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Instituto Superior de Agronomia
Universidade de Lisboa

New improvements on pesticide ecological risk assessment on the soil-water interface.

Tese apresentada para obtenção do grau de Doutor em Engenharia Agronómica

Sara Pais de Almeida da Silva Leitão

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Acronyms list

½RD	Half of the Recommended dosage
2RD	twice the recommended dosage
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
AZO	azoxystrobin
BR	between crop rows area
CAP	Common Agriculture Policy
CAS	Chemical Abstracts Service
CI	Confidence Interval
CLO	chlorothalonil
DIN	Deutsches Institut für Normung
DT_{50}	Half-life in soil
ECx	Effect Concentrations
EDx	Effect Dosages
EFSA	Eur. Food Saf. Author
EQS	Environmental Quality Standards
ERA	Environmental Risk Assessment
ETO	ethoprophos
EU	European Union

FOCUS	Forum for the Co-ordination of pesticide fate models and their use
Gm	geometric mean
GUS	Groundwater Ubiquity Score
H	Henry's law constant
HCx	Hazard Concentrations
HS-GC/MS/MS	headspace-gas chromatography-mass spectrometry/ mass spectrometry
ICP-MS	inductively-coupled plasma mass spectrometry
ISO	International Organization for Standardization
Koc	organic-carbon sorption coefficient
K-S	Kolmogorov–Smirnov
LC-MS/MS	liquid chromatography/mass spectrometry/mass spectrometry
LCx	Lethal Concentrations
LDx	Lethal dose
LE/GC-MS	liquid extraction/cleanup followed by gas chromatography/mass
LOEC	Lowest Observed Effect Concentration
LOEL	Lowest Observed Effect Level
log Kow	Octanol-water partition coefficient
LOQ	Limits of Quantification
MAC	Maximum Allowable Concentrations
NOEAEC	No Observed Ecologically Adverse Effects Concentration
NOEC	No Observed Effect Concentration
NOEL	No Observed Effect Level
NPK	Nitrogen: Phosphorous: Potassium
OECD	Organisation for Economic Co-operation and Development
OM	organic matter content
p	significance level
PAF	potentially affected fraction
PEC	Predicted Environmental Concentration
PED	Predicted Environmental Distribution
PNEC	Predicted No Effect Concentrations
PRC	principal response curves
QS	quality standards
R	crop row area
RD	recommended dosage
RDA	Redundancy Analysis
RH	relative humidity
SPA	Special Protection Areas
SPE/GC-MS	solid phase extraction followed by gas chromatography/mass spectrometry
SSD	Species Sensitivity Distribution
SW	solubility in water
SWS	soil-water simulator
T_{rel}	relative tolerance
UNESCO	United Nations Educational, Scientific and Cultural Organization
US-EPA	United States Environmental Protection Agency
VP	vapour pressure
WHC	water holding capacity

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RESUMO

A necessidade de avaliar e reduzir o impacto de pesticidas no ambiente é fundamental para o seu uso sustentável. Com o objetivo de aumentar a relevância ecológica na avaliação de risco ambiental (ARA) de pesticidas, neste estudo adotou-se uma abordagem inovadora integrando a exposição e efeitos de pesticidas em organismos aquáticos e terrestres não-alvo que habitam a interface solo-água, baseada em cenários agrícolas em condições Mediterrânicas para os quais existe uma lacuna de informação. Neste trabalho foram realizados estudos integrando níveis de complexidade crescente de ARA: um primeiro nível refinado utilizando solo natural em testes ecotoxicológicos laboratoriais, substituindo o convencional solo artificial; um nível intermédio com simulações de cenários agrícolas baseados em culturas de regadio de milho, batata e cebola com a aplicação dos fungicidas azoxistrobina e clorotalonil, e do inseticida etoprofos, utilizando uma nova metodologia de semi-campo; finalmente, um nível superior em campo incorporando as interações entre organismos e dinâmica das populações que habitam a interface solo-água e fatores ambientais que influenciam os efeitos dos pesticidas em condições de campo e as usuais práticas agrícolas. Os resultados obtidos contribuirão para aumentar o conhecimento na ARA de pesticidas e na tomada de decisões para o uso sustentável dos pesticidas.

Palavras-chave: clima Mediterrânico; solo natural; avaliação de risco ambiental de pesticidas; cenários agrícolas; ecotoxicologia aquática e terrestre.

ABSTRACT

Improving knowledge to evaluate and reduce pesticide impacts in the environment is a present concern to achieve their sustainable use. With the aim of increasing ecological relevance on the environmental risk assessment of pesticides (ERA), an integrated approach was undertaken linking pesticide fate and effects on aquatic and terrestrial non-target organisms under irrigated crop-based scenarios in Mediterranean realistic conditions, for which there is a lack of studies. Pesticides fate and effects were assessed by adopting an innovative approach embracing different levels of ERA complexity: a refined first-tier with the use of natural soil in ecotoxicological testing, instead of the conventional artificial soil; a refined higher-tier level performing simulations of crop-based agricultural scenarios of maize, potato and onion crops, with the application of the fungicides azoxystrobin and chlorothalonil and the insecticide ethoprophos, using a new semi-field methodology; and an higher tier field study incorporating biological interactions and dynamics of soil fauna communities and environmental factors that determine the effects of pesticides in the field under realistic agricultural practices. This study will increase the knowledge on ecological risks of pesticides under field situations improving decision making towards a sustainable use of pesticides and ecological protection.

Key-Words: Mediterranean conditions; natural soil; ERA pesticides; crop-based scenarios; aquatic-terrestrial ecotoxicology.

CHAPTER I

General Introduction

1. Targeting for a sustainable agriculture and environmental protection in 2020. Pesticides use and water, soil and biodiversity protection.

Agriculture plays a major role on European Union (EU) economics and society, and the use of plant protection products is seen as one of the most important ways to protect plants and their products against harmful organisms, including weeds, and of improving agricultural production (EFSA, 2010a). However, exploitation of natural resources, land-cover conversion and intensification of land use with investments on drainage, fertilizers and pesticides typically leads to disturbance and changes in the diversity of species and habitats (SOER, 2010), and to a depletion on the provision of several ecosystem services.

The Common Agriculture Policy (CAP) was confronted with a set of challenges towards Europe 2020 strategy goals (EC, 2010a) for a smart, sustainable and inclusive economic growth that requested European Union to make a strategic choice for the long-term future of its agriculture, within the context of economic policies and sustainable public finances (EC, 2010b). As a result, the CAP becomes a strong common policy structured around two complementary pillars focused on agriculture productivity and sustainability. The first pillar consists on the growth and more equitably distributed of the greener agricultural sector, and the second pillar focus more on competitiveness and innovation, climate change and the environment. Its strategic aims are to preserve the food production potential on a sustainable basis throughout the EU to face the growing world food demand (that is expected by Food and Agricultural Organization to increase by 70% by 2050), and to support the producing farming communities in line with the environmental, water, animal health and welfare, plant health and public health requirements (EC, 2010b). Managing the natural resources actively by sustainable farming, maintains the rural landscape, combat biodiversity loss and contributes to mitigate and to adapt to climate change. On the other hand, several farming practices have the potential to put pressure on the environment leading to soil depletion, water shortages, pollution, and loss of wildlife habitats and biodiversity (EC, 2010b). Therefore, the CAP towards the 2020 goals have to respond to new challenges, namely, to enhance the sustainable management of natural resources such as water, air,

biodiversity and soil, taking into account climate action, to contribute to viable food production and to balance territorial development by allowing structural diversity in the farming systems, among others. Within this policy framework, environment, climate change and innovation are guiding themes. As such, environmental measures should be related namely to the specific needs of regions and even local areas, such as Natura 2000 areas, and other measures related to help sustaining the potential of rural areas allowing for innovative ideas for business and local governance (EC, 2010b). With this favorable increase of sustainable agriculture demands, the control of the use of pesticides becomes of great importance. The adopted EU 6th **Environment Action Programme (**EC, 2001b**)** recognized that the impact of pesticides on human health and the environment must be further reduced, as such, the use of good agriculture practices, minimizing the use of pesticides, and the combat of over-cropping were several of its main goals. As to prevent any additional negative impacts caused by agricultural activity, the EU established the **Thematic Strategy on the sustainable use of pesticides** (CEC, 2006b) composing future measures taking into consideration economic, social, health and environmental points of view. These measures are included in the Directive 2009/128/EC (EC, 2009) establishing a framework for Community action to achieve the sustainable use of pesticides and in the new Regulation (EC) Nº 1107/2009 concerning the placing of plant protection products on the market repealing the Council Directive 91/414/EEC (EEC, 91). This legislative package intends to ensure a high level of environmental protection, to improve its functioning by a sustainable use of pesticides and promoting the use of integrated pest management, while improving agricultural production. Additionally it also promotes significant overall reduction in risks of the use of pesticides consistent with the necessary level of protection against pests. These good practices will also support the achievement of "good ecological and chemical status" under the Water Framework Directive (EC, 2000) and the proposed Soil Directive (CEC, 2006c) by protecting ecosystems.

As a result of inadequate agriculture practices the detection of pesticide residues in water and their effects on the aquatic environment have been accounted for in the Water Framework Directive (EC, 2000) under its objective of preventing **water pollution** and environmental protection. Among others, they aim at reducing pollution from discharges and emissions of hazardous substances into surface waters and protect

groundwater by preventing its pollution and deterioration. As such this Framework Directive defined a list of priority substances selected from among the ones which present a significant risk to or via the aquatic environment at European level, which include several pesticides (EC, 2001a). By listing these hazardous substances, the EU intends to progressively reduce its discharges, emissions and losses and if possible its cessation to water bodies. This list is revised by the Commission regularly and updated as new information on the environmental risk of these substances and new ones is developed (EC, 2008). In order to **protect the surface water** and aiming to achieve a "good chemical status", the compliance of emission limit values and environmental quality standards (EQS) must be attained (EC, 2000). Maximum allowable concentrations (MAC) for priority substances and certain other pollutants are established to protect against short-term exposure from chemical pollution and EQS are established to protect against long-term exposure, which are based on acute and chronic effects data respectively (EC, 2008). Currently a new directive amending Directives 2000/60/EC and 2008/105/EC as regard to the inclusion of new priority substances in the field of water policy and their EQS is being proposed as well as the introduction of biota standards for several substances (EC, 2011c). To achieve consistent levels of **protection of groundwater**, quality standards (QS) and thresholds for pollutants were established by the EU (EC, 2006). Pesticides are identified as major pollutant agents and their QS enunciate that: active substances in pesticides including their relevant metabolites, degradation and reaction products should not be higher than 0.1µ L^{-1} as individual substances and 0.5µ L^{-1} as the sum of all individual pesticides present in groundwater (EC, 2006). However recent studies (Daam *et al.,* 2010) have raised the question if the actual standard of 0.1µ L^{-1} is in fact protective of groundwater ecosystems because an ecotoxicological base is missing. Although the established value appears to be sufficiently protective for the majority of the pesticides, it may not fully protect groundwater life from several insecticides (Daam *et al.,* 2010). Groundwater is the most sensitive and the largest body of freshwater in the European Union and, in particular, also a main source of public **drinking water** supplies in many Regions (EC, 2006). When groundwater is used for human consumption, it is protected against deterioration by the appliance of quality standards so that is free from any polluting substances (CD 98/83/EC). Pesticides are among these substances and should not be

higher than the parametric values of 0.1μ L^{-1} and 0.5μ L^{-1}, as established for groundwater bodies in general, previously referred (EC, 2006). Currently, groundwater threshold values for each Member State for the purpose of assessing the "good chemical status" taking into account human toxicology and ecotoxicology knowledge, are being defined (Annex 3 of Directive 2006/118/EC, 2010). The interaction of these legislative frameworks, the sustainable use of pesticides and water policies, will make the matter of harmonizing the limit values of great importance in order to better protect the aquatic compartment.

Soil is generally defined as a very dynamic system that supports the plant and animal ecosystem above it (CEC, 2006c; Pierzynski *et al.,* 2000). Agriculture is directly related to soil and intensive or inadequate agricultural practices cause soil degradation, loss of fertility and biodiversity, and impairment of functions within the nutrient cycles, water-retention capacity and the capability of degrading contaminants. As soil formation and regeneration is an extremely slow process, soil is considered a non-renewable resource (Pierzynski *et al.,* 2000). Taking this need for soil protection into consideration, the EU implemented a Thematic Strategy for Soil Protection (CEC, 2006a) integrating environmental concerns into agriculture in order to protect the soil while using it in a sustainable way to prevent its further degradation and to preserve its functions. As a result, a **Soil Framework Directive** was proposed (CEC, 2006c) aiming to fulfill the lack of specific protection policy for soil ecosystems at a Community level defining, namely, measures to limit the introduction of dangerous substances into the soil that may pose a risk to human health and the environment, and setting up an inventory of contaminated sites, a soil status report, and establishing a national strategy for remediation of the contaminated sites identified. Since the adoption of the Strategy, several research works have been done relating to soil issues and in order to contribute to the knowledge base for action (EC, 2012). The integration of soil protection in different EU policies play a key role towards the goal of sustainable use of soil, namely on the Common Agricultural Policy reform in 2020 (EC, 2010b) within the Good Agricultural and Environmental Conditions on organic matter protection, and including a ban on arable stubble burning and an obligation not to plough wetlands and carbon rich soils (EC, 2012). This EU position on increasing and/or preserving soil productivity and decreasing risks to the environment and human health, namely by promoting

sustainable agriculture, it still stands today as an important goal to achieve. Land degradation in its various forms is a fundamental and persistent problem around Europe and it tends to increase (SOER, 2010).

Soil biodiversity provides various essential services such as transforming organic matter into nutrients that can be used by plants and other organisms, nutrient cycling, purifying water by removing contaminants and pathogens and are also implicated in several regulatory services such as climate regulation, the hydrological cycle and flood control, detoxification and pest regulation (EC, 2012; Vandewalle, *et al.,* 2010). Intense agricultural activity (e.g. cereals and industrial crops and horticulture) and high population density are a threat to soil biodiversity and the consequent increase of soil degradation (EC, 2012; Postma-Blaauw *et al.,* 2012). Agriculture has been a major contributor to Europe's **biodiversity of farmland species** due to diverse farming traditions that have resulted in the wide range of agricultural landscapes across Europe. However, intense farming and highly mechanized practices cause direct negative impacts on farmland biodiversity as well as land abandonment (EC, 2011a). In order to prevent any further biodiversity loss due to agriculture and other activities, the EU is committed to the protection of biodiversity by 2020 with the implementation of the B**iodiversity Strategy** (EC, 2011b) which is also an integral part of the Europe 2020 Strategy (EC, 2010a). This 2020 policy Biodiversity framework aims at restoring biodiversity in the EU by, namely, reinforcing the established Natura 2000, the world's largest network of protected areas, and accelerate the full implementation of the Birds and Habitats Directives i.e. reaching favorable conservation status of all habitats and species of European importance. Additionally, it also aims at restoring ecosystems functions by taking into account land use and management such as irrigation schemes, tillage, pesticide use, nature protection and restoration (van Oudenhoven *et al.,* 2012). Given that these processes influence the ecosystem properties, processes and components that are the basis of the ecosystems service provision, consequently all terrestrial and aquatic fauna integrating the ecosystems and their services will be protected and not only the most relevant species referred in the directives towards environmental protection (de Groot, *et al.,* 2010; van Oudenhoven *et al.,* 2012).

In order to achieve its goals, the EU is integrating biodiversity needs into the current reform of the Common Agriculture Policy that by preventing environmental

degradation and pursuing sustainable economic growth, it contributes to the "green growth" in the agricultural sector (EC, 2010b). The Biodiversity Strategy aims at reducing key major pressures on EU biodiversity by ambitioning a long-term vision of enhancing the positive contribution of a sustainable agriculture, fishery and forestry, maximizing coherence between biodiversity protection objectives and those of these policies contributing to improve a sustainable management of natural resources **(**EC, 2011b).

To reach these European 2020 policy framework targets, the EU will require the full implementation of existing environment legislation, as well as action at national, regional and local levels (EC, 2011b). Additionally, a necessity of national research programs aimed at determining the impacts of pesticide use on the environment and biodiversity, and programs to provide improved information and awareness regarding the risks and the potential acute and chronic effects of pesticides on non-target organisms, are also one of the key points of the Thematic Strategy on the sustainable use of pesticides (EC, 2009).

2. Current research needs on ecological risk assessment of pesticides. Placing this thesis into context.

Exposure of non-target organisms to pesticides may vary according to the natural properties of the ecosystem, namely due to differences in climate and soil characteristics, among others (Bending *et al.,* 2006; Chelinho *et al.,* 2011; De Silva *et al.,* 2009; Domene *et al.,* 2011; Jørgensen *et al.,* 2012; Koděsova *et al.,* 2009, 2011). Historically, higher tier studies for the **environmental risk assessment (ERA)** of pesticides in Europe have been performed mainly in the Central Europe and results have been extrapolated to other climatic regions including the Mediterranean (López-Mancisidor *et al.,* 2008; Daam *et al.,* 2011a). In the new Regulation concerning the placing of plant protection products on the market (Regulation (EC) Nº 1107, 2009), the European Union established three zones in Europe (North, Centre and South) making exposure assessment scenarios for the ERA of pesticides more realistic according to specific edapho-climatic conditions. However, the ERA of pesticides is still based on

generic FOCUS (Forum for the Co-ordination of pesticide fate models and their use) scenarios to simulate Predicted Environmental Concentrations (PECs), that are developed under northern and central Europe conditions where available monitoring data suggest that these simulations are able to reproduce the general characteristics of measured pesticide concentrations in water bodies (Brock, *et al.,* 2010). Nevertheless, when used in a generalized way under **Mediterranean conditions** where soil characteristics, climatic conditions and biota are substantially different, the generic scenarios do not allow a proper assessment for the Mediterranean region possibly leading to risk misestimates (Brock, *et al.,* 2010; Daam *et al.,* 2011a; López-Mancisidor *et al.,* 2008; Ramos *et al.,* 2000; Vanderborght *et al.,* 2010). As such, there is an increasing necessity to develop or improve scenarios for ERA in this region. The European Food Safety Authority is taking these concerns into consideration during current revisions of existing legislation and new uprising topics by incorporating Mediterranean scenarios (EFSA, 2010b; 2012). This is particularly true when looking at specific transfer pathways of pesticides in the **soil-water interface of agricultural fields** (leaching and drainage) due to the site hydrology as well as agricultural irrigation and rain events (Berenzen *et al.,* 2005; Dousset *et al.,* 2010; Tang *et al.,* 2012). Under Mediterranean scenarios, pesticide driven surface water contamination is strongly associated to soil erosion and runoff resulting from rain events (Berenzen *et al.,* 2005; Tarazona 2005). Pesticide transport is in fact influenced by multiple factors including pesticide and environmental properties as soil structure, organic matter, clay and iron oxides content, climatic and hydrogeological conditions, and agricultural management such as the time of application and land use (Ariaz-Estevez *et al.,* 2008; Dousset *et al.,* 2010; Fenoll *et al.,* 2011; Koděsova *et al.,* 2009; Tang *et al.,* 2012). When evaluating the risk of water pollution both chemical and site characteristics do need to be taken into account, since the retention of a pesticide by soil can prevent its short-term access to surface or groundwater and its effects on aquatic non-target organisms (Ariaz-Estevez *et al.,* 2008; Dousset *et al.,* 2010). Therefore, the need to study pesticide fate in natural environments, namely in the soil-water interface where there is an urgent request for a better understanding of water and pesticide fluxes in soils under intense rain events (Ariaz-Estevez *et al.,* 2008), and its effects on biota under Mediterranean conditions, is of critical importance due to the limited information that is currently available (Daam *et*

al., 2011a). Moreover, due to the known **vulnerability of soils** in the Mediterranean region (e.g. loss of organic matter and the consequent impairment of soil retention function) groundwater contamination by pesticides has a higher probability to occur (Gonçalves *et al.,* 2007; Silva *et al.,* 2006). This aspect becomes of greater importance when pesticides are applied in regions where the water input is high (irrigated crops) and in areas with very permeable soil surfaces lead to higher risks of pollution of the aquatic environment by soil transport or run-off waters (CEC, 2006d). **In Portugal,** several agricultural areas are identified as "contaminated" concerning exposure of superficial (including run-off) and groundwater to pesticides, especially when these sites are regularly under pesticides use, irrigation and located in particularly vulnerable areas (Batista *et al.,* 2002; Cerejeira, *et al.,* 2000, 2003, 2005; Gonçalves *et al.,* 2007; Silva *et al.,* 2006, 2012 a, b). The importance of linking environmental pesticide fate and effect assessment is relevant for the environmental assessment and ecosystems protection (Balderacchi and Trevisan, 2010; Brock, *et al.,* 2010). The environmental risk assessment schemes that support the registration of plant protection products are based on a tiered approach that starts with a conservative assessment and follows to an additional and more complex work if necessary, implying appropriate protection, internal consistency, cost effectiveness and address the problem with an increasing accuracy and precision when going from lower to higher tiers (EC, 2002a, b). The **protection of terrestrial ecosystems** at a first-tier level of the ERA of pesticides was until very recently assessed using only the earthworm acute test with *Eisenia fetida sensu lato* (*E. fetida* and *E. andrei*) at an initial stage (EC, 2002b). Tests using other non-target organisms could be performed if they were believed to be at risk, e.g. tests with Collembola on a case-by-case basis depending on the type of the pesticide and its application method (EC, 2002b). Although earthworms are key species of terrestrial ecosystems as decomposers contributing significantly to organic matter decomposition, nutrient cycling and soil formation (Cortet *et al.,* 1999; EFSA, 2009), there is a need for extending the available battery of toxicity tests with soil organisms to better illustrate the different trophic levels, taxonomic, physiological and/or functional groups of organisms in the terrestrial ecosystem, as well as sub-lethal effects in order to improve the ERA of chemicals in soil with the final aim to protect the structure and functioning of ecosystems (Daam *et al.,* 2011b; EFSA, 2010a; Frampton *et al.,* 2006; van Gestel,

2012; Römbke and Moser, 2002). The EU is **revising the ERA procedures for pesticides** to further update of the ecotoxicological risk assessment guidance documents SANCO/3268/2001 and SANCO/10329/2002 (EC, 2002a, b)[1] and suggest defining specific protection goals at a population level for specific group of organisms (microbes, algae, non-target vascular plants, aquatic invertebrates, terrestrial non-target arthropods, non-arthropod invertebrates and vertebrates) that play a key role in the ecosystems and are potentially impacted by pesticides in **agricultural landscapes** (EFSA, 2010a). This will take into account, for some key drivers, that temporary impacts on population size or structure resulting from pesticide use may be considered acceptable if the impacts are temporary and local, and recovery occurs (Nienstedt *et al.,* 2012). Although there is a growing concern about the potential adverse effects of pesticides in the environment, there is a lack of ecotoxicity data available for non-target terrestrial invertebrates (Daam *et al.,* 2011b; Frampton *et al.,* 2006).

The ERA of pesticides for terrestrial organisms uses standardized ecotoxicological tests traditionally performed in standard artificial soil e.g. OECD (ISO, 1998), or in standard natural soil (e.g. LUFA2.2) that often do not possess the characteristics of **agricultural natural soils**, therefore not mimicking realistic soil biota exposure to pesticides under field conditions (van Gestel, 2012; Kuperman *et al*., 2006). It has been documented that differences in soil properties such as organic matter content may influence pesticide bioavailability by soil-dwelling organisms (collembolans, enchytraeids and earthworms), and its persistence in soil (Amorim et al 2002a, 2002b; De Silva et al, 2009; Domene *et al.*, 2012; Kuperman *et al*., 2006; van Gestel, 2012). Compared to standard soils, natural soils may have properties supporting higher bioavailability of test chemicals than artificial soil, so their use considerably improves the relevance of laboratory ecotoxicological data for field conditions (Kuperman *et al.*, 2006; Van Gestel *et al.,* 2011, 2012). The use of natural soil in ecotoxicological testing makes them more ecologically relevance, enabling a more sound extrapolation of the test results to environmental conditions (Schaeffer *et al.,* 2011). Therefore the importance of using natural soils in ecotoxicity testing is supported by the need to develop more realistic

[1] A new regulation setting out the data requirements for active substances (Commission Regulation (EU) Nº 283/2013) has been adopted by the EU. New "Technical Guidance" documents are currently being developed by EFSA.

chemical toxicological evaluations for terrestrial ecosystems in the ERA of pesticides among European regions (Chelinho *et al.,* 2011; van Gestel, 2012).

The level of uncertainties raised over time have illustrated the difficulty of **extrapolating results from laboratory experiments to the field scale under outdoor conditions** (Boesten and Gottesbüren, 2000; Bouraoui, 2007). This has encouraged the use of different methodologies, such as semi-field methods to assess pesticide fate and behaviour in soil and water, as well as their effects on terrestrial and aquatic biota, increasing ecological relevance. The ERA of pesticides can be refined by including semi-field studies as potential tool for higher-tier ERA, with its selection depending on the research or risk assessment requirements and objectives to be addressed taking into account fate and behavior of the test substance (Schaeffer *et al.,* 2011). As a future research need, given the relative scarcity of standardized test protocols for soil organisms in contrast to the great diversity in their ecological strategies, test requirements should allow for the inclusion of non-standardized test systems where standardized ones are unavailable (Schaeffer *et al.,* 2011). The development and implementation of higher-tier procedures to refine estimates of pesticide exposure may lead to the decrease of uncertainty in a risk assessment, and may warrant improvement of the validation status of fate models and chemical monitoring procedures currently applied (Brock *et al.,* 2006; Tang *et al.,* 2012).

Determinations of **soil quality** using single and multi-species approaches to assess pesticide effects have been effective (Bezchlebová *et al.,* 2007; Engenheiro *et al.,* 2005; Lopes, *et al.,* 2007; Natal da luz, 2004; Sousa *et al.,* 2000) but they are not as reliable as **community studies** (Edwards, 2002; Fountain *et al.,* 2007; Frampton & Van den Brink, 2007; Schaeffer *et al.,* 2011). The most environmentally realistic way of evaluating the fate and effects of pesticides is at ecosystem level under **natural field conditions (**Schaeffer *et al.,* 2011**).** For example, despite their evident contribution to Environmental Risk Assessment (ERA) of pesticides, model ecosystems do not fully consider all biological interactions, environmental factors and stressors that determine the effects of pesticides in the field (Liess *et al.,* 2008). On the other hand, the lack of means/resources to distinguish these factors from natural variation often implied that previous field observation studies were difficult to interpret in terms of causal effects (Liess *et al.,* 2008). Moreover, historical records concerning soil organisms are

relatively limited and, as such, quantifying any changes which may have occurred in their prevalence and distribution is problematic (Gardi *et al.,* 2013).

As such, the EU emphasizes the importance of **linking exposure and effect assessments** and the **relevance of ecological scenarios** for appropriate pesticide risk assessment (Balderacchi and Trevisan, 2010; EFSA 2010a). By integrating ecological field data (including physical and chemical characteristics) of the landscape elements that are intended to be protected and by incorporating representative ecosystem properties in the scenarios and tools for both exposure and effects assessment, the tiered risk assessment approach is improved (EFSA, 2010a). The EU aims to develop robust environmental risk assessment procedures which provide the highest achievable protection to human health and the environment. Therefore, in order to meet the protection goals under **good plant protection practices in agricultural scenarios**, the risk assessment methodology should account for **realistic conditions** of use and variability in local conditions reflecting ecological, landscape and climate aspects (Balderacchi and Trevisan, 2010). This would result in a realistic worst case cropping system against which the use of the product, according to the proposed label instructions, is assessed (EFSA, 2010a). Agriculture scenarios under Mediterranean climate, due to the particular conditions of this area, and indirect and long-term effects associated with the use of pesticides, are critical elements for assessing the real impact of these chemicals at community level and ecosystems (Tarazona, 2005). Such as these environmental factors and stressors determine the effects of pesticides in the field, the process of recolonization in the agricultural ecosystems by local populations has to be considered when evaluating effects at a community level (Liess *et al.,* 2008). In order to protect **soil biota related to crop-based assessments** of agricultural areas, future clarifications are needed to define specific protection goals for in-crop and off-crop areas (including the crops planted in rows) for several key organisms such as terrestrial non-target vascular plants, non-target arthropods and non-target invertebrates (Nienstedt *et al.,* 2012). This will increases the knowledge of the environmental risks of pesticides under field situations improving decision making towards a sustainable use of pesticides.

3. Main objectives

In order to fulfill the gaps discussed above, a research plan following an integrated approach was developed linking fate and effects of pesticides on aquatic and terrestrial communities inhabiting the soil-water interface of agricultural fields under Mediterranean conditions. One of the main objectives of this study was to associate the influence of run-off and leaching as pathways of pesticide contamination into surrounding water bodies on the soil-water interface area during agricultural irrigation, since studies embracing the two compartments (soil and water) are very limited. As a second major aim, side-effects of pesticides on terrestrial and aquatic biota were also evaluated. In line with this and with the aim to increase ecological and realistic relevance of the ERA of pesticides, simulations of realistic exposure conditions using a new semi-field methodology that allows the use of natural soil, pesticide application and irrigation, were performed. These simulations were conducted under "worst case scenarios" of pesticide application for several pesticides in crop-based agricultural scenarios using agricultural natural soil, with the aim to provide realistic knowledge on pesticide risk assessment under an ecologically relevant condition. As a final main objective, a study of pesticide effects on non-target invertebrates in irrigated crop areas located at a relevant study-site under Mediterranean conditions was conducted during the entire crops cycle.

Having a bird's eye in the major goals of this thesis, pesticide side-effect assessment embraced different levels of environmental complexity: i) a first-tier level with laboratory terrestrial single species tests using natural soil and standard test organism in order to increase realistic exposure conditions; ii) a refined higher-tier by conducting crop-based simulations using a semi-field methodology, and iii) a higher-tier field study (evaluating effects at community level) aiming at providing ecological realism by incorporating information on biological interactions, environmental factors and stressors in determining the effects of pesticides under field conditions.

3.1 Work scheme and outline of the thesis.

To attain the main objectives of this thesis several actions/steps, each with a specific goal, were delineated: **Step 1)** selection of a relevant study site of Portuguese agricultural areas taking into consideration type of crops and agricultural practices particularly with respect to pesticide applications; this step included also the selection of pesticides to be used throughout the study taking into account agricultural use and ecological effects; **Step 2)** environmental assessment of the selected pesticides in the soil-water interface from irrigated crops based on crop-based simulations. This was done both by conducting single species tests with standard soil test species (after a prior study comparing the sensitivity of earthworms as compared to other non-target terrestrial organisms) and also by using a new semi-field laboratory methodology mimicking field conditions, taking into account pesticide application under a realistic "worst case scenario", and irrigation in relation to soil and water exposure and fate, and side-effects on non-target terrestrial and aquatic organisms; **Step 3)** higher-tier risk assessment of pesticides on indigenous terrestrial communities by conducting a field study on irrigated crops under Mediterranean conditions during a crop cycle.

Step 1 - Selection of study site and pesticides

Objective: Selection of a relevant agricultural site and crops in a Mediterranean area, and selection of a group of pesticides with different types of action to be used under laboratory and semi-field approaches in order to refine the environmental risk assessments under Mediterranean scenarios.

For the collection of natural soil to be used in Step 2, a reference site was selected taking into consideration the history of the site, particularly the absence of pesticide application (see section 1.1 of Chapter II). The selection of a study site relevant of Portuguese agricultural areas was performed taking into consideration several agricultural factors including: type of crop; pesticides use; agricultural practices particularly irrigation; soil characteristics; its vicinity to surface and groundwater bodies and particularly located in hydrogeological vulnerable areas (see section 1. of Chapter II).

A group of pesticides (active ingredients and formulated products) was selected from available scientific data and specific databases, taking into consideration several criteria: pesticide particularly used on irrigated crops (mainly horticultural and cereal) of Mediterranean countries; pesticide application methods (e.g. soil direct application); pesticides physico-chemical properties, namely environment partition coefficients; persistency in soil; ecotoxicological characteristics, particularly toxicity to aquatic and terrestrial organisms; and predictive exposure on soil and water (see section 2. of Chapter II).

Step 2 – Risk assessment of pesticides on the soil-water interface

Objective: Linking fate and effects of pesticides on soil and aquatic organisms through the simulation of realistic crop-based scenarios of pesticide application and agricultural practices (irrigation), using a newly developed semi-field laboratory methodology;

A – Comparing the sensitivity of earthworms as compared to other non-target terrestrial organisms to pesticides with different type of action.

To study the representativeness of the standard test organism, the earthworm *Eisenia fetida*, for the sensitivity of other non-target soil organisms, the Species Sensitivity Distribution (SSD) approach based on cumulative probability distributions of toxicity values for multiple species (Posthuma *et al.,* 2001) was applied. Information on pesticides ecotoxicity data for terrestrial organisms under a first tier level of ERA (species tested in laboratory single species tests) was compiled and treated according to several criteria towards ecological representativeness. The ecotoxicological information was grouped by substance type (e.g. insecticides, fungicides and herbicides) and taxonomic groups (e.g. Acari, Chilopoda, Coleoptera, Collembola). Differences in sensitivity between groups of organisms for the several pesticide types were established and implications for future studies on terrestrial risk assessment emphasized and discussed. The outputs were used for the selection of the terrestrial test species to be used on the evaluation of effects of the selected pesticides in laboratory and semi-field studies described on the following steps. Results are presented in Chapter III as a scientific paper entitled: Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*.

B – Effects of the selected pesticides on the reproduction of non-target terrestrial invertebrates using natural soil.

To study the impact of the application of the selected pesticides on soil communities, several ecotoxicity standard tests with species having a key role in ecosystem functioning, with different exposure modes and from different trophic groups, were performed. Effects were assessed using single species ecotoxicity tests: determination of effects on reproduction and survival of the echytraeid *Enchytraeus crypticus* (ISO, 2004); inhibition of reproduction of the collembolan *Folsomia candida* (ISO, 1999); and effects of pollutants on the earthworm *Eisenia andrei* (ISO, 1998). The selected pesticides were tested independently using different concentration gradients, and in order to assess the effects under realistic conditions, the tests were performed with natural soil from the "reference site" selected in Step 1. The results from these tests were taken into account for the selection of the terrestrial organisms to be used on the semi-field simulations studies described on the following work action.

Results are presented in Chapter III in the scientific paper: Effects of azoxystrobin, chlorothalonil and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil.

C - Side-effects of pesticides on non-target aquatic and terrestrial species exposed via different contamination pathways using a semi-field methodology.

A "worst case scenario" was established for each of the selected pesticides according to their agricultural use, using a semi-field methodology (soil-water simulator), that allows studying the soil-water interface as a representation of the real environmental conditions of defined crops (see section 1.2 of Chapter II). Pesticides fate in water and soil compartments was assessed focusing on the soil-water transfer pathways during crop irrigation (runoff and leaching), in order to differentiate the relevance of the main soil-water routes of pesticide entry into the aquatic compartments (surface water and groundwater). Soil samples and leaching and run-off waters, as well as elutriates, were analyzed for pesticide residues by independent laboratories. To study the impact of the selected pesticides application on non-target biota several ecotoxicological tests on single aquatic and terrestrial key species from different trophic levels were performed

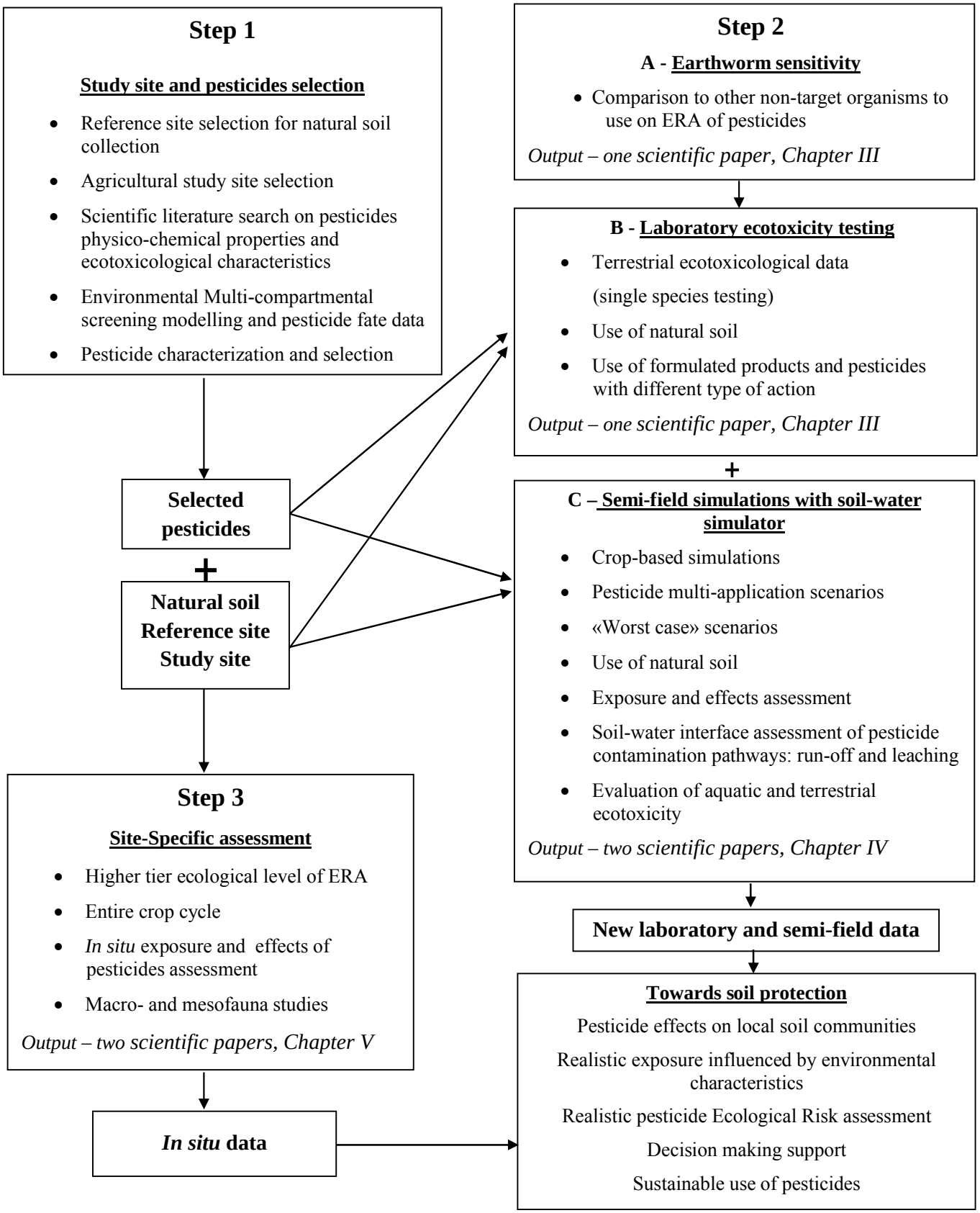
using water and soil samples from the simulated scenarios. Acute and chronic effects on aquatic communities were assessed using the cladoceran *Daphnia magna*: Daphtoxkit F Magna (MicroBioTests, 2000); and the 21-day reproduction test (OECD, 1998). Effects on soil communities were assessed using the terrestrial ecotoxicity standard tests described above, according to the evaluation of the results of Step 2B, in order to assess the most susceptible organisms to be affected by each pesticide. These results are presented in Chapter IV as two scientific papers, according to the pesticides type of action.

Step 3 - Site-Specific risk assessment

Objective: To evaluate pesticide effects at higher tier level on local terrestrial communities of a Mediterranean agricultural field over a crop cycle.

Taking into consideration a more relevant ecological perspective of the impact resulting from pesticide application on natural soil communities, a field study was conducted during an entire crop cycle. The reference study site and the irrigated crop sites were selected under Step 1. The evaluation focused on soil mesofauna (by collecting soil cores) and macrofauna (by installing pitfall traps). Samples were collected during the entire crop's cycles taking into account the timings of application of pesticide formulated products and fertilizers. The collected soil organisms were identified at morphospecies level and the effects observed were complemented with the study of soil fauna feeding activity in situ using the bait-lamina method (Hamel *et al.*, 2007). The knowledge resulting from this study is intended to provide useful information on the community structure of agricultural terrestrial invertebrates and its function and relate that with the its resistance and resilience towards agricultural practices, namely pesticide application under Mediterranean crop scenarios. The resulting data are presented in Chapter V as future two scientific papers for the selected crops.

Schematic representation of thesis Steps



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CHAPTER II

General Methodology.

This chapter focuses on global methodological aspects that are not included in the scientific papers presented on the following chapters, but are important to explain on the context of this thesis.

1. Agricultural main area, study site selection and description.

"Ribatejo e Oeste", located in Central Portugal, is one of the major agriculture regions with intensive agriculture in Portugal (EA, 2012). This region, together with "Alentejo" region, contribute with their high dimension agricultural holdings with more than 2/3 for the regional Standard Output ("Valor da Produção Padrão" - VPP), and are responsible for half of the national agricultural Total Standard Output ("Valor da Produção Padrão Total" - VPPT) (RA, 2011). This region is characterized by different edafo-climatic and socio-economic characteristics presenting a wide variety of agricultural production systems, being horticulture, maize and potato the most relevant (RA, 2011). Maize production in Portugal exceeded 830,000 tonnes and increased in production over the last years by 33% (EA, 2012). Potato is a main crop in "Ribatejo e Oeste" region with its 5.5 million ha of crop area (RA, 2011). As such, maize (*Zea mays* L.) and potato (*Solanum tuberosum* L.) were selected as main crops to be represented on the "crop-based simulation" studies, as well as onion (*Allium cepa* L.) by its importance as one of the main horticulture crops in Portugal (EA, 2012).

1.1 Natural soil and reference site.

In order to increase realism in laboratory terrestrial ecotoxicity tests with the selected pesticides and to extrapolate the results to the semi-field simulations study, the use of natural soil is of paramount importance. Although soil properties may influence terrestrial invertebrate physiological mechanisms such as reproduction (Chelinho *et al*, 2011; Domene *et al.*, 2011), the use of natural soil is recommended instead of the use of the Organisation for Economic Co-operation and Development (OECD) artificial soil when the objective is to predict the effects of harmful substances in real-world

situations (CSTEE, 2000; Römbke and Amorim, 2004; Römbke *et al.*, 2006). For site-specific studies in both prospective and retrospective Environmental Risk Assessment (ERA), the use of natural soil to act as control with matching soil properties of the contaminated soil is decisive to attain results of ecological relevance (Natal-da-Luz *et al.*, 2008).

A search for a natural soil and reference site in the region of "Ribatejo e Oeste" near the study site was undertaken. Several soil samples were taken from the field and several criteria had to be attained: absence of pesticide residues; similarity to the study site soil (see section 1.3) in terms of soil properties; acceptability, also in terms of soil properties, to act as reference material for the performance of the ecotoxicological tests with the soil organisms (e.g., survival, growth, and reproduction) used in the ecotoxicity evaluation during Step 2 (actions B and C). This last criteria was evaluated for all test organisms prior to the tests, since some of the pedological characteristics of soils might act as stress factors for the organisms (Amorim *et al.*, 2005; Chelinho *et al*., 2011; Jänsch *et al*, 2005), thus influencing the test results (Chelinho *et al*., 2011; Natal-da-Luz *et al.*, 2008).

A sandy clay loam soil (Table II.1), classified as Eutric cambisol (EuDASM, 2012; ANNEX I), never cultivated or used for farming, was selected. No pesticide residues were quantified after a broad spectrum pesticide analysis using a multi-method detection analysis (ASU L, 1999). The soil intrinsic physical and chemical properties, and the analytical methodologies adopted in an independent laboratory are summarised on Table II.1.

Table II.1: Natural soil intrinsic characteristics and respective analytical methodologies.

Natural soil		Methods	Natural soil		Methods
Particle size distribution		Hydrometer of Boyoucos, IM	OM content ($g\ kg^{-1}$)	57.4	Dry combustion ISO 10694:1995
Sand ($g\ kg^{-1}$)	544		Chemical parameters		
Silt ($g\ kg^{-1}$)	221		P_2O_5 ($mg\ kg^{-1}$)	99	Egner-Rhiem ICP-OES, IM
Clay ($g\ kg^{-1}$)	235		K_2O ($mg\ kg^{-1}$)	> 200	=
Soil texture	Sandy clay loam*		Mg ($mg\ kg^{-1}$)	> 125	Ammonium acetate 1M pH=7 FAAS, IM
pH (H_2O)	5.9	Potentiometry (20±2ºC) IM, LAS.PL.20.V01, 2009	$CaCO_3$ (%)	0	ISO 10693, 1995
pH (1M KCl)	5.0	ISO 10390, 1994**	Fe ($mg\ kg^{-1}$)	> 80	AAAc - EDTA (Lakanen) /FAAS, IM
Moisture (%)	11	ISO 11268-2.2, 1998**	Mn ($mg\ kg^{-1}$)	38	=
WHC max (% dry weight)	54.4	ISO 11268-2.2, 1998**	Zn ($mg\ kg^{-1}$)	1.8	=
Cation exchange capacity ($cmol_{c/kg}$)	9.12	ammonium acetate 1M pH=7 FAAS (Ca & Mg) and FAES (K & Na) Titration IM	Cu ($mg\ kg^{-1}$)	3	=
Sum of base exchange ($cmol_{c/kg}$)	6.72		B ($mg\ kg^{-1}$)	0.69	Boiling water ICP-OES, IM
Sum of exchangeable cations ($cmol_{c/kg}$)	73.7	=	N ($g\ kg^{-1}$)	2.97	Dry combustion ISO 13878,1998

IM – Internal method; WHC - water-holding capacity; FAAS – Flame atomic absorption spectrometry; FAES – Flame atomic emission spectrometry; OM – Organic matter; ICP-OES – Inductively coupled plasma optical emission spectrometry. * Soil particle size classification according to Pierzynski *et al*, 2000, ** see references for methodology.

1.2 Crop-based scenarios for the risk assessment of pesticides on the soil-water interface.

The semi-field approach methodology was applied to mimic pesticide application under realistic "worst case" scenarios of irrigated crops under Mediterranean conditions. In agricultural environments contamination can occur when pesticides are used intensively, affecting non-target organisms (Cerejeira *et al.*, 1999; Baptista *et al.*, 2002; Frampton and Van den Brink, 2007; Leitão *et al.*, 2007). When this contamination occurs close to national parks, reserves or sensitive areas, this indicates that it may affect the biota of the agricultural ecosystem itself and of the surrounding fields becoming a major threat to biodiversity. The use of pesticides in protected areas pays a special attention to what is specified on the directive of the Sustainable use of pesticides (EC, 2009) focusing on the need to establish necessary biodiversity conservation measures. Taking this into consideration, the selected study site to mimic in the simulations under the semi-field

approach was an agricultural field located in the Centre Portugal, at Ribatejo region (EA, 2012), referred previously. Pesticide contamination of the surface water is of paramount importance for this area due to its proximity to the Protected Area and Natural Reserve "Paul do Boquilobo" with an area of 432.78 ha (Figure II.1) inserted in this main agricultural area and incorporating agricultural fields in its area. This protected reserve is also part of the (i) Biogenetic Reserves (Council of Europe) characterized by one (or more) typical, unique, endangered or rare habitats, biocenoses or ecosystems; (ii) Ramsar Sites – Wetlands of International importance for conservation and wise use of its resources; (iii) Biosphere Reserves (MAB/UNESCO), sites covered by the Convention Concerning the Protection of World Cultural and Natural Heritage (UNESCO), sites of excellence that seek to reconcile conservation of biological, cultural diversity, economic and social development through practices to manage nature and human activities to sustainable development from local to international scales; and (iv) Special Protection Areas (SPA) nº 10 PTZPE0008 designated under the Birds Directive included on the "REDE NATURA 2000" by the great importance of its superficial waters for bird conservation (ICNF, 2013a).

Figure II.1: Agricultural landscape nearby the Special Protection Area (SPA nº 10) "Paul do Boquilobo" (ICNF, 2013b) and located in the Tagus river vulnerable zone.

This protected area also includes agricultural fields, as previously referred, where emphasis is made on ensuring that future management is both ecologically and economically sustainable (EC, 2013). The fact that "Paul do Boquilobo" SPA is located at lower quota than the surrounding agricultural fields, becomes prone to contamination of the water compartment resulting from run-off events and other contamination pathways that may occur during Tagus river floods. This occurrence makes this region important to study pesticide fate and effects due to its location in the agricultural area.

The study area is located in the Almonda river basin, the main water line in "Paul do Boquilobo" Special Protection Area, a Tagus sub basin (see ANNEX II), and is also surrounded by the Tagus hydrogeological vulnerable area ("ZV Tejo"), the largest of the country (see ANNEX III) with 2 416.86 km^2 (Portaria nº 164/2010). This area is characterized by having water polluted with nitrates from agricultural sources (EEC, 1991b). This important agricultural area was also chosen due to its present and continuous water contamination (surface and ground water) by pesticides registered in several studies over the last decade (Batista *et al.*, 2002; Cerejeira *et al.*, 1995a, b, 2000, 2003, 2005; Silva *et al.*, 2012a, b).

The used semi-field methodology, a soil-water simulator, was adopted to mimic the selected pesticides application under realistic "worst case" scenarios of irrigated crops, previously referred. The soil-water simulator (Figure II.2) is a transportable soil flume system of two articulated platforms recently developed at University of Coimbra allowing the simulation of pesticide application, mimicking a field situation (Chelinho *et al.*, 2012). The soil-water simulator has multiple functions among its design, e.g., (i) the possibility to use field soil (reference soil) to study pesticide fate mimicking field realistic conditions (ii) to adjust the slope according to field topography, (iii) to allow different irrigation methods and pesticide application types; (iv) to allow the collection of soil samples and of run-off and leaching waters that may result from irrigation scenarios and rain events simulations. Due to the possibility to control the slope of each platform independently, the system allows the simulation of worst case scenarios for the main soil-water pathways of pesticide entrance into the water system (to see more details about these different scenarios see Chelinho *et al.*, 2012).

