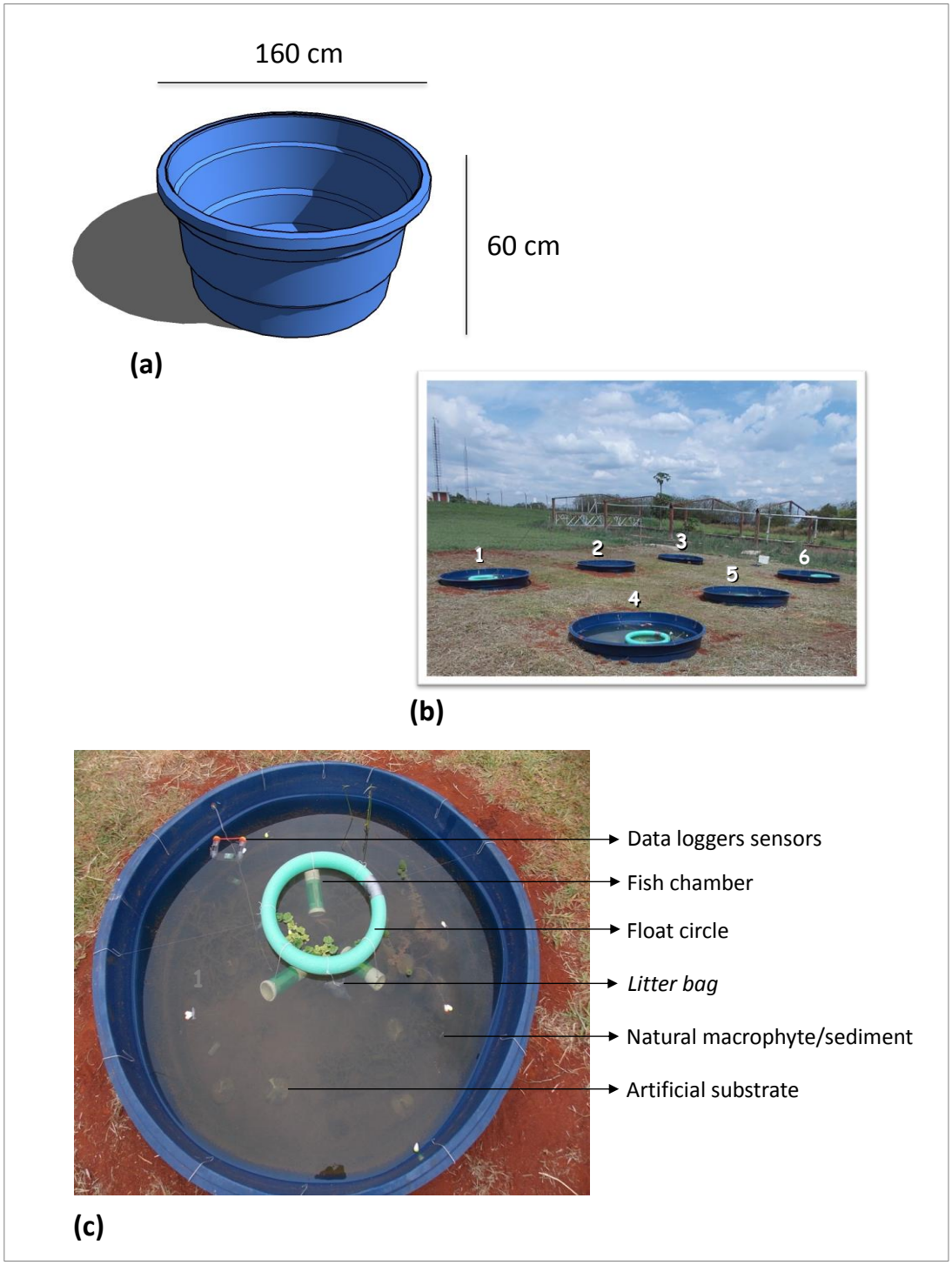

Figure 9. Overall view of aquatic mesocosms with the highlights the polypropylene 1500 liters water tanks dimensions of 160 cm diameter and 60 cm height (a), the arrangement of the outdoor tanks as control (2, 5 and 6) and contaminated with fungicide (1, 3 and 4) (b) and the interior layout of the experimental tanks with data loggers sensors, fish chamber (data not shown), float circle, litter bag, natural macrophyte and sediment and the artificial substrate for colonization (c) (Pictures: André Luís Sanchez).

Figure 10. Experimental photographic register of the outdoor mesocosms with the sediment sampling (1 and 2), the random arrangement of the sediment in the tanks (3), the water sampling point of the Lobo reservoir through water pump (4), the tanks with natural water and sediment (5), the artificial substrate with rocks (6), the stabilized tanks after 6 months the water filling and before the contamination (7), litter bags for the decomposition experiments (8), the float circle support the experiments in the tanks (9), solution preparation of the commercial formulation Mythos® (10), fungicide application on the water surface (11 and 12), overall view of one mesocosms (13), colonization sampling (14) decomposition experiment weighing (15) (Pictures: André Luís Sanchez).

APPENDIX II

Ecological risk assessment calculations

Model I - Triad approach, based on the triad of the line of evidence (chemical, ecotoxicological and ecological), with the steps calculations for the integrated risk based on Jensen & Mesman (2006) risk assessment model.

Table 1. Terrestrial toxicity values for the scaling results of the Ecotoxicological LoE for the fungicide Pyrimethanil.

	% as Control				Step 1. R1=(100-X)/100				Step 2. R2= (X-Ref) / (1-Ref)				Scaling results			
SAMPLES	**Ref0**	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Germination7	100	1,25	0,5	0,5	0,00	0,99	1,00	1,00	0,00	0,99	1,00	1,00	0,00	0,99	1,00	1,00
Germination14	100	4,25	8,5	5,75	0,00	0,96	0,92	0,94	0,00	0,96	0,92	0,94	0,00	0,96	0,92	0,94
Germination21	100	82,05	87,18	58,97	0,00	0,18	0,13	0,41	0,00	0,18	0,13	0,41	0,00	0,18	0,13	0,41
Seedling	100	25,87	28,25	28,15	0,00	0,74	0,72	0,72	0,00	0,74	0,72	0,72	0,00	0,74	0,72	0,72
Roots	100	29,11	25,46	30,82	0,00	0,71	0,75	0,69	0,00	0,71	0,75	0,69	0,00	0,71	0,75	0,69
Leaves	100	24,29	35,76	6,25	0,00	0,76	0,64	0,94	0,00	0,76	0,64	0,94	0,00	0,76	0,64	0,94
Biomass (g)	100	15,04	13,01	14,23	0,00	0,85	0,87	0,86	0,00	0,85	0,87	0,86	0,00	0,85	0,87	0,86
Germination7	100	200,00	66,67	1,00	0,00	-1,00	0,33	0,99	0,00	-1,00	0,33	0,99	0,00	0,00	0,33	0,99
Germination14	100	97,30	91,89	91,89	0,00	0,03	0,08	0,08	0,00	0,03	0,08	0,08	0,00	0,03	0,08	0,08
Germination21	100	89,19	91,89	81,08	0,00	0,11	0,08	0,19	0,00	0,11	0,08	0,19	0,00	0,11	0,08	0,19
Seedling	100	51,48	58,28	62,81	0,00	0,49	0,42	0,37	0,00	0,49	0,42	0,37	0,00	0,49	0,42	0,37
Roots	100	47,62	66,85	80,74	0,00	0,52	0,33	0,19	0,00	0,52	0,33	0,19	0,00	0,52	0,33	0,19
Leaves	100	98,26	102,13	93,02	0,00	0,02	-0,02	0,07	0,00	0,02	-0,02	0,07	0,00	0,02	0,00	0,07
Biomass (g)	100	53,64	57,27	69,09	0,00	0,46	0,43	0,31	0,00	0,46	0,43	0,31	0,00	0,46	0,43	0,31
F. candida	100	13,0	34,2	101,9	0,00	0,87	0,66	-0,02	0,00	0,87	0,66	-0,02	0,00	0,87	0,66	0,00

Negative values are converted in zero

Table 2. Terrestrial Ecotoxicological LoE values for the fungicide Pyrimethanil.

	Scaling				Step 1. R1= log (1-X)			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Germination7	0,00	0,99	1,00	1,00	0,00	-1,90309	-2,30103	-2,30103
Germination14	0,00	0,96	0,92	0,94	0,00	-1,37161	-1,07058	-1,24033
Germination21	0,00	0,18	0,13	0,41	0,00	-0,08591	-0,05959	-0,22934
Seedling	0,00	0,74	0,72	0,72	0,00	-0,58726	-0,54904	-0,5506
Roots	0,00	0,71	0,75	0,69	0,00	-0,53591	-0,59422	-0,51119
Leaves	0,00	0,76	0,64	0,94	0,00	-0,61465	-0,44656	-1,20412
Biomass (g)	0,00	0,85	0,87	0,86	0,00	-0,82273	-0,88579	-0,84687
Germination7	0,00	0,00	0,33	0,99	0,00	0	-0,17609	-2
Germination14	0,00	0,03	0,08	0,08	0,00	-0,0119	-0,03672	-0,03672
Germination21	0,00	0,11	0,08	0,19	0,00	-0,04969	-0,03672	-0,09108
Seedling	0,00	0,49	0,42	0,37	0,00	-0,28835	-0,2345	-0,20197
Roots	0,00	0,52	0,33	0,19	0,00	-0,32225	-0,17491	-0,09292
Leaves	0,00	0,02	0,00	0,07	0,00	-0,00764	0	-0,03141
Biomass (g)	0,00	0,46	0,43	0,31	0,00	-0,27054	-0,24205	-0,16058
E. Andrei (avoi)	0,00	0,99	0,87	0,67	0,00	-2	-0,88606	-0,48149
F. candida (avoi)	0,00	0,68	0,61	0,54	0,00	-0,49485	-0,40894	-0,33724
H. nitidus (avoi)	0,00	0,80	0,55	0,41	0,00	-0,69897	-0,34679	-0,23136
F. candida	0,00	0,87	0,66	0,00	0,00	-0,88636	-0,46545	0

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	0	-0,60843	-0,49528	-0,58601

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,7536	0,6803	0,7406

This is the terrestrial Risk for Ecotoxicological LoE

Negative values are converted to zero

Table 3. Terrestrial Ecological LoE values for the fungicide Pyrimethanil.

	Bait lamina score				Step 1. R1=X/100				Step 2. Calculate absolute values of log (R1)							
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Baitlam1	100	21,4	29,4	34,9	1	0,21	0,29	0,35	0,00	-0,67	-0,53	-0,46	0,00	0,67	0,53	0,46
Baitlam2	100	99,2	77,3	41,4	1	0,99	0,77	0,41	0,00	0,00	-0,11	-0,38	0,00	0,00	0,11	0,38
Baitlam3	100	74,3	79,3	70,4	1	0,74	0,79	0,70	0,00	-0,13	-0,10	-0,15	0,00	0,13	0,10	0,15

Positive values are converted to zero

Step 3. Calculate sum of all values and multiply with –1. R3 = -1 * Σ (R2)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0,00	-0,80	-0,74	-0,99

Step 4. Calculate number of endpoints. R4 = N				
SAMPLES	Ref0	Immediate	Middle	Short
R4	30,00	30,00	30,00	30,00

Step 5. Apply formula: 1-10^(R3/R4)				
SAMPLES	Ref0	Immediate	Middle	Short
R5	0,00	0,060	0,056	0,073

This is the terrestrial Risk for Ecological LoE

Negative values are converted to zero

Table 4. Terrestrial Chemical LoE (Toxic pressure) values for the fungicide Pyrimethanil.

Step 1. Measured concentration (mg Kg -1)					Step 2. Generic SSL (mg Kg -1)					Step 3. For each contaminant, calculate toxic pressure (PAF) per compound. R3=1/(1+exp^((logHC50-logR1)/0,4)				
SAMPLES	Ref0	Immediate	Middle	Short	SAMPLES	Ref0	Immediate	Middle	Short	SAMPLES	Ref0	Immediate	Middle	Short
Pyrimethanil mg.L-1	0,000	72	57	38	R2 - HC50 (F candida EC50)	18	18	18	18	R3	0,000	0,818	0,778	0,692

Step 4. Correct for background concentrations. R4=(R3-R3ref)/(1-R3ref)					Step 5. Calculate msPAF (TPMM) = 1- (1-R5(1))*(1-R5(2))* …. * (1-R5(n))					Step 6. Scale msPAF values R6= (Sample-REF)/(1-REF)				
SAMPLES	Ref0	Immediate	Middle	Short	SAMPLES	Ref0	Immediate	Middle	Short	SAMPLES	Ref0	Immediate	Middle	Short
R4	0,000	0,818	0,778	0,692	msPAF	0,000	0,818	0,778	0,692	R6	0,000	0,818	0,778	0,692

This is the terrestrial Risk for Chemical LoE

Negative values are converted to zero

Table 5. Terrestrial Integrated risk values for the fungicide Pyrimethanil.