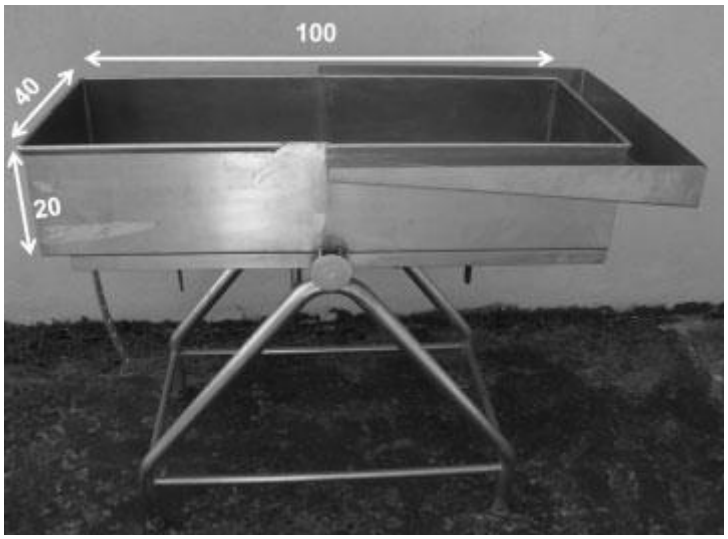

Figure II.2: Semi-field scale laboratory simulator adopted in the present study to evaluate soil-water interface environment (photo adapted from Chelinho *et al.*, 2012).

1.3 Geographical location and characteristics of the agricultural site used for the field experiment.

In order to evaluate, under a higher tier level of ERA, the effects of pesticides on local terrestrial meso and macrofauna communities under Mediterranean conditions (Step 3), an agricultural field was selected in the "Ribatejo e Oeste" region previously referred, based on: vicinity to vulnerable areas; pesticides demand for crop protection; irrigation; possibility of collecting soil organisms; farmers availability and responsibility to provide experimental data concerning crops management. The selected agricultural field is located in the limit of Tagus hydrogeological vulnerable area in Torres Novas County and was explored for maize, potato and onion, crops with high water needs with medium values of 300 mm to more than 700 mm per year (AGROMAIS, 2013). The good relation established with the local farmer allowed the monitoring, during the entire crop cycle, of the three selected crops, and the collection of correct and exact data concerning pesticide and fertilizers application.

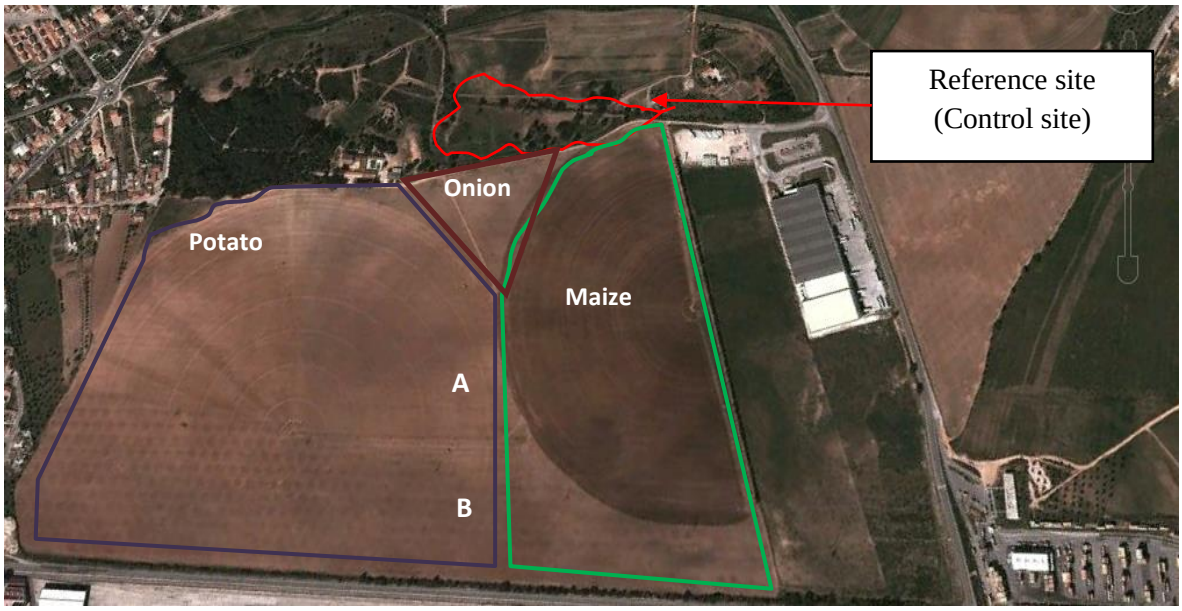

Figure II.3: Selected agricultural field, with two soil areas (A and B) for the site-specific assessment and reference soil site as control site.

The agricultural field (Figure II.3) has a total area of 52.7 ha and the three crops are planted under a rotation cycle every year (in 2010, maize crop occupied 34 ha, potato crop 14 ha and onion crop 4.7 ha), so the soil is usually exposed to different pesticides in different years.

The field soil is a sandy loam soil (Table II.2) with slight differences between two areas (A and B). The crops cycle irrigation was performed by center-pivot (automated sprinkler that rotates in a half a circle area) and by sprinklers. Pesticide spraying under recommended dosages was performed by the appropriate spraying equipment (Tagri 600L).

Table II.2: Intrinsic characteristics of the two areas in the agricultural field in 2010 and respective analytical methodologies.

Agricultural soil	A	B	Methods
Particle size distribution			
Sand (g kg^{-1})	614	704	Hydrometer of Boyoucos, IM
Silt (g kg^{-1})	201	171	
Clay (g kg^{-1})	185	125	
Soil texture	Sandy loam*		
pH (H_2O)	5.8	6.5	Potentiometry (20±2ºC) IM, LAS.PL.20.V01, 2009
Cation exchange capacity ($cmol_{c/kg}$)	4.38	3.74	ammonium acetate 1M pH=7 FAAS (Ca & Mg) and FAES (K & Na) Titration IM
Sum of base exchange ($cmol_{c/kg}$)	2.68	2.94	
Sum of exchangeable cations ($cmol_{c/kg}$)	61.2	78.6	=
OM content (g kg^{-1})	16.8	20.8	Dry combustion ISO 10694:1995
Chemical parameters			
P_2O_5 (mg kg^{-1})	153	> 200	Egner-Rhiem ICP-OES, IM
K_2O (mg kg^{-1})	> 200	> 200	=
Mg (mg kg^{-1})	112	> 125	Ammonium acetate 1M pH=7 FAAS, IM
$CaCO_3$ (%)	0	0	ISO 10693, 1995
Fe (mg kg^{-1})	55	53	AAAc - EDTA (Lakanen) /FAAS, IM
Mn (mg kg^{-1})	61	14	=
Zn (mg kg^{-1})	1.8	1.3	=
Cu (mg kg^{-1})	0.3	0.6	=
B (mg kg^{-1})	0.29	0.54	Boiling water ICP-OES, IM
N (g kg^{-1})	0.96	1.04	Dry combustion ISO 13878,1998

IM – Internal method; WHC - Water-holding capacity; FAAS – Flame atomic absorption spectrometry; FAES – Flame atomic emission spectrometry; OM – Organic matter; ICP-OES – Inductively coupled plasma optical emission spectrometry. * Soil particle size classification according to Pierzynski *et al*, 2000.

The area that was used as control site (Figure II.3) to compare terrestrial communities with the agricultural field is located next to the cultivated area, but at a higher quota and slope towards North which prevented any contamination from the selected field.

The detailed methodology adopted in this study is presented in Chapter V.

2. Pesticides selection and characterization.

Pesticides were selected based on their use to control important diseases and pests on irrigated crops existing in the "Ribatejo e Oeste" region. For the evaluation of pesticide effects on non-target terrestrial communities (Step 2), of all the pesticide types, only fungicides and insecticides were taken into consideration for their expected toxicity to soil organisms (Frampton *et al.*, 2006; Daam *et al.*, 2011; Wang *et al.*, 2012). The fungicides and insecticides registered in Portugal in 2009 for the selected crops and their target organisms are presented in the following tables.

Table II.3: Fungicides registered in Portugal for the three selected crops in 2009 (http://www.dgv.min-agricultura.pt).

Crop	Diseases	Fungicides (active ingredient)
Onion	Early Blight *Alternaria* sp.	folpet mancozeb
	Leaf blight *Stemphylium vesicarium* (Wallr.) E.G. Simmons	azoxystrobin
	Onion rust *Puccinia allii* Castagne	mancozeb
	Downy mildew *Peronospora destructor* [Berk.] Casp.	azoxystrobin copper(oxychloride) + iprovalicarb folpet mancozeb

Table II.3: Fungicides registered in Portugal for the three selected crops in 2009 (Cont.)

Crop	Diseases	Fungicides (active ingredient)	
Potato	Early Blight *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout	captan chlorothalonil folpet	mancozeb metiram
	Anthracnose *Colletotrichum coccodes* (Wallr.) S. Hughes	folpet	
	Downy mildew *Phytophthora infestans* (Mont.) de Bary	benalaxyl + mancozeb benalaxyl-M + mancozeb captan cyazofamid cymoxanil + copper (oxychloride) cymoxanil + famoxadone cymoxanil + folpet cymoxanil + folpet + mancozeb cymoxanil + folpet + metalaxyl cymoxanil + mancozeb cymoxanil + metiram cymoxanil + copper oxychloride cymoxanil + copper oxychloride + propineb cymoxanil + propineb chlorothalonil copper (hydroxide) copper (oxychloride) + iprovalicarb	copper (oxychloride) + metalaxyl copper (sulphate) copper (copper and calcium sulphate - *"bordalesa" mixture*) dimethomorph + mancozeb fenamidone + mancozeb fluazinam folpet mancozeb mancozeb + metalaxyl mancozeb + metalaxyl-M mancozeb + propamocarb (hydrochloride) mancozeb + zoxamide metalaxyl + copper (oxychloride) metiram copper oxychloride + propineb propineb

Table II.4: Insecticides registered in Portugal for the three selected crops in 2009 (http://www.dgv.min-agricultura.pt).

Crop	Pest	Insecticides (active ingredient)
Maize	Wireworms *Agriotes* spp. and *Athous* spp.	chlorpyrifos ethoprophos tefluthrin
	white grub *Melolontha* spp.	ethoprophos
	corn borer *Pyrausta nubilalis* and *Sesamia nonagrioides* Hbn.	indoxacarb
	Cutworm *Agrotis segetum* (Dennis & Schiffermuller) and *Agrotis ipsilon* (Hufganel)	beta-cyfluthrin ethoprophos lambda-cyhalothrin tefluthrin
	european corn borer *Ostrinia nubilalis* (Hubner)	lambda-cyhalothrin
	Scutigerella *Scutigerella immaculata* (Newport)	chlorpyrifos tefluthrin

Table II.4: Insecticides registered in Portugal for the three selected crops in 2009 (cont.)

Crop	Pest	Insecticides (active ingredient)	
Onion	onion fly *Delia antiqua* (Meigen)	Chlorpyrifos	
	onion thrips *Thrips tabaci* Lindeman	acrinathrin	
Potato	Aphids *Macrosiphum euphorbiae* Thomas	cypermethrin + chlorpyrifos pirimicarbe thiamethoxam	
	green peach aphid *Myzus persicae* Sulzer	thiamethoxam	
	Wireworms *Agriotes* spp. (Linnaeus)	Chlorpyrifos	
	colorado potato beetle *Leptinotarsa decemlineata* Say	acetamiprid alpha-cypermethrin azadirachtin beta-cyfluthrin cyfluthrin cyfluthrin + imidacloprid Cypermethrin cypermethrin + chlorpyrifos Chlorpyrifos chlorpyrifos-methyl + deltamethrin	deltamethrin phosmet imidacloprid lambda-cyhalothrin lufenuron spinosad thiacloprid thiamethoxam
	serpentine leafminers *Liriomyza* spp.	cyromazine	
	white grub cockchafer *Melolontha melolontha* (L.)	Chlorpyrifos	
	Cutworm *Agrotis segetum* (Dennis & Schiffermuller) and *Agrotis ipsilon* (Hufganel)	cyfluthrin Chlorpyrifos lambda-cyhalothrin	
	Scutigerella *Scutigerella immaculata* (Newport)	Chlorpyrifos	

Note: The active ingredient ethoprophos is also used as nematicide in potato crop against potato cyst nematode (*Globodera rostochiensis* (Wollenweber) Behrens and *G. pallide* (Stone) Behrens).

The pesticides were selected according to several **specific criteria**. The study of pesticides risk assessment in the soil-water interface involves mainly two environmental compartments, as such priority was given to the **water and soil compartment** in terms of pesticide fate in the environment. Pesticides should have **relevant fate for the water and soil compartment** (affinity to the soil and water compartments), **medium water**

solubility, to **not adsorb strongly to the soil** and **present leaching potential** to groundwater so that movement would occur. Taking into account the **ecotoxicological data,** pesticides should have **relevant toxicity for aquatic organisms** (fish and aquatic invertebrates), as well as for **terrestrial earthworms** due to the objective of studying pesticides side effects on the reproduction of non-target terrestrial organisms. Moreover those pesticides that could be applied only under a mixture were discarded for selection since the main objective of the work was to evaluate fate and effects of individual pesticides. In the following paragraphs, the information on fate and ecotoxicity used to select the pesticides adopted in this study is exposed in more detail.

Fate and transport of organic compounds in the environment are affected by various parameters (Lyman, 1990a). As such, a characterization of the pesticides specified in Tables 3 and 4 (fungicides and insecticides) was performed based on a group of physico-chemical properties (SW, VP and H), particularly the environmental partition coefficients (Kow and Koc) and persistence (DT50) in soil, considered key parameters to evaluate their fate in the environment (Lyman, 1990b). These parameters were compiled from scientific literature and specific data bases (Tables II.5 and II.6).

The chemical's final distribution and concentrations in the various environmental media are the result of numerous highly complex and interacting processes developed by fate models, based on the mass balance principle and developed to simulate transport among and transformation within multiple environmental media (Cowan *et al.*, 1995). To assess the relevance of targeted environmental compartments exposure to pesticides, a first level of a multi-compartmental environmental fate model was used: Fugacity Model (Mackay, 2001). The multi-media fate model simulates a situation in which a chemical achieves equilibrium between a number of phases of different composition and volume and is useful in chemical fate assessments as a first indication of where a chemical is likely to partition (Mackay *et al.*, 2009). The model uses key chemical properties as molecular mass, temperature, water solubility, vapor pressure and log Kow, but more chemical data may be necessary accordingly to the different chemical categories that are based on vapour pressure and water solubility variations (Chemical Type I – measurable in all phases; and Type II – insolubility in air but measurable in all other phases) (Mackay *et al.*, 1996). Results are given as a Predicted Environmental Distribution (PED) among the several environmental media as the chemical's

partitioning tendency (PED < 20% very low affinity; 20% ≤ PED < 40% low affinity; 40% ≤ PED < 60% average affinity; 60% ≤ PED < 80% high affinity; PED ≥ 80% very high affinity; Mackay, 2001). The various environmental media that play a role in the chemical partitioning are: air and water in which there is true dissolution; organic biological media such as organisms and vegetation that are either alive or have much the same composition as when they were alive (PED aquatic biota); and solid inorganic phases that include soil minerals (PED soil), bottom sediment minerals (PED sediment), aerosol particles (PED aerosol), and suspended inorganic matter in water (PED suspended solids) (Mackay *et al.*, 1996).

To evaluate the leaching potential of pesticides, the Groundwater Ubiquity Score (GUS) was applied (Gustafson, 1989). GUS is based on the environmental fate properties of the chemical such as the soil degradation half-life (DT50) and the organic-carbon sorption coefficient (Koc) where: GUS = log(DT50) x (4 - log (Koc)). The results given discriminated the pesticides into three classes of leaching potential: if GUS > 2.8 pesticide is likely to leach; if GUS < 1.8 pesticide is unlikely to leach; if GUS 1.8 - 2.8 leaching potential is transitional. This parameter is only a screening indicator to provide a general indication of potential leaching, since detailed environmental conditions are not taken into account.

Tabela II.5: Fungicides active ingredient characteristics: physico-chemical properties, persistence and potential fate[1]

Fungicides (a.i.)	Sw 20ºC (mg L^{-1})	VP (mPa)	H (Pa m^3 mol^{-1})	Kow	Koc (mL g^{-1})	$DT50_{lab}$ soil (d) (20ºC)	$DT50_{field}$ soil (d)	PED Water	PED Soil	PED Sediment	PED Suspended solids	PED Aquatic biota	PED Air	PED Aerosol	GUS
azoxystrobin	6.7	1.10E-07	1.10E-07	2.5	589	84.5	180.7	43.4	55.3	1.23	0.0384	1.09E-05	6.51E-08	7.68E-03	2.53
captan	5.2	0.0042	3.00E-04	2.5	200	0.8	3.7	77.7	21.8	0.484	0.0151	1.23E-03	0.006	5.26E-03	-0.16
chlorothalonil	0.81	0.076	2.50E-02	2.94	850	15.7	44	55.7	43	0.955	0.0299	2.43E-03	0.285	2.08E-03	0.7
cyazofamid	0.114	0.0133	4.03E-02	3.2	736-2172 [2]	10	4.5	40.9	57.4	1.27	0.0398	3.24E-03	0.318	0.133	0.87
fluazinam	0.135	7.5	25.9	4.03	1705 – 2316 [2]	72.5	16.4	6.24	59.2	1.32	0.0411	3.34E-03	33.1	0.056	1.73
folpet	0.8	2.10E-02	8.00E-03	3.02	304	4.7	3	51.3	47.5	1.06	0.033	2.68E-03	0.0819	0.0119	1.02
mancozeb	6.2	0.013	5.90E-04	1.33	998	0.1	18	98.1	1.86	0.0413	1.29E-03	1.05E-04	0.0117	3.12E-03	-1
metiram	2	0.01	5.40E-03	1.76	500 000	1	7	94.9	4.84	0.107	3.36E-03	2.73E-04	0.106	0.0545	0
propineb	10	0.16	8.00E-08	-0.26	-	3	-	99.9	0.0486	1.08E-03	3.38E-05	2.75E-06	9.51E-08	3.51E-03	-

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; SW – Solubility in water at 20ºC (SW ≤ 50 Low; 50 - 500 Moderate; > 500 High; FOOTPRINT, 2012); VP - Vapour pressure at 25ºC (VP < 1 Non-volatile; 1 – 1 x 104 Intermediate state; > 1 x 104 Volatile; FOOTPRINT, 2012); H - Henry's law constant at 25ºC (H > 100 Volatile; 0.1 - 100 Moderately volatile; < 0.1 Non-volatile; FOOTPRINT, 2012); Kow - Octanol-water partition coefficient at pH7, 20ºC as Log P (< 2.7 Low bioaccumulation; 2.7 – 3 Moderate; > 3.0 High; FOOTPRINT, 2012); Koc - Organic carbon sorption coefficient (Koc < 15 Very mobile; 15 - 75 Mobile; 75 - 500 Moderately mobile; 500 - 4000 Slightly mobile; > 4000 Non-mobile; FOOTPRINT, 2012); DT50 – Half-life in soil at 20ºC under aerobic conditions (DT50 < 30 Non-persistent; 30 - 100 Moderately persistent; 100 – 365 Persistent; > 365 Very persistent; FOOTPRINT, 2012; EC, 2000).; PED - Predicted Environmental Distribution (%) according to Mackay (2001) - Mackay fugacity model ('level I, version 3.00, 2004, Trentu University, Canada'); GUS - Groundwater Ubiquity Score (Gustafson, 1989).

Table II.6: Insecticides active ingredient characteristics: physico-chemical properties, persistence and potential fate[1]

Insecticides (a.i.)	Sw 20ºC (mg L^{-1})	VP (mPa)	H (Pa m^3 mol^{-1})	Kow	Koc (mL g^{-1})	$DT50_{lab}$ soil (d) (20ºC)	$DT50_{field}$ soil (d)	PED Water	PED Soil	PED Sediment	PED Suspended solids	PED Aquatic biota	PED Air	PED Aerosol	GUS
acetamiprid	2950	1.73E-04	5.30E-08	0.8	200	2.6	3	99.4	0.556	0.0123	3.86E-04	3.14E-05	2.66E-07	2.97E-05	0.94
acrinathrin	0.002	4.40E-05	1.80E-02	6.3	127500-319610[2]	42.8	22	0.0552	97.6	2.17	0.0678	5.51E-03	1.35E-04	0.0886	-1.09
azadirachtin[2]	260	3.6E-06	-	-	-	25	-	-	-	-	-	-	-	-	
chlorpyrifos	1.05	1.43	0.478	4.7	8151	76	21	2.15	95.4	2.12	0.0663	5.39E-03	0.211	0.0104	0.15
cyfluthrin	0.0066	3.0E-04	5.30E-02	6	123930	51	33	0.11	97.6	2.17	0.0678	5.51E-03	4.46E-04	0.0455	-1.66
beta-cyfluthrin	0.0012	5.6E-05	8.10E-03	5.9	64300	27.8	13	0.699	97.1	2.16	0.0674	4.12E-07	4.58E-07	2.39E-04	-0.9
cypermethrin	0.009	2.3E-04	2.00E-02	5.3	156250	68	69	0.549	97	2.16	0.0674	5.48E-03	0.0012	0.188	-2.12
alpha-cypermethrin	0.004	3.4E-04	6.90E-02	5.5	57889	100	35	0.347	97.2	2.16	0.0675	5.49E-03	0.00252	0.214	-1.18
cyromazine	13000	4.48E-04	5.80E-09	0.069	200[3]	31.8	9.7	99.9	0.104	0.0023	7.20E-05	5.85E-06	1.17E-07	2.73E-07	2.73
deltamethrin	0.0002	1.24E-05	3.10E-02	4.6	10240000	26	21	4.42E-03	97.8	2.17	6.79E-02	2.03E-09	2.25E-08	4.21E-05	-3.35
ethoprophos	700 [2]	78	1.35E-02	3.59 [2]	70	17	23	22.1	76.1	1.69	0.0528	0.00429	0.12	1.25E-06	2.41
imidacloprid	610	4.0E-07	1.7E-10	0.57	-	187	174	99.9	0.0505	0.00112	3.50E-05	5.70E-06	3.49E-09	5.92E-05	3.76
indoxacarb	0.2	0.006	6.00E-05	4.65	6450	5	20	2.24	88.4	1.97	0.0614	4.99E-03	7.26	0.03	0.23
lambda-cyhalothrin	0.005	2.0E-04	2.00E-02	7	330000	65	25	0.011	97.7	2.17	0.0679	5.52E-03	4.07E-05	0.0124	-1.67
lufenuron	0.046	4.0E-03	3.41E-02	5.12	38 (mg/g o.c.)[2]	20.8	256	0.83	96.9	2.15	0.0673	5.47E-03	7.57E-03	7.19E-03	-0.75
phosmet	15.2	0.065	1.36E-03	2.96	820[3]	3.1	7	54.7	44.2	0.938	0.0307	0.0025	0.0152	8.37E-03	0.24
pirimicarbe	3100	0.43	3.30E-05	1.7	23[3]	86	9	95.7	4.25	0.0943	2.95E-03	2.40E-04	6.49E-04	3.45E-05	2.73
spinosad[2]	235	3.0E-05	-	4	-	9.4 - 17.3	< 0.5	-	-	-	-	-	-	-	
tefluthrin	0.016	8.4	2.00E02	6.4	112900	37	27.1	0.0431	95.8	2.13	0.0665	0.00541	1.94	0.0157	-2.46
thiacloprid	184	3.0E-07	5.00E-10	1.26	615[2]	1.3	18	98.4	1.59	0.035	0.0011	8.95E-05	8.13E-09	2.60E-04	1.44
thiamethoxam	4100	6.6E-06	4.70E-10	-0.13	70	121	39	86.6	13.1	0.291	9.09E-04	1.13E-06	8.36E-09	9.62E-06	3.66

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; SW – Solubility in water at 20ºC (SW ≤ 50 Low; 50 - 500 Moderate; > 500 High; FOOTPRINT, 2012); VP - Vapour pressure at 25ºC (VP < 1 Non-volatile; 1 – 1 x 104 Intermediate state; > 1 x 104 Volatile; FOOTPRINT, 2012); H - Henry's law constant at 25ºC (H > 100 Volatile; 0.1 - 100 Moderately volatile; < 0.1 Non-volatile; FOOTPRINT, 2012); Kow - Octanol-water partition coefficient at pH7, 20ºC as Log P (< 2.7 Low bioaccumulation; 2.7 – 3 Moderate; > 3.0 High; FOOTPRINT, 2012); Koc - Organic carbon sorption coefficient (Koc < 15 Very mobile; 15 - 75 Mobile; 75 - 500 Moderately mobile; 500 - 4000 Slightly mobile; > 4000 Non-mobile; FOOTPRINT, 2012); DT50 – Half-life in soil at 20ºC under aerobic conditions (DT50 < 30 Non-persistent; 30 - 100 Moderately persistent; 100 – 365 Persistent; > 365 Very persistent; FOOTPRINT, 2012; EC, 2000).; PED - Predicted Environmental Distribution (%) according to Mackay (2001) - Mackay fugacity model ('level I, version 3.00, 2004, Trentu University, Canada'); GUS - Groundwater Ubiquity Score (Gustafson, 1989).

Environmental risk assessment (ERA) of pesticides aims to protect surface waters, groundwater, air and soil taking into account locations distant from its use following long-range environmental transportation, as well as, non-target species including its sustainability, biodiversity in general and the ecosystems (EFSA, 2010).

Pesticides side-effects on non-target aquatic and terrestrial biota were assessed for all the pesticides listed above and ecotoxicological data (Tables II.7 to II.10) compiled from specific databases. For the aquatic populations evaluation, toxic endpoints (EC50 – Effect Concentration, LC50 – Lethal Concentration and NOEC – No Observed Effect Concentration) from acute and chronic ecotoxicological tests were considered for fish, aquatic invertebrates and algae because these are the organisms, considered on the data requirements for pesticides environmental risk (EEC, 1991a; Regulation (EC) Nº 1107/2009), that live in the water column and not in the sediment. Effects on birds (acute oral toxicity) and bees (acute oral or contact toxicity) were also taken into consideration as ecotoxicological relevant information if the product is volatile. Terrestrial ecotoxicological information on earthworms was given priority due to the type of pesticides screened (mainly the fungicides) (Daam *et al.*, 2011; Frampton *et al.*, 2006) and for being abundant in literature since its considered the main data requirement for terrestrial pesticides ERA (EEC, 1991a; Regulation (EC) Nº 1107/2009).

Table II.7: Fungicides active ingredient aquatic ecotoxicological data [1].

Fungicides	Fish Species	96 h LC_{50} (mg L^{-1})	Fish Species	21d NOEC (mg L^{-1})
azoxystrobin	*Oncorhynchus mykiss*	0.47	*Pimephales promelas*	0.147
	Lepomis macrochirus [2]	1.1		
	Cyprinodon variegatus [2]	0.66		
captan	*Oncorhynchus mykiss*	0.186	*Oncorhynchus mykiss*	0.18
	Salvelinus fontinalis [2]	0.034		
chlorothalonil	*Oncorhynchus mykiss*	0.038	*Oncorhynchus mykiss*	0.003
	Lepomis macrochirus [2]	0.059		
cyazofamid	*Salmonidae*	0.56	*Oncorhynchus mykiss*	0.13
	Oncorhynchus mykiss [2]	> 0.510		
fluazinam	*Oncorhynchus mykiss*	0.036	*Oncorhynchus mykiss*	0.012
folpet	*Oncorhynchus mykiss*	0.233	-	-
mancozeb	*Oncorhynchus mykiss*	0.074	*Oncorhynchus mykiss (34d)*	2.20E-03
	Lepomis macrochirus [2]	> 3.6		
metiram	*Oncorhynchus mykiss*	0.33	*Oncorhynchus mykiss*	0.022
propineb	*Oncorhynchus mykiss*	0.4	*Oncorhynchus mykiss*	0.1

	Aquatic invertebrates		Algae		
	***D. magna* 48 h EC_{50} (mg L^{-1})**	***D. magna* 21 d NOEC (mg L^{-1})**	**Species** [3]	**test duration**	**EC_{50} (mg L^{-1})**
azoxystrobin	0.23	0.044	*Pseudokirchneriella subcapitata*	72 h	0.36
			Selenastrum capricornutum [2]	120 h	0.12
captan	7.1	0.56	*Raphidocelis subcapitata*	72 h	1.18
chlorothalonil	0.084	0.009	*Raphidocelis subcapitata*	72 h	0.21
	0.07		*Navicula pelliculosa*		5.10E-03
cyazofamid	0.19	0.11	*Raphidocelis subcapitata*	72 h	0.025
fluazinam	0.22	0.0125	*Pseudokirchneriella subcapitata*	96 h	0.16
folpet	0.68	0.002 (LOEC)	*Scenedemus subspicatus*	72 h	>10
mancozeb	0.073	0.0073	*Pseudokirchneriella subcapitata*	72 h	0.044
			Selenastrum capricornutum [2]	120 h	0.044
metiram	0.77	0.0043	*Pseudokirchneriella subcapitata*	72 h	0.063
			Chlorella sp.	96 h	0.3
propineb	4.7	0.026	Unknown species	72 h	2.68

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; [3] the species *Selenastrum capricornutum* and *Raphidocelis subcapitata* correspond to the presently renamed *Pseudokirchneriella subcapitata* due to taxonomics adjustments in the classifications.

Table II.8: Fungicides active ingredient terrestrial ecotoxicological data [1].

	Birds		Honey bees		Earthworms (*Eisenia fetida*)	
Fungicides	**Species**	**LD_{50} (mg kg^{-1})**	**exposure**	**48 h LD_{50} (µg bee^{-1})**	**14 d LC_{50} (mg kg^{-1})**	**14 d NOEC (mg kg^{-1})**
azoxystrobin	*Colinus virginianus*	> 2000	Oral	25	283	3
	Anas platyrhynchos [2]	> 2000				
captan	*Anas platyrhynchos*	> 2000	Oral	> 100	>519	12.2
	Colinus virginianus [2]	2000 - 4000				
chlorothalonil	*Coturnix japonica*	> 2000	Oral	> 40	268.5	*25 (5% OM)*
	Anas platyrhynchos [2]	> 4640				
cyazofamid	*Colinus virginianus*	> 5000	Contact	> 100	> 1000	4 (8 week)
fluazinam	*Colinus virginianus*	1782	Oral	> 100	> 1000	< 0.35 [4]
	Anas platyrhynchos [2]	> 4190				
folpet	*Colinus virginianus*	> 2510	Contact	> 200	> 500	5.18
	Anas platyrhynchos [2]	> 2000				
mancozeb	*Median across species*	> 2000	Oral	140.6	> 299.1	20
	Passer domesticus [2]	> 1290				
metiram	*Colinus virginianus*	> 2150	Contact	> 16	> 1000 [3]	-
propineb	unknown species	> 5000	Oral	> 70	> 700 [3]	-
	Coturnix japonica [2]	> 5000				

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; [3] Earthworm not specified; [4] Test performed with *Eisenia andrei.*

European community Risk Classification based on toxicological and ecotoxicological information was compiled for the fungicides and insecticides listed above according to the Directive 67/548/EEC (amended by European Commission Directive 2001/59/EC) and the new Regulation (EC) No 1272/2008 on classification, labeling and packaging of substances and mixtures (ANNEX IV and V). Other observations concerning ecotoxicity and fate, considered relevant information were also taken into account (ANNEX IV and V).

Table II.9: Insecticides active ingredient aquatic ecotoxicological data [1].

	Fish		Fish		Aquatic invertebrates		Algae	
Insecticides	**Species**	**96 h LC_{50} (mg L^{-1})**	**Species**	**21d NOEC (mg L^{-1})**	***D. magna* 48 h EC_{50} (mg L^{-1})**	***D. magna* 21 d NOEC (mg L^{-1})**	**Species [4]**	**72h EC_{50} (mg L^{-1})**
acetamiprid	*Salmonidae*	> 100	*Pimephales promelas*	19.2 (32 d)	49.8	5	*Scenedemus subspicatus*	> 98.3
acrinathrin	*Oncorhynchus mykiss*	0.0061	*Oncorhynchus mykis*	0.00083 (28 d)	0.000022	0.0000032	*Selenastrum capricornutum*	0.0035
azadirachtin [3]	*trout*	8.8 ml/l						
chlorpyrifos	*Oncorhynchus mykiss*	0.0013	*Oncorhynchus mykiss*	0.00014	0.0001	0.0046	Unknown species	0.48
	Lepomis macrochirus[2]	0.002			0.0017[2]			
cyfluthrin	*Oncorhynchus mykiss*	0.00047	*Oncorhynchus mykiss*	0.00001	0.00016	0.00002	*Scenedemus subspicatus*	> 10
beta-cyfluthrin	*Salmonidae*	0.000068	*Oncorhynchus mykiss*	0.00001	0.00029	0.00014	*Scenedemus subspicatus*	> 10
cypermethrin	*Salmo gairdneri*	0.0028	*Pimephales promelas*	0.00003 (34 d)	0.0003	0.00004	*Pseudokirchneriella subcapitata*	> 0.1
	Oncorhynchus mykiss[2]	0.00069						
alpha-cypermethrin	*Oncorhynchus mykiss*	0.0028	*Pimephales promelas*	0.00003	0.0003	0.00003	*Raphidocelis subcapitata*	0.1
cyromazine	*Oncorhynchus mykiss*	> 100	*Oncorhynchus mykiss*	> 1	> 100	4.6	*Scenedemus subspicatus*	124
deltamethrin	*Oncorhynchus mykiss*	0.00026	*Oncorhynchus mykiss*	< 0.032	0.00056	0.0041	Selenastrum capricornutum[2]	> 9.1
ethoprophos	*Lepomis macrochirus*	0.32	*Oncorhynchus mykiss*	0.064	0.2	-	Unknown species	28.3
	Oncorhynchus mykiss[2]	13.8						
imidacloprid	*Oncorhynchus mykiss*	211	*Oncorhynchus mykiss*	9.02	85	1.8	*Scenedemus subspicatus*	> 10
							Pseudokirchneriella subcapitata[2]	> 100
indoxacarb	*Oncorhynchus mykiss*	0.65	*Oncorhynchus mykiss*	0.15	0.6	0.042	*Raphidocelis subcapitata*	0.11
lambda-cyhalothrin	*Lepomis macrochirus*	0.00021	*Oncorhynchus mykiss*	0.00025	0.00036	0.3	*Raphidocelis subcapitata*	> 0.3
lufenuron	*Lepomis macrochirus*	> 29	*Pimephales promelas*	0.02	0.0013	0.0001	*Pseudokirchneriella subcapitata*	8.8
phosmet	*Oncorhynchus mykiss*	0.23	*Oncorhynchus mykiss*	0.032	0.002	0.00078	Unknown species	0.07
	Lepomis macrochirus[2]	0.07						
pirimicarbe	*Pimephales promelas*	> 100	*Oncorhynchus mykiss*	< 18	0.017	0.0009	*Pseudokirchneriella subcapitata*	140
	Lepomis macrochirus	55						
spinosad	japanese carp[2]	3.5			14[2]		Selenastrum capricornutum[2]	> 105.5
	Oncorhynchus mykiss[2]	30					Navicula pelliculosa [2]	0.09
tefluthrin	*Oncorhynchus mykiss*	0.00006	-	-	0.00007	0.000008	*Pseudokirchneriella subcapitata*	> 1.05
thiacloprid	*Oncorhynchus mykiss*	30.2	-	-	85.1	-	*Raphidocelis subcapitata*	60.6
	Lepomis macrochirus[2]	25.2						
thiamethoxam	*Oncorhynchus mykiss*	> 125	*Oncorhynchus mykiss*	20 (88 d)	> 100	> 100	*Pseudokirchneriella subcapitata*	*> 100*

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; [3] Copping, 2004; [4] the species *Selenastrum capricornutum* and *Raphidocelis subcapitata* correspond to the presently renamed *Pseudokirchneriella subcapitata* due to taxonomics adjustments in the classifications.

Table II.10: Insecticides active ingredient terrestrial ecotoxicological data [1].

	Birds		Honeybees		Earthworms (*Eisenia fetida*)	
Insecticides	**Species**	**acute LD_{50} (mg kg^{-1})**	**exposure**	**48 h LD_{50} (µg bee^{-1})**	**14 d LC_{50} (mg kg^{-1})**	**14 d NOEC (mg kg^{-1})**
Acetamiprid	*Anas platyrhynchos*	98	Contact	8.09	9	1.26
Acrinathrin	*Anas platyrhynchos*	> 2000	Oral	0.077	> 1000	1.6
azadirachtin [3]	*Anas platyrhynchos*	*	-	-	-	-
Chlorpyrifos	*Colinus virginianus*	13.3	Contact	0.059	129	12.7
	Anas platyrhynchos[2]	490				
Cyfluthrin	*Colinus virginianus*	> 2000	Contact	0.001	> 1000	-
beta-cyfluthrin	*Colinus virginianus*	> 2000	Contact	0.001	> 1000	> 0.133
Cypermethrin	*Anas platyrhynchos*	> 10000	Contact	0.02	> 100	-
alpha-cypermethrin	*Colinus virginianus*	> 2025	Contact	0.033	> 100	-
Cyromazine	*Colinus virginianus*	> 1785	Oral	186	> 1000	333 (58 d)
Deltamethrin	*Colinus virginianus*	> 2250	Contact	0.0015	> 1290	-
	Anas platyrhynchos[2]	> 4640				
ethoprophos	*Colinus virginianus*	6.04	Contact	5.56	39.6	8.3
imidacloprid	*Coturnix japonica*	31	Oral	0.0037	10.7	0.178
	Colinus virginianus[2]	152				
indoxacarb	*Colinus virginianus*	98	Contact	0.094	> 625	7.8
lambda-cyhalothrin	*Anas platyrhynchos*	> 3950	Contact	0.038	> 1000	-
lufenuron	*Anas platyrhynchos*	> 2000	Oral	> 197	> 500	-
phosmet	*Colinus virginianus*[2]	507	Contact	0.22	52	-
pirimicarbe	*Colinus virginianus*	20.9	Oral	4	> 60	-
spinosad	*Colinus virginianus*[2]	> 2000	Topical	0.0029	> 1000	
tefluthrin	*Passer domesticus*	> 267	Oral	0.28	0.32[2]	-
thiacloprid	*Coturnix japonica*	49	Oral	17.32	105	62.5
	Colinus virginianus[2]	2716				
thiamethoxam	*Anas platyrhynchos*	576	Oral	*0.005*	> 1000	5.34
	Colinus virginianus[2]	1552				

[1] FOOTPRINT, 2012; [2] Tomlin, 2006; [3] Copping, 2004. * Daily oral administration at 1-16mg kg^{-1} induced no negative effects over a 14 day test period.

The analysis of the collected information showed that all the **fungicides** available to be selected (Table II.3) have low solubility in water, have a slight to moderate tendency to adsorb to soil particles and are not likely to vaporize. In spite of these characteristics, there is a group of substances that are predicted to have a high to very high affinity to the water compartment (captan, mancozeb, metiram and propineb) but are not likely to leach to ground water. These substances were set aside because the aim of the study was to evaluate the behavior of pesticides between the terrestrial and aquatic compartments; as such, fluazinam was also set aside for its potential to volatile at ambient temperature and have very low affinity to the water compartment. Chemicals that are likely to be

gases and which have low water solubility and low adsorptive tendencies are less likely to transport and persist in soils and water (Verschueren, 1983). The remaining substances have fairly the same properties among them. However, azoxystrobin presents the highest leaching potential of all the substances, according to the GUS index, and also an average affinity to both relevant environmental compartments. The remaining fungicides, chlorothalonil, cyazofamid and folpet, have also similar affinity for the water and soil environment in spite of the unlikely potential to leach. Taking into account the ecotoxicological data of these fungicides (Tables II.7 and II.8) chlorothalonil was the most toxic for aquatic organisms and for terrestrial earthworms (lower endpoints values) as well as azoxystrobin. Folpet and cyazofamid were the least toxic for aquatic and terrestrial organisms, and as such were not selected. Taking into consideration the fate and effect data, **azoxystrobin and chlorothalonil were selected as the fungicides to study** on the soil-water interface risk assessment. Azoxystrobin is applied on onion crop against leaf blight and downy mildew (Table II.3) and may be persistent in soil (Table II.5) and sorb mainly to the top layer (ANNEX IV) where terrestrial organisms inhabit (Tu *et al.*, 2011). Chlorothalonil is applied on potato crop against early blight and downy mildew, and both fungicides are classified (ANNEX IV) as very toxic to the aquatic environment under Acute (H400) and Chronic Hazard (H410) with the probability to cause long-term adverse effects to aquatic organisms.

Regarding the **insecticides** applied on the three crops can all be applied individually and not in mixtures, contrary to what happens with some fungicides, so they were all valid for selection. The two biopesticides, azadirachtin, a plant (Neem, *Azadirachta indica* A. Juss) derived insecticide, and spinosad, a micro-organism (mixture of secondary metabolites of the soil Actinomycete *Saccharopolyspora spinosa* Mertz & Yoa) derived insecticide (Copping, 2004) were not taken into consideration for their lack of information on environmental fate and effects on non-target organisms (Tables II.6, II.9 and II.10). Pesticides that would preferentially occur in water and in soil and with potential leaching characteristics were selected, e.g. ethoprophos, phosmet and thiamethoxam. Insecticides with very low or very high water solubility, high Koc values, indicating strong adsorption to soil, and unlikely to leach to groundwater, were discarded due to their very strong affinity to only one environmental compartment, as

also estimated by the predicted environmental distribution for each substance (Table II.6). Highly soluble chemicals tend to have low adsorption coefficients for soils and sediments, and tend to be more readily biodegradable by microorganisms in soil and surface water (Lyman, 1990a). Soil sorption is a major process affecting pesticide pollution potential (Hornsby, 1996), where pesticides that are strongly adsorbed by soil or sediment particles are likely to be more persistent due to the binding mechanisms that protect them from chemical or biological degradation and volatilization. They will also not readily leach to ground water, and may be washed off the surface of fields in rain water under "runoff" events under erosive conditions where they will be attached to moving soil particles (Hornsby, 1996). As such, pesticides with very low potential to leach (non-mobile), related to movements between the relevant environmental compartments (water and soil), were not selected (e.g. acetamiprid, acrinathrin, chlorpyriphos and thiacloprid). Most of the insecticides are toxic to aquatic invertebrates as expected (Maltby *et al.*, 2005). Observing the terrestrial ecotoxicity data (Table II.10), in spite of showing toxicity to aquatic invertebrates, lufenuron is not toxic to terrestrial organisms including earthworms, an important factor for the selection as mentioned above. Thiamethoxam and thiacloprid were not selected for being less toxic to aquatic and terrestrial organisms (Tables II.9 and II.10). Ethoprophos and phosmet have similar aquatic and terrestrial ecotoxicity data but **ethoprophos was selected as the insecticide** to include in the batch of pesticides to be evaluated during the study, because it was one of the most commonly used insecticides in the study site (Pereira, 2008). Phosmet is rapidly broken down in soil (ANNEX V), as indicated by its degradation time and adsorbs more to soil particles than ethoprophos (Table II.6). The fact that there is a lack of mainly terrestrial ecotoxicological information for ethoprophos is of major importance since it is applied directly to the soil against soil insects (as prescribed).

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CHAPTER III

Evaluation of pesticides toxicity towards terrestrial biota

1. Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*

Based on the following manuscript:

Comparing the sensitivity of soil invertebrates to pesticides with that of Eisenia fetida. Michiel Daam, Sara Leitão, Mª José Cerejeira and José Paulo Sousa. Chemosphere (2011) 85: 1040–1047.

1.1. Abstract

The sole routine testing of the standard earthworm *Eisenia fetida* for the terrestrial risk assessment of pesticides has been under much debate since other soil invertebrates may be more sensitive than this standard test species. However, the very low availability of laboratory toxicity data for taxa other than *E. fetida* has greatly hampered sensitivity comparisons. In the present study, the relative tolerance (T_{rel}) approach was used to enable comparing toxicity thresholds obtained from the US-EPA ECOTOX database, for main terrestrial taxonomic groups and pesticidal types of action (insecticides, fungicides, herbicides, other) separately. Analyses confirmed previously reported lower and higher sensitivity of collembolans to fungicides and insecticides, respectively. However, various other discrepancies in susceptibility relative to *E. fetida* were encountered as indicated by species sensitivity distributions and/or calculated 95% confidence intervals of T_{rel} values. Arachnids and isopods were found to be more sensitive to insecticides, and nematodes to fungicides, as compared to *E. fetida*. Implications of study findings for the terrestrial risk assessment of pesticides are discussed.

Keywords: Environmental Risk Assessment; Soil invertebrates; *Eisenia fetida*; Relative tolerance; Terrestrial ecotoxicology.

1.2. Introduction

The first-tier ecotoxicological effect assessment of pesticides is usually based on toxicity values derived from laboratory toxicity tests using a limited number of standard test organisms (e.g., Solomon *et al.*, 2008). These organisms are intended to serve as sensitive surrogates for all species in a given environmental compartment, and were chosen based on their sensitivity to a wide range of compounds, well-known biology, and ease to keep/culture in the laboratory, among other reasons (e.g., Van Leeuwen, 1995). For example, current pesticide risk assessments for soil invertebrates in the EU are largely based on routine testing of earthworms (EC, 2002a; EPPO, 2003). Earthworms have indeed been considered as the most important invertebrates in most

soils worldwide, standardized sampling methods are available, and their taxonomy is well known (Römbke *et al.*, 2005). However, after reviewing laboratory studies into the effects of pesticides on soil invertebrates, Frampton *et al.* (2006) concluded that the standard test earthworm *E. fetida* sensu lato (*E. fetida* and *E. andrei*) was the least sensitive species to insecticides based on acute mortality (i.e., LC_{50} values). Soil arthropods (e.g., the standard Collembolan test species *Folsomia candida*) appeared to be more sensitive to compounds with a broad range of (especially insecticidal) toxic modes of action, indicating that soil arthropods should also be tested routinely in regulatory risk assessments (Frampton *et al.*, 2006).

Frampton *et al.* (2006) conducted their study by constructing species sensitivity distributions (SSD) based on a minimum of five species. Availability of toxicity data for soil invertebrates is very limited with a low number of species tested. Furthermore, the type of toxicity value and/or the unit in which they are expressed vary substantially among studies (see e.g. Figure 1). Subsequently, SSDs could only be constructed for 11 (2 herbicides, 2 fungicides and 7 insecticides) out of the total of 250 pesticides for which toxicity data was available (Frampton *et al.*, 2006). Furthermore, only acute mortality data (i.e., LC_{50}) sufficed to construct SSDs and these could also not be constructed for individual taxonomic groups (e.g., Collembola, Lumbricidae and Nematoda) separately.

The first aim of the present study was to evaluate the sensitivity of *E. fetida* relative to other soil invertebrates for a greater number of compounds and endpoints using (an adapted version of) the relative tolerance (T_{rel}) approach as used by Wogram and Liess (2001) to compare sensitivity of aquatic macroinvertebrates with that of *Daphnia magna*. T_{rel} was calculated by dividing the toxicity threshold value of a particular species with that of *E. fetida*. A T_{rel} of one thus indicates a relative tolerance equal to that of E. fetida. For species more sensitive than E. fetida, T_{rel} is less than one and for less sensitive species it is greater than one.

The development and application of several basic environmental risk evaluation concepts has often been discussed to be focussed on the aquatic compartment (e.g., Tarazona *et al.*, 2000; Jänsch *et al.*, 2007; Baird and Van den Brink, 2007). Therefore, a second aim of the present paper was to evaluate the applicability of various concepts developed in aquatic risk evaluation studies for the terrestrial compartment. Thirdly, implications of study findings for the environmental risk assessment of soil

invertebrates are discussed. This includes an evaluation of the protectiveness of predicted no effect concentrations (PNECs) based on one or more standard test organisms for other (non-standard) species using the T_{rel} approach.

1.3. Materials and methods

1.3.1. Database construction

Toxicity data were obtained from the US Environmental Protection Agency (US-EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/), the largest database of its kind available. On 29 November 2009, the entire database (date of last update by EPA on 16 September 2009) was downloaded as several delimited ASCII data files and subsequently reconstructed into one Microsoft Excel spreadsheet. Database reconstruction was successfully verified for 10 random compounds by comparing results from the reconstructed database with online database queries. Subsequently, data for which no dose unit and/or Latin species name was recorded, and/or resulting from tests not carried out in the laboratory, were omitted.

1.3.2. Representativeness of the database

The extent by which the taxonomic diversity in the database corresponded with that in natural terrestrial ecosystems was evaluated as done by Baird and Van den Brink (2007) for the aquatic ECOTOX database of US-EPA. To this end, the relative number of species tested from a given taxonomic group was compared with the relative abundance of species in nature based on estimates given in Wilson (1992). Since many species within a taxonomic group may have been tested few times and/or few species tested often, the same was done for the relative number of toxicity values generated per taxonomic group as to obtain an estimate for how often taxonomic groups were evaluated.

1.3.3. Relative tolerance calculations

To enable a comparison of threshold values from different compounds, the threshold concentrations had to be "normalised". This was done by transforming these concentrations to relative tolerance (T_{rel}) values by dividing them by the (geometric mean of) threshold value(s) of *E. fetida* sensu lato. To this end, the following steps were undertaken:

1. In accordance with Jänsch *et al*. (2006), only data for euedaphic (soil-dwelling) invertebrates were accepted.
2. The resulting database was divided in four separate spreadsheets, separating no-observed-effect thresholds (i.e., NOEL and NOEC) from thresholds indicating 50% population effect (e.g, ED50), and sublethal (e.g., avoidance behaviour, growth) from lethal (i.e., mortality) endpoints. Data for other thresholds (e.g., LOEL and EC25) were omitted and the four spreadsheets were analysed separately (see legend of Figure 1 for spelled-out acronyms).
3. T_{rel} values were calculated by dividing the lowest geometric mean (gm) toxicity value of a non-standard test species by the lowest gm toxicity value of *E. fetida* sensu lato. Subsequently, toxicity data for compounds for which no toxicity data were available for *E. fetida* sensu lato and at least one non-standard test species were omitted.
4. T_{rel} values were only calculated by dividing toxicity data of standard and non-standard taxa if expressed in the same dose units. In this regard, values expressed in kg/ha were converted to mg/kg using the equation reported in Jänsch *et al*. (2006): MC5 = 1.33*D*, where MC5 is the maximum concentration of a compound in the top 5 cm soil (in mg kg^{-1}) and *D* is the application concentration (in kg ha^{-1}). Subsequently, if no toxicity data for the standard taxon (or taxa) and a given non-standard taxon with comparable dose units were available for a given compound, no T_{rel} was calculated.
5. When multiple datapoints were available for the same taxon, compound and with the same dose unit, the gm of those values was taken.
6. If more than one T_{rel} could be calculated for the same taxon and compound, e.g. since both standard and non-standard taxa had toxicity values with more than one comparable dose unit (i.e., toxicity values were available for both *E. fetida* sensu

lato and another soil invertebrate expressed in for example mg kg^{-1} dry soil and ppm), only the lowest T_{rel} was included.

7. After finishing the analysis of the four spreadsheets (see step 2), calculated T_{rel} values were pooled and presented collectively

Studies using toxicity data sets often apply additional selection criteria besides those mentioned under (2) and incorporated under (4) (e.g., Daam *et al*. 2010) to their data as to account for differences in experimental conditions (e.g., exposure duration, determined endpoints) under which the data were generated. No such additional selection criteria were used in the present study, since i) data availability for soil invertebrates was already rather low; ii) Frampton *et al*. (2006) reported little influence of data selection approaches on LC_{50} estimates of *E. fetida*; and iii) Including all data has the advantage (over e.g. only including data applying standard test procedures) that it includes (the range of) more ecologically representative soils and exposure conditions (Frampton *et al.*, 2006).

1.3.4. T_{rel} PNEC

In the environmental risk assessment (ERA) procedure in the EU, uncertainty factors of 10 and 5 are applied to the acute and chronic toxicity values of *E. fetida*, respectively (EC, 2002a), to establish the predicted-no-effect-concentration (PNEC). To evaluate whether these uncertainty factors suffice to protect all other taxa included in the analyses, "T_{rel} PNECs" were calculated accordingly, i.e. by dividing toxicity values of non-standard test species for the different compounds by their corresponding PNEC values. In accordance with the ERA procedure in the EU, these PNECs were calculated by dividing the acute and chronic toxicity data for *E. fetida* with 10 and 5, respectively. A T_{rel} PNEC based on for example chronic NOEC data would thus be calculated using the following formula:

T_{rel} PNEC = gmNOEC non-standard test species / (gmNOEC *E. fetida* / 5)

Hence, a T_{rel} PNEC greater than 1 for a given non-standard test species indicates that the uncertainty factors applied to the toxicity data of *E. fetida* sufficiently protects this species, whereas a T_{rel} PNEC lower than 1 indicates that this may not be the case.

In addition, T_{rel} PNECs were calculated by considering the sensitivity of both *E. fetida* and *F. candida*, i.e. by using the lowest toxicity value of these organisms. In other words, the gmNOEC value of *E. fetida* in the previous formula would be replaced by that of *F. candida* if the gmNOEC value of *F. candida* was lower than that of *E. fetida*. Although the PNEC in the EU risk assessment is strictly based on lethal (mortality) acute data and sublethal (reproduction) chronic data, both lethal and sublethal data were included in the analysis since number of data points would otherwise be rather low. However, analysis of the data was done separately for no-observed-effect thresholds and thresholds indicating 50%, as well as sublethal and lethal endpoints, in the same way as described in section 2.3. Since no uncertainty factors are defined in EU legislation for laboratory threshold values of *Folsomia candida,* the same uncertainty factors as established for *E. fetida* were applied.

1.3.5. Species Sensitivity Distributions

The T_{rel} values calculated as described above were grouped for compound type (insecticides, fungicides, herbicides, and other) and taxonomic groups as used by Frampton *et al*. (2006; Acari, Chilopoda, Coleoptera, Collembola, Diplopoda, Enchytraeidae, Isopoda, Lumbricidae and other earthworm families, and Nematodes). Subsequently, if more than five T_{rel} values were available for a given taxonomic group and compound type (e.g., T_{rel} based on insecticides for Collembola), distribution curves of these T_{rel} values were constructed as described in Daam *et al.* (2010). In brief, log-normal distributions of the T_{rel} values were derived using the ETX computer program version 2.0 (Van Vlaardingen *et al.,* 2004). If lognormality was not accepted by the Anderson-Darling Test included in the ETX software package, the BurrliOz program (Campbell *et al.,* 2000) was used to fit a Burr type III distribution that best fitted the available data (log-logistic, log-normal, log-triangular, Weibull). The BurrliOZ software calculates confidence intervals for hazard concentrations (HC) values using a bootstrap technique, implying that confidence intervals may vary with subsequent re-runs (Hose, 2005). Therefore, each HC limit (i.e., lower and upper limits of HC5 and HC50) was estimated 10 times using 1000 permutations (separately for lower and upper limits) and the geometric mean of those 10 calculations was used as a best estimate (after Hose and Van den Brink, 2004). BurrliOZ does not include software to indicate how well the datapoints fit the curves. Hence, in accordance with Daam *et al.* (2010), r2 values were

calculated by applying linear regression in Microsoft Excel on PAF (potentially affected fraction) values indicated by the curve and actual PAF values of the individual T_{rel} values as a measure of how well the curve fitted the datapoints.

1.4. Results and Discussion

1.4.1. Data availability

After omitting those data for which no species name, dose unit and/or threshold type were recorded, the reconstructed US-EPA terrestrial ECOTOX database yielded 83229 entries. The variety in reported threshold values and units of these entries is visualized in Figure III.1. Interestingly, although in aquatic studies availability of NOEC values is often reported to be very limited (e.g., Daam *et al.*, 2010), NOEL was the most reported toxicity threshold for the terrestrial database (Figure III.1). Furthermore, a great variety in units used to express toxicity thresholds was noted (Figure III.1), which was not the case for the part of the aquatic US-EPA ECOTOX database used to conduct the study described in Daam *et al.* (2010), where "µg L^{-1}" was the unit used to express the vast majority of toxicity values.

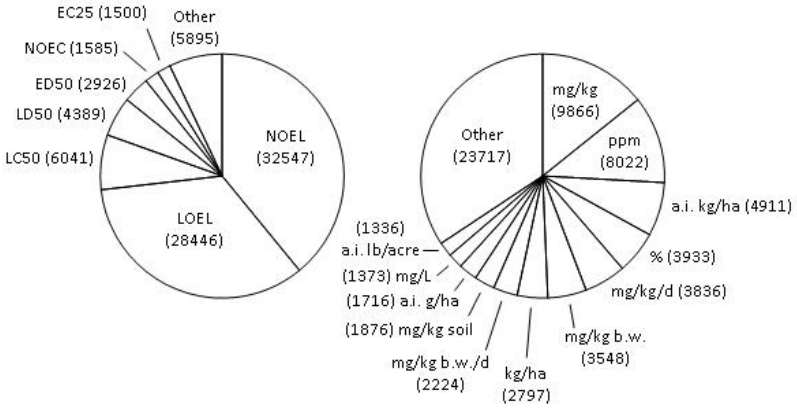

Figure III.1: Variety in threshold value type (left) and unit (right) of the data entries in the reconstructed US-EPA terrestrial ECOTOX database (after exclusion of those entries for which species Latin name, threshold type or unit was not recorded). Threshold types and units for which less than 1000 entries were encountered, were included in "other". Number of entries are provided in brackets. NOEL = no-observed-effect-level; LOEL = lowest-observed-effect-level; LC_{50} = lethal concentration to 50% of the test organisms; LD_{50} = lethal dose to 50% of the test organisms; ED_{50} = effective dose for 50% of the test organisms; NOEC = no-observed-effect-concentration; EC_{25} = effective concentration to 25% of the test organisms.

Evidently, this great variety in both threshold types and their units greatly hampers construction of "traditional" SSDs, i.e. based on different taxa with the same threshold type and unit for the same compound, as a result of incompatibility of the toxicity data, even though some toxicity values expressed in different units could be converted (e.g., a.i. g ha^{-1} and a.i. kg ha^{-1}). In the present study, this limitation was intended to be significantly reduced by applying the T_{rel} approach as to allow incorporating as much data as possible. Indeed, since Frampton *et al.* (2006) only considered LC_{50} data and constructed SSDs for individual compounds, no separate SSDs for the different taxonomic groups could be included. Hence, reported greater or lower sensitivity of a given taxonomic group was based on the fact that a limited number of datapoints were positioned in the lower or upper tail, respectively. In the present study, however, separate SSDs could be constructed for various taxonomic groups to compare sensitivity to compounds grouped for toxic type of action (insecticidal, herbicidal, fungicidal, and other; see below). In addition, SSDs could be constructed based on three to five times as many different compounds compared to the relatively low number of compounds included in the analysis by Frampton *et al.* (2006): 21 *versus* 7 insecticides, 7 *versus* 2 fungicides, and 11 *versus* 2 herbicides, respectively (Table III.1).

Table III.1: Total number of TUs (calculated by dividing the threshold value of a given species by the threshold value of *Eisenia fetida* sensu lato for the same compound) that could be calculated in the present study, sorted by compound type and taxonomic groups (after Frampton *et al.,* 2006).

Sorted by	Type/Taxonomic group	No. different pesticides/taxa	Total Nº. TUs
Compounds	Insecticides	21	58
	Fungicide	7	59
	Herbicide	11	20
	Other	35	112
	Total	74	249
Taxa	Acari	4	7
	Chilopoda	-	-
	Coleóptera	3	3
	Collembola	9	62
	Diplopoda	-	-
	Enchytraeidae	4	30
	Isopoda	3	10
	Lumbricidae	21	110
	Nematoda	18	27
	Total	62	249

TU – Toxic Units

1.4.2 Limitations of the analysis

The representativeness of the database in terms of taxonomic composition was evaluated by comparing the relative number of invertebrate species tested and toxicity data generated within the database for main taxonomic groups with those known to occur in nature (after Wilson, 1992). As can be seen in Figure III.2, insects are clearly under-represented in the database. As also discussed by Baird and Van den Brink (2007) for the aquatic US-EPA ECOTOX database, this is evidently not intended as a criticism towards US-EPA, but simply reveals the lesser attention that has (erroneously, as will be discussed below) been attributed to establishing toxicity values for insects. This is also reflected in the data that could be used to calculate T_{rel}. Almost half of all T_{rel} values (110 out of 249) were calculated for earthworms (Lumbricidae), for which also the greatest number of different taxa (21) were included (Table III.1; Figure III.2). Interestingly, although T_{rel} values could be obtained for a relatively great number of nematode taxa, total number of T_{rel} values were relatively low for this taxonomic group, indicating that many nematode species are tested very few times. Contrarily, only four enchytraeid taxa (*Cognettia sphagnetorum*, *Enchytraeus albidus*, *E. crypticus*, and *Enchytraeus* sp.) were in total tested 30 times (Table III.1).

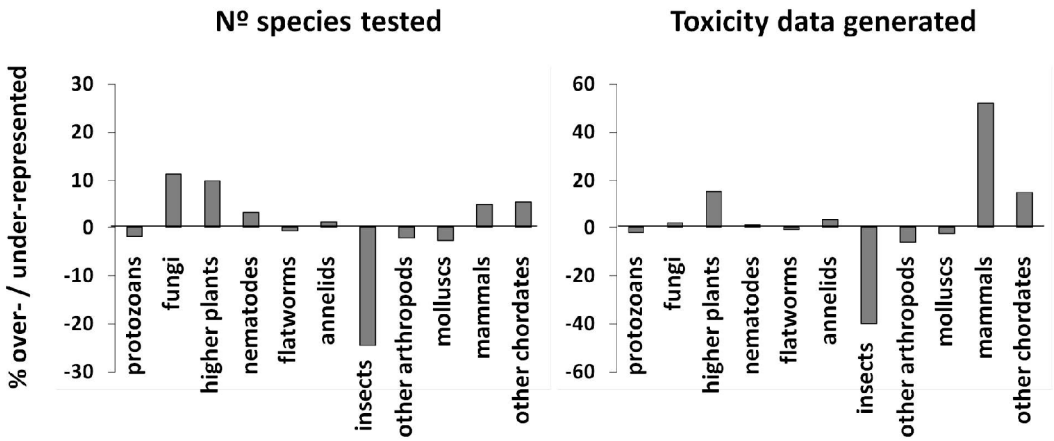

Figure III.2: Visualization of the relative number of invertebrate species tested (left) and toxicity data generated (right) in the US-EPA terrestrial ECOTOX database as compared to the relative abundance of species in nature as estimated by Wilson (1992). A negative percentage indicates that a group is under-represented in the database, whereas a positive percentage indicates that a group is over-represented (after Baird and Van den Brink, 2007).

For arthropods, only collembolans were tested relatively frequently, whereas for other groups (including the insect order Coleoptera) very few or no toxicity data were available that were suitable for T_{rel} calculations (Table III.1). As discussed by Wogram and Liess (2001), this indicates that species for which an above-average number of T_{rel} values could be calculated are overemphasized. Similarly, compounds that have been tested more frequently have a greater weight in the overall analysis of the pesticide type to which they belong. However, Wogram and Liess (2001) also concluded that the error introduced by alternatively taking a secondary mean at the order level to outweigh frequently tested taxa would probably be greater than the error resulting from overweighing individual species.

Due to the relatively low data availability and the great variety in test conditions (e.g., test duration, organism strain, and sublethal endpoints), no additional selection criteria were applied after separating the dataset in sublethal / lethal and 50% effect / no-observed-effect thresholds. Evidently, differences in experimental design will ultimately influence threshold levels. For example, Frampton *et al.* (2006) discussed that standard OECD soil has a higher organic content than most natural soils, implying a lower bioavailability and hence higher threshold values. Contrarily, longer exposure durations will logically lower threshold concentrations. To obtain an idea of the variation in toxicity values in the database as a result of differences in experimental design, the spread in toxicity values was evaluated by applying the method used by Brock *et al.* (2008) and Daam *et al.* (2009) to calculate the spread in NOECecosystem values derived in model ecosystem studies. To this end, 95% confidence intervals were calculated for those data for which at least three toxicity values, derived for the same species and compound but under different experimental conditions, were available. Subsequently, the ratio of the upper and lower limits of these intervals was used as an indication of the spread in toxicity values for that taxon-compound combination. Resulting average spreads (with 95% confidence intervals) were 5.3 (3.6-7), 8.5 (-1.6-19) and 7.1 (2.7-12) for 50% effect thresholds indicating mortality, 50% effect thresholds indicating sublethal effects and no-observed sublethal effect thresholds, respectively. For no-observed lethal effect thresholds not enough data were available to calculate a spread. These high values are not surprising considering that a ringtest with earthworm toxicity tests based on 18 participating laboratories, all using the same experimental conditions, resulted in a spread in LC_{50} values of up to a factor 5 (Moser *et*

al., 2009). To date, only few studies have been performed to clarify the influence of soil properties on the fate (e.g., bioavailability) and toxicity of organic chemicals to soil invertebrates (Sousa *et al.*, 2000; Frampton *et al.*, 2006; Römbke *et al.*, 2007; Chelinho *et al.*, 2011). The need for such studies appears evident given the spreads in toxicity values discussed above, and may be further stressed by the indication given in the Sixth Community Environment Action Programme that regional and local environmental differences should be considered in the Community's environmental policy-making (EU, 2002).

Due to the discussed low data availability, differences in experimental conditions under which the toxicity data were derived could not be accounted for in the presented analyses. Hence, sensitivity comparisons as visualized in Figure III.3 would have been biased by such differences in case experimental conditions of a certain taxonomic group would as a rule differ from that of *E. fetida*.

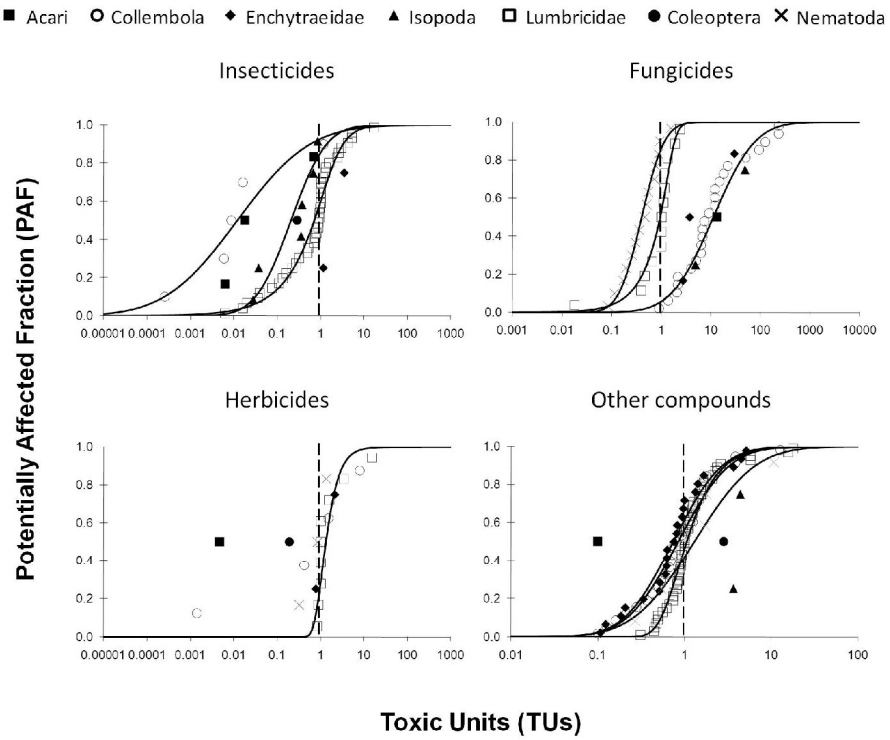

Figure III.3: Species sensitivity distributions (SSD) comparing the sensitivity of different taxonomic groups to insecticides, fungicides, herbicides and other compounds with that of *E. fetida* sensu lato using the toxic unit approach. The vertical dashed line at $T_{rel} = 1$ indicates the sensitivity of *E. fetida* sensu lato. A $T_{rel} < 1$ and a $T_{rel} > 1$ indicate a greater and lower sensitivity relative to *E. fetida* sensu lato, respectively. T_{rel} = relative tolerance.

Table III.2: Estimates of the 5% (P5) and 50% (P50) percentiles (with 95% confidence intervals) derived from the species sensitivity distributions (SSDs) of the toxic unit (TU) values. PAF = Predicted Affected Fraction.

		P5	**P50**	**PAF at TU = 1**	**SSD constructed with /fit to curve ****
Insecticide	Lumbricidae	0.022 (0.0062-0.076)	0.71 (0.49-1.03)	60%	BurrliOz Burr III / r=0.98 ($p < 0.01$; n=38)
	Collembola	0.000057 (0.000000038-0.0010)	0.012 (0.00068-0.21) *	93%	ETX lognormal / accepted (n=5)
	Isopoda	0.016 (0.00088-0.057)	0.020 (0.062-0.7) *	86%	ETX lognormal / accepted (n=6)
Fungicide	Lumbricidae	0.12 (0.018-0.59)	0.93 (0.62-1.25)	54%	BurrliOz Burr III / r=0.97 ($p < 0.01$; n=13)
	Collembola	0.86 (0.32-1.73)	11 (6.4-19) *	6%	ETX lognormal / accepted (n=24)
	Nematoda	0.097 (0.046-0.16)	0.39 (0.27-0.58) *	87%	ETX lognormal / accepted (n=15)
Herbicide	Lumbricidae	0.66 (0.62-0.75)	1.3 (1.1-1.6)	29%	BurrliOz Reciprocal Weibull / r=0.89 ($p < 0.01$; n=9)
Other compounds	Lumbricidae	0.45 (0.39-0.57)	1.05 (0.92-1.23)	47%	BurrliOz Burr III / r=0.99 ($p < 0.01$; n=50)
	Collembola	0.14 (0.078-0.22)	0.84 (0.60-1.2)	57%	ETX lognormal / accepted (n=29)
	Enchytraeidae	0.13 (0.0069-0.22)	0.73 (0.51-1.1)	62%	ETX lognormal / accepted (n=23)
	Nematoda	0.14 /0.012-0.43)	1.29 (0.45-3.6)	42%	ETX lognormal / accepted (n=6)

* considered significant since TU = 1 not in 95% CI; ** SSDs were constructed with the ETX program, which includes the Anderson-Darling Test to evaluate the fit to curve, or the BurrliOz software package, for which the fit to curve was determined by calculating the correlation coefficients (For details, please refer to the Materials and Methods section).

For example, consider the case where exposure durations of the tests evaluating insecticides conducted with collembolans are significantly longer than those carried out with *E. fetida*. This would indicate that the differences between collembolans and *E. fetida* (Tables III.2 and III.3; Figure III.3) did not result from a greater sensitivity of the former, but would merely be the result of these differences in experimental design.

Although there is no direct reason that indicates that this would be the case, it was verified for the T_{rel} calculations of Lumbricidae (both insecticides and fungicides) since the greatest differences with *E. fetida* were obtained for this taxonomic group. The exposure duration and organic matter content in studies used to calculate the T_{rel} values were verified as potential confounding parameters by dividing the values for collembolans by those of *E. fetida*. Average (with 95% confidence interval) ratios for exposure duration were 2.3 (0.4-4.2) for fungicides and 1.3 (0.8-1.8) for insecticides. Data to calculate this ratio for organic matter were only available for fungicides: 1.3 (0.5-2.1). As anticipated, no consistent trend could be demonstrated, although exposure duration appears slightly higher for collembolan tests evaluating fungicides. However, this would imply lower toxicity values, whereas a lower sensitivity of collembolans for fungicides was noted. Hence, difference in sensitivity between collembolans and *E. fetida* to fungicides might have been even slightly greater than indicated by the presented analysis (Figure III.3; Tables III.2 and III.3) if similar test conditions would have been considered.

Table III.3: Mean toxic unit (TU) values (with 95% confidence intervals) for the different taxonomic groups and compound types. - = no data; NP = not possible to calculate a 95% CI since not enough data available (< 3 datapoints). In the latter case, the single or two TUs are presented.

	Insecticide	**Fungicide**	**Herbicide**	**Other compounds**
Acari	0.24 (-0.21-0.69) *	13 (NP)	0.0047 (NP)	0.1
Chilopoda	-	-	-	-
Coleoptera	0.29 (NP)	-	0.19 (NP)	2.8 (NP)
Collembola	0.24 (0.22-0.70) *	37 (9.9-64) *	2.5 (-1.2-6.2)	1.5 (0.65-2.4)
Diplopoda	-	-	-	-
Enchytraeidae	1.16; 3.51 (NP)	12 (-5.3-30)	0.78; 2.1 (NP)	1.2 (0.64-1.8)
Isopoda	0.39 (0.12-0.65) *	4.9; 49 (NP)	-	3.7; 4.4 (NP)
Lumbricidae	1.56 (0.65-2.48)	1.1 (0.71-1.39)	2.9 (-0.22-6.1)	1.9 (1.008 - 2.8) *
Nematoda	1.3 (0.72-1.9)	0.53 (0.33-0.73) *	0.84 (0.26-1.4)	2.7 (-0.54-5.9)

* considered significant since TU = 1 not in 95% CI

1.4.3. Sensitivity of E. fetida sensu lato *compared to other soil invertebrates*

In Figure III.3, the sensitivity of soil invertebrates by taxonomic group are compared with that of *E. fetida* sensu lato. The greater and lower sensitivities of collembolans to insecticides and fungicides, respectively, as noted by Frampton *et al.* (2006; laboratory single species tests) and Jänsch *et al.* (2006; (semi) field tests), are confirmed (see also Tables III.2 and III.3). However, overall greater sensitivity of the standard collembolan *Folsomia candida* to a broad range of toxic modes of action (e.g., herbicidal), as discussed by Frampton *et al*. (2006), could not be demonstrated. This may be partly due to the fact that only 4 T_{rel} values could be calculated for collembolans based on herbicides. Although paraquat dichloride (T_{rel} = 0.0014) and pendimethalin (T_{rel} = 0.42) indicated a greater sensitivity of collembolans, they appear less sensitive to pentachlorophenol (T_{rel} values of 1.5 and 8). Contrarily, the SSD constructed by Frampton *et al.* (2006) for the latter compound indicated a (slightly) greater sensitivity for collembolans as compared to *E. fetida*. This may be related with the fact that Frampton *et al.* (2006) constructed their SSD based on LC_{50} data, whereas the two T_{rel} values for pentachlorophenol in the present study were based on sublethal NOEC and EC50 values.

Besides the anticipated differences in sensitivity between collembolans and *E. fetida* described above, the SSDs also revealed that isopods were more sensitive to insecticides, and nematodes to fungicides, as compared to *E. fetida* (Figure III.3; Table III.2). Since SSDs could only be constructed for a limited number of taxonomic-compound group combinations, 95% confidence intervals (CI) of T_{rel} values from these combinations were calculated, which are presented in Table III.3. These additional analysis also indicated significant (i.e., the value 1 is not covered by the 95% CI) greater sensitivity of Acari to insecticides, and nematodes to fungicides (Table III.3). This greater vulnerability of arthropods to insecticides, as demonstrated for Acari, Collembola and Isopoda, and indicated by the single T_{rel} value of 0.29 for Coleoptera (Table III.3), has also previously been demonstrated for aquatic organisms (e.g., Maltby *et al.*, 2005). Logically, pesticides developed to kill insect pest organisms (e.g., by inhibiting acetylcholinesterase or chitin production) are also more likely to exert side-effects on non-target insects and taxonomically-related taxa. Similarly, the lower sensitivity of the arthropods, as indicated by the SSD of collembolans (Table III.2; Figure 3) and individual T_{rel} values for Acari and Isopoda (Table III.3) compared to *E.*

fetida, could also be anticipated based on aquatic studies into fungicide toxicity. For example, Van Wijngaarden *et al.* (1998) and Cuppen *et al.* (2000) reported greatest sensitivity of "worm-like" taxa to the fungicide carbendazim in single species tests and a microcosm study, respectively, although the underlying reason for this is unclear. Frampton *et al.* (2006) discussed that a surprising finding of their analysis was that SSDs for insecticides could only be calculated for oligochaets despite the expected greater sensitivity of arthropods. Similarly, much more T_{rel} values based on insecticidal toxicity data could be calculated in the present study for Lumbricidae than for arthropods (Figure III.3). Another surprising observation arising from Figure III.3 is that, despite the discussed greater sensitivity of collembolans to insecticides, T_{rel} availability for these organisms is approximately 5 times higher for fungicides than for insecticides. These findings thus imply an overall poor selection of test compound (or test species) in the soil toxicity assays included in the database.

The SSD of Nematodes indicated a greater sensitivity than *E. fetida* for fungicides, which was based on toxicity values of 14 nematode taxa to copper sulphate and cupric chloride. Interestingly, studies evaluating the sensitivity of a single nematode species to copper compounds reported that obtained toxicity values were comparable (Boyd *et al.*, 2001), slightly lower (Kammenga *et al.*, 1996) or even slightly greater (Peredney and Williams, 2000) than those of *E. fetida*. Korthals *et al.* (1996) derived toxicity thresholds for a total of 14 nematode taxa from different feeding and life-history strategy groups to copper. Based on these tests, they concluded that *K*-strategist nematodes were among the most sensitive taxa (Korthals *et al.*, 1996). Interestingly, *E. fetida* has been considered a typical *r*-strategist in its life history traits (Lukkari *et al.*, 2005), which may thus be related with its low sensitivity to copper as compared to nematodes. This appears not to hold true, however, for all compound types, since Kammenga *et al.* (1994) concluded that slow colonizing nematodes (*K*-strategists) were not more sensitive to cadmium and pentachlorophenol than opportunistic nematode species (*r*-strategists). Sensitivity of *E. fetida* sensu lato appeared to be similar or slightly greater (for herbicides) compared to other Lumbricidae (Figure III.3; Tables III.2 and III.3).

1.4.4. Implications for the terrestrial risk assessment of toxic compounds

After reviewing the sensitivity of soil arthropods in single species, model ecosystem and field studies, Frampton *et al*. (2006) and Jänsch *et al*. (2006) concluded that the standard collembolan test species *Folsomia candida* should be included in regulatory risk assessments. Based on the analysis demonstrated in Figure III.4, the need for this seems justified: PNECs based on only *E. fetida* sensu lato do not fully protect a great number of other test organisms, whereas this is not the case when including *F. candida* in PNEC calculations (Figure III.4). Similarly, toxicity testing of a chironomid larvae (Insecta) is required in the aquatic environmental risk assessment of insecticides if side-effects on these organisms are to be expected (EC, 2002b).

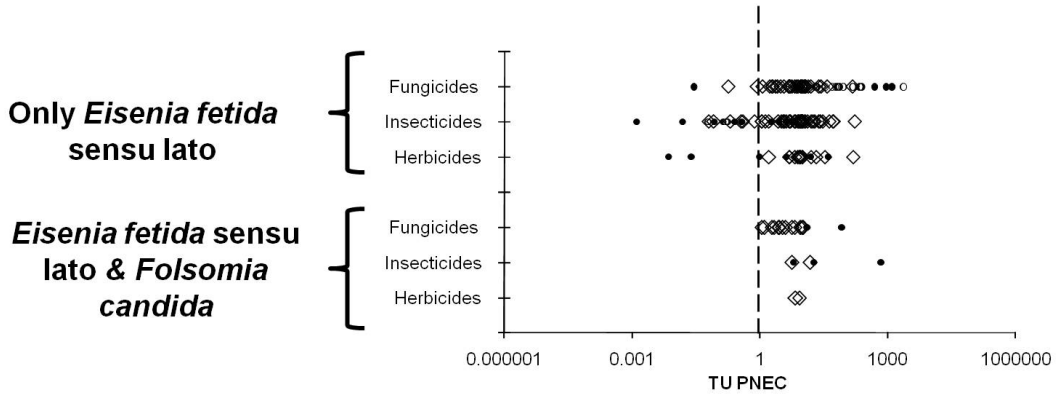

Figure III.4: Protectiveness of predicted no effect concentrations (PNEC) for *E. fetida* sensu lato alone, and in combination with *Folsomia candida*, for other test organisms included in the database (For details, see text). Taxonomic groups were grouped in arthropods (black dots) and annelids & nematodes (open diamonds). The vertical dashed line at T_{rel} PNEC = 1 indicates the PNEC of *E. fetida* sensu lato. A T_{rel} PNEC < 1 indicates that the corresponding PNEC value(s) for the standard test species considered is/are not sufficiently protective, whereas a T_{rel} PNEC > 1 indicates that the PNEC value(s) for the standard test species considered is/are sufficiently protective. T_{rel} = relative tolerance.

Only few T_{rel} PNEC values could be calculated when considering both *E. fetida* sensu lato and *F. candida* due to constraints in data availability and the fact that at least three (*E. fetida* sensu lato, *F. candida* and a third species) toxicity values for the same compound expressed in the same dose unit had to be available. Especially for arthropods few T_{rel} PNECs could be calculated, and was limited to a maximum of three values: Acari (1), Coleoptera (2), Collembola other than *F. candida* (3), and Isopoda (3). Furthermore, various T_{rel} PNEC values lay close to 1, especially for fungicides (Figure III.4), for which three T_{rel} PNEC values between 1 and 2 were obtained for three different nematode taxa. Furthermore, a T_{rel} PNEC of 0.96 was calculated for the enchytraeid *Enchytraeus crypticus* exposed to manganese sulphate. Also considering that several $T_{rel} < 1$ were obtained for Acari, Isopoda and Nematoda (Figure III.3; Tables III.2 and III.3), it may thus be questionable whether sole testing of *E. fetida* sensu lato and *F. candida* for the first-tier risk assessment covers the range of other potentially sensitive taxa. For the same reason, a battery of tests using a range of test organisms has previously been recommended (e.g., Jänsch *et al.,* 2007; Römbke *et al.,* 2005). Representatives of the organism groups indicated in the present study to contain sensitive taxa, have also previously been recommended as test organisms in laboratory toxicity testing, e.g. predatory Acari (Frampton and Van den Brink, 2007; Jänsch *et al.,* 2007), Isopoda (Caseiro *et al.,* 2000; Ribeiro *et al.,* 2001), Enchytraeidae (Jänsch *et al.,* 2005), and Nematoda (Kammenga *et al* 1996; Sochová *et al.,* 2006). Regarding Nematodes, Boyd *et al*. (2001) reported that the nematode *Caenorhobditis elegans* is especially suitable to assess toxicity associated with porewater exposures because it resides in water within the soil matrix. As further discussed by Boyd *et al*. (2001), among other authors, soil sorption (i.e. the capacity of soil particles to bind chemical substances) may alter the bioavailability of contaminants in soils and soil porewaters and influence the results of soil toxicity tests. Furthermore, chemical bioavailability in Organisation for Economic Co-operation and Development (OECD) artificial soil may contrast with bioavailability in natural soils and produce ecotoxicological benchmarks that are not representative of species exposure conditions in the field, indicating that toxicity testing should include studies with natural soils in addition to OECD soil to better reflect exposure conditions in the field (Römbke *et al.,* 2007; Chelinho *et al.,* 2011). In these regards, it should be noted that in the present study the representativeness of standard test organisms was only studied on a first-tier level, i.e.

by evaluating whether PNEC values for these species cover the sensitivity of other species tested in laboratory single species tests. Jänsch *et al.* (2006) made an effort to validate as to whether first-tier toxicity values suffice to protect terrestrial ecosystems under real-world (semi) field conditions. They concluded that for eight pesticides, higher-tier effect concentrations were within or below the 90% CI of the HC5 from SSDs constructed from first-tier toxicity values (Jänsch *et al.,* 2006). However, in most cases there was insufficient data from field studies and/or insufficiently low test concentrations were included to allow NOEC estimations, hampering the validation of risk predictions based on first-tier testing. This emphasizes the urgent need for higher-tier studies into the risk evaluation of pesticides in terrestrial (model) ecosystems. Besides the reasons discussed above, the need for this may be further stressed by the importance to evaluate functional endpoints, which may be more sensitive than structural effects (Jänsch *et al.* 2007). Furthermore, only model ecosystem or field studies will allow i) an environmental realistic evaluation of the influence of complex mixtures, usually present in natural contaminated soils (Sousa *et al.,* 2008), and ii) coping with interactions between species and the role of pesticide stress on this (indirect effects) as well as the recovery potential of affected terrestrial communities (Schaeffer *et al.,* 2010).

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2. Effects of azoxystrobin, chlorothalonil and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil.

Based on the following manuscript:

Effects of azoxystrobin, chlorothalonil and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. Sara Leitão, Mª José Cerejeira, Paul J. Van den Brink and José Paulo Sousa (*Submitted to the journal Applied Soil Ecology; under revision*).

2.1. Abstract

The potential terrestrial toxicity of three pesticides, azoxystrobin, chlorothalonil and ethoprophos was evaluated using reproduction ecotoxicological tests with non-target species from different trophic groups: the collembolan *Folsomia candida*, the earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus*. All reproduction tests were performed with natural soil from a Mediterranean agricultural area (with no pesticide residues) in order to improve the relevance of laboratory data to field conditions. Controls were performed with natural and standard artificial soil (OECD 10% OM). The fungicide azoxystrobin showed the highest toxicity to earthworms (EC_{50} = 42.0 mg a.i. kg^{-1} dw soil). Collembolans were the most sensitive taxa followed by the earthworms in terms of sublethal effects of chlorothalonil with an EC_{50} of 31.1and 40.9 mg a.i. kg^{-1} dw soil, respectively. The insecticide ethoprophos was the most toxic to collembolans affecting their reproduction with an EC_{50} of 0.027 mg a.i. kg^{-1} dw soil. Enchytraeids were generally the least sensitive of the three species tested for long-term effects. Earthworms were not always the most sensitive species, emphasizing the need to increase the number of mandatory assays with key non-target organisms in the environmental risk assessment of pesticides.

Keywords: Pesticides; non-target soil organisms; natural soil; Mediterranean conditions; ecotoxicity.

2.2. Introduction

The environmental risk assessment (ERA) of pesticides is based mainly on scenarios developed for northern and central European conditions. This may pose a problem when used for Mediterranean conditions where soil properties, climatic conditions, biological communities, agricultural practices and crops are substantially different (Daam *et al*, 2011a; Ramos *et al*, 2000). These generic scenarios can over- or underestimate the real risks of pesticides when applied to a typical Mediterranean environment (Ramos *et al*, 2000). Therefore, the use of natural soils is becoming more and more important when performing relevant regional ERA among European regions (Chelinho *et al.,* 2011).

Pesticides ERA for terrestrial organisms uses standardized ecotoxicological tests traditionally performed in standard artificial soil (e.g. OECD; ISO, 1998), or in standard natural soil (e.g. LUFA2.2) that often do not possess the characteristics of agricultural natural soils, therefore not mimicking realistic exposure conditions to pesticides for soil biota in the field (Kuperman *et al*, 2006). It has been documented that differences in soil properties such as organic matter content may influence pesticide persistence in soil and bioavailability to soil-dwelling organisms (enchytraeids and earthworms) (Amorim *et al* 2002a, 2002b; De Silva *et al*, 2009; Kuperman *et al*, 2006). Compared to standard artificial soils, natural soils may have properties supporting higher bioavailability of test chemicals, so their use considerably improves the relevance of laboratory ecotoxicological data for field conditions (Kuperman *et al*, 2006; Van Gestel *et al.,* 2011). Therefore, the importance of using natural soil is supported by the need to develop more realistic ecotoxicological evaluations for terrestrial ecosystems.

Until the implementation of the new data requirements setup according to the new Pesticide Regulation 1107/2009 (EU, 2013), the protection of terrestrial ecosystems at a first-tier level is assessed in the ERA of pesticides using only the earthworms acute test with *Eisenia fetida sensu lato* (*E. fetida* and *E. andrei*) (EC, 2009; SANCO, 2002). Tests using other non-target organisms can be performed if non-target arthropods are believed to be at risk, e.g. tests with Collembola and mites, and are performed on a case-by-case basis depending on the type of the pesticide and its application method (SANCO, 2002). Although earthworms are key species of terrestrial ecosystems as decomposers contributing significantly to organic matter decomposition, nutrient cycling and soil formation (Edwards and Bohlen, 1992; EFSA, 2009a), there is a need for further tests evaluating sub-lethal effects on soil organisms from different trophic levels, taxonomic, physiological and/or functional groups in order to improve the ERA of chemicals in soil (Daam *et al.,* 2011b; EFSA, 2010b; Frampton *et al.,* 2006; Römbke and Moser, 2002). Although there is a growing concern about the potential adverse effects of pesticides in the environment, there is a lack of sub-lethal ecotoxicity data available for non-target terrestrial invertebrates (Daam *et al.,* 2011b; Frampton *et al.,* 2006).

Thus to overcome these limitations, this study aimed at: i) evaluate sub-lethal effects of pesticides with different toxic types of action (two fungicides and one insecticide) on the reproductive performance of non-target soil invertebrates from different trophic

levels: collembolan, enchytraeids, and earthworms; ii) to increase knowledge on pesticides behaviour in the environment, by using a natural soil from a Mediterranean agricultural area, and iii) to perform a first-tier risk characterization for the three pesticides by comparing the obtained toxicity data with reported exposure data, whenever possible, eliciting the importance of using natural soil when evaluating exposure and effects on terrestrial organisms.

2.3. Material and methods

2.3.1. Pesticide selection, characterization, spiking and analytical procedures

Two fungicides, azoxystrobin and chlorothalonil, and the insecticide ethoprophos were chosen after a selection from a list of pesticides authorized on irrigated crops (onion, maize and potato) in Portugal. A preference was given to insecticides and fungicides with high expected toxicity to soil organisms (Frampton *et al.,* 2006; Wang *et al.,* 2012). In term of effects, the selection was based mainly on ecotoxicity data to terrestrial organisms, namely to earthworms, due to the lack of information on collembolans and enchytraeids. Relevant intrinsic physical and chemical characteristics such as water solubility, capacity to adsorb to soil particles, volatilization and persistence in soil were also taken into account (Table III.4). Information on environmental fate, such as the potential for leaching into groundwater and the predicted environmental distribution (PED) (Table III.4), focusing on the soil and water compartments, was assessed using the Groundwater Ubiquity Score and the Mackay fugacity model, respectively (Gustafson, 1989; Mackay, 2001). The application mode (e.g. direct soil application) was also taken in account.

Azoxystrobin (CAS 131860-33-8; methyl (*E*) – 2 - {2- [6- (2-cyanophenoxy) pyrimidin -4 -yloxy] phenyl} -3-methoxyacrylate) is a strobilurin fungicide with protectant, curative, eradicant, translaminar and systemic properties. Its mode of action focuses on inhibiting mitochondrial respiration, spore germination and mycelial growth and also showing antisporulant activity. It possesses a broad spectrum of activity against the four major groups of fungi: Ascomycota, Oomycota, Deuteromycota and Basidiomycota (Bartlett *et al.,* 2002; MacBean, 2012). It has been identified as low toxic to birds,

mammals, bees, and other non-target terrestrial organisms (arthropods and earthworms) (Bartlett *et al.,* 2002; Gullino *et al.,* 2000).

Chlorothalonil (CAS 1897-45-6; tetrachloroisophthalonitrile) is a chloronitrile fungicide with a non-systemic broad-spectrum mode of action and foliar action with some protectant properties. It is a broad spectrum organochlorine fungicide effective against fungal diseases like Potato Late Blight Agent and Fungus *Phytophthora infestans* (Mont.) de Bary and *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout. Chlorothalonil acts also by preventing spore germination and zoospore motility (Sakkas *et al.,* 2002; MacBean, 2012). Although effects on earthworms have been registered (Potter *et al.,* 1994; Tu *et al.,* 2011), information on other non-target organisms is scarce.

Ethoprophos (CAS 13194-48-4; *O*-ethyl *S*,*S*-dipropyl phosphorodithioate) is a broad spectrum organophosphate insecticide and nematicide with moderate residual activity and is not phytotoxic. It is an acetylcholinesterase inhibitor and is a non-systemic nematicide and soil insecticide with contact action. Ethoprophos is effective against potato nematodes (*Globodera rostochiensis* (Wollenweber) Behrens, *G. pallide* (Stone) Behrens) and soil insects (Agriotes spp., Agrotis spp. and Melolontha spp.) on maize crop (Karpouzas *et al* 1999a, 1999b; MacBean, 2012). Effects on non-target soil organisms are not well known and the information available is related to artificial soil (EFSA, 2006), although effects on terrestrial arthropods may be expected due to the pesticide type of action (Frampton *et al.,* 2006). Adverse effects on the abundance and biomass of earthworms are known (reduction of 88 to 95% and 83 to 96%, respectively, 3 weeks after the application of 5.6 kg a.i. ha^{-1} of Mocap10G in turf soil) (Potter *et al.,* 1994).

Table III.4: Pesticides physico-chemical characteristics, environmental potential fate (Groundwater Ubiquity Score - GUS and Predicted Environmental Distribution - PED), and pesticides ecotoxicity data for terrestrial earthworm (all data from MacBean, 2012 unless indicated otherwise).

	azoxystrobin	**chlorothalonil**	**ethoprophos**
Sw (mg L^{-1})	6.0	0.81 (25ºC)	700
VP (mPa)	1.10E-07	0.076 (25 ºC)	78[f]
Koc (ml g^{-1})	690[a]	850[d]	111[f]
Log Kow	2.5 (20 ºC)	2.9 (25ºC)	3.59 (21 ºC)
DT_{50}lab soil (d)	279[b]	0.3 – 87[d]	10 – 25[f]
DT_{50} field soil (d)	14	18 – 70[d]	4 – 25[g]
GUS	2.84	2.08 (DT_{50}lab soil 87)	2.73 (DT_{50}lab soil 25)
PED (%)			
Soil	**49.5**	**43**	**76.1**
Air	7.39E-08	0.285	0.122
Aerossol	8.72E-03	2.08E-03	5.91E-05
Water	**49.3**	**55.7**	**22.1**
Sediment	1.10	0.955	1.69
Suspended solids	0.0344	0.0299	0.0528
Aquatic biota	1.23E-05	2.43E-03	4.29E-03
Earthworms (lethal tests) LC_{50} (14d) (mg kg^{-1})	283	> 404 / 268.5 (5% OM)[d]	39.6[f]
NOEC (14 d) (mg kg^{-1})	20[c]	25 (5% OM)[d] / 1,65 (5% OM)[de]	<1.67 (56 d)[f]

Sw – Solubility in water at 20ºC; Kow – Octanol-water partition coefficient at pH7; Koc – Organic carbon sorption constant; VP - Vapor pressure at 20ºC; DT50 – Half life in soil at 20ºC under aerobic conditions; GUS = log(DT50)x(4-log(Koc)) - GUS > 2.8: leacher; 1.8 < GUS < 2.8: transition; GUS < 1.8: improbable leacher (Gustafson, 1989); PED - Predicted Environmental Distribution according to Mackay (2001) - Mackay fugacity model ('level I, version 3.00, 2004, Trentu University, Canada') PED < 20%: very low affinity; 20% ≤ PED < 40%: low affinity; 40% ≤ PED < 60%: average affinity; 60% ≤ PED < 80%: high affinity; PED ≥ 80%: very high affinity; OM – organic matter; [a] EFSA, 2010a, value for sandy clay loam soil; [b] EC, 1998, average value resulting from different soils; [c] FOOTPRINT, 2012; [d] EC, 2006; [e] EC, 2006, test with chlorothalonil 500 g L^{-1} SC; [f] EFSA, 2006; [g] EFSA, 2006, representative range for southern and central Europe locations.

In order to evaluate the environmental impact of the pesticides under realistic application in the agricultural fields, azoxystrobin and chlorothalonil were tested as the concentrated suspension formulation ORTIVA® (250g a.i. L^{-1}) and BRAVO 500® (500g a.i. L^{-1}), respectively. Ethoprophos was tested as pure compound (Dr. Ehrenstorfer 93.0% purity) because the available formulation in Portugal (MOCAP 10G®) consists of microgranules which poses a limitation in terms of nominal concentration calculation since it remains active in soil against insects for 2 to 4 months. For spiking procedures, specific amounts of the aqueous solution of each pesticide were prepared with distilled water to attain a moisture content of the natural soil of 50% of the Water Holding Capacity (WHC). The soils were spiked on day one of the start of the

experiments and the aqueous solutions used for spiking the soil were stored in refrigerated conditions (4 to 6°C) until pesticide residue analysis. Azoxystrobin and chlorothalonil residues in water were analysed by an independent laboratory, through solid phase extraction followed by gas chromatography/mass spectrometry (SPE/GC-MS) and ethoprophos residues through by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) according to DIN 38407-F 2 (1993) and ISO 10695 (2000). Limits of quantification (LOQ) were 0.1µg ml^{-1}, 0.3µg ml^{-1} and 0.05µg L^{-1} for azoxystrobin, chlorothalonil and ethoprophos, respectively.

2.3.2. Test organisms and culture conditions

Three different soil organisms were used: springtails *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae), the potworm *Enchytraeus crypticus* (Westheide & Graefe, 1992) (Oligochaeta: Enchytraeidae) and the earthworms *Eisenia andrei* (Bouché, 1972) (Oligochaeta: Lumbricidae). All organisms used in the experiments originated from laboratory cultures maintained at a constant temperature of 20 ± 2°C with a photoperiod of 16:8h light:dark. Springtails were cultured in plastic containers lined with an 11:1 mixture of plaster and activated charcoal. A small amount of granulated dry yeast was added as a food source once a week to avoid spoilage by fungi and mouldy food was removed when it was detected. The organisms were synchronized to be 10 to 12 days old at the start of the test. The Enchytraeid *E. crypticus* is listed in the ISO protocol 16387 (2004) as an alternative to *E. albidus* and was chosen for this study due to its better performance on natural soils with pH, organic matter content (OM), and clay characteristics similar to the test soil. It is also the preferred species when assessment objectives include natural soil types that support higher bioavailability of chemicals (Kuperman *et al.,* 2006). The enchytraeids were cultured in aerated plastic containers using uncontaminated garden soil which was free of additives as compost of fertilizers and pesticides, and was defaunated before use by deep-freezing cycles. The soil was moistened at 50% WHC and verified weekly to maintain the moisture content. The organisms were fed weekly with finely ground dry oat placed under soil particles to prevent fungal growth and facilitate availability of food for small juveniles (Römbke and Moser, 2002). Before the performance of the experiments the test soil was checked for its suitability using a few individuals and their response behaviour observed for a period of more than 2 weeks (Römbke and Moser, 2002). The organisms used in the

tests were carefully removed from the soil with the help of tweezers and placed on Petri dishes with distilled water for selection under a stereomicroscope, as possessing clitella and a body size between 10 and 12 mm long. Earthworms were kept in aerated plastic containers with a mixture of horse manure and peat as substrate. This mixture was moistened periodically to maintain the moisture content between 40 and 60% of the WHC. The organisms were fed twice a month with oat porridge. The earthworms used in the tests were synchronized to be more than one month old and before the start of the experiments the adults with clitella were separated and acclimated to the uncontaminated test substrate (natural soil and OECD 10% OM) for a period of between 24 and 48h. No mortality was observed during acclimation. After that, each organism was cleaned in water to remove soil particles, gently dried on absorbent paper, weighted (250 to 600 mg) and placed into plastic vessels covered with a lid in groups of ten.