Risk Values for each line of evidence				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0,000	0,818	0,778	0,692
Ecotox LoE	0,0000	0,7536	0,6803	0,7406
Ecological LoE	0,00	0,060	0,056	0,073

Step 1. R1= log (1-X)				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0	-0,74074	-0,65279	-0,51199
Ecotox LoE	0	-0,60843	-0,49528	-0,58601
Ecological LoE	0	-0,02671	-0,02481	-0,03308

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	0	-0,45863	-0,39096	-0,37703

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,652167	0,593518	0,580267

This is the terrestrial Integrate Risk

Table 6. Aquatic toxicity values for the scaling results of the Ecotoxicological LoE for the fungicide Pyrimethanil.

	% as Control				Step 1. R1=(100-X)/100				Step 2. R2= (X-Ref) / (1-Ref)				Scaling results			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
D similis	100	1	1	75	0,00	0,99	0,99	0,25	0,00	0,99	0,99	0,25	0,00	0,99	0,99	0,25
C. silvestrii	100	1	1	1	0,00	0,99	0,99	0,99	0,00	0,99	0,99	0,99	0,00	0,99	0,99	0,99
C. dubia	100	1	1	10	0,00	0,99	0,99	0,90	0,00	0,99	0,99	0,90	0,00	0,99	0,99	0,90
D similis	100	45	70	100	0,00	0,55	0,30	0,00	0,00	0,55	0,30	0,00	0,00	0,55	0,30	0,00
C. silvestrii	100	5	1	60	0,00	0,95	0,99	0,40	0,00	0,95	0,99	0,40	0,00	0,95	0,99	0,40
C. dubia	100	5	15	30	0,00	0,95	0,85	0,70	0,00	0,95	0,85	0,70	0,00	0,95	0,85	0,70
S. capricornutum	100	1	1	32	0,00	0,99	0,99	0,68	0,00	0,99	0,99	0,68	0,00	0,99	0,99	0,68
S. capricornutum	100	27	47	68	0,00	0,73	0,53	0,32	0,00	0,73	0,53	0,32	0,00	0,73	0,53	0,32
D. rerio 48h	100	1	100	100	0,00	0,99	0,00	0,00	0,00	0,99	0,00	0,00	0,00	0,99	0,00	0,00
D. rerio 96h	100	1	80	100	0,00	0,99	0,20	0,00	0,00	0,99	0,20	0,00	0,00	0,99	0,20	0,00

Negative values are converted in zero

Table 7. Aquatic Ecotoxicological LoE values for the fungicide Pyrimethanil.

	Scaling				Step 1. R1= log (1-X)			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
D similis	0,00	0,99	0,99	0,25	0	-2	-2	-0,12494
C. silvestrii	0,00	0,99	0,99	0,99	0	-2	-2	-2
C. dubia	0,00	0,99	0,99	0,90	0	-2	-2	-1
D similis	0,00	0,55	0,30	0,00	0	-0,34679	-0,1549	0
C. silvestrii	0,00	0,95	0,99	0,40	0	-1,30103	-2	-0,22185
C. dubia	0,00	0,95	0,85	0,70	0	-1,30103	-0,82391	-0,52288
S. capricornutum	0,00	0,99	0,99	0,68	0	-2	-2	-0,48818
S. capricornutum	0,00	0,73	0,53	0,32	0	-0,57152	-0,32698	-0,16969
D. rerio 48h	0,00	0,99	0,00	0,00	0	-2	0	0
D. rerio 96h	0,00	0,99	0,20	0,00	0	-2	-0,09691	0
D. rerio 4h	0,00	0,70	0,05	0,05	0	-0,52288	-0,02228	-0,02228
D. rerio 12h	0,00	0,45	0,99	0,99	0	-0,25964	-2	-2
L. latrans	0,00	0,48	0,18	0,25	0	-0,284	-0,08619	-0,12494
L. catesbeianus	0,00	0,16	0,18	0,18	0	-0,07572	-0,08619	-0,08619

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	0	-1,19019	-0,97124	-0,48292

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,9355	0,8932	0,6711

This is the aquatic Risk for Ecotoxicological LoE

Negative values are converted to zero

Table 8. Aquatic Ecological LoE values for the fungicide Pyrimethanil.

	Data				Step 1. R1=X/100			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Mdec1	208,2	179,6	148,5	127,1	1	0,86	0,71	0,61
Mdec2	188,9	173,8	143,9	127,2	1	0,92	0,76	0,67
Mdec3	191,8	160,5	137,9	122,0	1	0,84	0,72	0,64
ZTaxons	10	8	10	8	1	0,80	1,00	0,80
ZTaxons	15	8	11	14	1	0,53	0,73	0,93
ZTaxons	13	11	9	13	1	0,85	0,69	1,00
Zindiv	427	148	116	181	1	0,35	0,27	0,42
Zindiv	497	225	350	3171	1	0,45	0,70	6,38
Zindiv	534	117	249	619	1	0,22	0,47	1,16
Zshannon	1,35	1,75	1,6	1,2	1	1,30	1,19	0,89
Zshannon	1,88	1,5	1,8	0,91	1	0,80	0,96	0,48
Zshannon	1,25	1,9	0,82	1,44	1	1,52	0,66	1,15
ZMargalef	0,7	0,59	0,77	0,58	1	0,84	1,10	0,83
ZMargalef	1,06	0,57	0,78	0,87	1	0,54	0,74	0,82
ZMargalef	0,91	0,86	0,64	0,9	1	0,95	0,70	0,99
PTaxons	19	15	10	10	1	0,79	0,53	0,53
PTaxons	23	14	13	13	1	0,61	0,57	0,57
PTaxons	20	12	9	11	1	0,60	0,45	0,55
Pindiv	107	141	182	125	1	1,32	1,70	1,17
Pindiv	1198	1005	755	1131	1	0,84	0,63	0,94
Pindiv	419	944	678	937	1	2,25	1,62	2,24
Pshannon	2,27	1,51	1,31	1,75	1	0,67	0,58	0,77
Pshannon	1,03	0,46	1,13	1,34	1	0,45	1,10	1,30
Pshannon	1,21	0,77	1,47	1,3	1	0,64	1,21	1,07
PMargalef	3,85	2,8	1,73	1,86	1	0,73	0,45	0,48
PMargalef	3,1	1,9	1,81	1,7	1	0,61	0,58	0,55
PMargalef	3,15	1,6	1,27	1,46	1	0,51	0,40	0,46
McTaxon	3	4	3	3	1	1,33	1,00	1,00
McTaxon	4	3	3	4	1	0,75	0,75	1,00
McTaxon	5	7	4	4	1	1,40	0,80	0,80
McIndiv	87	85	76	123	1	0,98	0,87	1,41
McIndiv	138	103	104	79	1	0,75	0,75	0,57
McIndiv	158	171	368	97	1	1,08	2,33	0,61
McShannon	0,6909	0,7989	0,7533	0,6341	1	1,16	1,09	0,92
McShannon	0,264	0,5008	0,7063	0,4023	1	1,90	2,68	1,52
McShannon	0,9393	1,091	0,6349	0,7974	1	1,16	0,68	0,85
McMargalef	0,4478	0,6753	0,4618	0,4156	1	1,51	1,03	0,93
McMargalef	0,6089	0,4315	0,4306	0,6866	1	0,71	0,71	1,13
McMargalef	0,7901	1,167	0,5078	0,6558	1	1,48	0,64	0,83