2.3.3. Test soils

Artificial OECD soil with 10% organic matter content was prepared following the guideline instructions (OECD, 1984) and soil pH was adjusted to 6.0 ± 0.5 with $CaCO_3$. The natural soil used in this study, a eutric cambisol (EuDASM, 2011), is from an uncontaminated non-cultivated soil from an important agricultural area in Ribatejo, Central Portugal (see Chapter II section 1.). The uppermost soil layer (top 15-20 cm) was taken from the field and after major stones and vegetation were manually removed, the soil was air dried and sieved through a 2 mm mesh and submitted to several deep-freezing (-20°C) cycles to eliminate any existing fauna, and preserved at 4 to 6°C until used in the ecotoxicological tests. The soil was also tested for pesticide residues using a multi method ASU L 00.00- 34 GC detection analyses (ASU L, 1999). Soil parameters measured in the laboratory were soil pH (1M KCl), moisture content and water-holding capacity. Organic matter content, soil particle size distribution, cation exchange capacity, micronutrients concentrations and other chemical and physical characteristics were assessed by international and internal laboratorial standard methodologies. The characteristics of the natural soil and methodologies used are summarized in Table II.1. Both soils were moistened to 50% of the water holding capacity immediately before the start of the tests.

2.3.4. Experimental design of terrestrial ecotoxicity tests

All test treatments were performed with natural soil and two control soil types were used, one with natural soil for results comparison and other with OECD artificial soil for organism's performance validation. The ecotoxicological tests were performed under a controlled temperature of 20ºC ± 2ºC with a light:dark cycle of 16h:8h.

2.3.4.1 Collembolan reproduction test

Chronic toxicity tests followed ISO (1999) procedures. The following gradients of concentrations were selected to assess the full dose-response relationships for each pesticide : azoxystrobin (10, 15, 20, 35, 50, 80, 120, 200, 300, 450, 650 and 1000 mg a.i. kg^{-1} dw soil); chlorothalonil (0.5, 1, 1.5, 2.5, 5, 10, 20, 30, 50, 80, 150 and 200 mg a.i. kg^{-1} dw soil) and ethoprophos (0.015, 0.020, 0.030, 0.040 and 0.050 mg a.i. kg^{-1} dw soil). The 28d reproduction toxicity tests consisted of 10 synchronized springtails of 10-12 d old exposed to 30gr fresh weight soil per glass vessel, and fed with 2 mg of dry yeast at the start of the experiment. To reduce evaporation and prevent springtails from escaping, the containers were closed with a lid with small holes to allow aeration. Two replicates were used per test concentration and four for each of the control soils, except for the analysis of ethoprophos where four replicates were used at each concentration tested. An extra container without individuals and food was prepared for each combination and used for pH (1M KCl) and moisture determination at the end of the test (ISO, 1994, 1999). All replicates were aerated twice a week, and 14 days after the start of the test 2 mg of granulated dry yeast were added and moisture loss replenished according to total initial vessel weights, if needed. After 4 weeks, juveniles were assessed by flooding the vessels with water, by adding a few drops of ink and gentle stirring, after which the animals floating on the water surface were photographed and counted using the Image Tool software (Wilcox *et al*, 2002). The endpoint of the test was the total number of juveniles per test vessel at the end of the test; adult numbers were also registered.

2.3.4.2 Enchytraeids reproduction test

The reproduction tests were performed based on ISO 16387 (2004) guidelines with a few modifications. The test duration was four weeks instead of the six weeks indicated in ISO 16387 for the *E. albidus*, to accommodate the shorter reproductive cycle of *E. crypticus* (Kuperman *et al*, 2004). To assess the full dose-response relationships,

concentrations series of azoxystrobin (10, 15, 20, 35, 50, 80, 120, 200, 300, 450, 650, 1000 mg a.i. kg^{-1} dw soil), chlorothalonil (5, 10, 20, 30, 40, 60, 90, 150, 200, 250, 300 and 500 mg a.i. kg^{-1} dw soil) and ethoprophos (20, 30, 45, 65 and 100 mg a.i. kg^{-1} dw soil) were selected. The test started with the introduction of ten adult enchytraeids with well developed clitella in glass test containers, each containing approximately 20g of dry weight soil and 50 mg of finely ground dry oats of food covered with soil particles. Two replicates per pesticide treatment were used and four for each control soil. An extra container without individuals and food was prepared for each treatment concentration and used for pH (1M KCl) and moisture determination at the end of the test (ISO, 1994, 2004). All replicas were weighted weekly for moisture loss replenishment and fed with 25 mg of food if needed. At the end of the test all enchytraeids in soil (adults and juveniles) were collected by transferring all test containers content to a metal sieve (500μm) placed in a bowl and filled with water so that the soil was completed under the water. The organisms tended to stay at the surface of the soil and water and were collected with a plastic pipette. Each replicate group of organisms was fixed with alcohol and stained with Bengal red before counting. The measurement endpoint was the number of juveniles at the end of the test.

2.3.4.3 Earthworm reproduction test

The ecotoxicity tests followed the ISO 11268 - 2.2 (1998) guidelines. The following gradients of concentrations were selected to assess the full dose-response relationships for each pesticide: azoxystrobin (50, 100, 200, 300 and 500 mg a.i. kg^{-1} dw soil), chlorothalonil (5, 10, 20, 50 and 100 mg a.i. kg^{-1} dw soil) and ethoprophos (0.1, 0.3, 1, 3 and 12 mg a.i. kg^{-1} dw soil). At the beginning of the test, cylindrical plastic vessels (500 ml) with perforated transparent closing lids to facilitate air circulation were filled with 500g dry weight of soil. Fifteen grams of moistened dry finely ground horse manure were added to each test container and ten earthworms, previously weighted, were placed on each of the test replicates (four per concentration and controls). The groups of ten individuals were paired randomly with each replicate, and each group was weighted. The test containers were weighed for weekly moisture loss and replenished if needed. After four weeks of exposure, living adults were removed by hand sorting and each replicate's living individuals weighted for biomass variation. Mortality of adult individuals was assessed by counting the living organisms and any individuals not accounted for were considered dead. The soil and existing cocoons returned to the test

containers and 5g of food added, and incubated for another 4 weeks to allow cocoon development. At the end of the test, juveniles were extracted from the test soil using a water bath kept at 50/60°C and counted. The endpoints studied were adult mortality and change of biomass after 4 weeks and number of juveniles produced after 8 weeks. Soil pH (1M KCl) and moisture were determined at the beginning and at the end of the experiment for each concentration tested (ISO, 1994, 1998).

2.3.5 Calculations and statistical analyzes

Results were statistically analyzed according to EPS 1/RM/46 (2005) and using STATISTICA 7.0 (Stat Soft Inc., 2004).

Effect Concentrations of 50% and 20% at reproduction tests and corresponding 95% confidence limits were calculated through concentration-response relationships using nonlinear regressions. The nonlinear regression model was selected in order to best describe the concentration-response trend with the help of scatter plots or line graphs for each experiment distribution and the proportion of variance accounted for (r^2). Model used was: (i) Logistic: *juveniles=t/(1+(conc/x)^b)* where: *t* - y-intercept (control response); *x* - estimated EC value for the data set; *b* - a scale parameter (EPS 1/RM/46, 2005), with the estimation method of Levenberg-Marquardt. For the estimation of the ECx values, the normality for all test results was evaluated through a Q-Q plot of the residuals. The homogeneity of the variance was also evaluated after the analysis through a graphical distribution of the predicted versus the residual values.

On those tests where 4 replicates were used (Collembola tests with ethoprophos and Earthworms with all pesticides), NOEC (No Observed Effect Concentration) and LOEC Lowest Observed Effect Concentration) values could be estimated using a one way ANOVA using afterwards the Dunnett test. In this case normality of the distribution and homogeneity of the variance were tested using Kolmogorov–Smirnov (K-S) and Levene's tests, respectively. The same procedure was performed to evaluate significant differences among earthworm mortality and biomass variations results with the control values.

2.4. Results

2.4.1. Test soils

No pesticide residues were detected in the natural soil test which validates its use as a test soil for this study. The validity criteria of controls for each single species reproduction test were attained. The pH and moisture content in the natural soil controls of the three terrestrial ecotoxicity tests were on average 4.71 and 20% at the start of the tests respectively, and increased by an average of 0.1 units and 0.11%, respectively, at the end of the test. The average pH and moisture content of the artificial control soil decreased 0.1 units and 0.42%, respectively, from the initial values of 5.63 and 29%, respectively. Generally the three organisms reproduced twice as much in the natural soil control compared to the OECD artificial soil control (Table III.5).

Table III.5: Average number of juveniles in the controls at the end of the terrestrial ecotoxicity tests conducted with the different soil organisms using natural and artificial soil.

Organism tested	Pesticide tested	Natural soil (sandy loam)	Artificial soil (OECD 10%)
collembolans	azoxystrobin	262	250
	chlorothalonil	414	130
	ethoprophos	317	184
enchytraeids	azoxystrobin	1204	442
	chlorothalonil	1321	729
	ethoprophos	1373	1200
earthworms	azoxystrobin	77	36
	chlorothalonil	80	34
	ethoprophos	75	34

2.4.2. Exposure concentrations

The measured concentrations in the stock solutions were on average 97.8% and 93.5% of those of the nominal stock solutions used for spiking the soil on the terrestrial tests for azoxystrobin and chlorothalonil, respectively. Since the nominal and measured concentrations did not differ substantially, no adjustments for recovery were made when calculating the toxicity endpoints. Ethoprophos concentration could not be measured due to laboratory technical difficulties but since identical work procedures were used,

the risk of erroneous dosage in the present study was deemed minimal and the nominal concentrations were used for the toxicity endpoint assessment.

2.4.3. Assessment of pesticides effects to terrestrial organisms

In order to account for the differences in mass of each pesticide when comparing the results for the same organism between the pesticides, the active ingredient individual molar mass was used to transform the results values into mol of active ingredient (a.i.) per kg of dry weight of soil. This is the reason why results are shown in two types of units ('mg a.i. kg^{-1} dw soil' and 'mol a.i. kg^{-1} dw soil') in the text and Table III.6.

2.4.3.1. Collembolans

Adult collembolans showed a maximum of 10% mortality at the higher concentration (1000 mg a.i. kg^{-1} dw soil) during the reproduction tests with azoxystrobin. The highest chlorothalonil exposure concentration resulted in 35% mortality effect on adult collembolans after 4 weeks of exposure (150 mg a.i. kg^{-1} dw soil). No adult collembolans were observed at the two highest ethoprophos concentrations (0.040 and 0.050 mg a.i. kg^{-1} dw soil) and a 65% mortality rate was registered at 0.030 mg a.i. kg^{-1} dw soil. Ethoprofos had a significant effect on the reduction of juveniles at much lower concentrations (1000x less) compared to azoxystrobin and chlorothalonil test (Table III.6), with an EC_{50} of 1.11E-07 mol a.i. kg^{-1} dw soil. The EC_{50} of azoxystrobin (in mol a.i. kg^{-1} dw soil) was 2 times higher than that of chlorothalonil, herewith showing azoxystrobin to be less toxic for collembolans.

2.4.3.2. Enchytraeids

Enchytraeids showed to be affected by the three chemicals at comparable concentrations (Table III.6), with the EC_{50} values of 2.46 and 2.83E-04 mol a.i. kg^{-1} dw soil for azoxystrobin and ethoprophos, respectively, which is approximately half of the chlorothalonil value of 4.25E-04 mol a.i. kg^{-1} dw soil (Table III.6).

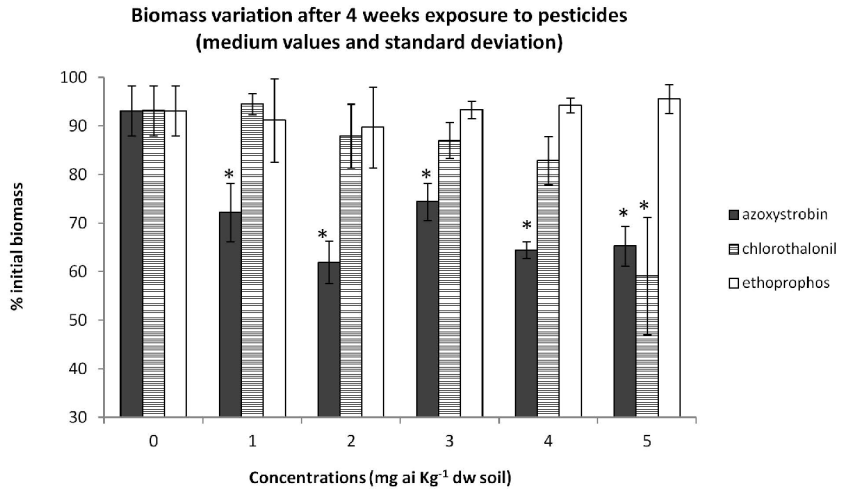
Table III.6: Pesticides molecular mass and results of statistical analysis for sub-lethal effects on terrestrial organism reproduction for each pesticide

Pesticide Mol mass (g mol^{-1})	**Organism**	**ECx (95% CI)** (mg a.i. kg^{-1} dw soil)	**ECx** (mol a.i. kg^{-1} dw soil)	**Model** r^2	**NOEC** **LOEC** (mg a.i. kg^{-1} dw soil)	**Normality** **Homogeneity**
AZO	*F. candida*	EC_{50} = 92.0 (57.9 – 126.1) EC_{20} = 54.9 (23.0 – 86.9)	EC_{50}= 2.28E-04 EC_{20}= 1.36E-04	0.88	-	-
(403.4)	*E. crypticus*	EC_{50} = 99.2 (73.3 – 125.7) EC_{20} = 42.6 (25.2 – 60.0)	EC_{50}= 2.46E-04 EC_{20}= 1.06E-04	0.95	-	-
	E. andrei	EC_{50} = 42.0 (23.2 – 60.8) EC_{20} = 12.2 (1.2 – 23.1)	EC_{50}= 1.04E-04 EC_{20}= 3.02E-05	0.96	< 50 50	K-S p> 0.20 Levene's p= 0.07
CLO	*F. candida*	EC_{50} = 31.1 (24.7 – 37.5) EC_{20} = 18.2 (12.0 – 24.5)	EC_{50}= 1.17E-04 EC_{20}= 6.84E-05	0.95	-	-
(265.9)	*E. crypticus*	EC_{50} = 112.9 (89.8 – 136.1) EC_{20} = 39.4 (25.6 – 53.3)	EC_{50}= 4.25E-04 EC_{20}= 1.48E-04	0.955	-	-
	E. andrei	EC_{50} = 40.9 (30.1 – 51.7) EC_{20} = 20.8 (11.0 – 30.5)	EC_{50}= 1.54E-04 EC_{20}= 7.82E-05	0.94	5 10	K-S p> 0.20 Levene's p= 0.09
ETO	*F. candida*	EC_{50} = 0.027 (0.024 – 0.031) EC_{20} = 0.021 (0.017 – 0.026)	EC_{50}= 1.11E-07 EC_{20}= 8.67E-08	0.944	0.020 0.030	K-S p> 0.10 Cochran C p= 1.00
(242.3)	*E. crypticus*	EC_{50} = 68.5 (42.9 – 94.1) EC_{20} = 41.2 (17.2 – 65.2)	EC_{50}= 2.83E-04 EC_{20}= 1.70E-04	0.77	-	-
	E. andrei	EC_{50} = 8.3 (3.6 – 13.0) EC_{20} = 3.5 (0 – 7.1)	EC_{50}= 3.43E-05 EC_{20}= 1.44E-05	0.76	3 12	K-S p> 0.20 Levene's p= 0.12

Mol mass - molecular mass (MacBean, 2012); AZO – azoxystrobin, CLO – chlorothalonil, ETO – ethoprophos; CI – Confidence interval; p – probability value.

2.4.3.3. Earthworms

The biomass of adult earthworms exposed to the control with natural soil for 4 weeks showed an average decrease of 7.0% compared to the initial biomass (Figure III.5). The exposure of *E. andrei* to azoxystrobin resulted in a significant weight loss (Dunnett test $p<0.05$) throughout the concentration gradient (Figure III.5). A 7.7% adult mortality was observed only at the higher concentration (LC_{50} > 500 mg a.i. kg^{-1} dw soil). Exposure to chlorothalonil resulted in a weight loss gradient (5.5 to 40.9%) with increasing pesticide concentration (Figure III.5) with only significant values for the highest concentration of 100 mg a.i. kg^{-1} dw soil. This decrease in biomass was accompanied with a mortality rate of 59.0% only at the highest concentration resulting in a LC_{50} for adults of approximately 95.0 mg a.i. kg^{-1} dw soil.



	1	2	3	4	5
AZO	50	100	200	300	500
CLO	5	10	20	50	100
ETO	0.1	0.3	1	3	12

Figure III.5: Adult earthworm (*E. andrei*) biomass variation after 4 weeks exposure to the tested pesticides (mean ± SD). * Significant differences with control ($p < 0.05$).

No adult earthworm mortality was observed after 4 weeks exposure to ethoprophos (LC_{50} > 12 mg a.i. kg^{-1} dw soil), and a slight (but not significant) gain of weight was registered (91.1 to 95.5% of initial biomass) along the concentration gradient (Figure III.5). The highest toxicity on earthworms' reproduction was found for ethoprophos resulting in an EC_{50} of 3.43E-05 mol a.i. kg^{-1} dw soil (Table III.6). The inhibition of

juvenile production by earthworms under azoxystrobin and chlorothalonil exposure resulted in similar EC_{50} toxicity values (1.04E-04 and 1.54E-04 mol ai.kg^{-1} dw soil, respectively). Nevertheless, EC_{20} values differed between these pesticides with azoxystrobin being more toxic (Table III.6). A significant reduction in juvenile numbers (Dunnett test $p<0.05$) was observed for all the three pesticides allowing LOEC and NOEC calculations (Table III.6). However, in azoxystrobin all tested concentrations were significantly different from the control ($p<0,005$) resulting in effects on earthworms, which did not allow for a NOEC value to be attained (NOEC < 50 mg a.i. kg-1 dw soil).

2.5. Discussion

The study focused on evaluating effects on reproduction for three commonly used pesticides in irrigated crops to non-target soil organisms using a Mediterranean natural soil. All the organisms presented different toxicity responses to the tested pesticides (Table III.6). This could be associated with the processes of chemical uptake by the organisms and the different type of action of the pesticides (Frampton *et al.,* 2006). Uptake of organic contaminants by terrestrial organisms is intimately associated with the soil pore water which is in general the dominant pathway (EFSA, 2009b; Styrishave *et al.,* 2008). Soft bodied soil organisms such earthworms and enchytraeids take pesticides up either through passive diffusion from pore water through the skin or by ingestion together with soil particles (De Silva *et al.,* 2009). Hard-bodied soil organisms take oxygen and water up through specialized organs, although collembolans tend to use the same route of uptake as soft bodied organisms since they are in constant contact with pore water satisfying their need of water by consuming humid food and possibly soil (EFSA, 2009b). In addition, pesticides bioavailability through the soil pore water can be influenced by soil properties such as organic matter (OM) and clay content (increase of OM and clay) that relates to sorption restraining the pesticide molecules in a form that is not available for organism uptake (EFSA, 2009; Kuperman *et al.*, 2006; Van Gestel, 2012). This fact has been reported by several authors for soil dwelling organisms such as enchytraeids and earthworms, for a number of compounds: organochlorine and carbamate insecticides, benzimidazole and polychlorinated

fungicides, among others (Amorim *et al*., 2002a, b; De Silva *et al*., 2009; Lanno *et al*., 2004; EFSA, 2009a; Patakioutas and Albanis, 2002).

2.5.1. Effects of azoxystrobin on soil biota

In spite of the low solubility of azoxystrobin in water, the distribution of the fungicide to the pore water may be expected due to its low soil sorption coefficient (Koc) and high potential to leach given by the GUS index (Table III.4). This affinity to the water compartment is also illustrated by the predicted environmental distribution (PED) values (Table III.4). Azoxystrobin is expected to have low environmental toxicity to earthworms and terrestrial arthropods due to its chemical group characteristics (strobilurin), as to be relatively readily degraded in the environment causing little potential for chronic exposure (Bartlett *et al.,* 2002). However, the present study revealed a higher sub-lethal effect response of azoxystrobin to earthworms (EC_{50} of 42.0 mg a.i. kg^{-1} dw natural soil) compared to collembolans and enchytraeids (Table III.6). Although a significant biomass decrease was observed for the lowest concentration (50 mg a.i. kg^{-1} dw soil) (Figure III.5), resulting in effects on the reproduction of the earthworms, only 7.7% mortality was registered at the highest concentration. Even though biomass and mortality are always registered together, Potter *et al*. (1994) verified that the loss in biomass was independent of the lethal effects of chemicals. The observed low lethal toxicity to earthworms with natural soil differ greatly from the reported results with OECD artificial soil tests showing LC_{50} values of 283 mg a.i. kg^{-1} soil (EFSA, 2010a) and 327.4 mg a.i. kg^{-1} soil (Wang *et al*, 2012). Nevertheless, the fact that the NOEC for earthworms test was not attained with the lowest concentration tested (50 mg a.i. kg^{-1} soil) is in agreement with the reported NOEC of 20 mg a.i. kg^{-1} soil for *E. foetida* (FOOTPRINT, 2012). Collembolans and enchytraeids results for azoxystrobin exposure are similar (Table III.6), supporting the observation that collembolans tend to take chemicals up from the soil pore water solution as soft bodied organisms do (EFSA, 2009).

2.5.2. Effects of chlorothalonil on soil biota

Chlorothalonil is not expected to distribute to the soil pore water due to its low solubility in water and high sorption constant facilitating adsorption to soil particles

(Table III.4). However, a slight movement to the pore water may occur due to the average affinity to the water compartment (PED) and potential to leach (GUS) dependent to soil characteristics (Table III.4). If present in the water fraction of the soil (soil pore water) the pesticide can be bioavailable for uptake by the soil organisms (Styrishave *et al.,* 2008). In terms of sub-lethal effects of chlorothalonil, collembolans were the most sensitive taxa followed by the earthworms (Table III.6). The enchytraeids were the least sensitive with an EC_{50} ratio of almost 3:1 of the other two organisms. This low sensitivity of enchytraeids towards chlorothalonil has also been reported for other pesticides such as a polychlorinated insecticide in specific and other fungicides of the same chemical group and insecticides in a broader evaluation (Bezchlebová *et al*, 2007; Daam *et al*, 2011b; Frampton *et al*, 2006). The NOEC for earthworms of 5 mg a.i. kg^{-1} dw soil is in agreement with the reported NOEC value of 1.65 mg a.i. kg^{-1} soil from tests with the same formulation (500 g a.i. L^{-1} SC), and a 5% OM OECD soil (EC, 2006), which is similar to the organic matter content of the natural soil used in this study (Table II.1). The 59% mortality of adult earthworms registered at the highest tested concentration of 100 mg a.i. kg^{-1} dw soil after 4 weeks exposure to chlorothalonil occurs at a concentration which is two times lower than the reported earthworms acute test effect concentration (LC_{50}) of 268.5 mg a.i. kg^{-1} soil (EC, 2006). Although this value is attained from a test with artificial soil with an organic matter content of 5% OM (EC, 2006) similar to the tested natural soil, this difference in the lethal effects results may be due to other factors associated to the natural soil such as pH and clay content influencing pesticide availability (EFSA, 2009).

2.5.3. Effects of ethoprophos on soil biota

Although the insecticide ethoprophos has a higher solubility in water than the fungicides tested, it also has a lower sorption coefficient, so the environmental potential fate values indicate a high affinity with the soil compartment (Table III.4). However, the pesticide may leach to the water compartment depending on soil characteristics (GUS = transition state; Table III.4), and become available for uptake by the soil organisms (Styrishave *et al.,* 2008). Collembolans were the most affected by ethoprophos with a low EC_{50} of 0.027 mg a.i kg^{-1} dw soil, which would be expected from the type of action of an insecticide towards arthropods (Daam *et al*, 2011b; Frampton *et al.,* 2006). The earthworms presented the second lowest EC_{50} value of 8.3 mg a.i. kg^{-1}dw soil and the

enchytraeids were the least sensitive with an EC_{50} value more than 8 times higher than the collembolan's (EC_{50}= 68.5 mg a.i. kg^{-1} dw soil). In spite of such, no mortality effects on adult earthworms were observed and a slight gain in weight was registered. This test results are in congruence with reported values of LC_{50} 39.6 mg a.i. kg^{-1} dw soil (EFSA, 2006) since the maximum tested concentration during this study was 12 mg a.i. kg^{-1} dw soil. The observed sub-lethal effects on cocoon production and viability may be a consequence of the pesticide intake by the adults that even at low dosages can cause adverse effects after long term exposures. The reported NOEC value of < 1.67 mg a.i. kg^{-1} dw soil (EFSA, 2006) from a test with artificial soil is lower than the study test results, which shows that different soils may cause different toxicity results, as referred above.

2.5.4. Sensitivity of the three invertebrate arthropods to the pesticides

The higher sensitivity of collembolans (*F. candida*) observed in this study as compared with the other organisms for two pesticides, the fungicide chlorothalonil and the insecticide ethoprophos, has been registered for a wide range of pesticides with different type of action, suggesting that the earthworms are not always the most sensitive species (Bezchlebová *et al*, 2007; Daam *et al*, 2011b; Frampton *et al*, 2006). However, care should be taken when making generalizations of effects of pesticides within the same chemical group where significantly different toxicities may occur in a single group of soil organisms (e.g. effects on earthworms among the strobilurin group (Wang *et al*, 2012)). Enchytraeids were mainly the least sensitive of the three species tested for reproductive effects. Although reports have shown that they are generally less sensitive than lumbricidae when assessing acute data such as LC_{50} (EFSA, 2009b), the results obtained in our study contradict the results reported by Römbke & Moser (2002) which report a similar sensitivity of the two organism groups regarding reproductive effects in different soil substrates (artificial and natural). These differences in long-term exposure tests reflect the difficulty in grouping pesticides effects on non-target organisms. This emphasises the need to include arthropods and other annelids as relevant organisms in the first tier of pesticide Environmental Risk Assessment in order to better represent and protect the terrestrial environment against the wide existing group of pesticides (Frampton *et al.,* 2006).

2.6. Conclusion

Results showed that the use of only the earthworm as a key species for the first tier terrestrial ERA of pesticides may not be enough to ascertain a significant protection level of the terrestrial ecosystem by not being the most sensitive organisms, especially for the tested insecticide. Moreover, the use of natural soil may lead to differences in toxicity values compared with OECD referenced values. This illustrates the importance of creating realistic scenarios under the first tier ERA, since artificial soils may not allow a realistic approach for the evaluation of pesticide toxicity. Natural soil variations are accounted for in the Guidance Document on Terrestrial Ecotoxicology (SANCO, 2002) under a risk assessment for earthworms. However, with the revision on the data requirements for active substances (EU, 2013), and the division of the EU territory into three zones (north, central and south) by the new regulation concerning the placing of plant protection products on the market (EC, 2009), understanding the different behaviour of pesticides and their availability in different soils types becomes of great importance.

2.7. Acknowledgements

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CHAPTER IV

Risk assessment of pesticides on the soil-water interface

1. Linking fate and effects of azoxystrobin and chlorothalonil using semi-field soil-water interface simulations under Mediterranean crop-based scenarios.

Based on the following manuscript:

Linking fate and effects of azoxystrobin and chlorothalonil using semi-field soil-water interface simulations under Mediterranean crop-based scenarios. Sara Leitão, Matilde Moreira-Santos, Paul J. Van den Brink, Rui Ribeiro, Mª José Cerejeira and José Paulo Sousa (*submitted to the journal Agriculture, Ecosystems and Environment*).

1.1. Abstract

The present study aimed at assessing the influence of pesticides application and agricultural practices on their environmental fate, transfer pathways in the soil-water system and effects on aquatic biota under simulated Mediterranean agricultural conditions using natural soil. Additionally, the study aimed to link pesticide exposure via leachate, runoff and elutriate waters through crop-based simulations, with their effects on aquatic ecosystems using ecotoxicological tests. A semi-field setup was used that mimicked "worst-case" azoxystrobin and chlorothalonil contamination in agricultural field situations including the simulation of irrigation practices. This setup applied twice the recommended dosage (2RD) of azoxystrobin and chlorothalonil for onion and potato crops, respectively. A rain event was simulated under a slope of 20º for both scenarios with collection of runoff waters. Soil and water samples were collected for analysis of pesticides residues. Toxicity of water samples was assessed by performing lethal and sublethal (reproduction) bioassays with the cladoceran *Daphnia magna*. Although the majority of the applied azoxystrobin sorbed to the top-layer soil, concentrations of this pesticide were detected in all water samples illustrating different pesticide transfer pathways through water movements (leachate, runoff and elutriate). Runoff proved to be an important transfer pathway of azoxystrobin to surface water since it resulted in the highest pesticide concentration (78 µg L^{-1}), although sublethal impacts on cladoceran populations were only observed for leachates at low concentrations (4.5 µg L^{-1}). Chlorothalonil sorbed to the soil and no residues were detected in the water samples above the level of quantification (0.05 µg L^{-1}) in either of the waters. However, lethal effects on the cladoceran were observed in runoff and elutriate samples after application of 2RD. Both simulated agricultural scenarios illustrated the relative importance of the different transfer pathways of pesticides to surface water in a soil-water interface as occurs in irrigated agricultural crops under Mediterranean conditions.

Keywords: fungicide; runoff; leaching; soil elutriate; aquatic effects.

1.2. Introduction

Exposure of non-target organisms to pesticides may vary according to the natural variability of the ecosystem, among others, due to differences in climate and soil characteristics (Jørgensen *et al.*, 2012; Koděsova *et al.*, 2011). In the new regulation concerning the placing of plant protection products on the market (ECR, 2009), the European Union established three zones in Europe (North, Centre and South) making exposure scenarios more realistic according to specific edapho-climatic conditions. However, since the Environmental Risk Assessment of pesticides is still based on generic FOCUS scenarios mainly developed considering conditions in northern and central Europe (Daam *et al.*, 2011), their use under Mediterranean conditions, where soil characteristics, climatic conditions and biota are substantially different, may lead to risk misestimates (Daam *et al.*, 2011; Ramos *et al.*, 2000). This is particularly true when looking at specific transfer pathways of pesticides through the soil-water interface. Under Mediterranean scenarios, pesticide driven surface water contamination is strongly associated to soil erosion and runoff resulting from rain events (Berenzen *et al.*, 2005; Tarazona 2005). Moreover, due to the loss of organic matter and the consequent impairment of soil retention function in irrigated agricultural fields, particularly in hydrogeological vulnerable areas in the Mediterranean region, groundwater contamination with agrochemicals can occur (Silva *et al.*, 2012a, 2012b). This aspect is emphasized by European authorities indicating that special attention must be given to the protection of groundwater when pesticides are applied in regions with vulnerable soil and/or climate conditions (EC, 2006). Therefore, the need to study pesticide fate and effects under Mediterranean conditions is of critical importance due to the limited information that is currently available (Daam *et al.*, 2011).

To address this knowledge gap, a semi-field crop-based experiment using a soil-water simulator was performed in the present study. This soil-water simulator developed in a previous study (Chelinho *et al.*, 2012) allows the collection of samples to evaluate exposure and effects on both terrestrial and aquatic compartments. The use of this experimental setup under controlled conditions decreases variability in collected data, which is often observed in field experiments, while maintaining the natural characteristics of the system under realistic field exposure conditions (e.g. soil type, slope, climatic condition, irrigation). With this approach, the risk of pesticide

applications can be evaluated for a particular agricultural area in an integrated way, taking into account not only the soil compartment but also the soil-water transfer pathways. In the present study this methodological approach was applied to mimic pesticide applications of the fungicides azoxystrobin and chlorothalonil under realist "worst-case" scenarios of irrigated crops (onion and potato, respectively) in a major agricultural area of Central Portugal (Ribatejo), under Mediterranean conditions. Evaluating pesticide contamination of surface and groundwater is of paramount importance in this area, due its proximity to the UNESCO biosphere reserve "Paul do Boquilobo" which contains surface waters that are of great importance for bird conservation and biodiversity protection. This reserve is nearby a hydrogeological vulnerable area where several pesticides have been detected in water at concentrations that may be expected to lead to environmental side-effects (Silva *et al.*, 2012a, 2012b).

The specific objectives of the present study were: i) to assess the fate of the two fungicides in the soil-water interface, particularly focusing on the soil-water transfer pathways (leaching, runoff and soil elutriates as a surrogate of the soil retention capacity (Chelinho *et al.*, 2012; EC, 2000), by performing pesticide applications mimicking realistic field conditions for the area using the soil-water simulator described earlier (Chelinho *et al.*, 2012); ii) to assess the ecotoxicological effects of the three different water matrices (leachate, runoff and elutriate) towards aquatic biota by performing lethal and sublethal (reproduction) toxicity tests with the standard cladoceran species *Daphnia magna*; iii) to compare the exposure and ecotoxicological results obtained in the different matrices, herewith assessing the relative importance of the different soil-water transfer pathways (leaching, runoff and elutriates) for the risk assessment of the water compartment.

The fungicides azoxystrobin and chlorothalonil are authorized in 27 and 24 countries in Europe, respectively (EPD, 2012). In Portugal azoxystrobin is registered for use in the onion crop and chlorothalonil for use in potato crop, among others. Azoxystrobin belongs to the fungicide strobilurin group (MacBean, 2012), and has been detected in water across Europe at low concentrations: 0.026 µg L^{-1} in surface waters of Danish agricultural areas (Warming *et al.*, 2009) and at levels of 11.1 and 29.7 µg L^{-1} in streams during runoff events in Germany (Berenzen *et al.*, 2005). Strobilurins originate from natural products (β-methoxyacrylic acid) produced by a range of Basidiomycete wood-rotting fungi and have been identified as low toxic to birds, mammals, bees, and

other non-target terrestrial organisms (arthropods, earthworms) (Rodrigues *et al.,* 2013). However, azoxystrobin is considered to be very toxic to aquatic organisms by European authority evaluations (EFSA, 2010; Rodrigues *et al.,* 2013). Azoxystrobin has been identified to also affect soil functions and processes through effects on soil microbial and fungal communities (Adetutu *et al.,* 2008).

Chlorothalonil is an organochlorine fungicide and has been detected in runoff waters from tomato fields (Arnold *et al.,* 2004). Chlorothalonil is classified by the EU as very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment (EC, 2006). This fungicide may cause lethal (96h LC_{50}) effects on fish at concentrations as low as 7.6 to 76 µg L^{-1} (EC, 2006; Sherrard *et al.,* 2003) and on planktonic crustacean with 48h LC_{50} of 38 to 169 µg L^{-1} (EC, 2006; Sánchez-Bayo, 2006; Sherrard *et al.,* 2003). Effects on the growth mechanisms of non-target submersed macrophytes at 189 and 615 µg L^{-1} have also been documented (Belgers *et al.,* 2009).

1.3. Materials and methods

An experimental setup mimicking crop-based pesticide applications under "worst-case" field scenarios was performed using natural soil. The application of twice the recommended dosage for Portugal of the formulated products of two fungicides was used as "worst-case" representing a possible misuse by farmers. Moreover, two applications of both pesticides were performed during the study (see section 1.3.4) according to the maximum number of applications authorized per crop cycle.

1.3.1. Fungicides

Azoxystrobin (CAS 131860-33-8; methyl (*E*) – 2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) is a strobilurin fungicide. Azoxystrobin has low solubility in water and is non-volatile (see Table III.4). The organic carbon sorption coefficient indicates that the pesticide is moderately sorbed to soil and has a low mobility in water (EFSA, 2010). The octanol-water partition coefficient indicates low bioaccumulation potential (Log Kow < 2.7; FOOTPRINT, 2012). Azoxystrobin may be persistent in soil when tested in laboratory, although under field conditions it proved to

be less persistent (EC, 2000). The leaching potential of azoxystrobin (GUS; see Table III.4) is considered likely to leach to groundwater depending on the field conditions, and according to the predicted environmental distribution azoxystrobin shows an average affinity for the soil and water compartments (see Table III.4).

Chlorothalonil (CAS 1897- 45-6; tetrachloroisophthalonitrile) is a chloronitrile. Chlorothalonil has low solubility in water, is non-volatile and may sorb to soil (Table III.4). Chlorothalonil's octanol-water partition coefficient indicates a moderate potential for bioaccumulation (FOOTPRINT, 2012). The fungicide is not persistent in soil with a half-life in field soil of less than 3 months (EC, 2000) and has a marginally (GUS; see Table III.4) leaching potential to groundwater (Gustafson, 1989). The predicted environmental distribution indicates that chlorothalonil has a potential affinity to the water and soil compartment (see Table III.4).

1.3.2. Soil - water simulator experimental setup

The soil-water simulator (SWS) consisted of a stainless steel transportable soil flume system of 0.4 m^2 with a controllable depth (maximum of 100 x 40 x 20 cm; length, width, and height, respectively), with two articulated platforms that can move independently allowing to work under different slopes (Chelinho *et al.*, 2012; see Figure IV.1a). Three SWS were setup in the horizontal position to guarantee the same conditions of plant growth and pesticide fate in the entire 0.4 m^2 area. One SWS was used as the control with no pesticide application (Control SWS) and two other under the established scenarios for azoxystrobin (AZO SWS) and chlorothalonil (CLO SWS) applications.

1.3.3. Natural soil

The natural soil used in this study is a sandy clay loam soil from an agricultural area at Ribatejo, Central Portugal that was never cultivated (i.e., an uncontaminated reference soil) as previously referred (see Chapter II section 1.). For soil collection, preparation and testing for the absence of pesticide residues see section 2.3.3 of Chapter III.

1.3.4. Soil-water simulator study design

Each SWS was setup by placing a 5-cm layer of glass beads (1 cm diameter) at the bottom of the perforated platforms to avoid dogging and facilitate leachate percolation (Figure IV.1b). On top of the glass beads, a 15-cm layer of soil was placed up to the edge of the platforms so that the SWS frame would not pose an obstacle during the runoff event (Figure IV.1c). The soil was left to settle and stabilize its structure for 33 days, to become as similar as possible to the field soil. After this period, the soil was prepared by maintaining its moisture via sprinkling 7.143 L m^{-2} (mm) of water every second day for 9 days, corresponding to the irrigation practices used in Portugal for that area and the crop needs. After these 9 days of irrigation, seeding and planting took place.

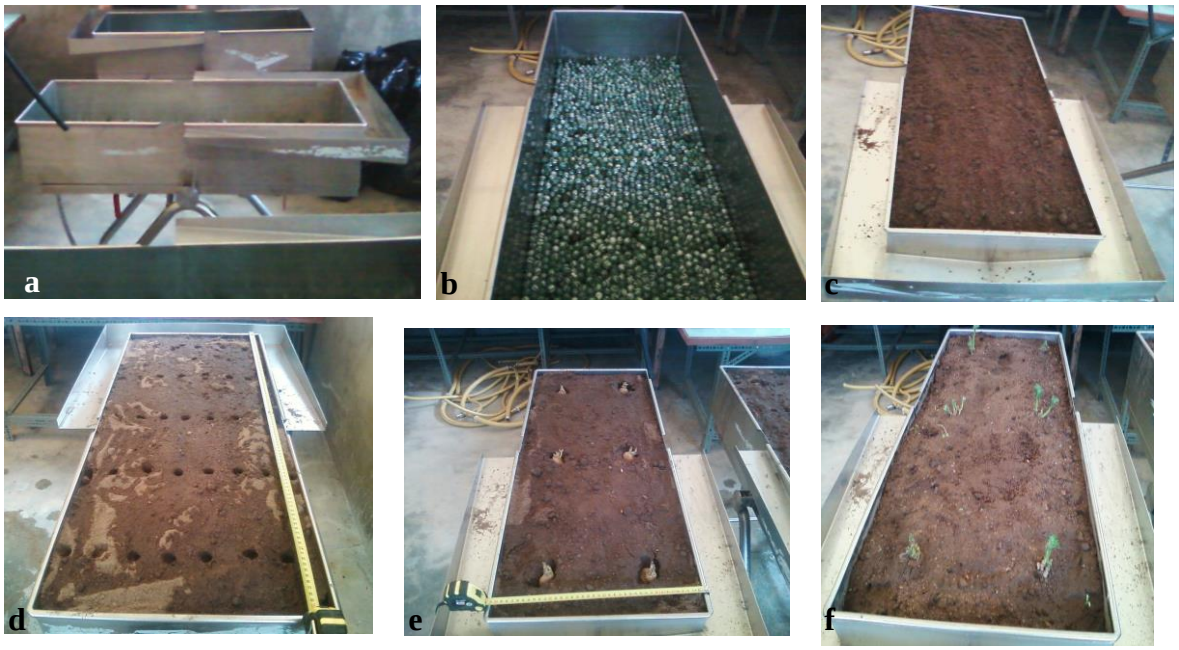

Figure IV.1: a) Soil-Water simulator (SWS); b) SWS with a first layer of glass beads; c) SWS with the natural soil layer; d) AZO SWS with onion seeds; e) CLO SWS with potatoes during planting; f) CLO SWS 3 days after planting.

On the AZO SWS, onion seeds of the variety 'Paudero' (*Allium cepa*, Lot 456711-M EXPRESSION F1) were seeded individually every 8 cm at a depth of 1 cm along 5 lines which were 20 cm apart, aligned perpendicular to the SWS major axis. A total of 35 seeds were placed and all germinated after 15 days (Figure IV.1d). On the CLO SWS, six young potatoes of the variety 'Hermes' were placed individually along two lines with 3 potatoes each (20 cm distance between lines and 40 cm between potatoes within each line), at a depth that they would be covered by a thin layer of soil (approximately 1

cm); all potatoes started to germinate after 3 days (Figure IV.1e and f). Irrigation, performed as described above, continued for the following 19 days after seeding and planting, till onion plant leafs developed and potato plants grew about 50 cm tall with small leafs unable to cover the soil. The Control SWS was prepared with the onion crop to illustrate the soil exposure "worst-case" scenario during the fungicides applications, due to the small size of the onion plants at the time of the pesticide application creating a higher probability of the fungicides to reach the soil.

The experiment started with the first application of the fungicides (day 0), after the appearance of the first leafs according to pesticide application indications, i.e., 22 days after seeding and planting (Figure IV.2a). Both fungicides were applied at twice the recommended dosage (2RD) by spraying the application solution prepared in 500 ml of distilled water, evenly over each SWS. The Control SWS was also sprayed with 500 ml of distilled water with no pesticide residues. Fungicides were applied in the morning and left to dry on leafs until late afternoon, time at which irrigation took place and leachates were collected after a waiting period of approximately 30 minutes (Figure IV.2b).

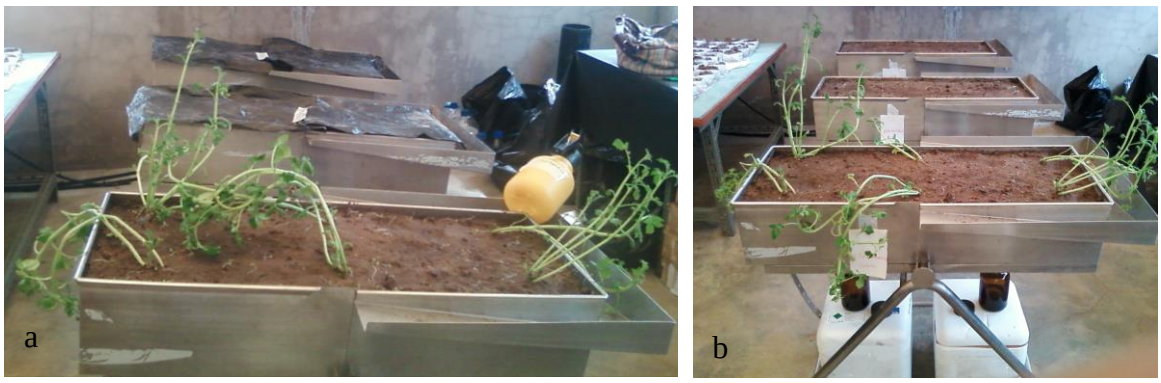

Figure IV.2: a) SWS during pesticides applications showing the sprayer; b) SWS during leachates collections showing dark glass vials under the SWS.

Azoxystrobin was applied on onion crop (AZO SWS) as a concentrated suspension containing 250 g active ingredient (a.i.) per L of the formulated product (f.p.) ORTIVA®: 2RD = 400 g a.i. ha^{-1} (RD = 0.8 L f.p. ha^{-1} corresponding to 200 g a.i. ha^{-1}). Chlorothalonil was applied on potato crop (CLO SWS) also as a concentrated suspension containing 500 g a.i. per L of the f.p. BRAVO500®: 2RD = 3 kg a.i. ha^{-1} (RD = 3 L f.p. ha^{-1} corresponding to 1.5 kg a.i. ha^{-1}). Dose concentrations in soil were calculated taking into account the natural soil density of 1.25 g cm^{-3}, previously

calculated, and a pesticide incorporation up to 15-cm depth. After the first application of the fungicides, irrigation continued until the end of the experiment exactly as described above. The second pesticide application, also of 2RD, was performed 7 days after the first application, which is the minimal time interval allowed between pesticide applications during a crop cycle; irrigation and leachates collection were performed in the same way as after the first application. The experiment was performed in a greenhouse with natural sun light, and air temperature and humidity were registered daily throughout the experiment with a RH/Temp Data Logger EL-USB-2T, whereas soil pH and moisture in all SWS were registered before each irrigation with a Kelway Soil Tester (Kelway soil® acidity and moisture tester Model HB-2).

The experiment ended 2 days after the second fungicide application (9 days after the first application) with the simulation of a rain event (see section 1.3.5) using a sprinkler and under a slope of 20º mimicking the site study higher quota in relation to the UNESCO biosphere reserve, to assess potential surface water contamination through runoff. Runoff waters resulting from the rain event were kept in glass vials at 4 to 6ºC in darkness until pesticide residue analysis and bioassays performance. Before the rain event, a side-to-side 20-cm row of the top side of the SWS was isolated with plastic and after the rain event, soil samples were collected from the upper 10-cm soil layer as simple composite samples for analysis of pesticide residues (kept frozen at -20 ˚C) and for elutriates preparation (stored at 4 to 6 ˚C in darkness for 24 hours).

1.3.5. Rain event

The simulated rain event was of 41.6 L m^{-2} (mm) in accordance with the highest monthly precipitation during the time when the product must be applied, observed in the year 2010 (IM, 2008). To obtain the precipitation value for one day, the monthly precipitation value was divided by three to simulate that the 2010 rain event occurred in just three days. A stock solution of 1 L of artificial rain water was prepared by mixing micronutrients in distilled water ($(NH_4)_2SO_4$ - 925 mg; NaCl - 386 mg; $CaCO_3$ - 200 mg; $MgSO_4$ - 180 mg; KCl - 37 mg; KH_2PO_4 - 14 mg; $NaNO_3$ - 40 mg; HNO_3 (3.5M) - 2.0 ml and HCl (1.0M) - 1.0 ml) according to the Standard Technical Procedure for Terrestrial Model Ecosystems (STP, 2000).

1.3.6. Pesticide residues analysis in water and soil samples

Azoxystrobin and chlorothalonil residues were analyzed in all water samples (runoff, leachates and elutriates) from both SWS by gas chromatography/mass spectrometry after solid phase extraction (SPE/GC-MS) with a limit of quantification (LOQ) of 0.05 µg L^{-1} for azoxystrobin and for chlorothalonil (DIN, 1993-2; ISO, 2000). Soil samples from both SWS were also analysed for azoxystrobin and chlorothalonil residues through liquid extraction/clean-up followed by gas chromatography/mass spectrometry (LE/GC-MS) with limits of quantification of 0.06 mg kg^{-1} and 0.015 mg kg^{-1}, respectively (ASU L, 1999).

1.3.7. Water matrices and ecotoxicity evaluation

Three types of water matrices/samples were used for the ecotoxicity evaluation toward aquatic organisms. Daily leachates were collected after each irrigation event, from day 0 until day 9, and kept separately in glass vials at 4 to 6 ºC in darkness. At the end of the experiment, a representative leachate composite sample was prepared by mixing similar volumes of the leachates collected each day. The latter was left to settle under refrigerated conditions and then decanted so that only a representative sample of the soluble fraction of the pesticide was used. Runoff water samples collected after the simulated rain event (day 9) were centrifuged (20 min at 4500 rpm) at room temperature, for suspended solids removal not to interfere with the cladoceran physiological feeding mechanism (Friberg-Jensen *et al.,* 2010), and the supernatant was collected and deep frozen at -20 ºC until use for bacterial growth control (Gao *et al.,* 2006). To evaluate the toxicity toward aquatic organisms of the pesticide water soluble components in the soil pore water, due to its potential mobilization to aquatic systems by the soil-water pathway, elutriates were prepared according to DIN 38 414-S4 (1984); a mixture of soil and ASTM reconstituted hard water (1:10 ratio, w/v, based on soil dry weight) was shaken in a magnetic stirrer during 24 hours centrifuged as described above and the supernatant was collected as elutriate and stored at 4°C in the dark until use.