Table 9. Aquatic Ecological LoE values for the fungicide Pyrimethanil.

	Step 2. Calculate absolute values of log (R1)							
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Mdec1	0,00	-0,06	-0,15	-0,21	0,00	0,06	0,15	0,21
Mdec2	0,00	-0,04	-0,12	-0,17	0,00	0,04	0,12	0,17
Mdec3	0,00	-0,08	-0,14	-0,20	0,00	0,08	0,14	0,20
ZTaxons	0,00	-0,10	0,00	-0,10	0,00	0,10	0,00	0,10
ZTaxons	0,00	-0,27	-0,13	-0,03	0,00	0,27	0,13	0,03
ZTaxons	0,00	-0,07	-0,16	0,00	0,00	0,07	0,16	0,00
Zindiv	0,00	-0,46	-0,57	-0,37	0,00	0,46	0,57	0,37
Zindiv	0,00	-0,34	-0,15	0,00	0,00	0,34	0,15	0,00
Zindiv	0,00	-0,66	-0,33	0,00	0,00	0,66	0,33	0,00
Zshannon	0,00	0,00	0,00	-0,05	0,00	0,00	0,00	0,05
Zshannon	0,00	-0,10	-0,02	-0,32	0,00	0,10	0,02	0,32
Zshannon	0,00	0,00	-0,18	0,00	0,00	0,00	0,18	0,00
ZMargalef	0,00	-0,07	0,00	-0,08	0,00	0,07	0,00	0,08
ZMargalef	0,00	-0,27	-0,13	-0,09	0,00	0,27	0,13	0,09
ZMargalef	0,00	-0,02	-0,15	0,00	0,00	0,02	0,15	0,00
PTaxons	0,00	-0,10	-0,28	-0,28	0,00	0,10	0,28	0,28
PTaxons	0,00	-0,22	-0,25	-0,25	0,00	0,22	0,25	0,25
PTaxons	0,00	-0,22	-0,35	-0,26	0,00	0,22	0,35	0,26
Pindiv	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pindiv	0,00	-0,08	-0,20	-0,02	0,00	0,08	0,20	0,02
Pindiv	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pshannon	0,00	-0,18	-0,24	-0,11	0,00	0,18	0,24	0,11
Pshannon	0,00	-0,35	0,00	0,11	0,00	0,35	0,00	0,00
Pshannon	0,00	-0,20	0,00	0,00	0,00	0,20	0,00	0,00
PMargalef	0,00	-0,14	-0,35	-0,32	0,00	0,14	0,35	0,32
PMargalef	0,00	-0,21	-0,23	-0,26	0,00	0,21	0,23	0,26
PMargalef	0,00	-0,29	-0,39	-0,33	0,00	0,29	0,39	0,33
McTaxon	0,00	0,12	0,00	0,00	0,00	0,00	0,00	0,00
McTaxon	0,00	-0,12	-0,12	0,00	0,00	0,12	0,12	0,00
McTaxon	0,00	0,15	-0,10	-0,10	0,00	0,00	0,10	0,10
McIndiv	0,00	-0,01	-0,06	0,15	0,00	0,01	0,06	0,00
McIndiv	0,00	-0,13	-0,12	-0,24	0,00	0,13	0,12	0,24
McIndiv	0,00	0,03	0,37	-0,21	0,00	0,00	0,00	0,21
McShannon	0,00	0,06	0,04	-0,04	0,00	0,00	0,00	0,04
McShannon	0,00	0,28	0,43	0,18	0,00	0,00	0,00	0,00
McShannon	0,00	0,07	-0,17	-0,07	0,00	0,00	0,17	0,07
McMargalef	0,00	0,18	0,01	-0,03	0,00	0,00	0,00	0,03
McMargalef	0,00	-0,15	-0,15	0,05	0,00	0,15	0,15	0,00
McMargalef	0,00	0,17	-0,19	-0,08	0,00	0,00	0,19	0,08

Positive values are converted to zero

Table 10. Aquatic Ecological LoE values for the fungicide Pyrimethanil.

Step 3. Calculate sum of all values and multiply with –1. R3 = -1 * Σ (R2)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0,00	-4,95	-5,44	-4,23

Step 4. Calculate number of endpoints. R4 = N				
SAMPLES	Ref0	Immediate	Middle	Short
R4	30,00	30,00	30,00	30,00

Step 4. Apply formula: 1-10^(R3/R4)				
SAMPLES	Ref0	Immediate	Middle	Short
R5	0,00	0,316	0,342	0,277

This is the aquatic Risk for Ecological LoE
Negative values are converted to zero

Table 11. Aquatic Chemical (Toxic pressure) LoE values for the fungicide Pyrimethanil.

Step 1. Measured concentration (mg Kg -1)				
SAMPLES	Ref0	Immediate	Middle	Short
R1	0	1	0,8	0,53

Step 2. Generic SSL (mg Kg -1)				
SAMPLES	Ref0	Immediate	Middle	Short
R2 - HC50 (*D. magna* EC50)	5	5	5	5

Step 3. For each contaminant, calculate toxic pressure (PAF) per compound. R3=1/(1+exp^((logHC50-logR1)/0,4)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0,000	0,148	0,120	0,080

Step 4. Correct for background concentrations. R4=(R3-R3ref)/(1-R3ref)				
SAMPLES	Ref0	Immediate	Middle	Short
R4	0,000	0,148	0,120	0,080

Step 5. Calculate msPAF (TPMM) TPMM = 1- (1-R5(1))*(1-R5(2))* …. * (1-R5(n))				
SAMPLES	Ref0	Immediate	Middle	Short
msPAF	0,000	0,148	0,120	0,080

Step 6. Scale msPAF values R6= (Sample-REF)/(1-REF)				
SAMPLES	Ref0	Immediate	Middle	Short
R6	0,000	0,148	0,120	0,080

This is the aquatic Risk for Chemical LoE
Negative values are converted to zero

Table 12. Aquatic integrated risk values for the fungicide Pyrimethanil.

Risk Values for each line of evidence				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0,000	0,148	0,120	0,080
Ecotox LoE	0,0000	0,9355	0,8932	0,6711
Ecological LoE	0,00	0,316	0,342	0,277

Step 1. R1= log (1-X)				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0	-0,06975	-0,05566	-0,03641
Ecotox LoE	0	-1,19019	-0,97124	-0,48292
Ecological LoE	0	-0,1649	-0,18149	-0,14096

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	0	-0,47494	-0,4028	-0,2201

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,664992	0,604449	0,397573

This is the aquatic Integrated Risk

Table 13. Ecosystem toxicity values for the scaling results of the Ecotoxicological LoE for the fungicide Pyrimethanil.

	% as Control				Step 1. R1=(100-X)/100			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Germination7	100	1,25	0,5	0,5	0,00	0,99	1,00	1,00
Germination14	100	4,25	8,5	5,75	0,00	0,96	0,92	0,94
Germination21	100,00	82,05	87,18	58,97	0,00	0,18	0,13	0,41
Seedling	100,00	25,87	28,25	28,15	0,00	0,74	0,72	0,72
Roots	100,00	29,11	25,46	30,82	0,00	0,71	0,75	0,69
Leaves	100,00	24,29	35,76	6,25	0,00	0,76	0,64	0,94
Biomass (g)	100,00	15,04	13,01	14,23	0,00	0,85	0,87	0,86
Germination 7	100,00	200,00	66,67	1,00	0,00	-1,00	0,33	0,99
Germination 14	100,00	97,30	91,89	91,89	0,00	0,03	0,08	0,08
Germination 21	100	89,19	91,89	81,08	0,00	0,11	0,08	0,19
Seedling	100	51,48	58,28	62,81	0,00	0,49	0,42	0,37
Roots	100	47,62	66,85	80,74	0,00	0,52	0,33	0,19
Leaves	100	98,26	102,13	93,02	0,00	0,02	-0,02	0,07
Biomass (g)	100	53,64	57,27	69,09	0,00	0,46	0,43	0,31
D similis	100	1	1	75	0,00	0,99	0,99	0,25
C. silvestrii	100	1	1	1	0,00	0,99	0,99	0,99
C. dubia	100	1	1	10	0,00	0,99	0,99	0,90
D. similis	100	45	70	100	0,00	0,55	0,30	0,00
C. silvestrii	100	5	1	60	0,00	0,95	0,99	0,40
C. dubia	100	5	15	30	0,00	0,95	0,85	0,70
S. capricornutum	100	1	1	32	0,00	0,99	0,99	0,68
S. capricornutum	100	27	47	68	0,00	0,73	0,53	0,32
E. andrei	100	0	13	33	0,00	1,00	0,87	0,67
F. candida	100	32	39	46	0,00	0,68	0,61	0,54
H. nitidus	100	20	45	58,7	0,00	0,80	0,55	0,41
F candida	100,0	13,0	34,2	101,9	0,00	0,87	0,66	-0,02
D. rerio 48h	100,0	1	100	100	0,00	0,99	0,00	0,00
D. rerio 96h	100,0	1	80	100	0,00	0,99	0,20	0,00
D. rerio 4h	100,0	30	95	95	0,00	0,70	0,05	0,05
D. rerio 12h	100,0	55	0	0	0,00	0,45	1,00	1,00
L. latrans	100,0	52	82	75	0,00	0,48	0,18	0,25
L. catesbeianus	100,0	84	82	82	0,00	0,16	0,18	0,18

Table 14. Ecosystem toxicity values for the scaling results of the Ecotoxicological LoE for the fungicide Pyrimethanil.

Step 2. R2= (X-Ref) / (1-Ref)					Scaling results			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Germination 7	0,00	0,99	1,00	1,00	0,00	0,99	1,00	1,00
Germination 14	0,00	0,96	0,92	0,94	0,00	0,96	0,92	0,94
Germination 21	0,00	0,18	0,13	0,41	0,00	0,18	0,13	0,41
Seedling	0,00	0,74	0,72	0,72	0,00	0,74	0,72	0,72
Roots	0,00	0,71	0,75	0,69	0,00	0,71	0,75	0,69
Leaves	0,00	0,76	0,64	0,94	0,00	0,76	0,64	0,94
Biomass (g)	0,00	0,85	0,87	0,86	0,00	0,85	0,87	0,86
Germination 7	0,00	-1,00	0,33	0,99	0,00	0,00	0,33	0,99
Germination 14	0,00	0,03	0,08	0,08	0,00	0,03	0,08	0,08
Germination 21	0,00	0,11	0,08	0,19	0,00	0,11	0,08	0,19
Seedling	0,00	0,49	0,42	0,37	0,00	0,49	0,42	0,37
Roots	0,00	0,52	0,33	0,19	0,00	0,52	0,33	0,19
Leaves	0,00	0,02	-0,02	0,07	0,00	0,02	0,00	0,07
Biomass (g)	0,00	0,46	0,43	0,31	0,00	0,46	0,43	0,31
D. similis	0,00	0,99	0,99	0,25	0,00	0,99	0,99	0,25
C. silvestrii	0,00	0,99	0,99	0,99	0,00	0,99	0,99	0,99
C. dubia	0,00	0,99	0,99	0,90	0,00	0,99	0,99	0,90
D similis	0,00	0,55	0,30	0,00	0,00	0,55	0,30	0,00
C. silvestrii	0,00	0,95	0,99	0,40	0,00	0,95	0,99	0,40
C. dubia	0,00	0,95	0,85	0,70	0,00	0,95	0,85	0,70
S. capricornutum	0,00	0,99	0,99	0,68	0,00	0,99	0,99	0,68
S. capricornutum	0,00	0,73	0,53	0,32	0,00	0,73	0,53	0,32
E. andrei	0,00	1,00	0,87	0,67	0,00	1,00	0,87	0,67
F. candida	0,00	0,68	0,61	0,54	0,00	0,68	0,61	0,54
H. nitidus	0,00	0,80	0,55	0,41	0,00	0,80	0,55	0,41
F. candida	0,00	0,87	0,66	-0,02	0,00	0,87	0,66	0,00
D. rerio 48h	0,00	0,99	0,00	0,00	0,00	0,99	0,00	0,00
D. rerio 96h	0,00	0,99	0,20	0,00	0,00	0,99	0,20	0,00
D. rerio 4h	0,00	0,70	0,05	0,05	0,00	0,70	0,05	0,05
D. rerio 12h	0,00	0,45	1,00	1,00	0,00	0,45	1,00	1,00
L. latrans	0,00	0,48	0,18	0,25	0,00	0,48	0,18	0,25
L. catesbeianus	0,00	0,16	0,18	0,18	0,00	0,16	0,18	0,18

Negative values are converted in zero

Table 15. Ecosystem Ecotoxicological LoE values for the fungicide Pyrimethanil.