Aquatic bioassays were conducted with the cladoceran *Daphnia magna,* a planktonic crustacean forming the base of the ecological structure in freshwaters environments, occupying an important position in food webs due to its high grazing potential (Friberg-Jensen *et al.,* 2010; Sánchez-Bayo, 2006; Warming *et al.,* 2009), and easily handled and

cultured under laboratory conditions, being for these reasons, the main standard organisms used in aquatic risk assessment. Lethal and sublethal (reproduction) assays were performed with leachate, runoff and elutriate samples originated from the Control, AZO and CLO SWS. Due to low sample volume availability, priority was given to reproduction assays and lethal assays were performed when possible. All assays were incubated under the same conditions of temperature (19-21 ºC) and light (14:10-h light:dark cycle).

For sublethal assays, all water samples from the three SWS were tested at 100 % (v/v) and 50 % (v/v) to mimic the 2RD and RD of the fungicides, respectively. This procedure was also adopted for the control SWS to be able to discriminate other potential stress factors associated with the soil matrix from those due to the pesticide (e.g., turbidity). For each combination of water sample (leachate, runoff or elutriate) and pesticide, a 21-days *D. magna* reproduction test consisting of a standard control plus the two SWS treatments (Control and AZO or Control and CLO) at the 100 and 50% (v/v) concentrations was conducted according to the OECD guidelines (OECD, 1998). For each treatment ten replicates, each containing 50 ml of test solution and one neonate less than 24 hour old were setup. During testing solutions were renewed three times per week and every day organisms were fed with a green algae solution and number of juveniles released were counted. After the 21-days exposure, reproduction was determined as the number of juveniles released per female. For each sublethal assay, reproduction was examined for significant differences among the control and respective doses (2RD and RD) using one-way analysis of variance followed, when necessary, by the Dunnet's test to identify differences between the control and each dose, or by Students t-test when 100% mortality occurred at the highest dose. Prior to the latter analysis, the assumptions of normality (Shapiro-Wilkinson test) and homogeneity of variance (Bartlett's test) were checked.

A lethal assay was performed also for each same combination of water sample and pesticide by determining the inhibition of the mobility of *D. magna* exposed for 48 h according to Daphtoxkit F^{TM} Magna protocol (2000), using a gradient concentration range of 100, 50, 25, 12.5 and 6.25% (v/v). During testing no food was provided and no medium renewal was performed. When possible, median lethal concentrations (LC_{50}) and correspondent 95% confidence intervals were calculated using the Trimmed Spearman-Karber Method (Hamilton *et al.,* 1978).

No terrestrial ecotoxicity assays for both fungicides were performed given that previously calculated EC_{50} values (Table III.6) from terrestrial laboratory tests for three organisms (*Folsomia candida*, *Eisenia andrei* and *Enchytraeus crypticus*) with the same natural soil and formulated products as the present study (AZO: 92, 42 and 99 mg a.i. kg^{-1} dw soil and CLO: 31, 41 and 113 mg a.i. kg^{-1} dw soil, respectively) were much higher than the here applied concentration of azoxystrobin (0.426 mg a.i. kg^{-1} dw soil) and of chlorothalonil (3.2 mg a.i. kg^{-1} dw soil).

1.4. Results

1.4.1. Air and soil measurements

Mean values (± standard deviation) of room air temperature and relative humidity (RH) during the experiment were 30.2 ± 6.2 °C (33.6 ± 6.3 °C daytime 8h-20h, and 26.1 ± 2.4 °C night 21-7h), and 53.7 ± 11.7% RH (47.5 ± 12.1% RH daytime 8h-20h, and 61.0 ± 4.8% RH night 21-7h), respectively. Daily soil pH and moisture measurements (% relative saturation) on each of the SWS during the experiment are presented in Table IV.1.

1.4.2. Pesticide residues in water and soil samples

Azoxystrobin and chlorothalonil concentrations in soil at the end of the experiment were 0.63 and 2.8 mg a.i. kg^{-1} dw soil, respectively (Table IV.1). Taking into account the soil depth of 15 cm and the natural soil density of 1.25 g cm^{-3} the expected concentrations of azoxystrobin and chlorothalonil as a.i. after the two applications of 2RD were 0.426 mg a.i. kg^{-1} dw soil (1 application of 2RD = 0.213 mg a.i. kg^{-1} dw soil) and 3.2 mg a.i. kg^{-1} dw soil (1 application of 2RD = 1.6 mg a.i. kg^{-1} dw soil), respectively. Azoxystrobin concentrations in water samples (leachate, runoff and elutriate) varied between 4.5 and 78 µg L^{-1}. Chlorothalonil was not detected above the limit of quantification (LOQ , 0.05 µg L^{-1}) in none of the water samples at the end of the experiment (Table IV.1).

Table IV.1: Soil pH and moisture (mean ± standard deviation) during the soil-water simulator (SWS) experiment and pesticides concentrations in soil and water samples collected at the end of the experiment.

	Control	**Azoxystrobin**	**Chlorothalonil**
Soil			
pH	6.0 ± 0.1	6.1 ± 0.2	6.1 ± 0.2
Moisture (% relative saturation)	90 ± 8.9	72.5 ± 13.3	75.0 ± 13.8
Pesticide in soil (mg ai kg^{-1})	n.m	0.63	2.8
Pesticide in water (μg L^{-1})			
Leachate	n.m	4.5	< LOQ
Runoff	n.m	78	< LOQ
Elutriate	n.m	24	< LOQ

n.m. = not measured; LOQ (limit of quantification) = 0.05 μg L^{-1}

1.4.3. Lethal and sublethal ecotoxicity to Daphnia magna

The results of the lethal and sublethal *D. magna* assays were valid according to the criteria established in the guidelines. A summary of the results are presented in Table IV.2. In the lethal assay, leachate and elutriate samples from the Control SWS caused negligible effects on *D. magna*, i.e., a mortality of 10% and 15%, respectively, whereas Control SWS runoff caused a 100% lethal effects at the 100% concentration. In the reproduction assay, leachate and elutriate Control SWS samples at the 100% concentration showed also negligible lethal effects (within or close to the acceptability criteria of 20%; OECD, 1998); 30 and 20%, respectively. Thus, to test for toxic effects of leachate and elutriate samples all comparisons were made against the 100% Control SWS data. On the contrary, the runoff from the Control SWS caused 100% mortality at the 100% in the sublethal assay (as well as both the 100% concentration pesticide runoff samples). Thus, effects on the reproduction of *D. magna* were assessed only at the 50% concentration for both AZO SWS and CLO SWS, by comparisons with the respective 50% Control SWS concentration.

Table IV.2: Lethal LC_{50} (48 h concentration values with 95% confidence limits within brackets; LC_{50}) and 21-d reproduction (sublethal) effects (mortality of adult organisms within brackets) on *Daphnia magna* exposed to water samples from the different matrices (leachate, runoff, elutriate) originated from the soil-water simulator experiment with the fungicides azoxystrobin and chlorothalonil and tested at the 100, 50, 25, 12.5 and 6.25% (v/v) concentrations and at 100 and 50% (v/v) concentrations in the lethal and sublethal assays, respectively.

	Toxicity	Azoxystrobin	Chlorothalonil
Leachate	Lethal	LC_{50} > 100%	n.p. [a]
	Sublethal	Effect at 100% * (18% inhibition)	No effect on reproduction
Runoff	Lethal	LC_{50} > 100%	LC_{50} = 40.6 % (32.5 – 50.5)
	Sublethal (only tested at 50%) [b]	No effect	No effect on reproduction (60% mortality at 50%)
Elutriate	Lethal test	n.p. [a]	LC_{50} = 77.1 % (59.3 – 100.3)
	Sublethal test	No effect	No effect on reproduction (80% mortality at 50%, 100% mortality at 100%)

* significant different from the control ($p < 0.05$); n.p.[a] – not performed due to low samples volume; [b] due to 100% mortality in the 100% Control SWS and pesticide dilutions only the correspondent 50% dilutions were tested.

1.4.3.1. Azoxystrobin application scenario

Leachate and runoff samples contaminated with azoxystrobin did not cause lethal effects on *D. magna* during the 48-h exposure at the tested concentrations, as shown by the LC_{50} values > 100% (Table IV.2); for this pesticide no lethal assay was performed with elutriate samples due to water volume limitations.

In terms of azoxystrobin sublethal effects, the leachate had a significant effect on the reproduction of *D. magna* (1-way ANOVA: $F_{2,22} = 3.81$, $p = 0.038$), but only at the 2RD concentration a significant inhibition relatively to the control (only by 18%) was observed (Dunnet´s test: $p < 0.05$) (Table IV.2). No effects on *D. magna* reproduction were observed either for the runoff (only tested at the 50% concentration mimicking the RD, due to 100% mortality at the 2RD dose; Student *t*-test: $t_{17} = 1.51$, $p = 0.15$) and the elutriate water (1-way ANOVA: $F_{2,22} = 1.48$, $p = 0.25$) (Table IV.2).

1.4.3.2. Chlorothalonil application scenario

As stated above, lethal effects on *D. magna* were evaluated under water volume limitations; as such no results for leachate samples were obtained. Chlorothalonil lethal toxicity to *D. magna* was observed with both runoff and elutriate CLO SWS samples with LC_{50} values of 41 and 77% (v/v), respectively (Table IV.2). Leachate waters did not affect *D. magna* reproduction (Table IV.2), either at the assumed RD or 2RD concentrations (1-way ANOVA: $F_{2,23} = 2.95$, $p = 0.072$). CLO SWS runoff caused no effects on reproduction at the 50% concentration mimicking the RD (Student *t*-test: $t_{11} = 1.20$, $p = 0.25$), even though 60% mortality of the parental organisms occurred during the test (50% after the first 48 h of exposure and 10% added at day 6). For the elutriate reproduction tests, 80 and 100% mortality were observed on parental organisms at 50% and 100% concentrations, respectively. However, the 50% concentration did not affect the cladoceran reproduction (Student *t*-test: $t_8 = 2.58$, $p = 0.033$).

1.5. Discussion

1.5.1. Fate and behaviour of azoxystrobin in soil and water samples

After the simulation of the crop-based "worst-case" scenario with azoxystrobin, the measured concentration of the fungicide in the natural soil was 0.630 mg a.i. kg^{-1} dw soil (Table IV.1), a value slightly higher than the expected of 0.426 mg a.i. kg^{-1} dw soil for the 15-cm soil depth of the SWS. However, taking into account a 10 cm depth from where the soil was collected, and assuming that the entire pesticide amount would have stayed in this 10-cm topsoil, the expected concentration of azoxystrobin in soil after two applications of 2RD would be 0.640 mg a.i. kg^{-1} dw soil, a value similar to the one measured. Therefore the results indicate that azoxystrobin probably did not spread to the deepest part of the 15 cm soil stratum staying mostly within the 10 cm topsoil layer. Such a strong sorption of azoxystrobin to the soil was not expected on the basis of the fungicide characteristics. However, this behaviour has indeed been documented under different leaching assessment studies with different natural soils as well. Bending et al. (2006) found that azoxystrobin concentrations were maintained stable in sandy loam and silt-loam soils over the first month after the application of 5 mg kg^{-1} dw soil of the fungicide. Azoxystrobin applied at 625 g a.i. ha^{-1} under continuous water flow

conditions sorbed to the top 0–5 cm layer in sandy loam soil columns (Ghosh and Singh, 2009) while when applied under discontinuous flow conditions azoxystrobin was not detected in either of the leachate fractions collected over a period of 28 weeks (Ghosh and Singh, 2009). The same "non-leaching" behaviour of azoxystrobin was observed over a five years monitoring study with 3 applications of the pesticide at 250 g a.i. ha^{-1} to a sandy soil field, as a result of strong sorption to soil (Jørgensen *et al.*, 2012). The presence of a relatively high organic matter content (i.w. being 5.74%, Table II.1) in the natural soil used in the present study may have facilitated azoxystrobin sorption since azoxystrobin sorption is positively directly related to OM content, more than to pH (Koděsova *et al.,* 2011). The present results suggest that azoxystrobin staying mostly in the upper soil layer of the SWS agree with the observed differences in pesticide concentration among the three water matrices. Runoff showed the highest azoxystrobin concentrations (78 μg L^{-1}) followed by elutriate and finally the leachate with a value as small as 4.5 μg L^{-1} (Table IV.1). During the rain event, azoxystrobin may have been transported along with soil particles to the runoff water. The same may have occurred during the preparation of elutriates with soil collected from the top soil layer (10 cm) with the pesticide soluble particles moving to the water solution. However, at long term the fungicide is expected to degrade rapidly in soil under field conditions (DT_{50} = 14 d) due to degradation processes by microbial communities in natural soil (Adetutu *et al.*, 2008), and by photodegradation in surface water bodies (Boudina *et al.*, 2007; Zafar *et al.*, 2012).

1.5.2. Linking exposure and effects of azoxystrobin on the aquatic biota and evaluation of potential environmental risks

The observed absence of lethal effects of azoxystrobin in leachate and runoff waters is in agreement with the documented LC_{50} of azoxystrobin of 80 μg L^{-1} for *Daphnia* sp. (MacBean, 2012) since actual azoxystrobin concentrations were 4.5 μg L^{-1} and 78 μg L^{-1}, respectively (Table IV.1). The fungicide concentration in runoff was similar to the reported LC_{50} value of 80 μg L^{-1} (MacBean, 2012) which may explain the 45% immobilization attained at 2RD (100% concentration) in the lethal assay. Unexpectedly, and contrary to what was observed in the lethal assay, runoff resulting from the 2RD Control SWS application scenario caused high mortality of *D. magna* in the sublethal test, indicating that the observed lethal effect at the AZO SWS 100% runoff in both

lethal and sublethal assays (45 and 100% mortality) may be associated to other stressors than the pesticide. Given that Control SWS elutriate samples were also prepared by centrifugation to remove excess suspended soil particles and showed negligible mortality (see section 1.4.3), an effect due to the suspended solids originated from the natural soil towards *D. magna* (Friberg-Jensen *et al.,* 2010) may be dismissed as the cause of this additional stress. Possible the deep freezing of the runoff samples for approximately one week was not enough to control for the presence of bacteria/fungi originated mainly from the top soil and thus expected in higher amounts in runoff than in elutriates or leachates (Gao *et al.,* 2006). Nevertheless, sublethal effects on aquatic invertebrate communities, inhabiting surrounding aquatic ecosystems, of runoff waters with 78 μg L^{-1} of azoxystrobin would be expected, taking into consideration the lower no observed ecologically adverse effects concentration (NOEAEC) of 10 μg L^{-1} accepted by EFSA (2010) for azoxystrobin risk assessment on aquatic invertebrate communities. The fact that the highest concentrations of azoxystrobin was observed in the runoff samples validates the importance of runoff waters resulting from rain events under Mediterranean climate as a transfer pathway of pesticides to possible surface water contamination (Berenzen *et al.*, 2005; Tarazona, 2005).

The 18% inhibition of the *D. magna* reproduction observed in the present study at the 100% leachate with an actual pesticide concentration one order of magnitude lower than that detected in runoff and elutriate samples (Table IV.1), was unexpected since no reproduction effects were observed with both these latter water samples. In addition, the documented no observed effect concentration for *D. magna* (21d-NOEC (no-observed-effect concentration) = 44 μg L^{-1}; FOOTPRINT, 2012) is higher than the azoxystrobin concentration detected in the leachate (4.5 μg L^{-1}) indicating that no effects would be expected. Nonetheless, significant clonal variation in the sensitivity of *D. magna* toward the fungicide tested has been documented (Warming *et al.,* 2009), and a sublethal sensitivity of the cladoceran group towards azoxystrobin exposure has been observed in mesocosmos studies at concentrations as low as 10 μg L^{-1} (EFSA, 2010; Zafar *et al.,* 2012) and 15 μg L^{-1} (Gustafsson *et al.,* 2010). In addition, sublethal stress through respiration measurements and life-table experiments on populations of daphnids has also been observed at concentrations of azoxystrobin as low as 0.026 μg L^{-1} in natural waters (Warming *et al.,* 2009). The presence of micronutrients derived from the soil in the runoff and elutriate waters, expected more in the latter than in leachates which are

obtained by slow percolation, may have counterbalanced the toxic effects. On the other hand, the absence of larger suspended particles (removed by the centrifugation procedure) from runoff and elutriate may have decreased pesticide availability. The observed sublethal effects may suggest that changes in the daphnid populations may occur at much lower concentrations of azoxystrobin in natural water bodies in agricultural areas than expected by the reported LC_{50} and NOEC values (FOOTPRINT, 2012; MacBean, 2012). This emphasises the need to used natural waters to assess realistic environmental effects of pesticides; for instance, toxicant exposure may be enhanced in leachates through its small suspended soil particles. Thus, the present study results show that leaching may play an important role as a transfer pathway of azoxystrobin to groundwater contamination (Ghosh *et al.,* 2009; Jørgensen *et al.,* 2012) particularly under irrigated conditions.

No toxicity is expected from the use of the formulated product (Ortiva®) since it does not contain other ingredients indicated as toxic to aquatic and terrestrial organisms (Syngenta, 2011b), and in terms of azoxystrobin degradation, there is a lack of information on the environmental toxicity of those environmental relevant metabolites but were described as less toxic (Rodrigues *et al.,* 2013), with a documented 48h-EC_{50} of >180 000 μg L^{-1} for *D. magna* (FOOTPRINT, 2012), and consequently not posing a risk to the aquatic ecosystem under this crop-based scenario.

1.5.3. Fate and behaviour of chlorothalonil in soil and water samples

At the end of the crop-based "worst-case" simulation with chlorothalonil only the soil samples showed chlorothalonil residues. The measured peak-concentration of 2.8 mg a.i. kg^{-1} dw soil is comparable with the expected concentration of 3.2 mg a.i. kg^{-1}dw soil, as calculated from the amount derived from two applications of 2RD and based on the soil volume and density. The fact that no fungicide was detected above the limit of quantification (LOQ = 0.05 μg L^{-1}) in either the water samples (leachate, runoff and elutriate) may suggest that the pesticide did not move to the water compartment. This behaviour has previously been documented in laboratory column leaching studies where chlorothalonil did not leach and remained in the top 5 cm soil layer (EC, 2006), and also in a three years field leaching study using lysimeters where this fungicide also remained in the upper soil layer (15 cm) not being detected in groundwater (EC, 2006). Nevertheless, a slight movement to the water compartment would be expected due to

the physico-chemical properties of chlorothalonil and its predicted environmental potential fate (see section 1.3.1).

1.5.4. Linking exposure and effects of chlorothalonil on the aquatic biota and evaluation of potential environmental contamination.

Two applications of two times the simulated recommended dosage of chlorothalonil under the potato crop-based scenario with daily irrigation would cause slight and marked lethal effects on the cladoceran exposed to elutriate and runoff samples, respectively, with 48h LC_{50} values of 77 and 41%, respectively. In terms of sublethal effects, leachate, runoff and elutriate samples did not cause any negative effect on the cladoceran reproduction, either on the RD and 2RD scenarios. Nonetheless, 60 and 80% mortality of the parental organism occurred in runoff and elutriate samples at the 50% concentration mimicking the RD, whereas all parental organisms died at the 100% concentration corresponding to 2RD (Table IV.2). The observed toxicity would not be expected given that chlorothalonil residues in all water samples were lower than the LOQ (0.05 µg L^{-1}), a value much lower than the documented values for *D. magna* lethal toxicity of 48h-EC_{50} = 84 µg L^{-1} (EC, 2006), 48h-LC_{50} = 70 µg L^{-1} (MacBean, 2012), and 48h-LC_{50} = 129 µg L^{-1} (Sherrard *et al.,* 2003), and sublethal toxicity of 21d NOEC = 8.5 µg L^{-1} (EC, 2006). The high toxicity observed on parental daphnids exposed to runoff samples from chlorothalonil and Control SWS at the 100% concentration, both with and without pesticide, suggests the presence of other stressors than the fungicide, as discussed on section 1.5.2 relatively to azoxystrobin. However the mortality observed in both the lethal and sublethal tests conducted with runoff and leachate samples may be related to the fungicide since no mortality effects were observed on the Control SWS samples. Although no pesticide was detected above the LOQ in the water samples, the observed toxicity results under the simulated agricultural scenario may show that the site hydrology as well as agricultural irrigation and rain events, take an important role in the pesticide movements to the water compartment, establishing water contamination pathways (Berenzen *et al.,* 2005; Jørgensen *et al.,* 2012) that may cause negative impacts on the aquatic communities inhabiting the water bodies.

Stressors resulting from the application of chlorothalonil as the formulated product BRAVO500® which contains propane-1,2-diol (CAS 57-55-6) as other ingredient, are

not expected since no risks for the environment are identified (Syngenta, 2011a). In addition, the formulation is indicated to be less toxic for aquatic invertebrates (*D. magna* 24h EC_{50} = 882 μg L^{-1}) than the active ingredient (Syngenta, 2011a). Nevertheless, although no degradation products were analysed in this study, it is known that chlorothalonil degrades in soil into a persistent (DT50 > 6 months) and mobile metabolite: hydroxychlorothalonil (4-hydroxy-2,5,6-trichloroisophthalonitrile) (Armbrust, 2001; FOOTPRINT, 2012). However, a rapid photodegradation is possible for this metabolite (Armbrust, 2001). Although there is a lack of environmental toxicity information for this compound, the reported LC_{50} value for *D. magna* (24 000 μg L^{-1}) for this metabolite (Armbrust, 2001) is much higher than those for chlorothalonil. Therefore effects to the aquatic ecosystem related to this metabolite would not be expected under this crop-based scenario.

1.6. Conclusions

The simulation of "worst-case" crop-based scenario of the application of the fungicide azoxystrobin showed that, under realistic agricultural procedures the application of twice the recommended dosage as possible misuse may cause toxic effects on the reproduction of aquatic cladocerans if exposed to leachate waters. Runoff waters proved to be an important transfer pathway of azoxystrobin to surface water contamination under Mediterranean conditions, as it resulted in the highest azoxystrobin water concentration. Chlorothalonil "worst-case" application scenario may cause lethal effects on aquatic cladoceran communities exposed to runoff and elutriates, in spite of the low pesticide concentrations detected in the present study in waters resulting from the contamination pathways associated with the transfer from the natural soil to the water compartment. The present study shows the importance of using natural soil in realistic simulations as it reveals unexpected pesticide fate behaviour (e.g. leaching) that may occur under real agricultural environmental conditions. Semi-field simulations based on crop scenarios under natural climate and soil conditions are a valuable tool for pesticide risk assessment linking pesticide fate and contamination pathways and resulting toxicity under realistically simulated pesticide stress. This semi-field approach is also capable of improving the practicality and acceptability of results (Arts *et al.*, 2006). Pesticides

metabolite's fate under field realistic environmental conditions and their toxicity to biota should be taken into account when conducting future work on pesticide fate and effects, to contribute to a sustainable use of pesticides.

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2. Ethoprophos fate on soil-water interface and effects on non-target terrestrial and aquatic biota under Mediterranean crop-based scenarios.

Based on the following manuscript:

Ethoprophos fate on soil-water interface and effects on non-target terrestrial and aquatic biota under Mediterranean crop-based scenarios. Sara Leitão, Matilde Moreira-Santos, Paul J. Van den Brink, Rui Ribeiro, Mª José Cerejeira and José Paulo Sousa (*accepted for publication at the journal Ecotoxicology and Environmental Safety following minor revisions*).

2.1. Abstract

The present study aimed at assessing the environmental fate of the insecticide and nematicide ethoprophos in the soil-water interface following the pesticide application in simulated maize and potato crops under Mediterranean agricultural conditions, particularly of irrigation. Focus was given to the soil-water transfer pathways (leaching and runoff), to the pesticide transport in soil between pesticide application (crop row) and non-application areas (between crop rows), as well as to toxic effects of the various matrices on terrestrial and aquatic biota. Soil elutriates were also used as surrogates of the soil retention capacity and thus potential of ethoprophos to be mobilized into aquatic systems. A semi-field methodology mimicking a "worst-case" ethoprophos application (twice the recommended dosage, 2RD, for maize and potato crops: 100% concentration) in agricultural field situations was used. A rain event was simulated under a slope of 20º for both crop-based scenarios. Soil and water samples were collected for the analysis of pesticide residues. Ecotoxicity of soil and aquatic samples was assessed by performing lethal and sublethal bioassays with organisms from different trophic levels: the collembolan *Folsomia candida,* the earthworm *Eisenia andrei* and the cladoceran *Daphnia magna*. Although the majority of ethoprophos sorbed to the soil application area, pesticide concentrations were detected in all water matrices illustrating pesticide transfer pathways of water contamination (leachate, runoff and elutriate). Leaching to groundwater proved to be an important transfer pathway of ethoprophos under both crop-based scenarios, as it resulted in high pesticide concentration in leachates from maize scenario (130 μg L^{-1}) and potato crop scenario (630 μg L^{-1}). For the simulated RD (two times dilution of the original samples), and lower concentrations, ethoprophos application at the potato crop scenario caused more toxic effects on terrestrial and aquatic biota than at the maize scenario. This was an expected result since the RD of the pesticide as a nematicide for potato crop is 10 times higher than the dosage needed for treating maize crop against soil insects. In both crop-based scenarios, ethoprophos moved with the irrigation water flow to the soil between the crop rows where no pesticide was applied, causing also toxic effects on the terrestrial organisms. The two simulated agricultural crop-based scenarios proved to illustrate the importance of transfer pathways of pesticides from soil to groundwater through leaching and from

crop rows to the surrounding soil areas in a soil-water interface environment, which is representative for irrigated agricultural crops under Mediterranean conditions.

Keywords: insecticide; runoff; leaching; soil elutriate; terrestrial and aquatic toxic effects; natural soil.

2.2. Introduction

The natural variability of environmental conditions may influence the exposure of non-target organisms to pesticides due to differences in, among others, climate and soil characteristics (Chelinho *et al.,* 2011; De Silva *et al.,* 2009; Domene *et al.,* 2011). As such, in the new regulation concerning the placing of plant protection products on the market (ECR, 2009), the European Union established three zones in Europe (North, Centre and South), making exposure scenarios for the environmental risk assessment of pesticides more realistic according to specific edapho-climatic conditions. Under Mediterranean conditions, pesticide driven surface water contamination is mainly related to soil erosion and runoff ensuing from rain events (Tarazona, 2005). This becomes of great importance when looking at specific contamination pathways of pesticides in the soil-water interface of agricultural fields due to the site hydrology as well as agricultural irrigation and rain events (Berenzen *et al.,* 2005). Therefore, the need to study pesticide behaviour under realistic exposure scenarios in Mediterranean conditions is of critical importance, also due to the scarcity of information on pesticide fate under the natural environment and its effects on biota for this region (Daam *et al.,* 2011).

Extrapolating results from laboratory experiments to the field scale under outdoor conditions adds uncertainty to the environmental risk assessment (Boesten and Gottesbüren, 2000; Bouraoui, 2007). This has encouraged the use of different methodologies to assess pesticide fate in soil and routes of entry into the aquatic compartment and their effects on the biota. To address this knowledge gap, a semi-field crop-based experiment using a soil-water simulator was performed in the present study. This soil-water simulator was developed in a previous study (Chelinho *et al.,* 2012), and its use under controlled conditions decreases variability in collected data which is often observed in field experiments, while maintaining the natural characteristics of the

system under realistic field exposure conditions (e.g. soil type, slope, climatic condition, irrigation). With this approach, the environmental risk of pesticide applications can be assessed for a particular agricultural area in an integrated way taking into account not only the soil compartment but also specific soil-water transfer pathways such as leaching, runoff and soil elutriates as a measure of the soil retention capacity (Chelinho *et al.,* 2012), i.e., the potential of contaminants to be mobilized into aquatic systems through soil.

In the present study this semi-field methodological approach was applied to simulate the application of the pesticide ethoprophos on two irrigated crops (maize and potato) under a realist "worst-case" Mediterranean scenario of a major agricultural area of Central Portugal (Ribatejo) under Mediterranean conditions. Potential pesticide contamination of water bodies is of paramount importance for this area due to its proximity of the UNESCO biosphere reserve "Paul do Boquilobo" which contains surface waters that are of great importance for bird conservation and biodiversity protection (ICNF, 2013). This reserve is in the vicinity of a hydrogeological vulnerable area where several pesticides have been detected in surface and groundwater at concentrations that may be expected to lead to environmental side-effects (Silva *et al.,* 2012a, b).

The objectives of the present study were: i) to assess the fate of ethoprophos in the soil-water interface focusing both on transfer pathways from the soil to the water compartment through leaching, run-off and soil elutriates (as surrogates of the soil retention capacity), and on the mobility within the pesticide application and non-application areas (those between crop rows), by performing pesticide applications mimicking realistic field conditions; ii) to assess the pesticide ecotoxicological effects of soil samples from both crops areas (crop row and between row) to terrestrial biota by performing reproduction assays with the collembolan species *Folsomia candida* and the earthworm species *Eisenia andrei*, and of the different water matrices (leachates, runoff and soil extracts, i.e. elutriates from soils of both crops areas) to aquatic organisms by performing lethal and sublethal (reproduction) toxicity assays with the standard cladoceran species *Daphnia magna*; and iii) to compare and link exposure and ecotoxicological results from the soil and water matrices, herewith assessing the relative importance of the different soil-water transfer pathways (leaching, runoff and elutriates) for the risk assessment of the water compartment.

2.3. Materials and methods

An experimental semi-field methodology using natural soil similar to the one used for the study with the fungicides azoxystrobin and chlorothalonil (see section 1.) was performed to mimic a crop-based pesticide application under a "worst-case" field scenario for the insecticide ethoprophos. As "worst-case", an application of twice the recommended dosage established in Portugal for ethoprophos formulated product, as representing a possible misuse by the farmers, was also assumed.

2.3.1. Soil-water simulator experimental setup

A stainless steel transportable soil flume system of 0.4 m^2 (from here onward referred to as soil-water simulator - SWS), with a controllable depth (maximum of 100 x 40 x 20 cm; length, width and height, respectively), with two articulated perforated platforms that can move independently allowing to work under different slopes was used (see Figure II.2; Chelinho *et al.,* 2012). This methodology allows the collection of samples to evaluate exposure and effects on both the soil and the aquatic compartments. The experimental design followed in the present study consisted of three SWS that were setup in a horizontal position to guarantee the same conditions of pesticide fate in the entire 0.4-m^2 area: one SWS was used as the control with no pesticide application (Control SWS), and other two under the established scenarios of ethoprophos applications on maize crop (Maize SWS) and on potato crop (Potato SWS).

The natural soil used in the experiment was the same soil used under the fungicides experiment, see section 1.3.3. for more details. For soil collection, preparation and testing for the absence of pesticide residues prior to the experiment see section 2.3.3 of Chapter III. The soil was air dried and preserved at room temperature till SWSs setup. The experiment was prepared by setting up the SWSs placing first a 5-cm layer of glass beads (1-cm diameter) at the bottom of each of the three SWS platforms to avoid dogging and facilitate leachate percolation (Figure IV.1b). On top of the glass beads, a 15-cm layer of soil was placed up to the edge of the platforms so that the SWS frame would not pose as an obstacle during the runoff event (Figure IV.1c). The soil was left to settle and stabilize its structure for 30 days to become as similar to the field soil as possible. After this period, the soil was prepared with daily irrigation, to maintain its

moisture, by daily sprinkling 7.143 L m^{-2} (mm) of water for 7 days, according to crop needs and irrigation practices used in Portugal for that area.

All SWSs setup and the experiment were performed in a greenhouse with natural sun light, and air temperature and humidity were registered daily through the experiment using a RH/Temp DATA Logger EL-USB-2T, whereas soil pH and moisture in all SWS were registered before each irrigation with a Kelway Soil Tester (Kelway soil® acidity and moisture tester Model HB-2).

2.3.2. Crop-based simulations

The experiment started with the insecticide application (day 0) at twice the recommended dosage (2RD) for the two crop-based scenarios: Maize SWS and Potato SWS. Ethoprophos was applied directly to the soil as granules (GR) containing 10% w/w active ingredient (a.i.) using the formulated product (f.p.) MOCAP 10G® (biological efficacy period of 2 to 4 months) at 2RD for maize and potato crop, i.e. 25 kg f.p. ha^{-1} (2.5kg a.i. ha^{-1}) and 200 kg f.p. ha^{-1} (20 kg a.i. ha^{-1}), respectively. In the maize scenario, ethoprophos was applied together with maize grains (*FAO600 PR33Y74*), as it is applied during seeding stage (Figure IV.3a). Individual grains were planted every 17cm at 1-cm depth, mimicking field seedling, along a line (crop row area – R) in the middle of the SWS length, leaving at the sides two areas where no pesticide was applied (between rows area - BR) (Figure IV.4A). Under the potato scenario no potatoes were placed on the SWS because the pesticide is usually applied before planting. The Potato SWS area was divided in two equal areas, where the same pesticide dosage was applied but with different spatial distribution (Figure IV.3b and IV.4B). This was performed in order to be able to attain the volume of soil needed for the terrestrial ecotoxicity assays (i.e., evaluate pesticide effects on soil biota) while maintaining the required soil area to perform the simulation of a runoff event at the end of the experiment by placing the SWS at the requested slope (see below). At the soil sampling area (upper half of the simulator slope positioned during rain event at the end of the experiment) ethoprophos was applied in a strip of 20-cm width x 50-cm length mimicking crop application row (R), and incorporated into the soil at a depth of 5 cm by revolving the soil. The remaining area (20-cm width x 50-cm length) corresponded to the area between crop rows where no pesticide was applied (BR). For the simulation of

the rain event at the lower half of the Potato SWS, ethoprophos was applied on four equidistant points (Figure IV.4B) at a depth of 5 cm and covered with soil in order to allow the pesticide to disperse along the soil column.

Figure IV.3: a) Maize SWS; b) Potato SWS; c) Control, Maize and potato SWS in the greenhouse (see text for more details).

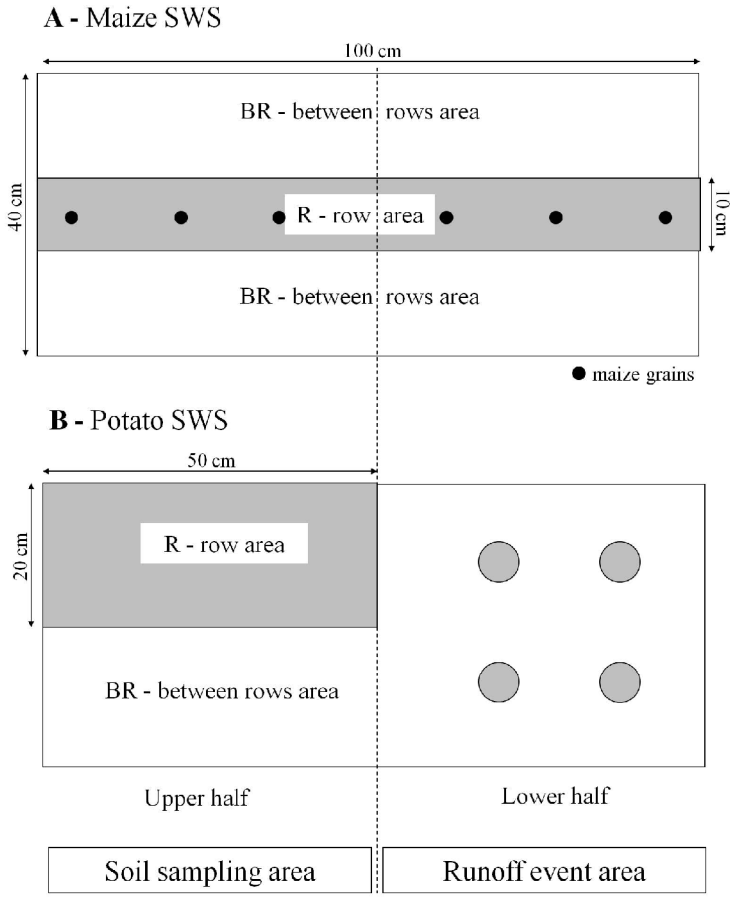

Figure IV.4: Spatial scheme of ethoprophos application on Maize soil-water simulator (SWS) (A) and Potato SWS (B). Shadow areas correspond to pesticide application area.

Pesticide application at both crop-based scenarios took place in the morning, followed by irrigation (as described above) in all three SWS in late afternoon. Leachates were collected after a waiting period of approximately 30 minutes. The same irrigation procedure and leachate collection continued daily for the following 9 days after the pesticide application. All leachates were kept separately at 4 to 6^{o}C in glass vials in darkness until pesticides residue analysis and bioassays performance.

The experiment ended ten days (day 10) after the insecticide application with the simulation of a rain event of 41.6 L m^{-2} (mm) to assess potential surface water contamination through runoff under a SWS slope of 20^{o} to mimic the study site (see section 1.3.5. for more details). The rain event was performed on the lower half of all three SWS (Figure IV.4) by isolating the upper half soil with plastic sheets placed in vertical till the glass beads level so that no water would get in contact with the soil. Runoff waters resulting from the rain event were kept in glass vials at 4 to 6^{o}C in darkness until pesticide residue analysis and bioassays performance.

After the rain event simulation, soil samples from the isolated upper half of all three SWS, for Potato and Maize SWS along both R and BR areas and for the Control SWS from the isolated soil area, were collected from the upper 10-cm soil layer as composite samples for pesticide analysis and ecotoxicity bioassays Soil samples for pesticide residue analysis were frozen to -20^{o}C until laboratory extraction and analysis through Liquid extraction/Cleanup followed by Gas Chromatography/Mass Spectrometry (LE/GC-MS), with a limit of quantification (LOQ) of 0.03 mg kg^{-1} (ASU L, 1999). Soil samples for elutriates and soil ecotoxicity bioassays were kept at 4 to 6^{o}C in darkness for 24 hours until use. Ethoprophos residues were also analysed in all water matrices (leachates, runoff and elutriates from soil at R and BR areas) from both SWS scenarios through Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) with a limit of quantification (LOQ) of 0.05µg L^{-1} (DIN, 1993-2; ISO, 2000). All pesticide residue analyses in soil and water samples were performed by an independent laboratory.

2.3.3. Pesticide characterisation

The pesticide ethoprophos (CAS 13194-48-4; *O*-ethyl *S*,*S*-dipropyl phosphorodithioate) is registered in Portugal for use in maize and potato crops as an insecticide and nematicide, respectively. Ethoprophos is a broad spectrum organophosphate nematicide

and insecticide with moderate residual activity and is not phytotoxic (MacBean, 2012; Karpouzas and Walker, 2000). It acts as an acetylcholinesterase inhibitor and is a non-systemic nematicide and soil insecticide with contact action (MacBean, 2012). Ethoprophos is effective against potato nematodes (*Globodera rostochiensis* (Wollenweber) Behrens, *G. pallide* (Stone) Behrens) and soil insects (*Agriotes* spp., *Agrotis* spp. and *Melolontha* spp.) on maize crop (Karpouzas and Walker, 2000), and is to be applied before and at the planting stage, respectively. Ethoprophos has high solubility in water and has potential to volatilize (Table III.4). The organic carbon sorption coefficient indicates that the pesticide sorbs moderately to soil and has a low mobility in water. The octanol-water partition coefficient indicates bioaccumulation potential (Log Kow > 3; FOOTPRINT, 2012). Ethoprophos is not persistent in soil ($DT50_{field}$ < 30d; EC, 2000), has the potential to leach to groundwater (GUS) depending on the field conditions, and shows a high affinity for the soil compartment according to its Predicted Environmental Distribution (Table III.4).

Ethoprophos concentrations in natural waters and soils are not documented. Ethoprophos effects on non-target soil and aquatic organism are not well known and the scarce information available for terrestrial organisms was obtained using artificial soil (EFSA, 2006), although effects on aquatic and terrestrial arthropods may be expected due to the pesticide type of action as an insecticide and nematicide (Frampton *et al.,* 2006; Maltby *et al.,* 2005). In fact, adverse effects of ethoprophos on the abundance and biomass of earthworms are known. Potter et al. (1994) observed a reduction in both endpoints of more than 80% observed 3 weeks after the application of 5.6 kg a.i. ha^{-1} of Mocap10G in turf soil.

2.3.4. Terrestrial ecotoxicity evaluation

Terrestrial ecotoxicity assays were performed with Collembola and Earthworms, two soil invertebrate groups that are important for soil functioning (Lavelle and Spain, 2001). Moreover, the species used, *Folsomia candida* and *Eisenia andrei* are widely used to evaluate the effects of different contaminants and used as standard organism in terrestrial risk assessment (Tiepo *et al.,* 2010; SANCO, 2002). In order to select the species to be used in this experiment, a study to determine the 50% Effect Concentration on terrestrial invertebrates using ethoprophos as active ingredient was performed for three standard soil species according to International Guidelines (ISO,

1998, 1999, 2004), using the same natural soil as in the SWS experiment (see section 2. of Chapter III) resulting in the following chronic effect endpoints: *Folsomia candida*: 28-d EC_{50}= 0.027 mg a.i. kg $^{-1}$dw soil; *Eisenia Andrei:* 8-weeks EC_{50}= 8.3 mg a.i. kg $^{-1}$dw soil and *Enchytraeus crypticus*: 4-weeks EC_{50}= 68.5 mg a.i. kg $^{-1}$dw soil. Taking into account the density of the natural soil (1.25g cm^{-3} previously calculated) used on both crop-based experiments and the established soil depth of 15 cm for all SWS, the expected concentrations of ethoprophos as active ingredient per kg of dry weight (dw) soil after the application of 2RD for Maize scenario would be 1.34 mg a.i. kg $^{-1}$dw soil (RD = 0.67 mg a.i. kg $^{-1}$dw soil) and 10.6 mg a.i. kg $^{-1}$dw soil (RD = 5.3 mg a.i. kg $^{-1}$dw soil) for Potato SWS scenario. On the basis of this information the collembolan *Folsomia candida* and the earthworm *Eisenia andrei* were selected. Sublethal assays with these two species were performed according to the International Guidelines referred above for all soil samples from both crop-based scenarios (at 2RD) including soil from crop row (R) and between crop row (BR) areas. Four replicates were used in each of the *F. candida* and *E. andrei* assays. To assess the effect of the recommended dosage (RD) on soil biota a concentration of 50% (v/v) was attained by mixing soil from Maize and Potato SWS (100% (v/v) concentration) with soil from the Control SWS in a 50:50 ratio, for each crop-based scenario. The controls of the ecotoxicological assays used natural soil from the Control SWS where no pesticide was applied. Collembolan and earthworm reproduction inhibition were accounted for in juvenile numbers after 4 and 8 weeks test duration, respectively. Collembolan adults were registered and adult earthworm biomass was also measured after 4 weeks of exposure. Bioassays results were analysed using one-way analysis of variance (ANOVA) to assess effects among the two study areas (R and BR) compared to the Control for each SWS crop scenarios (potato and maize) and for the 50% and 100% (v/v) concentrations which mimic the RD and 2RD, respectively. The assumptions of normality and homogeneity of variance were checked using Shapiro-Wilkinson and Bartlett's test, respectively. Post-hoc comparisons (Dunnett's test) were applied to verify the existence of significant differences from the control.

2.3.5. Water samples and aquatic ecotoxicity evaluation

Three types of water matrices/samples were used for the ecotoxicity evaluation toward aquatic organisms: leachates, runoff and elutriates, and prepared as described in section 1.3.7 at the end of the experiment for each SWS (Control, Maize and Potato). To evaluate the toxicity toward aquatic organisms of the pesticide water soluble components in the soil pore water, elutriates from R and BR areas soils of the crop-base scenarios and of the Control SWS were prepared as described in section 1.3.7.

All aquatic assays were conducted on the single leachate and runoff sample originated from each SWS (Control, Maize and Potato), on one elutriate sample from the Control SWS and two elutriate samples from each of the two pesticide SWS (Maize and Potato), prepared from the R and BR soil, as described above. Aquatic assays were conducted with the cladoceran *Daphnia magna,* a planktonic crustacean forming the base of the ecological structure in freshwater environments and occupying an important position in food webs due to its high grazing potential (Gustafsson *et al.,* 2010; Warming *et al.,* 2009). Additionally, *D. magna* is one of the most sensitive arthropod organisms in the aquatic environment (Warming *et al.,* 2009) and easily handled and cultured under laboratory conditions, being for these reasons the main standard organisms used in aquatic risk assessment. All water samples from the three SWS treatments (Control, Maize and Potato) were tested at least at 100% (v/v) and 50% (v/v) concentrations as an estimate for the potential effect that could be expected at the 2RD and RD of the insecticide, respectively, and also at 25% (mimicking ½RD) to increase the likelihood of discriminating toxic effects between the two crops. This procedure was also adopted for the control SWS to be able to discriminate other potential stress factors associated to the matrix from those due to the pesticide (e.g. turbidity).

Lethal assays were performed by determining the inhibition of the mobility of *D. magna* exposed for 48 h according to the Daphtoxkit F^{TM} Magna protocol (2000) using the following gradient concentration range for each type of water sample: 6.25; 12.5; 25; 50 (RD) and 100 % (v/v) (2RD). Additionally, a standard Control was performed with standard growth medium according to the protocol for test validity assessment. Test organisms were incubated at 19 to 21ºC with a 14:10-h light:dark cycle and no food was provided and no medium renewal was performed. Lethal results were analysed for percentage of effect and if possible LC_{50} and 95% confidence intervals calculated

according to ISO 6341 (2012) and using the Trimmed Spearman-Karber Method (Hamilton *et al.*, 1978).

To assess sublethal effects, a 21-days *D. magna* reproduction assay was performed for each water matrix, which consisted of a standard control plus the 100, 50 and 25% (v/v) concentrations, according to the OECD guidelines (OECD, 1998). A standard Control was also performed with standard growth medium according to the guidelines for test validity assessment. For each treatment, ten replicates each containing 50 ml of test solution and one neonate less than 24 hour old were used, which were incubated at 19 to 21ºC with a 14-h:10-h light:dark cycle and organisms were fed daily with an algae suspension. Because of the severe lethal effects that occurred after an exposure period as short as 72 h, the only test endpoint possible to evaluate under the conditions established for a sublethal assay was mortality. Survival results were evaluated by one-tailed Fisher's exact test for significant differences ($p < 0.05$) in mortality between the Control SWS and the respective pesticide concentration (2RD, RD and ½RD).

2.4. Results and Discussion

2.4.1. Air and soil measurements

Indoor air temperature and relative humidity(RH) mean values (± standard deviation) during the experiment were 17.8 ± 3.3 ^{o}C (19.4 ± 3.6 ^{o}C day time 8h - 20h, and 15.9 ± 1.3 ^{o}C night 21-7h), and 70.6 ± 10.5 % RH (67.2 ± 12.2 % RH day time 8h - 20h, and 74.7 ± 5.7 % RH night 21 - 7h), respectively. Daily soil pH and moisture measurements (% relative saturation) on each of the SWS are presented in Table IV.3.

2.4.2. Ethoprophos fate in soil and water samples

Ten days after the ethoprophos application in the crop-based "worst-case" scenario and daily irrigation, only soil from the row area (R) under Potato SWS scenario showed pesticide residues above the LOQ at 10.5 mg a.i. kg^{-1} dw soil (Table IV.3). These results observed in the potato scenario are in agreement with the predicted environmental potential fate distribution showing a high affinity to the soil compartment (Table III.4). In fact this residue concentration corresponds to 99.1% of the expected concentration in

soil after one application of 2RD for potato crop (10.6 mg a.i. kg^{-1} dw soil). Although ethoprophos has high solubility in water and a low soil sorption coefficient, may be the reason why no pesticide residue was detected at the Maize scenario where the dosages were ten times lower than those for the potato scenario. This strong soil sorption behaviour observed in the present study with a sandy clay loam natural soil has also been documented for sandy loam soils (Dowling *et al.,* 1994; Smelt *et al.,* 1977) and in sandy soils (Boesten and van der Pas, 2000; Bouraoui *et al.,* 2007). Under field conditions ethoprophos dissipated with a DT50 of 28 d in studies using the same formulated product at the same recommended dosage of the present study in a sandy loam soil (Boesten and Van der Pas, 2000). A similar DT50 of 22 d was also observed in a natural sandy soil and losses were attributed mainly to volatilization (Boesten and Van der Pas, 2000; Tiktak, 2000) since ethoprophos has potential to volatilize (Table III.4).

Table IV.3: Soil pH and moisture (mean ± standard deviation) during the soil-water simulator (SWS) experiment and ethoprophos concentrations in soil and water samples collected at the end of the experiment.

	Control	**Maize crop**	**Potato crop**
Soil pH	6.3 ± 0.2	6.3 ± 0.2	6.1 ± 0.2
Moisture (% relative saturation)	63 ± 27.5	58.5 ± 20	83 ± 14.8
Pesticide in soil (mg a.i. kg^{-1}dw soil)	n.m	R< LOQ BR < LOQ	R 10.5 BR < LOQ
Pesticide in water (µg L^{-1})			
Leachate	n.m	130	630
Runoff	n.m	44	19
Elutriate	n.m	R 130 BR 6.7	R 2200 BR 21

n.m. - not measured ; R – row area; BR – between rows area; LOQ = 0.03 mg kg^{-1}.