Scaling					Step 1. R1= log (1-X)			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Germination7	0,00	0,99	1,00	1,00	0	-1,90309	-2,30103	-2,30103
Germination14	0,00	0,96	0,92	0,94	0	-1,37161	-1,07058	-1,24033
Germination21	0,00	0,18	0,13	0,41	0	-0,08591	-0,05959	-0,22934
Seedling	0,00	0,74	0,72	0,72	0	-0,58726	-0,54904	-0,5506
Roots	0,00	0,71	0,75	0,69	0	-0,53591	-0,59422	-0,51119
Leaves	0,00	0,76	0,64	0,94	0	-0,61465	-0,44656	-1,20412
Biomass (g)	0,00	0,85	0,87	0,86	0	-0,82273	-0,88579	-0,84687
Germination7	0,00	0,00	0,33	0,99	0	0	-0,17609	-2
Germination14	0,00	0,03	0,08	0,08	0	-0,0119	-0,03672	-0,03672
Germination21	0,00	0,11	0,08	0,19	0	-0,04969	-0,03672	-0,09108
Seedling	0,00	0,49	0,42	0,37	0	-0,28835	-0,2345	-0,20197
Roots	0,00	0,52	0,33	0,19	0	-0,32225	-0,17491	-0,09292
Leaves	0,00	0,02	0,00	0,07	0	-0,00764	0	-0,03141
Biomass (g)	0,00	0,46	0,43	0,31	0	-0,27054	-0,24205	-0,16058
D. similis	0,00	0,99	0,99	0,25	0	-2	-2	-0,12494
C. silvestrii	0,00	0,99	0,99	0,99	0	-2	-2	-2
C. dubia	0,00	0,99	0,99	0,90	0	-2	-2	-1
D similis	0,00	0,55	0,30	0,00	0	-0,34679	-0,1549	0
C. silvestrii	0,00	0,95	0,99	0,40	0	-1,30103	-2	-0,22185
C. dubia	0,00	0,95	0,85	0,70	0	-1,30103	-0,82391	-0,52288
S. capricornutum	0,00	0,99	0,99	0,68	0	-2	-2	-0,48818
S. capricornutum	0,00	0,73	0,53	0,32	0	-0,57152	-0,32698	-0,16969
E. andrei	0,00	0,99	0,87	0,67	0	-2	-0,88606	-0,48149
F. candida	0,00	0,68	0,61	0,54	0	-0,49485	-0,40894	-0,33724
H. nitidus	0,00	0,80	0,55	0,41	0	-0,69897	-0,34679	-0,23136
F. candida	0,00	0,87	0,66	0,00	0	-0,88636	-0,46545	0
D. rerio 48h	0,00	0,99	0,00	0,00	0	-2	0	0
D. rerio 96h	0,00	0,99	0,20	0,00	0	-2	-0,09691	0
D. rerio 4h	0,00	0,70	0,05	0,05	0	-0,52288	-0,02228	-0,02228
D. rerio 12h	0,00	0,45	0,99	0,99	0	-0,25964	-2	-2
L. latrans	0,00	0,48	0,18	0,25	0	-0,284	-0,08619	-0,12494
L. catesbeianus	0,00	0,16	0,18	0,18	0	-0,07572	-0,08619	-0,08619

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	3,01352E-18	-0,86295	-0,70351	-0,54091

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,8629	0,8021	0,7122

This is the ecosystem Risk for Ecotoxicological LoE

Ngative values are converted to zero

Table 16. Ecosystem Ecological LoE values for the fungicide Pyrimethanil.

	Data				Step 1. R1=X/100			
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Mdec1	208,2	179,6	148,5	127,1	1	0,86	0,71	0,61
Mdec2	188,9	173,8	143,9	127,2	1	0,92	0,76	0,67
Mdec3	191,8	160,5	137,9	122,0	1	0,84	0,72	0,64
ZTaxons	10	8	10	8	1	0,80	1,00	0,80
ZTaxons	15	8	11	14	1	0,53	0,73	0,93
ZTaxons	13	11	9	13	1	0,85	0,69	1,00
Zindiv	427	148	116	181	1	0,35	0,27	0,42
Zindiv	497	225	350	3171	1	0,45	0,70	6,38
Zindiv	534	117	249	619	1	0,22	0,47	1,16
Zshannon	1,35	1,75	1,6	1,2	1	1,30	1,19	0,89
Zshannon	1,88	1,5	1,8	0,91	1	0,80	0,96	0,48
Zshannon	1,25	1,9	0,82	1,44	1	1,52	0,66	1,15
ZMargalef	0,7	0,59	0,77	0,58	1	0,84	1,10	0,83
ZMargalef	1,06	0,57	0,78	0,87	1	0,54	0,74	0,82
ZMargalef	0,91	0,86	0,64	0,9	1	0,95	0,70	0,99
PTaxons	19	15	10	10	1	0,79	0,53	0,53
PTaxons	23	14	13	13	1	0,61	0,57	0,57
PTaxons	20	12	9	11	1	0,60	0,45	0,55
Pindiv	107	141	182	125	1	1,32	1,70	1,17
Pindiv	1198	1005	755	1131	1	0,84	0,63	0,94
Pindiv	419	944	678	937	1	2,25	1,62	2,24
Pshannon	2,27	1,51	1,31	1,75	1	0,67	0,58	0,77
Pshannon	1,03	0,46	1,13	1,34	1	0,45	1,10	1,30
Pshannon	1,21	0,77	1,47	1,3	1	0,64	1,21	1,07
PMargalef	3,85	2,8	1,73	1,86	1	0,73	0,45	0,48
PMargalef	3,1	1,9	1,81	1,7	1	0,61	0,58	0,55
PMargalef	3,15	1,6	1,27	1,46	1	0,51	0,40	0,46
Baitlam1	100	21,4	29,4	34,9	1	0,21	0,29	0,35
Baitlam2	100	99,2	77,3	41,4	1	0,99	0,77	0,41
Baitlam3	100	74,3	79,3	70,4	1	0,74	0,79	0,70
McTaxon	3	4	3	3	1	1,33	1,00	1,00
McTaxon	4	3	3	4	1	0,75	0,75	1,00
McTaxon	5	7	4	4	1	1,40	0,80	0,80
McIndiv	87	85	76	123	1	0,98	0,87	1,41
McIndiv	138	103	104	79	1	0,75	0,75	0,57
McIndiv	158	171	368	97	1	1,08	2,33	0,61
McShannon	0,6909	0,7989	0,7533	0,6341	1	1,16	1,09	0,92
McShannon	0,264	0,5008	0,7063	0,4023	1	1,90	2,68	1,52
McShannon	0,9393	1,091	0,6349	0,7974	1	1,16	0,68	0,85
McMargalef	0,4478	0,6753	0,4618	0,4156	1	1,51	1,03	0,93
McMargalef	0,6089	0,4315	0,4306	0,6866	1	0,71	0,71	1,13
McMargalef	0,7901	1,167	0,5078	0,6558	1	1,48	0,64	0,83

Table 17. Ecosystem Ecological LoE values for the fungicide Pyrimethanil.

Step 2. Calculate absolute values of log (R1)								
SAMPLES	Ref0	Immediate	Middle	Short	Ref0	Immediate	Middle	Short
Mdec1	0,00	-0,06	-0,15	-0,21	0,00	0,06	0,15	0,21
Mdec2	0,00	-0,04	-0,12	-0,17	0,00	0,04	0,12	0,17
Mdec3	0,00	-0,08	-0,14	-0,20	0,00	0,08	0,14	0,20
ZTaxons	0,00	-0,10	0,00	-0,10	0,00	0,10	0,00	0,10
ZTaxons	0,00	-0,27	-0,13	-0,03	0,00	0,27	0,13	0,03
ZTaxons	0,00	-0,07	-0,16	0,00	0,00	0,07	0,16	0,00
Zindiv	0,00	-0,46	-0,57	-0,37	0,00	0,46	0,57	0,37
Zindiv	0,00	-0,34	-0,15	0,00	0,00	0,34	0,15	0,00
Zindiv	0,00	-0,66	-0,33	0,00	0,00	0,66	0,33	0,00
Zshannon	0,00	0,00	0,00	-0,05	0,00	0,00	0,00	0,05
Zshannon	0,00	-0,10	-0,02	-0,32	0,00	0,10	0,02	0,32
Zshannon	0,00	0,00	-0,18	0,00	0,00	0,00	0,18	0,00
ZMargalef	0,00	-0,07	0,00	-0,08	0,00	0,07	0,00	0,08
ZMargalef	0,00	-0,27	-0,13	-0,09	0,00	0,27	0,13	0,09
ZMargalef	0,00	-0,02	-0,15	0,00	0,00	0,02	0,15	0,00
PTaxons	0,00	-0,10	-0,28	-0,28	0,00	0,10	0,28	0,28
PTaxons	0,00	-0,22	-0,25	-0,25	0,00	0,22	0,25	0,25
PTaxons	0,00	-0,22	-0,35	-0,26	0,00	0,22	0,35	0,26
Pindiv	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pindiv	0,00	-0,08	-0,20	-0,02	0,00	0,08	0,20	0,02
Pindiv	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pshannon	0,00	-0,18	-0,24	-0,11	0,00	0,18	0,24	0,11
Pshannon	0,00	-0,35	0,00	0,11	0,00	0,35	0,00	0,00
Pshannon	0,00	-0,20	0,00	0,00	0,00	0,20	0,00	0,00
PMargalef	0,00	-0,14	-0,35	-0,32	0,00	0,14	0,35	0,32
PMargalef	0,00	-0,21	-0,23	-0,26	0,00	0,21	0,23	0,26
PMargalef	0,00	-0,29	-0,39	-0,33	0,00	0,29	0,39	0,33
Baitlam1	0,00	-0,67	-0,53	-0,46	0,00	0,67	0,53	0,46
Baitlam2	0,00	0,00	-0,11	-0,38	0,00	0,00	0,11	0,38
Baitlam3	0,00	-0,13	-0,10	-0,15	0,00	0,13	0,10	0,15
McTaxon	0,00	0,12	0,00	0,00	0,00	0,00	0,00	0,00
McTaxon	0,00	-0,12	-0,12	0,00	0,00	0,12	0,12	0,00
McTaxon	0,00	0,15	-0,10	-0,10	0,00	0,00	0,10	0,10
McIndiv	0,00	-0,01	-0,06	0,15	0,00	0,01	0,06	0,00
McIndiv	0,00	-0,13	-0,12	-0,24	0,00	0,13	0,12	0,24
McIndiv	0,00	0,03	0,37	-0,21	0,00	0,00	0,00	0,21
McShannon	0,00	0,06	0,04	-0,04	0,00	0,00	0,00	0,04
McShannon	0,00	0,28	0,43	0,18	0,00	0,00	0,00	0,00
McShannon	0,00	0,07	-0,17	-0,07	0,00	0,00	0,17	0,07
McMargalef	0,00	0,18	0,01	-0,03	0,00	0,00	0,00	0,03
McMargalef	0,00	-0,15	-0,15	0,05	0,00	0,15	0,15	0,00
McMargalef	0,00	0,17	-0,19	-0,08	0,00	0,00	0,19	0,08

Positive values are converted to zero

Table 18. Ecosystem Ecological LoE values for the fungicide Pyrimethanil.

Step 3. Calculate sum of all values and multiply with –1. R3 = -1 * Σ (R2)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0,00	-5,75	-6,19	-5,22

Step 4. Calculate number of endpoints. R4 = N				
SAMPLES	Ref0	Immediate	Middle	Short
R4	30,00	30,00	30,00	30,00

Step 4. Apply formula: 1-10^(R3/R4)				
SAMPLES	Ref0	Immediate	Middle	Short
R5	0,00	0,357	0,378	0,330

This is the ecosystem Risk for Ecological LoE
Negative values are converted to zero

Table 19. Ecosystem Chemical (Toxic pressure) LoE values for the fungicide Pyrimethanil.