Taking into account the observed ethoprophos fate results in soil at the potato scenario, movements to the water compartment would not be likely to occur (Smelt *et al.,* 1977; Tiktak, 2000). However, ethoprophos residues were detected in all water samples and with differences in concentrations among the three water matrices of both crop scenarios that agree with the soil fate results. This movement to the water compartment may be explained by the insecticide physico-chemical characteristics and GUS value showing a possible potential for leaching (Table III.4). Elutriates from the soil pesticide

application area (R) showed the highest ethoprophos concentrations (130 and 2200 μg L^{-1} for Maize and Potato SWS scenarios, respectively) followed by leachates and finally the runoff with values of 44 and 19 μg L^{-1} for Maize and Potato SWS scenarios, respectively (Table IV.3). Ethoprophos was also detected in elutriates from soil from BR area where no pesticide was applied (Table IV.3), which indicates that the insecticide moved away from where it was applied to the surrounding area. These observed higher values of ethoprophos concentration in leachates than in runoff waters at both crop-based scenarios, showing a leaching capacity to groundwater, has also been described in studies on arable fields in Italy also under Mediterranean conditions (Garratt *et al.,* 2002). Ethoprophos degradation in water under natural environmental conditions is not likely to occur given that no chemical hydrolysis ($DT50_{water}$ > 365 days at 25°C and pH 7) and/or photodegradation (stable to photolytic breakdown) are expected (EFSA, 2006).

Environmental fate models have only been predicting the strong sorption behaviour of ethoprophos in soil not considering other factors that may influence this behaviour such as water flows (e.g. irrigation), while pesticide leaching models tend to indicate that the leaching potential of ethoprophos is negligible (Garratt *et al.,* 2002; Pistocchi, 2010; Tiktak, 2000). By combining the current fate results of ethoprophos in soil (strong adsorption) and water (leaching potential), the present study emphasizes the need to use different methodologies (semi-field) to better illustrate realistic pesticide contamination pathways and validate pesticide fate and behaviour between several environmental compartments, namely soil and water compartment.

2.4.3. Linking exposure and effects on biota and evaluation of potential environmental risk

All terrestrial and aquatic assays proved to be valid according to the respective test validity criteria as recommended in the respective ISO guidelines and protocols described above (see section 2.3.4 and 2.3.5).

In the aquatic lethal assays, leachate and elutriate matrices from the 100% Control SWS caused low mortality of 15% and 5%, respectively. However, runoff samples from Control SWS caused 40% lethality at the 100% concentration, while the 50% Control SWS concentration caused a low mortality of 5%. Therefore, for leachates and elutriates

the Control SWS results, rather than the assay standard Control with reconstituted artificial medium, were used to calculate the lethal toxicity parameters. Runoff samples from both crop-based scenarios were only assessed for the concentration gradient of 6.25 to 50% due to the high mortality observed with the Control SWS runoff (which would preclude discriminating effects due to the pesticide from other effects associated to the runoff). At the aquatic 72-h exposure assay under the conditions of a sublethal assay, the Control SWS leachate and runoff samples caused a lethal effect after 48 and 72 h at the 100% concentration (50 and 90% mortality, respectively, for leachates and 100% mortality after 48-h exposure for runoff). Therefore, effects due to the pesticides via leachate and runoff can only be evaluated after 24 h for all concentrations and up to 72 h for all (25 and 50%) except the 100% concentration. On the contrary, no mortality was registered for the Control SWS elutriates, and thus effects due to the pesticides via elutriates were assessed for all three concentrations up to 72-h exposure.

2.4.3.1 Maize crop scenario- terrestrial biota and soil compartment

Although no pesticide residues were detected above the limit of quantification (LOQ = 0.03 mg kg^{-1}) in any soil sample from the maize crop-based scenario simulation (Table IV.3), significant effects on non-target terrestrial organisms were observed. The soil collected at crop row area (R) where the pesticide was applied, caused significant effects on collembolans reproduction (1-way ANOVA: $F_{8,27} = 155.4$, $P < 0.001$; Dunnett's test: $p < 0.05$), with a 100% mortality of adults and consequently with no offspring at the assumed recommended dosage (RD) and 2RD (50 and 100% concentrations, respectively; Figure IV.5). Earthworm reproduction was also significantly inhibited (1-way ANOVA: $F_{8,27} = 12.3$, $p < 0.001$; Dunnett's test: $p < 0.05$) in soil from R area at both tested concentrations mimicking the RD and 2RD (Figure IV.6). Although there is a lack of information on effects of ethoprophos on non-target terrestrial species, these results are in agreement with the previously calculated ethoprophos EC_{50} of 0.027 mg a.i. kg^{-1} dw soil for collembolan using the same natural soil (see section 2.3.4.), given that this EC_{50} value is similar to the LOQ of 0.03 mg a.i. kg^{-1} dw soil. For earthworms, the previously reported values of EC_{50} = 8.3 mg kg^{-1} dw soil (see section 2.3.4.), 14-d LC_{50} = 39.6 mg kg^{-1} dw soil and the 56-d NOEC value of < 1.67 mg kg^{-1} dw soil (EFSA, 2006), with the same formulated product, are higher than the measured concentration of ethoprophos (< 0.03 mg kg^{-1}), suggesting that the observed pesticide toxicity to earthworms may have been influenced by the

characteristics of natural soil and of realistic environmental conditions (EFSA, 2009; Lanno *et al.*, 2004).

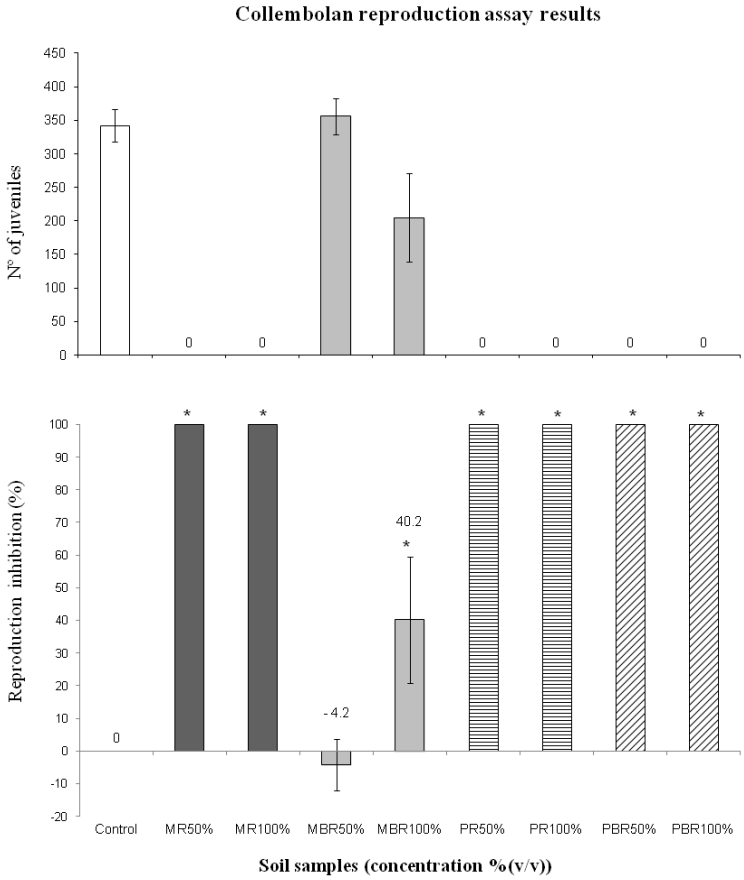

Figure IV.5: Effects of ethoprophos (mean values and standard deviation) on the reproduction (number of juveniles and reproduction inhibition) of collembolan exposed to soil from row area (R) and soil from between row (BR) from maize (M) and potato (P) scenarios. 50% - RD and 100% - 2RD (see details in the text); *Significant differences from control (p<0.05).

Soil from the BR area showed significant effects (1-way ANOVA: $F_{8,27} = 67.1$, $p < 0.001$; Dunnett's test: $p < 0.05$) at the 100% concentration corresponding to the applied 2RD on adult collembolan survival (30% mortality) and number of juveniles (40 % inhibition) (MBR100%, Figure IV.5). However, no significant effects on collembolan reproduction were observed at the RD (50% concentration), with a small increase of juveniles relatively to the control being observed (Figure IV.5). Significant negative effects were also observed on earthworm reproduction (1-way ANOVA: $F_{8,27} = 12.3$, $p < 0.001$; Dunnett's test: $p < 0.05$) in soil from BR area at both concentrations tested

(Figure IV.6). Adult earthworms biomass after 4 weeks exposure to soil from both areas (R and BR, Figure IV.7) was not significantly different from the control (Dunnett's test: $p > 0.05$). Even though ethoprophos is most probably redistributed more strongly in the vertical direction rather than in the horizontal direction (Smelt *et al.*, 1977), the fact that significant effects on collembolan and earthworms at 2RD, and assumed RD were observed in the area where no pesticide was applied, indicates that the pesticide also moved horizontally from the applied area to the surroundings due to water flows caused by irrigation (Garratt *et al.*, 2002).

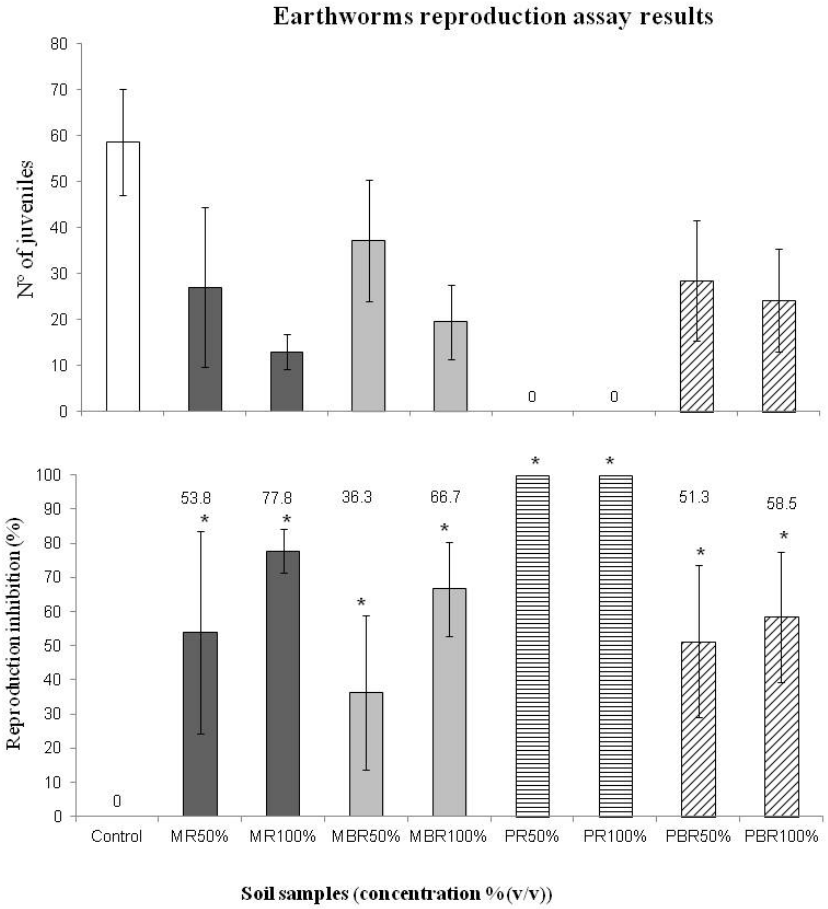

Figure IV.6: Effects of ethoprophos (mean values and standard deviation) on the reproduction (number of juveniles and reproduction inhibition) of earthworms exposed to soil from row (R) and from soil between row (BR) from maize (M) and potato (P) scenarios, 50% - RD and 100% - 2RD (see details in the text); * Significant differences from control ($p<0.05$).

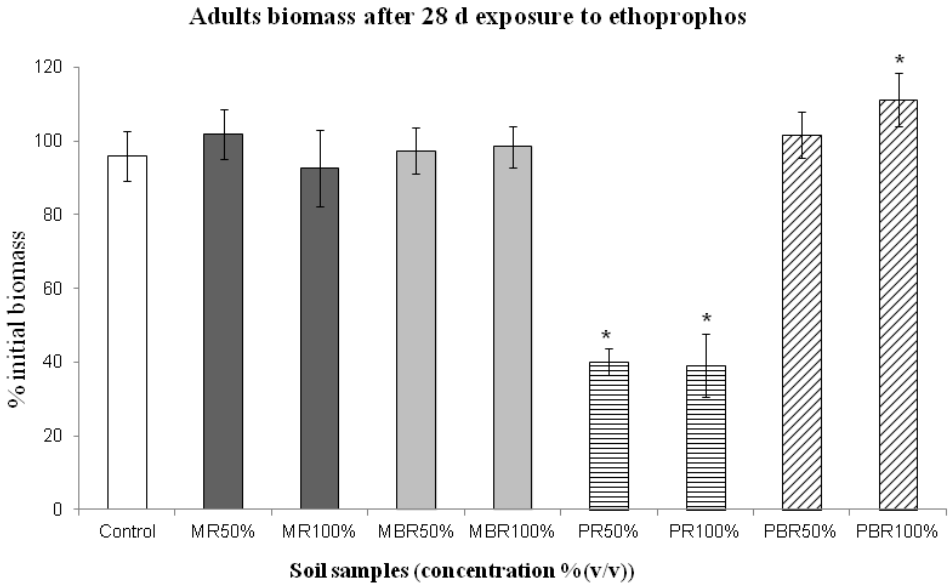

Figure IV.7: Initial biomass variation (mean values and standard deviation) of adult *Eisenia andrei* after 4 weeks (28d) exposure to soil from row area (R) and soil from between row (BR) from both Maize (M) and Potato (P) scenarios. 50% - RD and 100% - 2RD (see details in the text); *Significant differences from control ($p<0.05$).

As mentioned above, significant effects on the tested organisms were observed although no pesticide residues were detected in soil. Other stressors that may have influenced the observed toxicity are factors associated to the use of formulated product itself and metabolites. However, the formulated product used (MOCAP 10G ®) does not have any other toxic additional components than the active ingredient itself (Certis, 2011) and ethoprophos does not degrade in soil in any environmental relevant metabolites (EFSA, 2006). Only a minor metabolite is identified (O-ethyl-S-propylphosphorothioic acid - AE 0592496) that degrades into CO_2 and un-extractable residues as final degradation products (EFSA, 2006), and for which there is a lack of information on environmental toxicity.

2.4.3.2. Maize crop scenario- aquatic biota and water compartment

The absence of toxicity towards *D. magna* of leachate waters simulating a groundwater contamination pathway (Table IV.4), at the assumed ½ RD and RD after 48- and 72-h (one-tailed Fisher's exact test: $p > 0.15$) exposure and at 2RD after 48-h exposure (a maximum of 40% immobilization was observed) is in agreement with the reported value of 48-h LC_{50} = 200 µg L^{-1} for *D. magna* (EFSA, 2006), given that the latter is higher

than the pesticide concentration of 130 µg L^{-1} (Table IV.3) measured in the present study.

Table IV.4: Lethal 48-h LC_{50} (concentration values with 95% confidence limits) and mortality after 72h sublethal assay on *Daphnia magna* exposed to water matrices (leachate, runoff and elutriate) originated from Maize and Potato SWS scenarios treated with ethoprophos, and tested at 100, 50, 25, 12.5 and 6.25% (v/v) concentrations (except for runoff that the 100% concentration was not valid) and at 100 and 50% (v/v) concentrations in the lethal and sublethal assays, respectively.

	Leachate	**Runoff**	**Elutriate**
48-h lethal assay			
Maize SWS	LC_{50} >100%	LC_{50} > 50% [a]	R & BR LC_{50} >100%
Potato SWS	LC_{50} = 27.5% (22.7-33.2)	LC_{50} < 6.25% [b]	R LC_{50} = 10.6% (8.2-13.7) BR LC_{50} >100%
72-h sublethal assay			
Maize SWS	No effect (mortality 0-10%) [c]	No effect (mortality 10-30%) [c]	R Significant mortality (100% at 50 and 100% (v/v) after 72- and 48-h exposure, respectively) BR No effect (mortality 0%)
Potato SWS	Significant mortality (80-100% at 25 to 100 % (v/v) after 24-h exposure) [c]	Significant mortality (100% at 100 % (v/v) after 48-h exposure) [c]	R Significant mortality (100% at 25 to 100% (v/v) after 24-h exposure) BR No effect (mortality 0%)

CI – confidence interval; R – row; BR – between row; [a] No effect, a maximum of 5% effect at 50% concentration; [b] 100% effect at all concentrations; [c] due to elevated mortality in Control SWS, pesticide effects could only be evaluated after 24 h for all concentrations and up to 72 h for the 25 and 50% concentrations.

The same situation occurred with runoff waters simulating surface water contamination pathway, which after 48- and 72-h (one-tailed Fisher's exact test: $p > 0.15$) exposure at the assumed RD did not cause significant lethality and for which the measured concentration of ethoprophos in water was lower (44 µg L^{-1}) than that of the leachate (Table IV.3). Unexpectedly, runoff resulting from the Control SWS scenario caused high mortality of *D. magna* in the 48 and 72-h exposure assays at the 100%

concentration simulating the 2RD. However, given that Control SWS elutriate samples were also prepared by centrifugation to remove excess suspended soil particles and showed negligible mortality (see section 2.4.3.), an effect due to the suspended solids originated from the natural soil towards *D. magna* (Friberg-Jensen *et al.,* 2010) may be dismissed as the cause of this additional stress. Possibly the deep freezing of the runoff samples for approximately one week was not enough to control the presence of bacteria/fungi originated mainly from the top soil and thus expected in higher amounts in runoff than in elutriates or leachates (Gao *et al.,* 2006).

Toxicity towards the cladoceran with elutriates from soil in R area where ethoprophos was applied and between these areas (BR; Table IV.4) differed from the terrestrial toxicity results, where both areas proved to be toxic for collembolan and earthworms reproduction (see section 3.3.1). No effects on *D. magna* were observed with elutriates from the BR area soil after 48- and 72-h exposure at all tested concentrations as expected (Table IV.4), given that this water matrix from the BR area showed the lowest pesticide concentration (6.7 μg L^{-1}) detected among all water samples. In spite of the no observed toxicity with elutriates from the R area after 24 h of exposure, a significant toxicity towards the cladoceran was observed after 48- and 72-h (one-tailed Fisher's exact test: $p < 0.001$) of exposure at concentrations corresponding to the assumed 2RD and both RD and 2RD (Table IV.4), respectively, most likely due to the longer exposure period.

These results illustrate the importance of studying combined environmental compartments to increase ecological realisms in risk assessment evaluations on soil-water interface environments, as well as to evaluate the potential of contaminants to be mobilized into aquatic systems from the soil compartment.

2.4.3.3 Potato crop scenario – terrestrial biota soil compartment

After the application of ethoprophos at 2RD and daily irrigation during 10 days, the soil from the pesticide application area (R) caused 100% mortality on adult collembolans (1-way ANOVA: $F_{8,27} = 155.4$, $p < 0.001$; Dunnett's test: $p < 0.05$), and significant effects on adult earthworms survival (1-way ANOVA: $F_{8,27} = 53.8$, $p < 0.001$) originating no offspring at both concentrations tested (PR100 % - 2RD and PR50% - RD, Figure IV.5 and 4). The remaining adults showed a significant decrease of initial biomass (1-way ANOVA: $F_{8,27} = 58.7$, $p < 0.001$) of approximately 60% at both concentrations (R50% and R100%; Figure IV.7).

The same high toxicity results were observed at both tested concentrations for collembolan reproduction in soil from BR areas where no pesticide was applied, as well as significant negative effects on adult earthworm reproduction (1-way ANOVA: $F_{8,27} = 12.3$, $p < 0.001$) were observed with only an average of 20 to 30 juveniles at both concentrations *versus* the 59 juveniles in the Control SWS soil (Figure IV.6). These negative effects on the reproduction of earthworms are in agreement with the previously reported EC_{50} of 8.3 mg kg^{-1} dw soil (see section 2.3.4.**).** Although ethoprophos is referred by Smelt et al. (1977) as most probably redistributed in vertical direction using the same formulations as the present study, the observed toxicity results show otherwise and indicate that the pesticide may have moved from the application area to the surroundings, due to water flow caused by irrigation as also demonstrated by other studies (Berenzen *et al.,* 2005; Garratt *et al.,* 2001). In spite of no pesticide residues were detected in soil from BR area, its detection in elutriates may illustrate the availability of ethoprophos to terrestrial invertebrates through the soil pore water which is the main uptake source for these organisms (EFSA, 2009; Styrishave *et al.,* 2008), given that elutriates can be a measure of the soil retention function, i.e., on the potential of contaminants to move to the water compartment. The observed negative impact on collembolan from soil from the R and BR area are in agreement with the previously calculated ethoprophos EC_{50} of 0.027 mg kg^{-1}dw soil (see section 2.3.4.**)**, a value much lower than the measured concentration of 10.5 mg a.i. kg^{-1}dw soil in R area and the limit of quantification of 0.03 mg a.i. kg^{-1} dw soil for the BR area (Table IV.3).

In terms of lethal toxicity to adult earthworms after 4-weeks exposure to soil from R area, the mortality effects of 68% and 25% at 2RD and the assumed RD (100% and 50% concentrations, respectively) were observed at lower concentrations (10.5 mg a.i. kg^{-1}dw soil at the 100% concentration) than the reference value using the same formulated product (14-d LC_{50} = 39.6 mg kg^{-1}dw soil; EFSA, 2006). This may suggest that the natural soil properties and the mimicking of realistic environmental conditions of the experiment may have influenced the pesticide toxicity towards the tested organisms (EFSA, 2009; Lanno *et al.,* 2004) as well as chronic toxicity processes *per se* since the reference value was attained at a minor duration (14 days) than the present study assay (28 days).

At the soil area where no pesticide was applied (BR), adult earthworms survival was not significantly affected at RD and 2RD, as well as their initial biomass (Dunnett test p =

0.995) (Figure IV.7). Furthermore, a significant increase of initial biomass was observed at 100% concentration corresponding to the 2RD (PBR100%; Figure IV.7). Other authors have registered this positive effect on the earthworm biomass when studying short-term toxicity endpoints, such as survival that depends on dermal uptake, when exposed to organophosphates (De Silva *et al.,* 2009). Although classifying effects of pesticides within the same chemical group must be done with special attention to the pesticide individual performance and to the biology of the organism (Wang *et al.,* 2012), in the present study the low ethoprophos concentration in natural soil (< LOQ = 0.03 mg kg^{-1}) may have led to enhanced food intake resulting in biomass increase (Figure IV.7).

Soil from the R area proved to be more toxic for earthworm reproduction than the surrounding area (BR) and no evident differences were observed between the 50% and 100% concentrations, which mimic RD and 2RD, respectively (Figure IV.6).

2.4.3.4. Potato crop scenario - aquatic biota and water compartment

In spite of ethoprophos strong adsorption to soil particles, the soluble fraction component of the pesticide moved to the water compartment through leaching and runoff as well as elutriates (Table IV.3), illustrating the potential of the pesticide to be mobilized into the aquatic systems.

Leachate waters caused high toxicity to *D. magna* after the 48-h exposure assay with an LC_{50} of 27.5% (Table IV.4). Taking into consideration the measured pesticide concentration of 630 μg L^{-1} (Table IV.3) in leachate waters at the 100% concentration resulting from the application of 2RD, the observed 48-h LC_{50} of 27.5% may correspond to an 48-h LC_{50} value of 173 μg L^{-1} (143 – 209 μg L^{-1}). This ecotoxicity value is in agreement with the documented 48-h EC_{50} of 200 μg L^{-1} for *D. magna* (EFSA, 2006).

Nevertheless, the observed high toxicity of leachates (> 80% mortality) towards the cladoceran during the 72-h sublethal assay (after 24-h exposure at all concentrations and after 72-h exposure at 25% and 50% concentrations; one-tailed Fisher's exact test: $p <$ 0.0004) are corresponding with concentrations simulating the application of ½RD, RD and 2RD showing the high potential risk of groundwater contamination under the simulated realistic scenario for potato crop using the insecticide ethoprophos.

The application of the tested formulation (granules at 2RD - 20 kg a.i. ha^{-1}) can result in a quite persistent presence of ethoprophos in water (Robinson *et al.,* 1999) and as such, pose a threat to microcrustaceans that occupy an important position in food webs (Gustafsson *et al.,* 2010; Warming *et al.,* 2009). The observed high lethal effects (100% mortality) towards *D. magna* after the 48-h and 72-h exposure assays (one-tailed Fisher's exact test: $p < 0.001$) with runoff waters at the simulated 2RD (100% concentration) and less (Table IV.4) was not expected, since the documented 48-h EC_{50} of 200 μg L^{-1} for *D. magna* (EFSA, 2006) is much higher than the measured ethoprophos concentration in water of 19 μg L^{-1}. Other stressors than those involved directly with the pesticide use, such as freezing procedures related to sample treatment prior to the bioassays performance may have influenced the observed ecotoxic response, as discussed for the Maize scenario (see section 2.4.3.2.). However, a toxic response was observed and as such, runoff after a rain event as a surface waters contamination pathway simulated at a potato based exposure realistic "worst-case" scenario with the application of ethoprophos may possibly cause negative effects towards the aquatic cladoceran communities.

Taking into account the soil retention function through the aqueous extract (elutriates) where the soluble components of the pesticide are present, aquatic toxicity results (Table IV.4) differ from terrestrial results where both areas (R and BR) revealed to be toxic for collembolan and earthworms populations (see section 2.4.3.3). Only elutriates from soil R area showed high aquatic toxicity after 48- and 72-h exposure (48-h LC_{50} = 10.6% and 100% mortality, one-tailed Fisher's exact test: $p < 0.001$, respectively), while no effects on *D. magna* were observed on elutriate from BR soil area where no pesticide was applied (Table IV.4). Although ethoprophos residues were quantified at elutriates from BR area soil (21 μg L^{-1}, Table IV.3), the observed ecotoxicity results are in accordance with the documented ethoprophos 48-h EC_{50} of 200 μg L^{-1} for *D. magna* (EFSA, 2006). Given the measured pesticide concentration of 2200 μg L^{-1} in the elutriate from R area soil (Table IV.3), the observed 48-h LC_{50} of 10.6% corresponds to 233.2 μg L^{-1} (180 – 301 μg L^{-1}), which is in agreement with the documented effect value for *D. magna* of 48-h EC_{50} = 200 μg L^{-1} (EFSA, 2006).

2.5. Conclusion

The application of twice the recommended dosage of the insecticide ethoprophos representing possible misuse under the simulation of "worst-case" crop-based scenario for maize and potato crops caused effects on the reproduction of terrestrial organisms and on the survival of aquatic cladoceran if exposed to soil and leachate and elutriate waters, respectively. Leachate proved to be an important transfer pathway of groundwater contamination by ethoprophos under realistic Mediterranean agricultural practices and irrigation, as it resulted in the highest pesticide concentration in water samples from the maize and potato crop-based scenarios. Runoff was also considered as a relevant contamination pathway for the pesticide ethoprophos, although, the observed toxic effects on the aquatic cladoceran from low pesticide concentrations were possibly due to other factors than those resulting from the pesticide use. The ethoprophos exposure in the potato crop scenario to ethoprophos caused more toxic effects on non-target terrestrial and aquatic organisms than in the maize scenario at pesticide concentrations mimicking the recommended dosage and lower. This may be expected since the application dosage in potato is 10 times higher than the dosage needed for treating maize crops against soil insects. The observed pesticide movement associated with water flow during irrigation transporting the pesticide from row areas where the pesticide was applied to the surrounding area supports the idea that the pesticide moved horizontally and possibly causing toxic effects on the surrounding terrestrial non-target communities. The present study showed that groundwater may be at risk in irrigated agricultural fields and that terrestrial communities may be under threat when pesticide fate and effects are assessed using natural soil. Semi-field simulations based on crop scenarios under natural climate and soil conditions are a valuable tool for pesticide risk assessment linking pesticide fate and contamination pathways and resulting toxicity effects under realistically simulated pesticide stress. Pesticides fate under field realistic environmental conditions and their toxicity on biota should be taken into account when conducting future work on their fate and effects to contribute to a sustainable use of these pesticides.

2.6. Acknowledgments

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CHAPTER V

Effects of agricultural practices on the soil macro- and mesofauna communities of three crops in Mediterranean conditions.

Based on the following manuscripts:

Effects of agricultural practices on the soil macro- and mesofauna communities of potato and onion crops in Mediterranean conditions. Sara Leitão, Mª José Cerejeira, Paul J. Van den Brink and José Paulo Sousa (*in preparation*).

Effects of agricultural practices on the soil macro- and mesofauna communities of maize crop in Mediterranean conditions. Sara Leitão, Mª José Cerejeira, Paul J. Van den Brink and José Paulo Sousa (*in preparation*).

1. Abstract

Sustainable agricultural production relies on soil communities as the main actors in key soil processes necessary to maintain sustainable soil functioning. Soil biodiversity influences soil physical and chemical characteristics and thus the sustainability of crop and agro-ecosystems functioning. The present study aimed to study the influence of agricultural practices of three crops (potato, onion and maize) under Mediterranean climate conditions on soil macro- and mesofauna during their entire crop cycles. All crops are summer crops and were under tillage, mulching incorporation, as well as fertilizers and pesticides applications. As specific objectives the study aimed to: i) identify the composition of the soil macro- and mesofauna communities inhabiting the soil surface and soil *stratum*, respectively, and to ii) compare and link exposure and effects for each crop, assessing the relative impact of pesticides and other agricultural practices on the soil macro- and mesofauna communities. Soil macro- and mesofauna were collected using two methodologies through pitfall trapping and soil sampling. Soil feeding activity was also measured using the Bait Lamina strip method.

The community of soil macro- and mesofauna of the three crops field varied *versus* control site along the crops cycles. Main differences were due to arachnids, coleopterans, ants and adult Diptera presence and abundance. The feeding activity of soil fauna between control site and crop areas varied only for potato and onion crops vs. control site but not among crops. Concentration of pesticides residues in soil did not cause apparent negative effects on the soil communities. Significant differences of soil communities from potato and onion crops with the one from control site were observed at the beginning and during the crop cycle, but similarities were observed at the last sampling date after harvesting. The same was observed for the maize crop, indicating that soil communities recovered from the agricultural disturbances associated with crops management. An integrated approach such as the one adopted in present study, taking into consideration soil community's abundances, feeding activity and time variations along entire crop cycles of several crops under Mediterranean conditions, as well as soil exposure to pesticides residues in soil, may contribute to decision making towards a sustainable use of crop areas, including pesticide use and management practices.

Keywords: potato crop; onion crop; maize crop; irrigation; crop cycle; soil biodiversity; pesticide effects; agricultural sustainability.

2. Introduction

Most of the biodiversity of agricultural systems can be found in soil and is a keystone component for a sustainable crop production (Blanchart *et al.*, 2006; Brussaard *et al.*, 2007; Roger-Estrade *et al.*, 2010). The functions performed by soil biota through food web interactions have major direct and indirect effects on crop growth and quality, soil disease suppressions, nutrient cycling, soil structure and regulation of water cycle in soil, and, thus, on the sustainability of crop systems and agro-ecosystems functioning (Brussaard *et al.*, 2007; Carrillo *et al.*, 2011; Roger-Estrade *et al.*, 2010). Soil organisms, especially predators, also act through as beneficial organisms controlling several pest species and conferring resistance and resilience against disturbance and stress (Brussaard *et al.*, 2007).

Soil macrofauna has earthworms, ants and termites as the most important components of soil, as the importance of their activities has caused them to be designated "ecosystem engineers" moving through the soil changing its physical properties enhancing and mixing macroporosity and humidification, and building organo-mineral structures that promote microbial activity (Ayuke *et al.*, 2011; Domínguez *et al.*, 2010;). Other macrofauna groups, like isopods and diplopods, act as litter fragmenters, able to fragment litter enhancing microbial activity (Ayuke *et al.*, 2011; Domínguez *et al.*, 2010; Lavelle and Spain, 2001). Other macroarthropods, like spiders, carabids and chilopods are predators, exerting a top-down control of other macrofauna and mesofauna groups. Thus, these macrofauna groups also exert a direct or indirect influence on soil turnover rates, mineralization and humification of soil organic matter, soil texture, soil-water retention characteristics and C and N gas emission (Domínguez *et al.*, 2010). Soil mesofauna is mainly composed of microarthropods, such as Collembola, Acari (mites) and small Diptera and Coleoptera, and by small oligochaeta such as Enchytraeids. They belong to different trophic levels, but many species exert an important role as selective microbial grazers, facilitating microbial succession during organic matter decomposition (Lavelle and Spain, 2001).

Consequently, soil is no longer seen purely as a medium for plant growth, but also as a habitat for a number of organism community's actors in a plethora of multi-trophic interactions, and where biodiversity-ecosystem functioning relationships are complex. These soil organism communities are part of the biological resources of agro-ecosystems that must be preserved and taken into consideration in agricultural management decisions **(**Brussaard *et al.*, 2007; Roger-Estrade *et al.*, 2010**).** A consequence of this complexity is that a multidisciplinary approach is required to study it, embracing ecological concepts, knowledge of soil science, agronomy, ecophysiology and soil mechanics towards the decision on sustainable cropping systems minimizing pesticide use and other stressing agricultural practices (Brussaard *et al.*, 2007; Roger-Estrade *et al.*, 2010**).** The main management options comprise: tillage, crop rotation (and sequence) and organic matter management (Ayuke *et al.*, 2011; Brussaard *et al.*, 2007; Roger-Estrade *et al.*, 2010; van Cappelle *et al.*, 2012). Both soil meso and macrofauna are deeply affected by management and land-use changes (Blanchart *et al.*, 2006; Postma-Blaauw *et al.*, 2012). Effects of agricultural practices in soil mesofauna have been documented mainly for collembolan and mites (Dittmer and Schrader, 2000; Filser *et al.*, 2002; Frampton and Van den Brink, 2002; Heisler and Kaiser, 1995). Effects on arthropod communities of natural fields have also been registered by Filser *et al.*, (1996) and Loranger *et al.* (1999).

Soil tillage affects negatively or positively soil biodiversity and abundances, depending on the organism group, by modifying the relationships between organisms in the soil ecosystem (Roger-Estrade *et al.*, 2010). One of soil tillage positive effects is that it counteracts nutrient leaching and cleans the soil surface facilitating precise seeding (van Cappelle *et al.*, 2012). On the other hand, ploughing is often accompanied by the degradation of soil structure leading to soil surface sealing, erosion and a decrease in soil organic matter (van Cappelle *et al.*, 2012). Additionally, studies have observed that tilled agro-ecosystems with narrow crop rotation/short fallow management may lead to a decrease in species richness and dominance of some species, whereas, management characterized by no-tillage, crop rotations and organic amendments leads to an increase in species richness and overall density (Brussaard *et al.*, 2007).

In many cropping systems, organic matter is periodically returned to the soil (mulch-processing) either as litter, crop residues or as animal waste products, a major source of plant nutrients in soils with little inherent mineral fertility, enhancing soil fertility or

promoting soil rehabilitation (Ayuke *et al.*, 2011). This management action and the presence of soil fauna associated with plant and microorganisms and aboveground production, increases water and nutrient use efficiency sustaining the ecosystem functioning (Ayuke *et al.*, 2011; Brussaard *et al.*, 2007). However, soil cultivation and the amount and quality of organic matter applied can have either positive or negative effects on species richness in soil (Brussaard *et al.*, 2007). Additionally to this agricultural management options, irrigation and drainage that can influence positively the soil communities and agricultural sustainability depending on agro-ecological conditions (Brussaard *et al.*, 2007). The adoption of these practices should help increase food quality and quantity for the soil community and create a more suitable environment for their activities (Brussaard *et al.*, 2007). Pesticide effects evaluated under field conditions on natural soil fauna are not well documented, but effects on collembolan community's abundances and *taxa* (Çilgi *et al.*, 1993; Frampton, 1999) and in soil macro-, meso- and microfauna communities (Parfitt *et al.*, 2010) have been observed.

The present study intends to evaluate the influence of agricultural practices of three major crops (potato, onion and maize) under Mediterranean climate conditions on soil macro- and mesofauna during their entire crops cycles. These summer crops were commonly under tillage, mulching incorporation and particularly fertilizer and pesticide applications, being maize crop cycle longer than potato and onion. The main specific objectives of the present study were to i) identify the composition of the soil macro- and mesofauna communities inhabiting the soil surface and soil *stratum* ii) compare and link exposure and effects at each crop, assessing the relative impact of pesticides and other agricultural practices on the soil macro- and mesofauna communities.

3. Material and methods

3.1. Agricultural and Control sites and soil characteristics

The present study was carried out during the 2010 growing season at an agricultural field (39º26'25.66'' N and 8º29'51.53'' W, elevation 29 m above sea level) located in a

major agriculture region of Central Portugal, Ribatejo e Oeste (see section 1.3 of Chapter II). The average annual air temperature was 16ºC and the rainfall was 75 mm for this region (IM, 2010). The 2010 summer crop cycle (April to September) was characterized by an average air temperature of 21ºC, with an average rainfall of 18 mm at the agricultural field and a heavy rainfall of 63.8 mm in April prior planting, followed by a decrease in rain of 9.3 mm in May and 14.4 mm in June (SNIRH, 2013). The months of July, August and October were the driest in the last 20 years with almost no precipitation and high temperatures of more than 30ºC (IM, 2010). A reference site near the agricultural field site (39º26'31.08'' N and 8º30'15.30'' W, elevation 30 m above sea level) was selected as a Control site to compare the terrestrial communities' composition and variation along the crop cycle (Figure II.3). This control site is located at a slight higher quota and slope which prevented any contamination in terms of pesticide use from the selected agricultural field. For more details see section 1.1 of Chapter II. The maize crop field was divided in two areas (A and B) due to a clear visible presence of pebbles in one area (B) presenting a slight difference in terms of pH (potentiometric method, soil in water) with values of 5.8 and 6.5, respectively, and particle size distribution, although both were classified as sandy loam soils. The pedological properties of the study site soil areas are presented in Table II.2.

3.2. Cropping procedures

The agricultural field was composed of 14 ha of potato crop (*Solanum tuberosum* L.), 4.7 ha of onion crop (*Allium cepa* L.) and 34 ha of maize crop (*Zea mays* L.) (Figure II.3). Seedbed preparation for all crops was performed in early March by disc-harrowing to approximately 15/20-cm depth, to incorporate weeds and mulch from the previous year, and by mouldboard ploughing (20-cm depth), in which moist soil is inverted and a surface with little or no remaining plant residues is created (Figure V.1a). The soil preparation procedures ended with a second disc-harrowing at the same depth as the first.

Figure V.1: Agricultural site field in 2010: a) March; b) September; c) October.

Potato crop agricultural field (Figure V.2) was fertilized two days before planting with 700 kg ha^{-1} of NPK (13:13:21) fertilizer and 150 kg ha^{-1} of nitrogen fertilizer at 23% with trace elements S (15% SO_3) and Mg (4% MgO). Potato planting and furrowing took place in April 8th with young potatoes of the variety 'Hermes' placed individually along the rows (80-cm distance between rows and 25 cm between potatoes within each row). Planting was accompanied by the addition of an insecticide against wireworms and other soil organisms (Table V.1). The agricultural field/crop was fertilized for the last time in May 10th with 200 kg ha^{-1} of 30% nitrogen with 40% SO_2. The growing crop was treated with one herbicide to reduce annual grasses, five fungicide and two insecticide treatments by spraying under recommended dosages (Table V.1), with the appropriate spraying equipment (Tagri 600L). Potatoes were harvested in August 26th by revolving the top 25/30-cm soil layer, and the crop mulch was left in the field till soil incorporation in the next crop cycle.

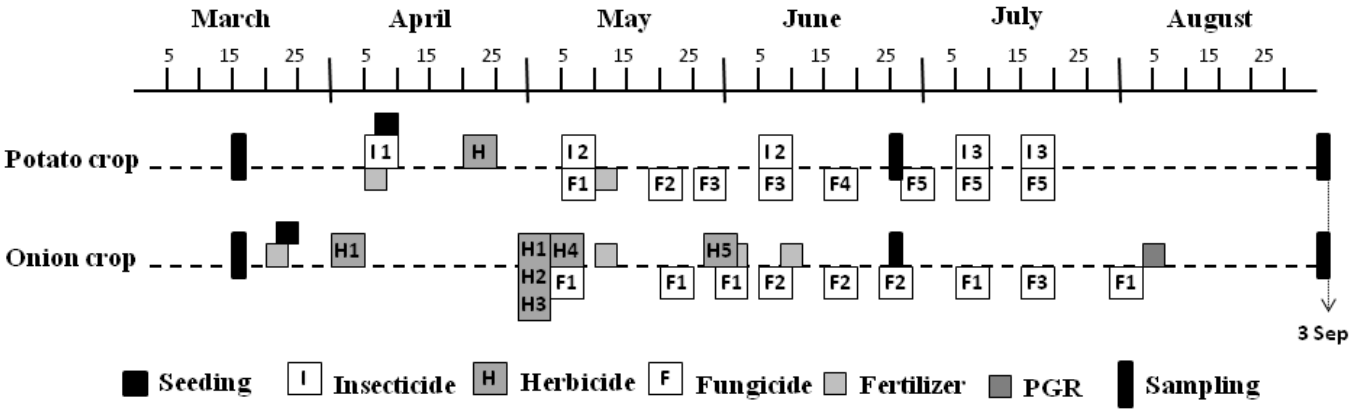

Figure V.2: Agricultural practices and sampling dates during the field management of potato and onion crops. For pesticides applications see Table V.1. PGR – Plant Growth Regulator.

Onion crop field (Figure V.2) was also subjected to fertilizer application two days before seeding with 300 kg ha^{-1} of a NPK (6:12:20) fertilizer, based on humic and enzymatic compounds to serve as bio-stimulation and phyto-hormonal agent and 200 kg ha^{-1} of a phosphate based fertilizer at 45% rate. Onion seeds of the variety 'Paudero' (*Allium cepa*, Lot 456711-M EXPRESSION F1) were seeded individually every 8 cm at a depth of 1 cm along rows of 20 cm apart in April 23rd. Three more fertilizer applications were performed (Figure V.2): one with a nitrogen (22%) based fertilizer at 200 kg ha^{-1} with the trace elements CaO, MgO and SO_3 at 6, 2 and 7%, respectively, followed by two applications of a N fertilizer at 150 and 100 kg ha^{-1} of Nitrogen at 32%. The growing crop had three more pesticides treatments (April 29th, May 6th and 29th) with several herbicides to reduce annual grasses and dicotyledon weeds (Table V.1). Fungicide treatments against downy mildew, among others, were regular till the end of the crop cycle with the application of three different active ingredients by spraying under recommended dosages with the appropriate spraying equipment (Tagri 600L). Fifteen days before onion harvesting (August 5th) a plant growth regulator (PGR - Maleic hydrazid as a potassium salt) was applied at 3.75 kg ha^{-1} (60% a.i. / f.p.) to suppress sprout and bud growth. Onions were harvested from August 20 to 23rd by revolving the 15-cm top soil layer and the mulch was also left in the field till next crop season.

Maize agricultural site field (Figure V.3) was fertilized one day before seeding with a potassium chloride fertilizer at 300 kg ha^{-1} with 60% K. Maize seeding and furrowing took place in April 26 to 28th with maize grains (*FAO600 PR33Y74*) placed in groups of 5 (so that at least one grain would germinate) every 17 cm at a depth of 1 cm along rows of 75 cm apart. Seeding was accompanied by the addition of 250 kg ha^{-1} of a NPK (18:46:0) fertilizer along planting rows. The growing crop had only one pre-emergent herbicide treatment to reduce annual grasses, and one insecticide treatment against abroad spectrum of insects by spraying under recommended dosages (Table V.1) with the appropriate spraying equipment (Tagri 600L). After seeding, weekly applications of a nitrogen based fertilizer at 32% were performed along with irrigation till July 7th (Figure V.3). Maize was harvested in September 20th and the mulch left in the field till soil incorporation in the next crop cycle.

Table V.1: List of pesticides applied to potato, onion and maize crops in 2010, dosage and target organism or disease.

	Application date	Type of action[a]	Active ingredient (a.i.)	a.i. / f.p. (% (p/p))	Dosage per ha	Target
Potato crop	8-Apr	I 1	chlorpyrifos	5	26 kg	Wireworms (*Agriotes* spp.), cutworm (*Agrotis segetum*), white grub cockchafer (*Melolontha melolonta*), scutigerella (*Scutigerella immaculata).*
	24-Apr	H	flufenacet + metribuzin	24 + 17.5	2 kg	Grasses and broad-leaved weeds
	7-May	F 1	cymoxanil + mancozeb	4+40	3 kg	Downy mildew *(Phytophthora infestans)*
	7-May, 7-Jun,	I 2	Thiametoxam	25	80 g	Aphids (*Macrosiphum euphorbiae)* and green aphid (*Myzus persicae)*, colorado potato beetle (*Leptinotarsa decemlineata*)
	20-May	F2	dimethomorph + mancozeb	7.5 + 66.7	2.4 kg	Downy mildew *(Phytophthora infestans)*
	28-May, 7-Jun	F3	Fluazinam	39 or 500g/L	400 ml	Downy mildew and with secondary action on *Botrytis cinerea.*
	16-Jun	F 4	Fluazinam	40 or 500g/L	400 ml	Downy mildew *(Phytophthora infestans)*
	29-Jun, 9, 19-Jul	F 5	mancozeb	75	2.5 kg	Downy mildew and early blight *(Alternaria solani)*
	9, 19-Jul	I 3	clorpyrifos	44 ou 480g/L	2 L	Colorado potato beetle (*Leptinotarsa decemlineata*)
Onion crop	3, 29-Apr	H 1	pendimethalin	33.8 or 330g/L	1.5 L	Annual grasses and dicotyledon
	29-Apr	H 2	oxyfluorfen	41 or 480g/L	150 ml	Annual grasses and dicotyledon
	29-Apr	H 3	ioxynil	21.2 or 225g/L	1 L	Dicotyledon weed species
	6-May	H 4	oxyfluorfen	41 or 480g/L	100 ml	Annual grasses and dicotyledon
	7, 21, 31-May, 9, 29-Jul	F 1	mancozeb	75	2.5 kg	Downy mildew *(Phytophthora infestans)*, onion rust (*Puccinia allii*) and early blight *(Alternaria sp.)*
	29-May	H 5	ioxynil	21.2 or 225g/L	1.2 L	Dicotyledon weed species
	8, 17, 28-Jun	F 2	azoxystrobin	23.1 or 250g/L	1 L	Downy mildew and leaf blight *(Stemphylium vesicarium)*
	19-Jul	F 3	copper (oxychloride) + iprovalicarb	40.6 + 8.4	1.5 kg	Downy mildew *(Phytophthora infestans)*,
Maize crop	30-Apr	H	s-metolachlor + terbuthylazin	28.9 +17.4 or 312.5g/L+187.5g/L	3.5 L	Annual grass weeds, annual dicotyledon weeds and yellow nutsedge (*Cyperus esculentus*)
	30-Apr	I	cypermethrin	10.9 or 100g/L	750 ml	Broad spectrum insecticide (pyrehtroid)

[a] Type of action: I – insecticide; H – herbicide; F – fungicide.

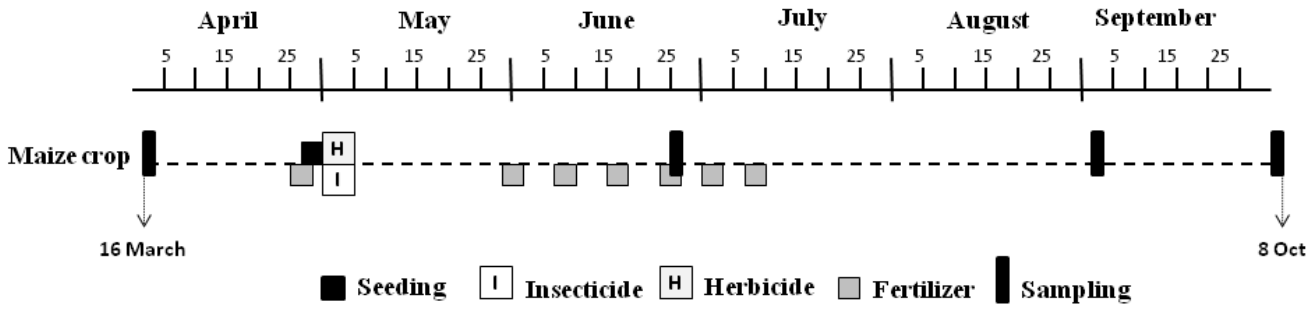

Figure V.3: Agricultural practices and sampling dates during field management of the maize crop. For pesticides applications see Table V.1.

The three crops were irrigated every two days during the entire crop cycle according to crops needs (7/8 mm), by center-pivot (automated sprinkler that rotates in a half a circle area) and by sprinklers, with the exception of the maize crop that was irrigated till August 31st. At the end of the crop cycle all crop fields returned to pasture (Figure V.1 b and c).

3.3. Sampling of soil, macro- and mesofauna and soil feeding activity

Three samplings were performed along potato and onion crops cycle consisting of a first sampling after soil preparation and before onion seeding and potato planting in March 16th (T0), followed by a second sampling during pesticide treatments in June 25th (T1) and the last sampling after harvesting in September 3rd (T2) (Figure V.2). At maize crop field, four sampling campaigns were performed along the crop cycle consisting of a first sampling after soil preparation and before maize seeding in March 16th (T0), followed by a second sampling after pesticide treatments in June 25th (T1) and a third after fertilizations and at the end of the crop cycle in September 3rd (T2). The last sampling was performed after harvesting in October 8th (T3) (Figure V.3). Ten sampling points were selected along a grid in potato and onion crop fields, with 60-m distance among them occupying an area of 2.5 ha and 3.5 ha, respectively, whereas at maize crop, two areas were delimited: A and B (with visibly more round pebbles in soil than A area, as previously referred), with 11 and 8 sampling points (80-m distance among them) occupying an area of 8.3 ha and 6.7 ha, respectively. At each sampling date for each crops one composite soil sample consisting of 5 random subsamples (top 10-cm soil

layer) along the respective sampling points were taken and preserved in plastic bags at -20ºC till pesticide residues analyses were performed (see section 3.5).

At each sampling point of all crops and Control site (with 8 sampling points; Figure V.4) the following samples were taken: i) a soil core (2-cm width and 10-cm long) was collected and preserved at 4 to 6ºC for 24 h till soil pH (1M KCl) (ISO, 1994) and moisture content (ISO, 1998) measurements; ii) a pitfall trap (a plastic vial with 10-cm diameter x 15-cm depth filled up to half with anti-freezing liquid and covered with a plastic lid suspended at 2-cm higher with wire strips (Figure V.5b) was placed for ten days to collect surface-dwelling macrofauna (Wardle *et al.*, 1999) ; and iii) one soil core sample (5-cm diameter x 7-cm depth) was collected to assess the soil dwelling fauna and maintained in plastic bags till organisms extraction during the following day. On the last two sampling dates for potato and onion crops and three sampling dates for maize crop, six bait lamina strips (Hamel *et al.*, 2007) filled with a paste made of cellulose, bran and activated charcoal were placed in soil for 10 days to evaluate soil fauna feeding activity in situ. An average of 4 sampling points (6 baits each) at the Control site, 6 at potato and onion fields, and 5 at maize crop areas A and B (different numbers of sampling points due to difficulties of placing the strips in the soil) were used per sampling date. The feeding activity was assessed by counting the number of holes in each strip that were half and /or totally eaten.

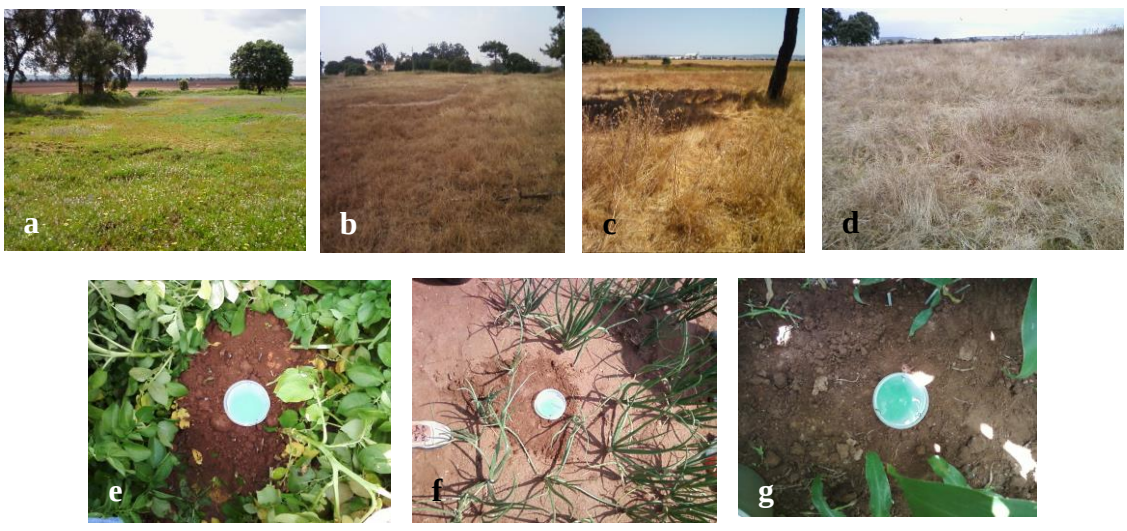

Figure V.4: Control site in: a) March; b) June; c) September; d) October. Pitfall trap in: e) potato crop; f) onion crop; g) maize crop.

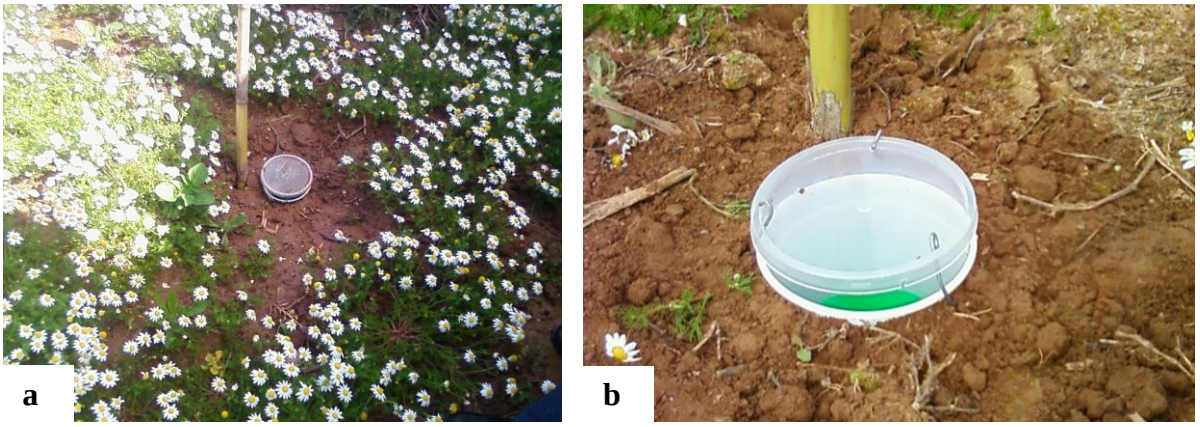

Figure V.5: Pitfall trap in control site (a) and pitfall trap detail (b).

3.4. Macro- and mesofauna extraction, sorting and identification

After ten days in the field, the total content of each pitfall trap was sieved in laboratory using a tight mesh cloth and preserved in 80% ethanol till sorting. Soil fauna from soil cores was extracted using a Macfadyen high-gradient extractor into small plastic vials containing 70% ethanol (a period of 10 days was adopted for extraction). Meso- and macrofauna were counted and sorted into broad taxonomic groups (Aracnidae, Collembola, Coleoptera, Acari, Diptera, Hemiptera, Hymenoptera and Dermoptera among others) and subsequently at family and morphospecies level (organisms that are morphologically similar), and preserved in 70% alcohol. A total of 143 morphospecies were considered among pitfall trapping (116) and soil sampling (45). All taxa were identified according to Quigley and Madge (1988) and Unwin (1984).

3.5. Pesticide characteristics, fate, ecotoxicity and residue analyses

Pesticides active ingredients applied to the three crops were characterized for their physico-chemical properties, particularly the environmental partition coefficients and persistence, from scientific literature and specific data bases (Table V.2). Pesticides potential fate in the environment (Table V.2) was evaluated by a first level of a multi-compartmental environmental fate model, Fugacity Model ('level I, version 3.00, 2004, Trentu University, Canada', Mackay, 2001) to assess the relevance of targeted environmental compartments exposure to pesticides and the Groundwater Ubiquity

Score (GUS) to assess their leaching potential (Gustafson, 1989). The Fugacity Model uses key chemical properties as molecular mass, temperature, water solubility, vapor pressure and log Kow and results are given in Predicted Environmental Distribution (PED) percentages. GUS is based on environmental fate properties of the chemical such as soil degradation half-life (DT50) and organic-carbon sorption coefficient (Koc). Pesticides ecotoxicity information on terrestrial organisms such as birds (50% Lethal Dose - LD_{50}), honey bees (LD_{50}) and earthworms (50% Lethal Concentration – LC_{50} and No Observed Effect Concentration - NOEC) and other environmental relevant information was compiled from scientific literature and specific data bases, (Table V.3). The latter organisms were selected for being considered relevant organism given that ecotoxicity tests with these organisms area required for the terrestrial pesticides environmental risk assessment (CR, 2013).

Table V.2: Pesticides active ingredient (a.i.) characteristics: physico-chemical properties, persistence and potential fate[1]

		a.i.	Sw 20ºC (mg L^{-1})	VP (mPa)	H (Pa m^3 mol^{-1})	Kow	Koc (mL g^{-1})	$DT50_{field}$ soil (d)	DT50 water-sed (d)	PED Water	PED Soil	PED Sediment	PED Suspended solids	PED Aquatic biota	PED Air	PED Aerosol	GUS
Potato and onion crops	**Insecticide**	chlorpyrifos	1.05	1.43	0.478	4.7	8151	21	36.5	2.15	95.4	2.12	0.0663	5.39E-03	0.211	0.0104	0.15
		thiamethoxam	4100	6.6E-06	4.70E-10	-0.13	70	39	40	86.6	13.1	0.291	9.09E-04	1.13E-06	8.36E-09	9.62E-06	3.66
	Herbicide	flufenacet	56	0.09	9.00E-04	3.2	401	40	81	41.1	57.6	1.28	0.04	3.25E-03	4.92E-03	1.73E-03	2.38
		ioxynil	64.3[2]	2.04E-03	1.5E-05	2.2	-	5	4.6	87.4	12.3	0.273	8.52E-03	6.93E-04	0.248E-04	0.160E-04	1.18
		metribuzin	1165	0.121	2.00E-05	1.65	-	19	50	96.5	3.40	0.0756	2.36E-03	1.92E-04	2.34E-04	4.16E-05	2.57
		oxyfluorfen	0.116	0.026	0.0238	4.86	2891-32381[2]	73	-	3.60	94.1	2.09	0.0654	5.31E-03	0.0615	0.0579	0.19
		pendimethalin	0.33	1.94	2.73E-03	5.2	17581	90	16	0.690	96.8	2.15	0.0673	5.47E-03	0.234	6.29E-03	-0.39
	Fungicide	azoxystrobin	6.7	1.10E-07	1.10E-07	2.5	589	180.7	205	43.4	55.3	1.23	0.0384	1.09E-05	6.51E-08	7.68E-03	2.53
		cymoxanil	780	0.15	3.80E-05	0.67	38-237[2]	3.5	0.3	99.6	0.412	9.17E-03	2.86E-04	2.33E-05	7.79E-04	2.40E-05	-0.37
		dimethomorph	28.95	9.85E-04	2.04E-05	2.68	290-566[2]	44	38	69.8	29.6	0.657	0.0205	1.67E-03	1.89E-04	1.52E-03	2.56
		fluazinam	0.135	7.5	25.9	4.03	1705 – 2316[2]	16.4	3.1	6.24	59.2	1.32	0.0411	3.34E-03	33.1	0.056	1.73
		iprovalicarb	17.8	7.9*-05	1.40E-06	3.2	106	15.5	181	81	18.6	0.412	0.0129	2.59E-05	2.15E-05	1.17E-03	2.35
		mancozeb	6.2	0.013	5.90E-04	1.33	998	18	76	98.1	1.86	0.0413	1.29E-03	1.05E-04	0.0117	3.12E-03	-1
Maize crop	**Herbicide**	s-metolachlor	480	3.7	2.20E-03	3.05 25ºC	226.1[3]	21	47.5	49.6	49.3	1.09	0.0342	2.78E-03	0.0219	7.10E-04	1.94
		terbuthylazine	6.6	0.12	3.24E-03	3.4	231[3]	22.4	70	30.5	67.9	1.51	0.0472	3.83E-03	0.0141	6.12E-04	3.07
	Insecticide	cypermethrin	0.009	2.3E-04	2.0E-02	5.3	26492-144652[2]	69	17	0.0277	97.7	2.17	0.0679	5.52E-03	1.18E-04	0.0213	-2.12

[1] FOOTPRINT, 2013; [2] MacBean C, 2012; [3] Kfoc; SW – Solubility in water at 20ºC; VP - Vapour pressure at 25ºC; H - Henry's law constant at 25ºC; Kow - Octanol-water partition coefficient as log P at pH7, 20ºC; Koc - Organic carbon sorption coefficient; DT50 – Half-life in soil at 20ºC under aerobic conditions; PED - Predicted Environmental Distribution (%) according to Mackay (2001) - PED < 20%: very low affinity; 20% ≤ PED < 40%: low affinity; 40% ≤ PED < 60%: average affinity; 60% ≤ PED < 80%: high affinity; PED ≥ 80%: very high affinity; GUS - Groundwater Ubiquity Score, GUS = log(DT50) x (4 - log (Koc)): if GUS > 2.8 pesticide is likely to leach; if GUS < 1.8 pesticide is unlikely to leach; if GUS 1.8 - 2.8 leaching potential is transitional. * Suppresses sprout and bud growth, absorbed by leaves and roots and translocated.

Table V. 3: Terrestrial ecotoxicological data [1] and other observations on the pesticides applied to potato, onion and maize crops.

		Birds		Honey bees		Earthworms (*Eisenia foetida*)		
		Species	**LD_{50} (mg Kg^{-1})**	**exposure**	**48 h LD_{50} (µg bee^{-1})**	**14 d LC_{50} (mg Kg^{-1})**	**14 d NOEC (mg Kg^{-1})**	**Other observations [1,2]**
Insecticides	chlorpyrifos	*Colinus virginianus*	13.3	Contact	0.059	129	12.7	Toxic to Collembola *Folsomia candida*, 35day LC_{50} Mortality 0.2 mg kg^{-1}. Harmful at 1 kg ha^{-1} to *Alphidius colemani* and *Typhlodromus pyri*. Harmful to Carabidae and Staphylinidae, Tenebrionidae.
		Anas platyrhynchos[2]	490					
	cypermethrin	*Anas platyrhynchos*	> 10000	Contact	0.02	> 100	-	Harmful to *Typhlodromus pyri.* Not toxic to Collembola.
	thiamethoxam	*Anas platyrhynchos*	576	Oral	0.005	> 1000	5.34	-
		Colinus virginianus[2]	1552					
Herbicides	flufenacet	*Colinus virginianus*	1608	Oral	> 170	219	>4.0	*Aphidius rhopalosiphi* 29% mortality effect at 0.06 kg ha^{-1}. Harmful at 0.06 kg ha^{-1} to *Typhlodromus pyri* with 100% mortality.
	ioxynil	*Colinus virginianus*	62	Oral	10.1	> 60	20.0	*Aphidius rhopalosiphi* and *Typhlodromus pyri*, 55.6% and 98.7% Mortality effect respectively at 0.625 kg ha^{-1}. No acute or chronic risks predicted by risk assessment.
	metribuzin	*Colinus virginianus*	164	Oral	53	427	> 5.25 (56d)	Moderately harmful at 1 kg ha^{-1} to *Aphidius rhopalosiphi* and *Typhlodromus pyri.*
	oxyfluorfen	*Colinus virginianus*	> 947	Contact	> 100	> 1000	24.09	*Folsomia candida* NOEC reproduction 1.25 mg kg^{-1}. Harmless at 1 kg ha^{-1} to *Typhlodromus pyri* and *Pardosa* spp.
	pendimethalin	*Anas platyrhynchos*	1421	Contact	100	> 1000	33.45	*Aphidius rhopalosiphi* and *Typhlodromus pyri*, 100% and 38.7% Mortality effect respectively, at 3.2 kg ha^{-1}.
	s-metolachlor	*Anas platyrhynchos*	> 2510	Oral	> 85	570	<2.54	Harmless to *Aphidius rhopalosiphi.*
	terbuthylazine	*Colinus virginianus*	> 1236	Oral	> 22.6	> 141.7	-	Harmful at 1 kg ha^{-1} to *Typhlodromus pyri* and *Aphidius rhopalosiphi.*
Fungicides	azoxystrobin	*Colinus virginianus*	> 2000	Oral	25	283	3	Harmless to non-target organisms, including predatory mites and bugs, spiders, lacewings, hoverflies, ladybirds, carabid beetles, parasitoid wasps and bees, under field conditions at field application rates. E.g. LR50 (48h) *Aphidius rhopalosiphi* > 1135 g ha^{-1}
		Anas platyrhynchos [2]	> 2000					
	copper oxychloride	*Colinus virginianus*	173	Oral	12.1	> 489.6	< 15 (as Cu 8w)	Harmful at 1 kg $ha^{-1}$to *Aphidius rhopalosiphi* and *Typhlodromus pyri* as Cu.
	cymoxanil	*Colinus virginianus*	> 2000	Oral	> 85.3	> 1000	6.6	-
	dimethomorph	*Colinus virginianus*	> 2000	Oral	> 32.4	> 500	60	Dimethomorph 150 g L^{-1} is harmless to various non-target arthropods
	fluazinam	*Colinus virginianus*	1782	Oral	> 100	> 1000	< 0.35	-
		Anas platyrhynchos [2]	> 4190					
	iprovalicarb	*Colinus virginianus*	> 2000	Oral	> 199	> 1000	3.37	No negative effects on soil organisms up to 4.95 kg ha^{-1}
	mancozeb	*Median across species*	> 2000	Oral	140.6	> 299.1	20	Mancozeb is of low toxicity to the majority of non-target and beneficial arthropods.
		Passer domesticus [2]	> 1290					
Plant growth regulator	maleic hydrazide (potassium salt) [2]	*Anas platyrhynchos*	> 2250	Oral	> 100	> 1000	-	Harmless to *Chrysoperla carnea*, *Poecilus cupreus*, *Aleochara bilineata* and *Pardosa* spp. Harmful to *Aphidius rhopalosiphi* and *Typhlodromus pyri* at 4 kg K salt ha^{-1}.

[1] FOOTPRINT, 2013; [2] MacBean C, 2012.

Pesticides residues in soil samples from potato crop field were analysed for (limit of quantification - LOQ in brackets): chlorpyriphos (0.015 mg kg^{-1}), cymoxanil (0.05 mg kg^{-1}), dimethomorph (0.05 mg kg^{-1}), fluazinam (0.03 mg kg^{-1}), flufenacet (0.05 mg kg^{-1}), mancozeb (0.5 mg kg^{-1}), metribuzin (0.05 mg kg^{-1}) and thiamethoxam (0.05 mg kg^{-1}). Soil samples from onion crop field were analysed for the following pesticides residues (quantification limits - LOQ in brackets): azoxystrobin (0.06 mg kg^{-1}), ioxynil (0.05 mg kg^{-1}), mancozeb (0.5 mg kg^{-1}), oxyfluorfen (0.06 mg kg^{-1}), pendimethalin (0.05 mg kg^{-1}) and copper (1.0 mg kg^{-1}). Maize crop soil samples from both field zones were analysed for s-metolachlor and terbuthylazin with a LOQ of 0.05 mg kg^{-1}, and for cypermethrin residues with a LOQ of 0.015 mg kg^{-1}. The pesticides cymoxanil, dimethomorph, flufenacet, metribuzin, pendimetalin and thiamethoxam were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS), whereas azoxystrobin, chlorpyriphos, fluazinam, oxyfluorfen and cypermethrin were analyzed by liquid extraction/cleanup followed by gas chromatography/mass spectrometry (LE/GC-MS). Mancozeb and copper residues in soil were analyzed by headspace-gas chromatography-mass spectrometry/ mass spectrometry (HS-GC/MS/MS) after an acidic hydrolysis to yield CS2 and by inductively-coupled plasma mass spectrometry (ICP-MS) acc. to DIN EN ISO 17294-2 after an *aqua regia* digestion, respectively. The herbicide ioxynil was analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) after an acidic extraction. S-metolachlor and terbuthylazin residues were analysed by liquid extraction followed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS). All pesticide residue analyses were performed by an independent laboratory.

3.6. Statistical analysis

To evaluate the changes of soil fauna (both meso- and macrofauna) between the three crops during agricultural practices and the Control site over time, a Principal response curves (PRC) analysis was performed using the multivariate analysis statistical program CANOCO (Ter Braak, 2009). This method is based on a Redundancy Analysis (RDA) and has the advantage of integrating both treatments (crop sites) and time and compare the relative differences between treatments and the control site over time.

Two data sets were used, maize crop vs. Control data and potato and onion crops vs. Control data, due to differences in the number of sampling dates (4 and 3, respectively). Prior to the analysis a log(x+1) transformation was applied to normalize the presence of a high number of null and high result values in both data sets. The significance of the analysis was evaluated by Monte-Carlo permutations ($p < 0.05$). To evaluate the species responsible for the observed variations by the agricultural practices for each crop and crop area in maize, the species scores on the first axis were used (b_k), assuming that a higher positive score implied a decrease in abundance of that species in crop areas in comparison to control, and vice-versa. Species with score around zero indicted species that were not positively nor negatively affected by the treatments.

To assess significant differences between the soil communities of the three crops and the Control site for each sampling date, a one-way analysis of variance (ANOVA) was performed using the sample scores on axis 1 from each RDA analysis, followed by Dunnett test. To assess differences among the crops potato and onion and maize areas A and B, respectively, a Tukey test ($p < 0.05$) was performed after each ANOVA for each sampling date and soil organisms results with the two sampling methodologies previously refereed. These analyses were performed using STATISTICA 7.0 (Stat Soft Inc., 2004). Before running the ANOVAs, homogeneity of the variance was tested for each sampling date (T0 to T3) using Levene's test. If the data did not meet the requirements for homogeneity of variance, a logarithm transformation was performed (log (x+1)), and if assumptions were violated even after data transformation, a non-parametric Kruskal-Wallis test followed by multiple comparisons with control was used.

Soil meso- and macrofauna abundances and number of taxa (morphospecies) from all study sites (agricultural and Control) and at each sampling date were tested for significant differences by performing a one-way analysis of variance (ANOVA) of a "within-between" subject design, followed by a Newman-Keuls test for multiple comparisons. A p of approximately < 0.01 level of acceptance was adopted as significantly relevant differences. Feeding activity results were evaluated for significant differences also for both data sets by a one-way analysis of variance (ANOVA) followed by a Newman-Keuls test for multiple comparisons ($p < 0.05$).

4. Results

4.1. Soil pH, moisture and pesticide residues

Agricultural soil pH and moisture for all crops and control site varied during the crop cycle between 4.8 to 6.4 and 0.9 to 17.0 % relative humidity respectively (Figure V.6).

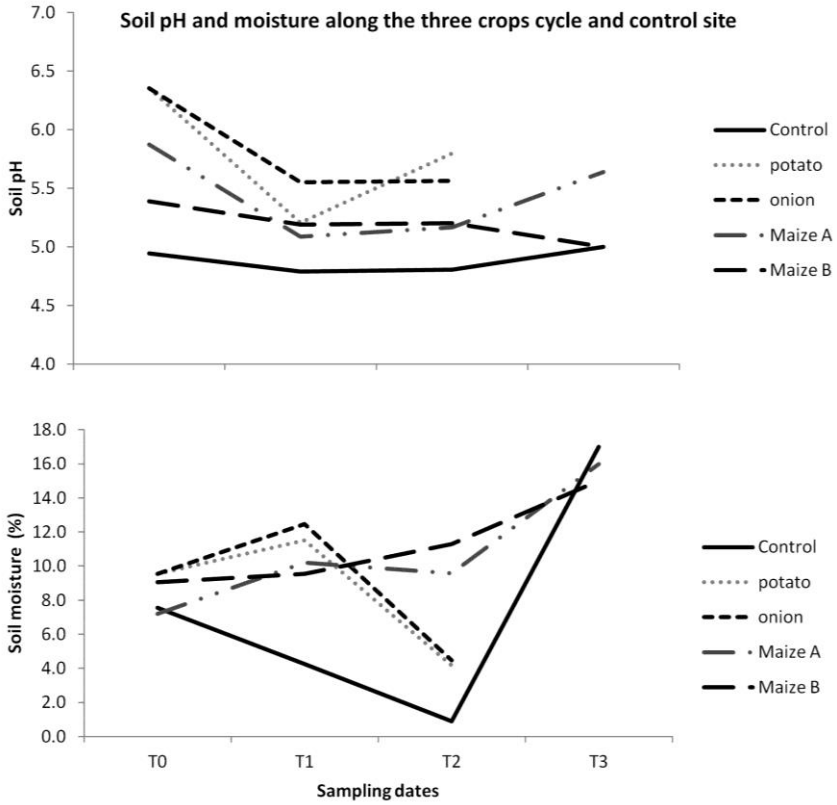

Figure V.6: Agricultural soil pH and moisture variation along the three crops cycles and control site.

No pesticide residues were detected and quantified in the soil from the Control site as expected. Pesticides concentrations in soil along the crop cycle from the three crops agricultural field are presented in the following table.

Table V.4: Pesticide concentrations in soil from potato, onion and maize (areas A and B) crops field during the 2010 agricultural season. *(Note: For complete list of pesticides see ANNEX VI).*

Crop	Sampling date	Type of action [a]	Pesticides	Concentration (mg kg^{-1})	
Potato	T2 - 3-Sep	F	dimethomorph	0.067	
		I	chlorpyrifos	0.063	
Onion	T1 - 25-Jun	F	copper	5.4	
	T2 - 3-Sep	F	copper	5.4	
		H	pendimethalin	0.14	
				A	**B**
Maize	T2 - 3-Sep	H	s-metolachlor	0.16	0.13
	T3 - 8-Oct	H	s-metolachlor	0.11	0.11

[a] H – herbicide; F – fungicide; I – Insecticide

4.2. Composition of the soil macro- and mesofauna communities

Soil **macrofauna** collected with pitfall traps at **potato and onion crop** fields during both crops cycle showed a dominance of arachnids, coleopterans and adult dipterans (Table V.5). Significant differences on macrofauna **abundances** were observed among crops ($F_{(2, 25)} = 10.04$, $p < 0.001$), although important differences could only be seen between Control site's *versus* onion crop, and between potato and onion crops during crops development and agricultural practices (T1). No significant differences in abundance were observed between the sampling dates ($F_{(2, 50)} = 0.630$, $p > 0.05$), nor in terms of **number of *taxa*** (morphospecies) between agricultural and Control sites ($F_{(2, 25)} = 3.15$, $p > 0.05$) and between sampling dates ($F_{(2, 50)} = 3.32$, $p < 0.05$).

Soil **mesofauna** extracted from soil samples at **potato and onion crops** field along the crops cycle showed a clear dominance of collembolans and mites (Acariformes) (Table V.6). Significant differences in mesofauna **abundances** were observed during crops development (T1) between Control site and onion crop's communities (Newman-Keuls test; $p < 0.001$), but not between crops as observed for macrofauna. However, significant differences were observed in terms of the **number of mesofauna *taxa*** collected during the crops cycle ($F_{(2, 50)} = 28.07$, $p < 0.001$) at the Control site during crop developments (T1) and after harvesting (T2) and between Control site and the onion crop before crop introduction in the field (T0).

At **maize crop** field, soil **macrofauna** collected with pitfall traps during the crops cycle showed higher abundances of arachnids, coleopterans and adult dipterans as at potato and onion crop, and occasionally of ants (Hymenoptera) (Table V.5). No significant differences in **abundance** of macrofauna communities were observed among the maize crop areas A and B and with Control site, with the exception of maize area A and Control site at late crop development (T2, Newman-Keuls test, $p < 0.01$). During the crop cycle, significant differences in macrofauna abundances ($F_{(3, 72)} = 125.08$, $p < 0.0001$) were observed at the Control between all sampling times except during crop development (T2) and before seeding (T0) and the early stages of crop growth (T1). At maize area A and B significant differences were observed for macrofauna abundances among the sampling dates during crop development (T1 and T2) and after harvesting (T3); and between before seeding (T0) and after harvesting (T3). Maize area A showed also a significant difference in macrofauna abundance before seeding (T0) and during crop development (T2). In terms of **macrofauna *taxa*,** also significant differences were found among sampling times within each treatment ($F_{(3, 72)} = 8.41$, $p < 0.001$), with very similar pattern described for the abundances. Additionally, significant differences on *taxa* richness were also observed between Control site and both maize areas during full crop development (T2) and at the latter stage of crop cycle (T3).

Soil **mesofauna** extracted from soil samples at **maize crop** along the crops cycle showed a clear dominance of collembolans and mites (Acariformes) as at potato and onion crops (Table V.6). No differences of mesofauna communities **abundances** were observed among the maize crop areas A and B as observed for macrofauna, but significant differences were observed at both maize areas with Control site ($F_{(2, 24)} = 5.51$, $p < 0.01$) at all sampling dates till harvesting (T0 to T2). During the crop cycle, significant differences in mesofauna abundances ($F_{(3, 72)} = 81.44$, $p < 0.0001$) were observed within each treatment except for T2 and T0 in Control and Maize area B and T2 and T0 and T1 in Maize area A. In terms of the **number of mesofauna *taxa*,** differences between treatments within the same time were less evident (only found between Control and both Maize areas at T2 and T3). The same was observed for differences during the crop cycle at each treatment ($F_{(3, 72)} = 19.23$, $p < 0.0001$); with the exception of Control site where differences were found among all sampling times (expect between T0 and T3), on the other two Maize areas significant differences were only observed between T0 and T1.

Table V.5: Composition of soil macrofauna collected with pitfall traps, expressed in total abundances (nº of taxa / morphospecies in brackets) of major taxonomic groups in each sampling date (T0 to T3 according to Figures V.2 and V.3).

Sampling date	**T0**				**T1**					**T2**					**T3**		
Crop site	**Control**	**P & O**	**MA**	**MB**	**Control**	**P**	**O**	**MA**	**MB**	**Control**	**P**	**O**	**MA**	**MB**	**Control**	**MA**	**MB**
Class Gastropoda	19 (2)	1 (1)	68 (1)	114 (2)	0	0	0	0	0	0	0	0	9 (1)	5 (1)	0	2 (1)	0
Order Araneae	213 (14)	57 (8)	36 (8)	60 (5)	163 (7)	124 (3)	47 (8)	93 (5)	119 (4)	32 (6)	11 (4)	27 (5)	101 (9)	67 (3)	78 (7)	5 (3)	4 (2)
Order Opiliones	0	0	0	7 (1)	1 (1)	0	1 (1)	4 (1)	8 (1)	0	1 (1)	0	11 (1)	15 (1)	13 (1)	6 (1)	19 (1)
Order Coleoptera	40 (16)	55 (10)	52 (10)	46 (14)	170 (14)	306 (15)	54 (12)	483 (13)	571 (7)	13 (5)	113 (11)	102 (12)	605 (9)	767 (12)	87 (20)	15 (7)	22 (4)
Order Diptera	93 (6)	176 (13)	164 (11)	54 (6)	125 (5)	366 (13)	159 (10)	107 (6)	117 (6)	38 (3)	1022 (7)	233 (8)	169 (9)	119 (6)	82 (9)	21 (6)	17 (6)
Order Hymenoptera	351 (1)	7 (4)	9 (3)	8 (4)	895 (3)	14 (5)	23 (1)	32 (4)	6 (2)	179 (3)	7 (4)	32 (3)	71 (8)	61 (6)	398 (4)	40 (3)	85 (3)
Order Hemiptera	9 (3)	5 (2)	7 (3)	1 (1)	75 (5)	4 (3)	12 (4)	7 (2)	23 (1)	9 (5)	3 (2)	6 (4)	102 (3)	55 (2)	16 (2)	2 (1)	2 (1)
Order Dermaptera	0	0	0	1 (1)	0	0	1 (1)	0	0	0	4 (1)	9 (1)	42 (1)	57 (1)	0	0	0
Class Chilopoda	1 (1)	0	0	1 (1)	0	5 (1)	0	0	2 (1)	0	1 (1)	1 (1)	2 (1)	4 (1)	0	0	0
Order Isopoda	10 (1)	0	0	2 (1)	0	0	1 (1)	0	0	0	0	0	0	0	8 (1)	0	0
Class Diplopoda	3 (1)	0	0	3 (1)	0	0	0	0	0	1 (1)	0	0	0	5 (1)	3 (1)	0	37 (1)
Order Siphonaptera	2 (1)	0	0	0	0	0	1 (1)	1 (1)	1 (1)	0	0	0	0	0	0	0	0
Order Lepidoptera	0	0	0	0	1 (1)	1 (1)	0	0	0	1 (1)	83 (1)	3 (1)	1 (1)	0	0	0	0
Order Orthoptera	0	0	0	0	7 (1)	0	2 (1)	0	0	1 (1)	0	1 (1)	3 (1)	3 (1)	21 (2)	0	0
Order Trichoptera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (1)
Order Psocoptera	0	0	0	0	1 (1)	0	0	0	0	0	0	0	0	0	0	0	0
Order Isoptera	6 (1)	0	0	0	0	0	0	0	0	26 (1)	0	0	0	0	0	0	0
Total abundance	747	294	336	297	1438	820	301	727	847	300	1245	503	1116	1158	706	91	187
Total taxa	47	37	36	37	38	41	40	32	23	26	32	36	44	35	47	22	19

P – Potato crop; O – onion crop; MA – maize crop area A; MB – maize crop area B.

Table V.6: Composition of soil mesofauna collected from soil samples, expressed in total abundances (nº of taxa / morphospecies in brackets) of major taxonomic groups in each sampling date (T0 to T3 according to Figures V.2 and V.3).

Sampling date	**T0**				**T1**					**T2**					**T3**		
Crop site	**Control**	**P & O**	**MA**	**MB**	**Control**	**P**	**O**	**MA**	**MB**	**Control**	**P**	**O**	**MA**	**MB**	**Control**	**MA**	**MB**
Oder Collembola	541 (6)	1666 (6)	1880 (6)	9031 (5)	6234 (3)	269 (5)	79 (4)	346 (4)	197 (4)	503 (1)	1762 (4)	604 (3)	626 (4)	893 (5)	2373 (7)	299 (5)	169 (5)
Order Araneae	0	0	0	0	2 (1)	0	0	0	0	0	0	0	0	0	0	0	0
Class Symphyla	0	0	0	1 (1)	0	0	0	0	0	0	0	0	1 (1)	0	0	1 (1)	0
Class Diplopoda	0	0	0	4 (1)	0	0	0	0	0	0	0	0	0	1 (1)	1 (1)	0	1 (1)
Class Chilopoda	3 (1)	0	1 (1)	0	0	5 (1)	1 (1)	0	1 (1)	0	0	0	0	0	7 (1)	0	0
Order Isopoda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23 (1)	0	0
Order Diptera	10 (2)	1 (1)	10 (1)	2 (1)	2 (1)	1 (1)	0	0	2 (2)	0	0	1 (1)	2 (2)	0	1 (1)	1 (1)	0
O. Pseudoscorpiones	0	0	0	0	1 (1)	0	0	0	0	0	0	0	0	0	0	0	0
Order Acariformes	261 (17)	236 (13)	422 (11)	141 (11)	239 (14)	371 (12)	353 (9)	415 (10)	178 (9)	50 (6)	268 (10)	283 (9)	1152 (13)	617 (12)	967 (15)	204 (10)	130 (12)
Order Hemiptera	1 (1)	0	0	0	44 (1)	0	0	0	0	0	0	0	0	0	0	0	0
Order Coleoptera	6 (4)	1 (1)	1 (1)	4 (1)	1 (1)	3 (2)	0	0	0	0	2 (1)	0	2 (1)	2 (1)	1(1)	1 (1)	0
Total abundance	822	1904	2314	9183	6523	649	433	761	378	553	2032	888	1783	1513	3373	506	300
Total taxa	31	21	20	20	22	21	14	14	16	7	15	13	21	19	27	18	18

P – Potato crop; O – onion crop; MA – maize crop area A; MB – maize crop area B.

4.3. Variations of soil macro- and mesofauna communities at agricultural crops

PRC results for **potato and onion crops** macrofauna communities are presented in Figure V.7. Monte-Carlo tests of the potato and onion crops revealed significant PRC curves (F = 27.93, p = 0.002). Treatment levels (crops x time) explained 36.9% of the variation in community composition. Prior to onion seeding and potato planting (T0), the one-way ANOVA revealed significant differences (Dunnett test, $p < 0.05$) between the soil communities of both crops with those from the Control site, but did not show significant differences between them (Tukey test, $p > 0.05$) (Table V.7). During the crop development and agricultural practices (T1) significant differences with Control site were observed for both crops and among crops (Table V.7). After harvesting at,the last sampling date (T2), we can notice a tendency for a recovery of the soil macrofauna community. However significant differences with control site were still observed for the potato crop (Dunnett test, $p < 0.05$). The morphospecies responsible for these differences, mainly for occurring in higher abundances in the Control site, were the ants (Hymenoptera), a Diptera, one coleopteran and several spiders (Araneae) morphospecies (see high b_K values in Figure V.7). On the other hand, morphospecies that showed higher abundances in the crops field soil comparing with the Control site were three dipterans and one coleopteran (see low b_K values in Figure V.7).

Soil mesofauna inhabiting both crops did not follow the same pattern as soil macrofauna (Figure V.7). In this case, treatments (crops x time) explained 25% of the variation in community composition but Monte-Carlo tests still showed significant PRC curves (F = 13.13, p = 0.002). Significant differences with control site were observed at the first sampling date and during agricultural practices (T0 and T1) as for macrofauna, but not after harvesting (T2), indicating a complete recovery of soil dwelling communities (Table V.7). The b_K scores revealed that morphospecies most responsible for the differences between crop areas and the control were four morphospecies of collembolans and four mite (Acariformes) morphospecies (see high and low b_K values in Figure V.7).

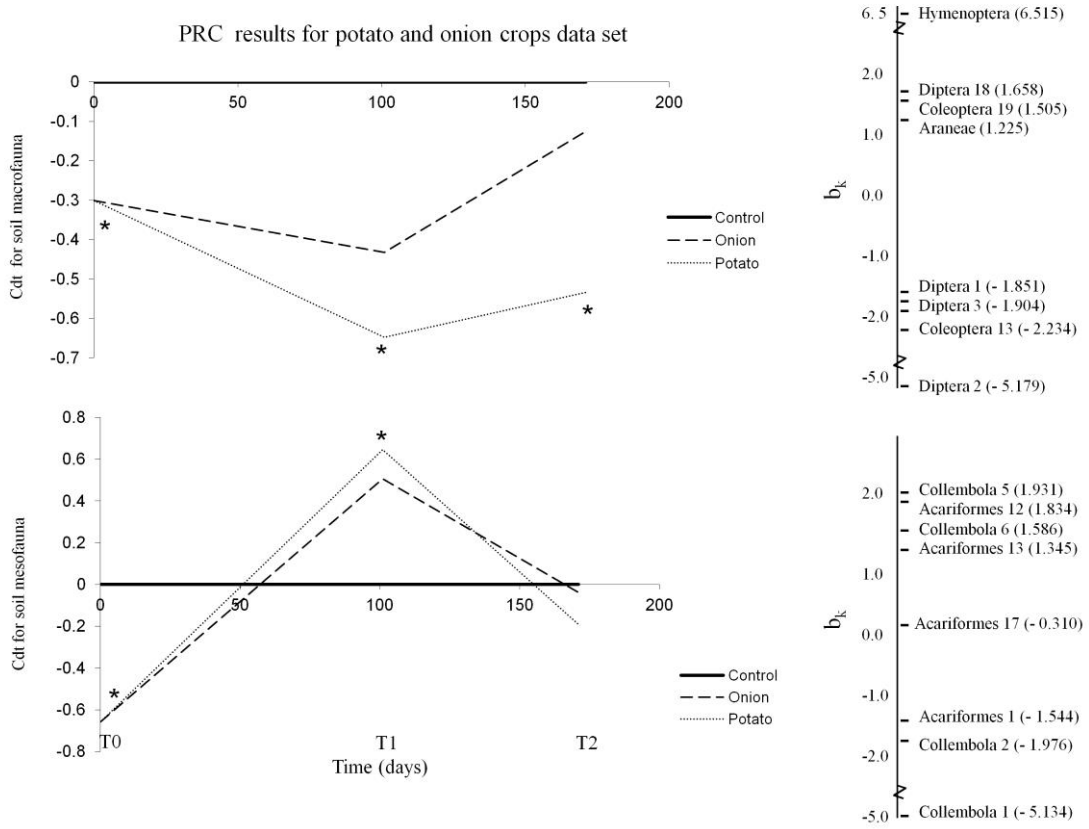

Figure V.7: PRC results on soil macro- and mesofauna of potato and onion crops along the crop cycle. For better perception of the sampling times according to the different management practices see Figure V.2. *Significant differences between treatments and control site.

Table V.7: One –way ANOVA results for macro- and mesofauna collected at potato (P) and onion (O) crops.

	P & O vs. Control		P vs.O	Homogeneity
Macrofauna		Dunnett´s test	Tukey test	
T0	$F_{2,25} = 49.7$, $p < 0.0001$ *	$p < 0.0001$*	$p = 1.000$	Levene's $p = 0.76$;
T1	$F_{2,25} = 119.8$, $p < 0.0001$ *	$p < 0.0001$*	$p = 0.0001$*	Levene's $p = 0.22$;
T2	$F_{2,25} = 95.5$, $p < 0.0001$ *	$p < 0.0001$* for P and $p = 0.057$ for O	$p = 0.0001$*	Levene's $p = 0.24$
Mesofauna				
T0	$F_{2,25} = 28.9$, $p < 0.0001$*	$p < 0.0001$*	$p = 1.000$	Levene's $p = 0.63$;
T1	H (2,28) = 15.1, $p = 0.0005$*[a]	$p < 0.04$* [b]	$p = 0.58$ [b]	
T2	$F_{2,25} = 28.9$, $p = 0.13$	-	$p = 0.249$	Levene's $p = 0.28$;

*Significant differences; [a] Kruskal-Wallis test value; [b] p values after multiple comparisons.

At **maize crop** field, significant variations with control over time (Monte-Carlo F = 31.80, p = 0.002) of soil macrofauna are shown in Figure V.8 for both monitoring areas. The analysis explained 32.8% of the data variation with significant differences of both maize areas with control site observed during all sampling dates (T0 to T2) except for the last one conducted after harvesting (T3) (Table V.8). No significant differences were observed among maize crop areas along the crop cycle (Table V.8). Soil macrofauna most responsible for the observed differences, mainly for inhabiting the control site under higher abundances were the dipteran 17 (b_k 1.459), the coleopterans 19 and 45 (b_k 1.415 and 1.218, respectively) and a general group of spider (Aranaea, b_k 1.345) morphospecies (Figure V.8).

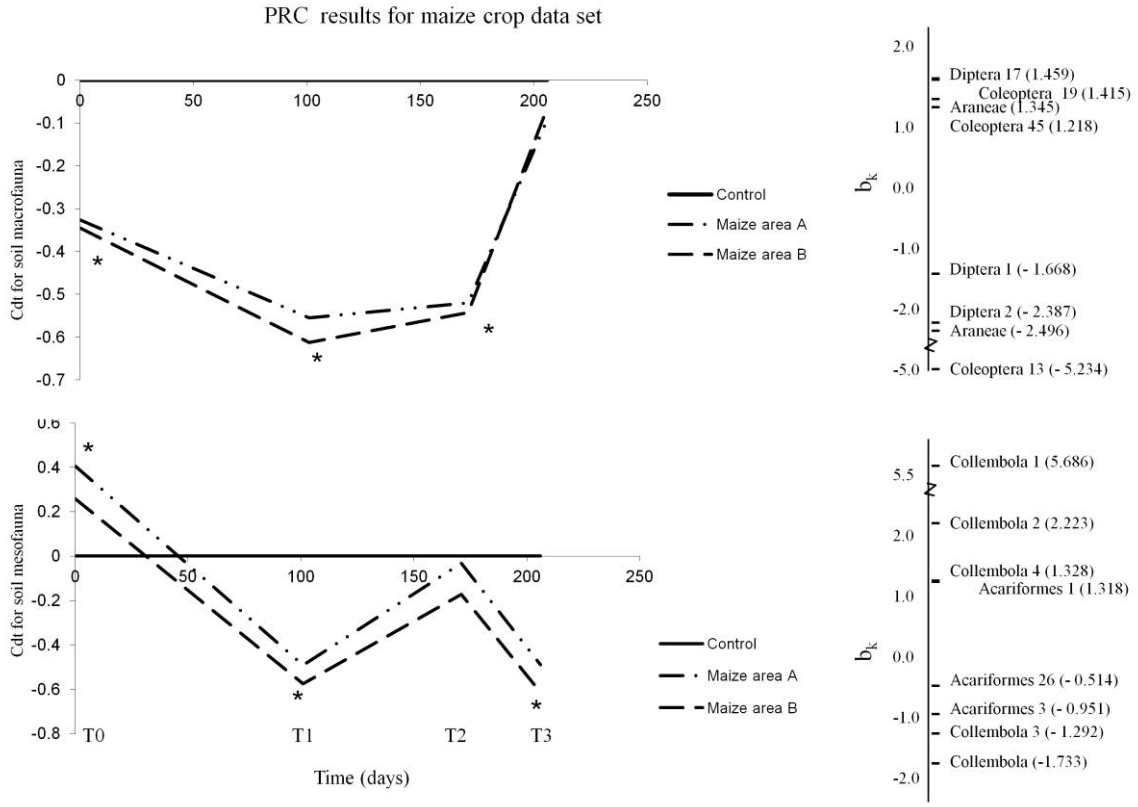

Figure V.8: PRC results on soil macro- and mesofauna of maize crop (areas A and B) along the crop cycle. For better perception of the sampling times according to the different management practices see Figure V.3. *Significant differences between treatments and control site.

Table V.8: One -way ANOVA results for soil macro- and mesofauna collected from soil samples and pitfall trapping at maize areas A and B.

	A & B area vs. Control		A vs. B area	Homogeneity
Macrofauna		Dunnett´s test	Tukey test	
T0	H (2,27) = 16.9, $p = 0.0002$*[a]	$p < 0.004$* [b]	-	-
T1	$F_{2,24} = 49.1$, $p < 0.0001$ *	$p < 0.0001$*	$p = 0.649$	Levene's $p = 0.81$;
T2	$F_{2,24} = 267.0$, $p < 0.0001$ *	$p < 0.0001$*	$p = 0.655$	Levene's $p = 0.74$;
T3	$F_{2,24} = 2.30$, *p= 0.122*	-	$p = 0.814$	Levene's $p = 0.60$;
Mesofauna				
T0	$F_{2,24} = 17.3$, $p < 0.0001$*	$p < 0.004$*	$p = 0.105$	Levene's $p = 0.09$;
T1	$F_{2,24} = 21.5$, $p < 0.0001$*	$p < 0.0001$*	$p = 0.483$	Cochran C $p = 0.10$
T2	$F_{2,24} = 1.36$, $p = 0.28$	-	$p = 0.389$	Levene's $p = 0.33$;
T3	$F_{2,24} = 71.0$ $p < 0.0001$*	$p < 0.0001$*	$p = 0.019$*	Levene's $p = 0.13$;

*Significant differences; [a] Kruskal-Wallis test value; [b] *p* values after multiple comparisons

As in potato and onion crops, also in maize areas the variation of soil mesofauna did not follow the same pattern of the soil macrofauna (Figure V.8). In this case 27.4% of the species data was explained by the analysis, with the Monte-Carlo test revealing a significant analysis ($F = 9.51$, $p = 0.002$). Only the third sampling date (T2) conducted at late maize crop development did not reveal any significant differences with control site, and significant differences among maize crop areas were only observed after harvesting (T3) (Table V.8). The b_K scores revealed that the soil mesofauna morphospecies that varied the most with the Control site were five morphospecies of collembolans (1, 2, 4, 3 and 6) and three mite (Acariformes 1, 26 and 3) morphospecies (see high and low b_K values in Figure V.8).

4.4. Feeding activity of soil fauna along crops cycles

At potato and onion crop, the feeding activity of soil fauna was significantly different among treatments ($F_{(2, 25)} = 6.56$, $p < 0.01$) and between time ($F_{(1, 25)} = 16.01$, $p < 0.001$), and both factors also showed a significant interaction between them ($F_{(2, 25)} = 7.86$, $p < 0.01$). Differences between onion and potato crops with Control site were only observed at during crop development and agricultural practices (T1; Figure V.9).

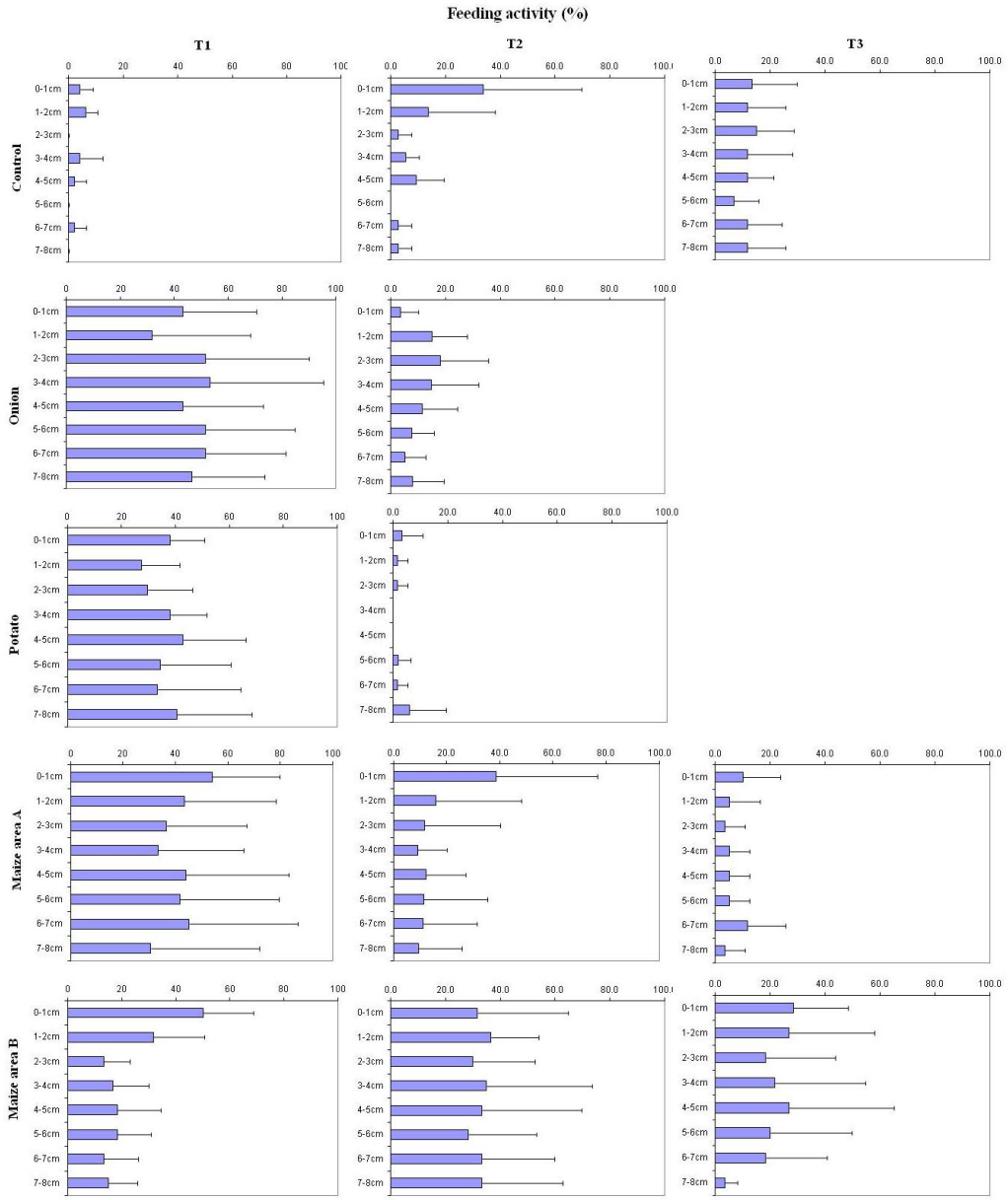

Figure V.9: Feeding activity of soil fauna of the three crops and Control site measured till 8-cm depth over the three crops cycles.