Step 1. Measured concentration (mg Kg -1)				
SAMPLES	Ref0	Immediate	Middle	Short
R1 terrestrial	0	1	0,8	0,53
R1 aquatic	0	72	57	38

Step 2. Generic SSL (mg Kg -1)				
SAMPLES	Ref0	Immediate	Middle	Short
R2 - HC50 (*D. magna* EC50)	5	5	5	5
R2 - HC50 (F candida EC50)	18	18	18	18

Step 3. For each contaminant, calculate toxic pressure (PAF) per compound. R3=1/(1+exp^((logHC50-logR1)/0,4)				
SAMPLES	Ref0	Immediate	Middle	Short
R3 terrestrial	0,000	0,148	0,120	0,080
R3 aquatic	0,000	0,818	0,778	0,692

Step 4. Correct for background concentrations. R4=(R3-R3ref)/(1-R3ref)				
SAMPLES	Ref0	Immediate	Middle	Short
R4 terrestrial	0,000	0,148	0,120	0,080
R4 aquatic	0,000	0,818	0,778	0,692

Step 5. Calculate msPAF (TPMM) = 1- (1-R5(1))*(1-R5(2))* …. * (1-R5(n))				
SAMPLES	Ref0	Immediate	Middle	Short
msPAF	0,000	0,845	0,804	0,717

Step 6. Scale msPAF values R6= (Sample-REF)/(1-REF)				
SAMPLES	Ref0	Immediate	Middle	Short
R6	0,000	0,845	0,804	0,717

This is the ecosystem Risk for Chemical LoE

Negative values are converted to zero

Table 20. Ecosystem integrated risk values for the fungicide Pyrimethanil.

Risk Values for each line of evidence				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0,000	0,845	0,804	0,717
Ecotox LoE	0,0000	0,8629	0,8021	0,7122
Ecological LoE	0,00	0,357	0,378	0,330

Step 1. R1= log (1-X)				
SAMPLES	Ref0	Immediate	Middle	Short
Chemical LoE	0	-0,81049	-0,70845	-0,5484
Ecotox LoE	0	-0,86295	-0,70351	-0,54091
Ecological LoE	0	-0,19161	-0,2063	-0,17403

Step 2. R2 = Average (X1… Xn)				
SAMPLES	Ref0	Immediate	Middle	Short
R2	0	-0,62168	-0,53942	-0,42111

Step 3. Retro-transform values R3= 1-(10^X)				
SAMPLES	Ref0	Immediate	Middle	Short
R3	0	0,761045	0,711212	0,620784

This is the ecosystem Integrated Risk

Model II - Deterministic approach, that the risk estimation compares exposure and effects data with the steps calculations for the integrated risk based on the United States Environmental Protection Agency (EPA, 2016).

Table 21. Terrestrial Risk Quotient values for the fungicide Pyrimethanil.

RISK ASSESSMENT	TERRESTRIAL		Toxicity	Exposure			$Risk\ Quotient\ (RQ) = (Exposure\ )/Toxicity$		
Atribute		Endpoint	EC50	Immediate	Middle	Short	RQ Immediate	RQ Middle	RQ Short
Plants	Germination21	Acute	1300	1,658	0,1067	0,00162	0,00000128	0,00000008	0,00000000
	Seedling	Acute	0,02	1,658	0,1067	0,00162	0,08290000	0,00533500	0,00008100
	Biomass	Acute	0,012	1,658	0,1067	0,00162	0,13816667	0,00889167	0,00013500
	Germination21	Acute	265	0,11	0,0061	0,0001	0,00000042	0,00000002	0,00000000
	Seedling	Acute	0,0023	0,11	0,0061	0,0001	0,04782609	0,00265217	0,00004348
	Biomass	Acute	0,0029	0,11	0,0061	0,0001	0,03793103	0,00210345	0,00003448
Terrestrial mesocosm	*E. fetida*	Mesocosm	3,29	40	27	1,4	12,15805471	8,20668693	0,42553191
	H. nitidus	Mesocosm	17,33	40	27	1,4	2,30813618	1,55799192	0,08078477
	P. fimata	Mesocosm	15,83	40	27	1,4	2,52684776	1,70562224	0,08843967
	P. minuta	Mesocosm	16,58	40	27	1,4	2,41254524	1,62846803	0,08443908
	F. fimetaria	Mesocosm	39,63	40	27	1,4	1,00933636	0,68130204	0,03532677
	H. aculeifer	Mesocosm	46,2	40	27	1,4	0,86580087	0,58441558	0,03030303
Terrestrial avoidance	*H. nitidus*	Avoidance	28,53	40	27	1,4	1,40203295	0,94637224	0,04907115
	E. andrei	Avoidance	0,0000036	1,60E-04	8,00E-05	1,00E-05	0,04444444	0,02222222	0,00277778
	F. candida	Avoidance	0,0000018	1,30E-03	6,70E-04	8,00E-05	0,72222222	0,37222222	0,04444444
Terrestrial	*F. candida*	Chronic	17,1	40	27	1,4	2,33918129	1,57894737	0,08187135

This is the terrestrial risk quotient values

Table 22. Aquatic Risk Quotient values for the fungicide Pyrimethanil.

RISK ASSESSMENT	AQUATIC		Toxicity	Exposure			$Risk\ Quotient\ (RQ) = (Exposure\ )/Toxicity$		
Atribute		Endpoint	EC50	Immediate	Middle	Short	RQ Immediate	RQ Middle	RQ Short
Elutriate	*D. similis* To	Acute	0,0017	1,658	0,1067	0,00162	0,975294118	0,062764706	0,000952941
	D. similis On	Acute	0,0016	0,11	0,0061	0,0001	0,06875	0,0038125	0,0000625
	C. silvestrii	Acute	4,10E-05	0,11	0,0061	0,0001	2,682926829	0,148780488	0,002439024
	C. dubia	Acute	6,10E-05	0,11	0,0061	0,0001	1,803278689	0,1	0,001639344
	S. capricornutum To	Chronic	1,25E-03	1,658	0,1067	0,00162	1,3264	0,08536	0,001296
	S. capricornutum On	Chronic	5,70E-04	0,11	0,0061	0,0001	0,192982456	0,010701754	0,000175439
Aquatic	*D. rerio* 48h	Acute	32,17	45	30	1	1,4	0,9	0,03
	D. rerio 96h	Acute	27,46	45	30	1	1,6	1,1	0,04
Aquatic avoidance	*D. rerio* 4h	Avoidance	1,1	1,4	0,7	0,2	1,3	0,6	0,182
	L. latrans	Avoidance	0,41	1,4	0,7	0,2	3,4	1,7	0,49
	L. catesbeianus	Avoidance	0,48	1,4	0,7	0,2	2,9	1,5	0,42

This is the aquatic risk quotient values