Differences between sampling time within each treatment were only observed at crop sites and not at the control site. At maize crop, no significant differences were observed neither among treatments (maize areas A and B vs. Control site) ($F_{(2, 34)} = 2.77$, $p > 0.05$), nor among sampling times within each treatment ($F_{(2, 34)} = 0.975$, $p > 0.05$).

5. Discussion

5.1 Potato and onion crops

At the beginning of the crop cycle (T0), after seedbed preparation and before onion seeding and potato planting, when no pesticides or fertilizers were applied to both crop fields, macro- and mesofauna communities at both crop fields differed significantly from control site communities (Figure V.7).

Although no significant differences in macrofauna total abundances (see section 4.2) among all study sites at the beginning of the crop cycle, the observed differences are caused by the presence in higher numbers of ants, spiders, and a coleopteran (morphospecie 19) belonging to the Anthicidae family at the control site (Figure V.7). These differences could be mainly attributed to the habitat configuration of the control site, with a dense shrub and tree cover, while the crop fields did not have any vegetation to serve as refuge to these organisms (Blanchart *et al.*, 2006; Brévault *et al.*, 2007).

Significant differences were also observed on the mesofauna communities (Figure V.7) between the two agricultural study sites and control, with the crop fields presenting higher total abundance of collembolans than the control site (Table V.6). Although, tillage practices such as soil preparation by ploughing and mulching incorporation, as performed in the present study, can exert opposite effects on these organisms, by either causing a decrease in their abundance or richness, or, in opposition facilitation refuge for them (Brévault *et al.*, 2007; Roger-Estrade *et al.*, 2010), results must be discussed under a case by case environment. The observed collembolan differences among study sites could be due to differences in the soil preparations for seedbed (not performed at the control site) that could create a favourable environment for these microarthropods. Soil texture loosening, as a result of tillage practices, exerts may act as benefic for mites and collembolans communities as these are less sensitive to mechanical injury and strongly dependent on sufficient pore spaces (Domínguez *et al.*, 2010; van Cappelle *et al*, 2012; Wardle *et al.*, 1999).

After the introduction of the crops (T1), significant differences with control site maintained for both crops and for both macro- and mesofauna communities (Figure

V.7), although, a significant difference between crops was observed only for macrofauna (Table V.7). Differences with control site are mainly due to lower abundances of macro- and mesofauna at the onion crop. The later treatment showed also a lower abundance when compared to with potato crop. On the other hand, differences of potato crop with control site may be due to the number of *taxa* of mesofauna communities observed (see section 4.2). The fact that at onion crop soil communities showed lower numbers, namely of arachnids and coleopterans as well as collembolans, in comparison to control site and potato crop (Table V.5 and V.6), may be a result of the plant cover of each treatment/crop. Plant cover in the control and high potato plant development in late June confers an advantage for soil macro- and mesofauna in comparison to onion crop that does not offer high number of habitats and predatory protection due to its low plant coverage (Figure V.4). Studies have shown that when a cover plant (e.g. legume) is present it favours the development of Coleoptera, Diptera larvae and Isopoda (Blanchart *et al.*, 2006) as observed here. Moreover other studies have shown that conventional tillage systems (as that in the present study) favours the increase in soil inhabiting predatory arthropods, especially ground beetles (Carabidae) and spiders (Brévault *et al.*, 2007) as observed here. The observed significant differences with Control were accompanied by differences of the soil organisms feeding activity. Both crops showed a similar activity while the control revealed a reduced feeding activity (Figure V.9). This feeding activity is due in general to soil dwelling organisms (mesofauna) and although mesofauna abundance is higher at the control site, the presence of irrigation could have caused a bias in the results. These type of contradictions illustrate the difficulty of assessing biological and community parameters under realistic scenarios.

At this stage of the crop development (T1), no pesticides were detected in soil at potato crop, but copper (applied as copper oxychloride acting as a fungicide) was detected in onion crop soil at 5.4 mg kg^{-1} (Table V.4). Copper is harmful to aphids and mites (Table V.3) at concentrations higher (1 kg ha^{-1}) than the applied, 0.609 kg ha^{-1} (Table V.1), and effects on earthworms would also not be expected due to the higher NOEC value (15 mg kg^{-1}). No significant differences were observed on soil mesofauna communities between both crops corroborating the hypothesis that no effects would be expected from copper concentrations in soil, since no copper was applied at potato crop. Addittionaly, other studies showed no negative effects in collembolan populatiosn with natural soil

with a concentration of copper higher (20 mg kg^{-1}) than the detected in the present study soil (Filser *et al.*, 1999).

Eight and eleven days after potato and onion harvesting (T2), respectively, both macro- and mesofauna communities at onion crop field where similar to control site's communities. However, potato crop macrofauna communities showed significant differences with Control site (Figure V.7), and although no significant differences were revealed for the macrofauna total abundances and number of *taxa* (see section 4.2) these differences in community composition may be due to low abundance of ants (Hymenoptera) and spiders (Aranaea) observed in potato crop (Figure V.7 and Table V.5). Although mesofauna abundance at potato crop did not show significant a difference with control site nor with onion crop, feeding activity of soil organisms was lower than in onion's or controls site's (Figure V.9). This may also be a result of several environmental and agricultural factors relating to crops management influencing the activity of soil organisms.

At T2 copper was again detected at onion crop, but since it was present at lower concentration values (5.4 mg kg^{-1}) than the documented (Table V.3) no negative effects on the soil communities would be expected as previously referred. However, the herbicide pendimethalin was also detected (Table V.4), but at a lower concentration (0.14 mg kg^{-1}) than the documented no observed effect concentration for earthworms (NOEC < 33.45 mg kg^{-1}) and since it was applied at a dosage (495gr ha^{-1}) lower than the referred as harmful for aphids and mites, 3.2 kg ha^{-1} (Table V.3), no negative effects would be expected on soil mesofauna communities. This is in agreement with the observed results of no significant differences between onion crop and control site, where no pesticides were applied. At the potato crop the insecticide chlorpyrifos was detected in soil, although at lower concentrations (0.063 mg kg^{-1}) than the registered to cause harmful effects on the soil organisms, namely to collembolans (*Folsomia candida*, 35-d LC_{50} = 0.2 mg kg^{-1}), mites (1 kg ha^{-1}) and coleopteran (Table V.3). As such, no effects would be expected on the soil communities. In fact, potato crop showed higher abundances for coleopterans than onion crop, where this insecticide was not applied (Table V.5) and soil mesofauna communities maintained the non significant differences between crops (Table V.7).

Onion crop field communities reached equilibrium with Control site at the end of the crop cycle, 11 days after harvesting when the fields returned to pasture, while potato

crop field macrofauna did not. The fact that the sampling was performed 8 days versus the 11 days at onion crop may be an influencing factor together with the type and quantity of mulch left in the potato field, the later not allowing for a complete reestablishment of fauna communities. Following a disturbance, the reestablishment or reorganization of an ecosystem may take a long time as it is influenced by spatial heterogeneity of source areas for re-colonization, as by dispersal abilities of organisms, being lower in the case of soil dwelling organisms when compared to aboveground biota (Brussard et la., 2007).

5.2. Maize crop

Along the crop cycle (T0 to T3) no significant differences among the two maize areas were observed (Table V.8), both in terms of soil macro- and mesofauna (except for T3) communities abundances and number of *taxa* (see section 4.2). This reveals that the slight differences in soil characteristics (Table II.2) and presence of pebbles (area B) did not influence both soil organism communities. The same results were observed for the feeding activity of mainly mesofauna along the crop cycle (see section 4.4).

After soil preparation and before maize seeding (T0) significant differences were observed in the soil communities from maize crop field and the Control site for both macro- and mesofauna (Figure V.8). As for potato and onion crops, these differences were due to the presence in higher numbers at the control site of spiders (Aranaea), two coleopteran morphospecies (nº 19 and 45) belonging to the Anthicidae and Silphidae family, respectively, and a dipteran morphospecies (Figure V.8). These differences could be attributed to the vegetation cover as previously referred (Blanchart *et al.*, 2006; Brévault *et al.*, 2007). Soil mesofauna was also responsible for these differences with Control site, with lower abundances of several collembolans in the control site at this sampling date, which may be due to some soil compaction causing a negative effect on fauna inhabiting the soil profile (Dittmer and Schrader, 2000; Domínguez *et al.*, 2010; van Cappelle *et al*, 2012; Wardle *et al.*, 1999). Under agricultural sites with high management intensity collembolans can have large populations (Filser 1995) and be less affected by farming practices than most of other soil animals are, such as earthworms and epigeic predators (Sabatini *et al.* 1997; Wardle 1995; Wardle *et al.* 1999).

During the crop development, T1 and T2 sampling dates, significant differences with control site were observed for both soil communities, with the exception of soil mesofauna (Figure V.8) at late crop development in September 3rd (T2), 17 days before harvesting (Figure V.3). This similarity of soil mesofauna of the maize crop with the one from the control site may be due to the lack of irrigation after July 7th, thus creating a similar soil moisture regime as in the control site. At late June (T1) no pesticides were detected in soil, however differences on both macro- and mesofauna were observed, the control site showed high total abundance of ants (hymenoptera) and lower abundances of coleopterans versus both maize areas (Table V.5), as well as very high total abundances of collembolans (Table V.6). The pesticides applied between T0 and T1 were the herbicide s-metolachlor and the insecticide cypermethrin, both were not detected in soil, as referred above, and would not be expected to cause negative effects to soil organisms for not being toxic to earthworms and collembolans and mites (Table V.3). In spite of the insecticide showing a very high affinity to the soil compartment (PED value) and having a half life (DT50) in soil of 69days (Table V.2), when in a water-sediment environment as the existing in irrigated crops such as maize (irrigation stopped in July), it's DT50 is only of 17 days (Table V.2). This late value corresponds to a period of time less than the occurred between the application time (April 30th) and the sampling time T1 in late June during the crops practices (56days), so the pesticide may have been degraded till then, would explain the no detection in the pesticide residue analysis. The observed differences in macro- and mesofauna may be due to other factors related to the presence of shrubs in the control site by serving as refuge these beneficial organisms (Brévault *et al.*, 2007; Roger-Estrade *et al.*, 2010), and that agricultural habitats showing high soil moisture levels, as occurs in the present study in both maize filed areas *vs.* control (Figure V.6) during irrigation, may not be preferred by most collembolan populations tending to decrease in numbers (Böckl *et al.*, 1998).

The following sampling date (T2), after the application of all pesticides and fertilizers, revealed the same significant differences with control site only for organisms inhabiting the soil surface (Figure V.9). Although the herbicide s-metolachlor was detected in soil at both maize areas (Table V.4), it was present at concentrations (0.16 mg kg^{-1}) lower to the documented no effect value for earthworms (NOEC < 2.54 mg kg^{-1}). This, and the fact that the herbicide is referred as harmless to aphids (Table V.3), indicate that no negative effects on the soil arthropods communities would be expected from this

substance (Daam *et al.*, 2011; Frampton *et al.*, 2006). The observed significant differences may be due to the general high abundances of soil organisms at the crop fields versus control site namely arachnids, coleopterans, dipterans and hemipterans (Table V.5) as a results of habitat availability among the maize crop plants and food resources provided by the incorporation of the crop mulches (Blanchart *et al.*, 2006; Brévault *et al.*, 2007; Roger-Estrade et la., 2010; Wardle *et al.*, 1999). At the end of the maize crop cycle (T3) communities inhabiting the soil surface reached a similar state as control site (Figure V.8). However, soil mesofauna communities did not, due to the high abundance of collembolan and acari in the control site (Table V.6). Although the herbicide s-metolachlor was also detected in soil (Table V.4), its presence at the measured concentration would not be expected to cause any negative effect on these organisms as previously referred.

After the harvesting of all crops, soil communities reached, in general, equilibrium similar to the Control site where no agricultural practices were performed. The fact that crops mulch is left in the fields may have favoured this resemblance creating similar environments with the control site (Roger-Estrade *et al.*, 2010) by serving as refuge these beneficial organisms (Brévault *et al.*, 2007; Roger-Estrade *et al.*, 2010). As such, agricultural intensification may not be consistently harmful to the soil fauna, as registered by other authors (Domínguez *et al.*, 2010; Wardle *et al.*, 1999), being highly dependent on the management actions adopted.

6. Conclusion

In general, soil arthropod communities from three crops reached, at the end of the crop cycle, a similar composition to the control site were no agricultural practices were performed. Pesticides concentrations in soil from the three corps may have not caused negative effects on the soil macro- and mesofauna communities inhabiting both the soil surface and the upper soil layer. Soil organisms feeding activity illustrated differences among potato and onion crops field and control site communities, but not at maize field, and were not always concordant to the observed differences in soil community's abundances.

The present study illustrated that differences in the soil macro- and mesofauna communities among crops are not as clear as differences due to agricultural practices such as ploughing and mulch incorporation. Other studies also verified that the kind of crop plays only a minor role regulating below-ground communities as these are less sensitive to mechanical injury and strongly dependent on sufficient pore spaces originated by tillage practices (Domínguez *et al.*, 2010; van Cappelle *et al*, 2012; Wardle *et al.*, 1999).

An integrated approach such as the one adopted in present study, taking into consideration soil communities, feeding activity and time variations along entire crop cycles of several crops under Mediterranean conditions, as well as pesticides detection in soil, may contribute to decision making towards a sustainable use of crop areas, including the use of pesticides and the management actions adopted. The difficulty of evaluating effects under realistic agricultural conditions must not be taken lightly but explored at as many levels as possible among its limitations under controlled variables, in order to better understand the factors influencing pesticide effects in biota under realistic environmental conditions.

7. Acknowledgements

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CHAPTER VI

Final remarks

1. Conclusions

The objectives of the present study in providing knowledge targeting at environmental protection under a sustainable agriculture including pesticides use, water, and soil and biodiversity protection, and to contribute to refine the ecological risk assessment of pesticides on the soil-water interface of irrigated crops under Mediterranean conditions, were accomplished as follows:

1. Prior to the assessment of side-effects of pesticides on terrestrial and aquatic biota inhabiting the soil-water interface, a study based on the selection of the terrestrial test species to be used on the evaluation effects of the three pesticides (the fungicides azoxystrobin and chlorothalonil and the insecticide ethoprophos) in laboratory and semi-field simulations studies was performed. A Species Sensitivity Distribution approach based on cumulative probability distributions of toxicity values for multiple species of main taxonomic groups (e.g. Acari, Chilopoda, Coleoptera, Collembola) and for different pesticide types of action (insecticides, fungicides and herbicides), was adopted. Results indicated that different organisms, when compared to earthworms, can display a lower and higher sensitivity to fungicides and insecticides, respectively, such as collembolans, isopods and acari, and be more sensitive to fungicides as nematodes. Consequently, a second study prior to the simulations studying the potential terrestrial toxicity of the three pesticides was evaluated using sub-lethal (reproduction) ecotoxicological tests with non-target species from different trophic groups: the collembolan *Folsomia candida*, the earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus* under laboratory conditions using natural soil, in order to improve the relevance of the laboratory data to field conditions during the simulations (semi-field environment). The fungicide azoxystrobin showed the highest toxicity to earthworms, whereas, collembolans were the most sensitive taxa to the fungicide chlorothalonil and the insecticide ethoprophos, followed by the earthworms.

   The fact that Earthworms were not always the most sensitive species in the two studies (sub-lethal laboratory data using artificial and natural soil instead of the standard lethal information) emphasizes the need to increase

the number of mandatory assays with key non-target organisms in the environmental risk assessment of pesticides, such as for e.g. collembolans and acari. In agreement with the present study results, early this year the EU adopted a new regulation setting out the data requirements for active substances, in accordance with the Regulation (EC) Nº 1107/2009 concerning the placing of plant protection products on the market (CR, 2013) implementing changes in the environmental risk assessment (ERA) of pesticides. In terms of terrestrial ERA, a relevant change introducing as mandatory the sub-lethal test using earthworms for testing effects on non-target soil fauna when the active substance is prone to contaminate the soil. Effects on other non-target organisms must also be conducted if soil contamination can occur or if concern is raised for the aphid *Aphidius rhopalosiphi* and the mite *Typhlodromus pyri*, using the collembolan *Folsomia* candida and the mite *Hypoaspis aculeifer* (CR, 2013). **The protection of terrestrial ecosystems is now at a higher level of safety and closer to represent a more realistic ecological environment of terrestrial ecosystems under field conditions.**

2. In order to evaluate the influence of run-off and leaching as pathways of pesticide contamination into surrounding water bodies on the soil-water interface during agricultural irrigation, several simulations of realistic exposure conditions using a new semi-field methodology were performed. These simulations were undertaken using natural agricultural soil, irrigation practices and realistic "worst-case" scenarios of pesticide application (azoxystrobin, chlorothalonil and ethoprophos), pesticides physical and chemical properties and ecotoxicological characteristics, as a way to increase ecological and realistic relevance on the ERA of pesticides. In order to provide realistic knowledge on pesticide exposure and effects under an ecologically relevant approach, three crop-based scenarios were adopted for potato, onion and maize crops.

   The results showed an unexpected behaviour of the fungicide azoxystrobin in soil and water on the basis of its physico-chemical characteristics. Azoxystrobin sorbed strongly to the topsoil but it also demonstrated a leaching capacity in agreement with the observed differences in pesticide concentration among the three water matrices (runoff > elutriate > leachate). Runoff proved to be an important transfer pathway of azoxystrobin to surface water, illustrating

that a strong sorption to soil may also lead, under some scenarios, to relevant water contamination. **For the fungicide chlorothalonil, in spite of the expected (and observed) strong sorption to soil particles, a leaching behaviour would also be expected due to its physico-chemical properties and predicted environmental potential fate. However, the later did not occur**. **The insecticide ethoprophos has also demonstrated an unexpected behaviour in soil** with its strong soil sorption behaviour; given that it has a high solubility in water and a low soil sorption coefficient. However, the results are in agreement with the predicted environmental potential distribution showing a high affinity mainly to the soil compartment. This behaviour of ethoprophos has also been documented in several natural soils (Boesten and van der Pas, 2000; Bouraoui *et al.*, 2007). Nevertheless, in spite of its strong sorption ability this insecticide was also detected in all water matrices with differences in concentrations that relate with the observed fate results in soil (elutriate > leachate > runoff). **Therefore, the present study results allowed verifying the influence of natural soil under realistic agricultural field conditions, namely irrigation, on pesticide fate as of great importance for exposure assessment in the ERA of pesticides.**

3. When relating the effects data of the three pesticides from the previous studies (point 1) and the pesticides "worst-case" applications dosages (twice the recommended dosage), it was concluded that terrestrial tests were to be performed only for the insecticide ethoprophos after the semi-field simulations (described at point 2). Only ethoprophos revealed a negative possibility of effects on earthworms and collembolans at concentrations lower than twice the recommended dosage, whereas the fungicides demonstrated effects at much higher concentrations than the applied during the simulations of realistic exposure conditions of a soil-water interface existing in irrigated agricultural fields. The assessment of lethal and sub-lethal side-effects of pesticides on aquatic biota inhabiting the soil-water interface was therefore performed for the three pesticides and sub-lethal effects on the terrestrial communities were only performed for the insecticide ethoprophos.

   The results concluded that only two times the recommended dosages for azoxystrobin, chlorothalonil and ethoprophos would cause sub-lethal effects on

the cladoceran communities inhabiting surface and groundwater and as such, possible of affecting the freshwater ecosystems viability given that they form the base of the ecological structure of freshwaters environments, occupying an important position in food webs due to its high grazing potential (Friberg-Jensen *et al.*, 2010; Warming *et al.*, 2009). Additionally, the observed sublethal effects may suggest that changes in the cladoceran populations may occur at much lower concentrations of azoxystrobin and chlorothalonil than expected in natural water bodies near agricultural areas. This emphasises the need to use natural water matrices to assess realistic environmental effects of pesticides; for instance, toxicant exposure may be enhanced in leachates through its small suspended soil particles. **The fact that effects were observed with water samples from the chlorothalonil and ethoprophos simulations where no pesticide residues were detected constitutes an important point for future studies. The presence and possible effects of degradation metabolites should also be addressed, given that studies on the behaviour under field realistic environmental conditions and their toxicity to biota are lacking**.

The application of the insecticide ethoprophos at only twice the recommended dosages caused significant effects on the reproduction of non-target terrestrial organisms (collembolan and earthworms) at the application area (crop rows) and surrounding areas where the pesticide was not applied (areas between crop rows). **These effects occurred at lower concentrations than expected, thus from this simulation it was also confirmed the importance of using natural soil given that may have influenced the pesticide availability under realistic field conditions**. **The fact that toxicity was observed in soil from areas where the pesticide was not applied but were surrounded by the application area (crop rows), validates an important observation that pesticide residues are transported by water flow caused by irrigation not only vertically but horizontally to the surrounding areas**. The aquatic compartment adjacent to the terrestrial compartment in the simulated maize crop soil-water interface environment was also affected, but did not reflected the terrestrial toxicity effects, e.g. toxicity to cladoceran was not observed with runoff, leachates and elutriates from between the rows soil areas. The same situation was observed in the potato simulated crop-based scenario in terms of elutriates. **These results**

illustrate the importance of studying combined environmental compartments to increase ecological realisms in risk assessment on soil-water interface environments, as well as to evaluate the potential of contaminants to be mobilized into aquatic systems from the soil compartment. Additionally, through the present study it was verified that semi-field simulations based on crop scenarios under natural climate and soil conditions are a valuable tool for pesticide risk assessment linking pesticide fate and contamination pathways and the resulting toxicity towards soil and aquatic biota under realistically simulated pesticide stress.

4. Finally, a field study with the aim of providing ecologically relevant data on soil fauna communities derived from different agricultural practices was performed in an agricultural field with three irrigated crops (potato, onion and maize) under Mediterranean conditions during the entire crops cycle. Results obtained provided valuable information on the join effects of pesticide application, irrigation conditions, tillage and incorporation of crop mulch on natural indigenous soil communities The later two agricultural practices influenced the soil communities structure, but they presented a fair resilience by reaching a similar status to those in the control site (not agricultural environment) at the end of the crops cycle when the fields return to pasture. **Long-term impacts of tillage systems on belowground biodiversity are poorly understood (van Cappelle *et al.*, 2012) and should be integrated in future studies in order to evaluate the potential interaction effects between tillage and agricultural practices and distinctive ecosystem properties, such as crop type as primary nutrient provider and soil texture as a structural habitat, to predict possible management options and solutions to sustainable use of soils and conservation of its biodiversity in agro-ecosystems**. Integrated management of soil biota, biodiversity and agricultural ecosystems is a holistic process that relies largely on locally available resources, climate, socio-economic conditions and, above all, direct involvement of farmers and other stakeholders in identifying and adapting management practices to their specific context (Brussaard *et al.*, 2006).

Therefore, the information gathered on the present thesis will contribute to a more realistic pesticide risk assessment taking account the different levels of complexity of agricultural ecosystems under Mediterranean climate where a lack in studies does exist (Daam *et al.*, 2011). **Linking the results of these two lines, exposure and effects, will provide information about pesticide fate on water bodies and natural soils and side-effects on aquatic and terrestrial biota under realistic crop-based Mediterranean conditions, as well as evaluating the effects of extensive pesticide use along full crop-cycle by using low tier (single species) and high tier (community level) methodologies**. This will additionally **contribute to create realistic input data for FOCUS (Forum for the Co-ordination of pesticide fate models and their use) scenarios used in pesticide exposure assessment in EU**. The output of this thesis will also **lead to a refinement of methodologies to assess quality standards under the effects assessment that will contribute to decision-making targeting at a sustainable use of pesticides towards water, soil and biodiversity protection, contributing to reduce soil degradation and water contamination at European level**.

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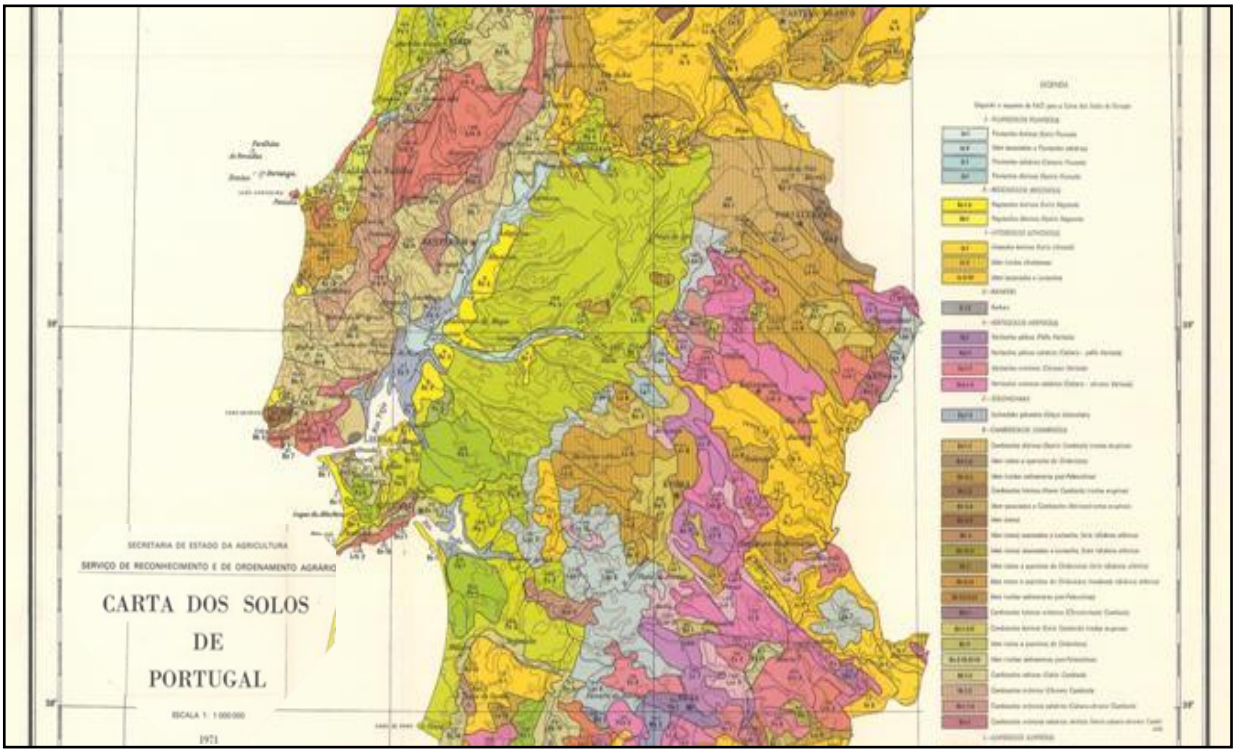
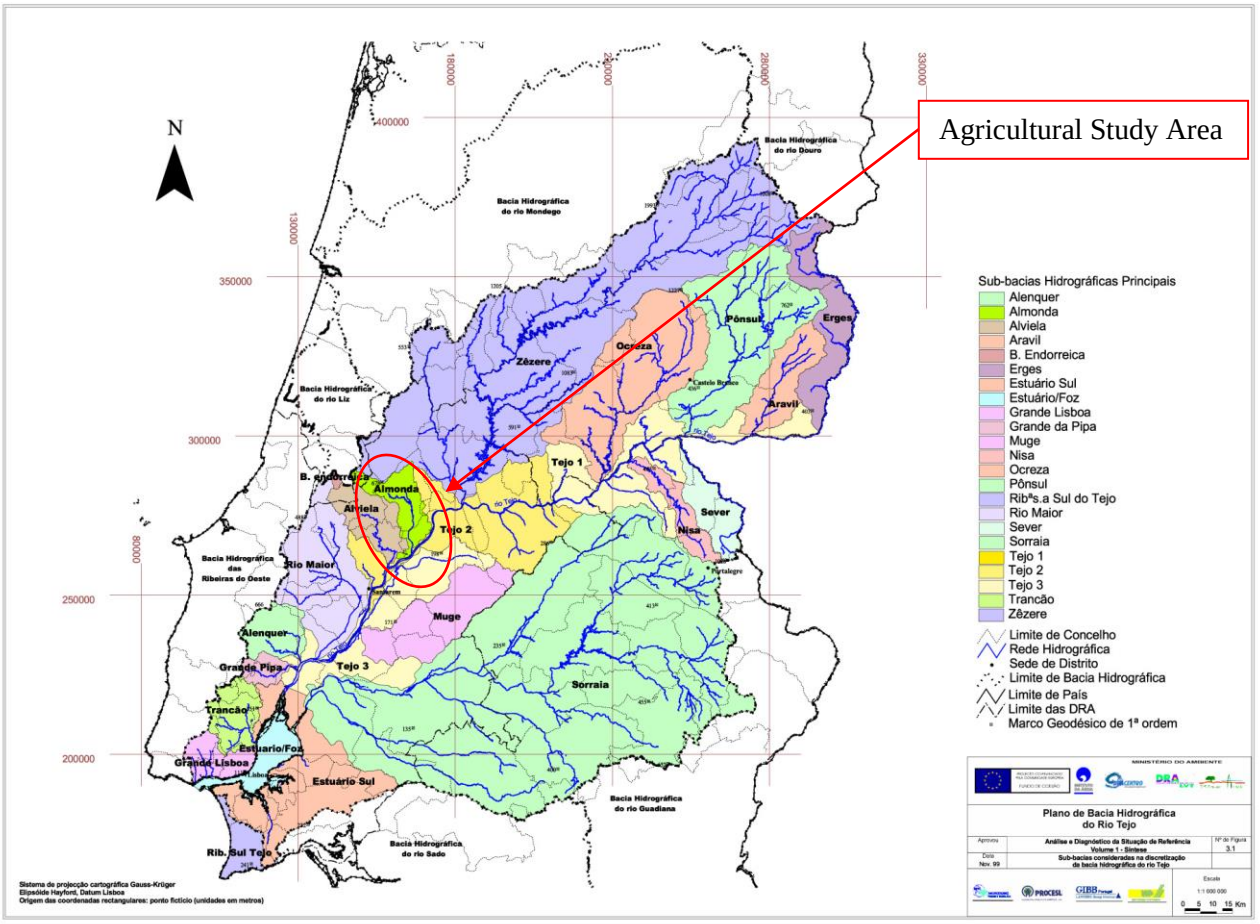
ANNEX

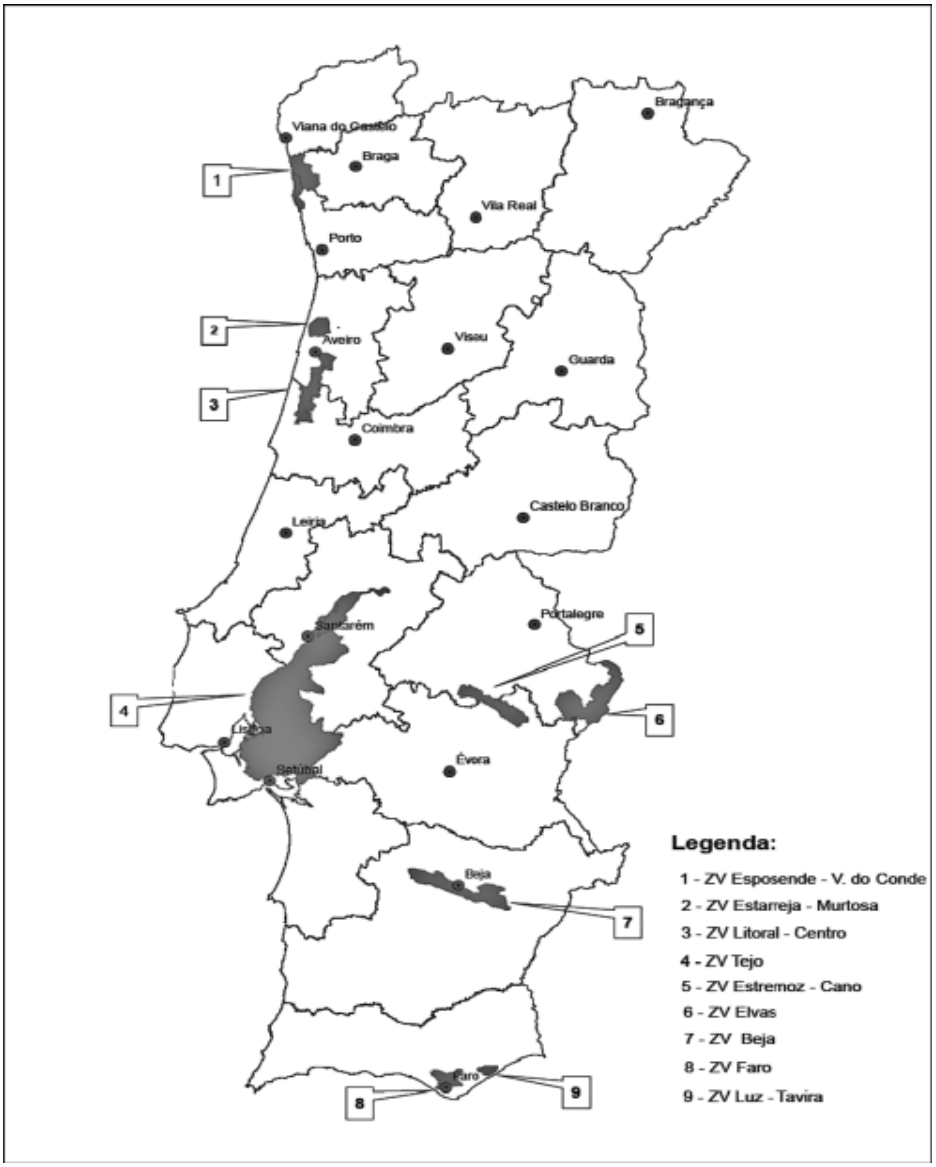
ANNEX I - Carta dos solos de Portugal (EuDASM, 2012).



ANNEX II - Tagus (Tejo) river basin and its sub-basins.



ANNEX III - Hydrogeological vulnerable areas (ZV) in continental Portugal (Portaria nº 164/2010).



ANNEX IV - Fungicides (applied individual) EC risk Classification [1] and other observations [2].

Fungicides	EC Risk Classification		Other observations
	EU Directive 67/548/EEC amended by EU Directive 2001/59/EC *	Regulation (EC) No 1272/2008 **	
azoxystrobin	[T, R23], [N, R50/53]	H331, H400, H410	Harmless to non-target organisms, including predatory mites and bugs, spiders, lacewings, hoverflies, ladybirds, carabid beetles, parasitoid wasps and bees, under field conditions at field application rates. E.g. LR50 (48h) *Aphidius rhopalosiphi* > 1135 g/ha. Field dissipation studies showed that neither azoxystrobin nor its major degrates were typically found in soil below the top 15 cm.
captan	[Carcinogen category 3: R40], [T, R23], [Xi, R41, R43], [N, R50]	H317, H318, H351, H331, 400	Moderately toxic to aquatic invertebrates
chlorothalonil	[Carcinogen category 3: R40], [T+, R26], [Xi, R37, R41, R43], [N, R50 /53]	H317, H318, H351, H330, H335, H400, H410	LR50 (7 d) *Typhlodromus pyri* > 18.75 kg~7ha; LR50 (48h) *Aphidius rhopalosiphi* > 18.75 kg/ha. Degradation is faster in biotic aquatic systems, typical DT50 (aerobic) <2h, (anaerobic) < 10d.
cyazofamid	[N, R50/53]	H400, H410	Harmless to *Typhlodromus pyri, Aphidius rhopalosiphi, Chrysoperla carnea* and *Aleochara bilineata*.
fluazinam	-	-	Degradation is faster in aerobic or anaerobic aquatic media. The degradation products appear to be relatively persistent under most conditions.
folpet	[Carcinogen category 3: R40], [Xn, R20], [Xi, R36, R43], [N, R50]	H317, H319, H351, H332, H400	Slightly harmful to *Coccinella septempunctata*, harmless to *Poecilus cupreus, Trichogramma cacoeciae, Typhlodromus pyri, Aphidius rhopalosiphi, Chrysoperla carnea* and *Aleochara bilineata*.
mancozeb	[Reproduction risk category 3: R63], [Xi, R43], [N, R50]	H317, H361, H400	Mancozeb is of low toxicity to the majority of non-target and beneficial arthropods. Mancozeb breaks down rapidly in soil, sediment and water; terminal metabolites are natural products and with mineralization to carbon dioxide.
metiram	-	-	The parent molecule is rapidly degraded.
propineb	[Xn, R20, R48/20 /22], R43, [N, R50]	H317, H373, H373, H332, H400	Effects on non-target insects are unlikely; only predatory mites are sensitive. Degradation is very rapid and can be classified as not mobile in soils.

[1]EU pesticide Database [assess online 20/11/2012; [2] Tomlin, 2006;

* ANNEX 2 Symbols and indications of danger for dangerous substances and preparations: T Toxic; T+ Very toxic; Xn Harmful; Xi Irritant; N Dangerous for the environment; ANNEX 3 Nature of special risks attributed to dangerous substances and preparations: R20 Harmful by inhalation; R22 Harmful if swallowed; R23Toxic by inhalation; R26 Very toxic by inhalation; R36Irritating to eyes; R37Irritating to respiratory system; R40Limited evidence of a carcinogenic effect; R41Risk of serious damage to eyes; R43May cause sensitisation by skin contact; R50 Very toxic to aquatic organisms; R48/20/22 Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed; R48/22 Harmful: danger of serious damage to health by prolonged exposure if swallowed; R50/53 Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment; R51/53Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

** ANNEX III Part 1: hazard statements: H302 Acute toxicity (oral), Hazard Category 4 - Harmful if swallowed; H317 Sensitisation - Skin, Hazard Category 1- May cause an allergic skin reaction; H318 Serious eye damage/eye irritation, Hazard Category 1- Causes serious eye damage; H319 Serious eye damage/eye irritation, Hazard Category 2 - Causes serious eye irritation; H330 Acute toxicity (inhalation), Hazard Category 1, 2 - Fatal if inhaled; H331 Acute toxicity (inhalation), Hazard Category 3 - Toxic if inhaled; H332 Acute toxicity (inhalation), Hazard Category 4 - Harmful if inhaled; H335 Specific target organ toxicity - Single exposure, Hazard Category 3, Respiratory tract irritation - May cause respiratory irritation; H351 Carcinogenicity, Hazard Category 2 - Suspected of causing cancer; H361 Reproductive toxicity, Hazard Category 2 - Suspected of damaging fertility or the unborn child; H373 Specific target organ toxicity - Repeated exposure, Hazard Category 2 - May cause damage to organs through prolonged or repeated exposure; H400 Hazardous to the aquatic environment - AcuteHazard, Category 1 - Very toxic to aquatic life; H410 Hazardous to the aquatic environment - Chronic Hazard, Category 1 - Very toxic to aquatic life with long lasting effects; H411 Hazardous to the aquatic environment - Chronic Hazard, Category 2 - Toxic to aquatic life with long lasting effects.

ANNEX V - Insecticides EC risk Classification [1] and other observations [2].

Insecticides	EC risk Classification		Other observations
	EU Directive 67/548/EEC amended by EU Directive 2001/59/EC *	Regulation (EC) No 1272/2008 **	
acetamiprid	[Xn, R22], [N, R52/53]	H302	Harmful to some arthropod species (*Aphidius rhopalosiphi, Typhlodromus pyri*). The primary degradation pathway is aerobic soil metabolism.
acrinathrin	-	-	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri*. Soil column leaching: <1% of aplied acrinathrin found in leachate.
azadirachtin	-	-	-
chlorpyrifos	[T, R25], [N, R50, R53]	H301, H400, H410	*Folsomia candida*, 35day LC_{50} Mortality 0.2 mg $kg^{-1.}$ Harmful at 1 kg ha-1 to *Alphidius colemani* and *Typhlodromus pyri*. Harmful to Carabidae and Staphylinidae, Tenebrionidae.
cyfluthrin	[T, R23], [T+, R28], [N, R50/53]	H300, H331, H400, H410	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri*. The metabolites are subjected to further microbial degradation to the point of mineralisation to CO_2.
beta-cyfluthrin	[T+, R26/28], [N, R50/53]	H300, H330, H400, H410	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri.* The metabolites are subjected to further microbial degradation to the point of mineralisation to CO2 in the soil.
cypermethrin	[Xi, R37], [Xn, R20/22], [N, R50/53]	H302, H332, H335, H400, H410	Under field conditions, fish are not at risk from normal agricultural usage. Field applications at recommended dosages do not put honeybee hives at risk. Not toxic to Collembola.
alpha-cypermethrin	[T, R25], [Xn, R48/22], [Xi, R37], [N, R50/53]	H301, H335, H373, H400, H410	No effect on earthworm reproduction was observed at treatment 300g/ha. Harmful at 1 kg ha-1 to *Alphidius rhopalosiphi* and *Typhlodromus pyri*
cyromazine	-	-	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri*.
deltamethrin	[T, R23/25], [N, R50/53]	H301, H331, H400, H410	Not toxic to fish under natural conditions. Low LD50 and LC50 values under laboratory conditions do not represented a significant hazard to aquatic fauna in normal field use.

[1] EU pesticide Database [assess online 20/11/2012]; [2] Tomlin, 2006;

* ANNEX 2 Symbols and indications of danger for dangerous substances and preparations: T Toxic; T+ Very toxic; Xn Harmful; Xi Irritant; N Dangerous for the environment; ANNEX 3 Nature of special risks attributed to dangerous substances and preparations: R21 Harmful in contact with skin; R22 Harmful if swallowed; R23Toxic by inhalation; R25Toxic if swallowed; R26 Very toxic by inhalation; R28 Very toxic if swallowed; R37Irritating to respiratory system; R43May cause sensitisation by skin contact; R50 Very toxic to aquatic organisms; R53May cause long-term adverse effects in the aquatic environment; R20/22 Harmful by inhalation and if swallowed; R21/22 Harmful in contact with skin and if swallowed; R23/25 Toxic by inhalation and if swallowed; R26/27 Very toxic by inhalation and in contact with skin; R26/28 Very toxic by inhalation and if swallowed; R48/22 Harmful: danger of serious damage to health by prolonged exposure if swallowed; R50/53 Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment; R52/53 Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

** ANNEX III Part 1: hazard statements: H300 Acute toxicity (oral), Hazard Category 1, 2 - Fatal if swallowed; H301 Acute toxicity (oral), Hazard Category 3- Toxic if swallowed; H302 Acute toxicity (oral), Hazard Category 4 - Harmful if swallowed; H310 Acute toxicity (dermal), Hazard Category 1, 2 - Fatal in contact with skin; H312 Acute toxicity (dermal), Hazard Category 4- Harmful in contact with skin; H317 Sensitisation - Skin, Hazard Category 1- May cause an allergic skin reaction; H330 Acute toxicity (inhal.), Hazard Category 1, 2 - Fatal if inhaled; H331 Acute toxicity (inhalation), Hazard Category 3 - Toxic if inhaled; H332 Acute toxicity (inhal.), Hazard Category 4 - Harmful if inhaled; H335 Specific target organ toxicity - Single exposure, Hazard Category 3, Respiratory tract irritation - May cause respiratory irritation; H373 Specific target organ toxicity - Repeated exposure, Hazard Category 2 - May cause damage to organs through prolonged or repeated exposure; H400 Hazardous to the aquatic environment - AcuteHazard, Category 1 - Very toxic to aquatic life; H410 Hazardous to the aquatic environment - Chronic Hazard, Category 1 - Very toxic to aquatic life with long lasting effects; H412 Hazardous to the aquatic environment - Chronic Hazard, Category 3 - Harmful to aquatic life with long lasting effects.

ANNEX V - Insecticides EC risk Classification [1] and other observations [2] (cont.).

Insecticides	EC risk Classification		Other observations
	EU Directive 67/548/EEC amended by EU Directive 2001/59/EC *	Regulation (EC) No 1272/2008 **	
ethoprophos	[T+, R26/27], [T, R25], R43, [N, R50/53]	H301, H317, H310, H330, H400, H410	-
imidacloprid	[Xn, R22], [N, R50/53]	H302, H400, H410	Harmful to honeybees by direct contact. Besides sunlight, the microbial activity of water/sediment system is na important factor for the degradation of imidacloprid.
indoxacarb	-	-	Moderately harmful at 1 kg ha^{-1} *Aphidius colemani* and *Typhlodromus pyri.*
lambda-cyhalothrin	[T+, R26], [T, R25], [Xn, R21], [N, R50/53]	H301, H312, H330, H330, H330, H400, H410	Intrinsic toxicity to aquatic organisms is greatly reduced by rapid loss from the water adsorption and degradadtion.. Toxic to some non-target arthropods. Effects under field conditions reduced, with rapid recovery.
lufenuron	R43, [N, R50/53]	H317, H400, H400, H410	Moderately harmful at 1 kg ha^{-1} to *Typhlodromus pyri* and *Coccinella semptempunctata*. Lufenuron was rapidly degraded in biologically active soils under aerobic conditions.
phosmet	[Xn, R21/22], [N, R50/53]	H302, H312, H400, H410	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri.* Rapidly broken down in the soil.
pirimicarb	[T, R25], [N, R50/53]	H301, H400, H410	Harmless at 1 kg ha^{-1} to *Alphidius rhopalosiphi* and *Typhlodromus pyri*. Not toxic to Collembola.
spinosad	[N, R50/53]	H400, H410	Not toxic to sucking insects, predacious insects, lacewings, big-eye bugs or minute pirate bugs. Rapidly degraded by u.v. light and soil microbes to naturally-occurring substances.
tefluthrin	-	-	Under field conditions, adsorption of tefluthrin on bottom and suspended sediments should prevent any hazard. At normal application rates, there was no effect on soil microflora or earthworms.
thiacloprid	-	-	Harmful at 1 kg ha^{-1} to *Alphidius rhopalosiphi*.
thiamethoxam	[Xn, R22], [N, R50/53]	H302, H400, H410	Aqueous photolysis occurs rapidly.

[1] EU pesticide Database [assess online 20/11/2012]; [2] Tomlin CDC, 2006;

* ANNEX 2 Symbols and indications of danger for dangerous substances and preparations: T Toxic; T+ Very toxic; Xn Harmful; Xi Irritant; N Dangerous for the environment; ANNEX 3 Nature of special risks attributed to dangerous substances and preparations: R21 Harmful in contact with skin; R22 Harmful if swallowed; R23Toxic by inhalation; R25Toxic if swallowed; R26 Very toxic by inhalation; R28 Very toxic if swallowed; R37Irritating to respiratory system; R43May cause sensitisation by skin contact; R50 Very toxic to aquatic organisms; R53May cause long-term adverse effects in the aquatic environment; R20/22 Harmful by inhalation and if swallowed; R21/22 Harmful in contact with skin and if swallowed; R23/25 Toxic by inhalation and if swallowed; R26/27 Very toxic by inhalation and in contact with skin; R26/28 Very toxic by inhalation and if swallowed; R48/22 Harmful: danger of serious damage to health by prolonged exposure if swallowed; R50/53 Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment; R52/53 Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

** ANNEX III Part 1: hazard statements: H300 Acute toxicity (oral), Hazard Category 1, 2 - Fatal if swallowed; H301 Acute toxicity (oral), Hazard Category 3- Toxic if swallowed; H302 Acute toxicity (oral), Hazard Category 4 - Harmful if swallowed; H310 Acute toxicity (dermal), Hazard Category 1, 2 - Fatal in contact with skin; H312 Acute toxicity (dermal), Hazard Category 4- Harmful in contact with skin; H317 Sensitisation - Skin, Hazard Category 1- May cause an allergic skin reaction; H330 Acute toxicity (inhal.), Hazard Category 1, 2 - Fatal if inhaled; H331 Acute toxicity (inhalation), Hazard Category 3 - Toxic if inhaled; H332 Acute toxicity (inhal.), Hazard Category 4 - Harmful if inhaled; H335 Specific target organ toxicity - Single exposure, Hazard Category 3, Respiratory tract irritation - May cause respiratory irritation; H373 Specific target organ toxicity - Repeated exposure, Hazard Category 2 - May cause damage to organs through prolonged or repeated exposure; H400 Hazardous to the aquatic environment - AcuteHazard, Category 1 - Very toxic to aquatic life; H410 Hazardous to the aquatic environment - Chronic Hazard, Category 1 - Very toxic to aquatic life with long lasting effects; H412 Hazardous to the aquatic environment - Chronic Hazard, Category 3 - Harmful to aquatic life with long lasting effects.

ANNEX VI - Pesticides active ingredient concentrations in soil from potato, onion and maize (areas A and B) crops field during the 2010 agricultural season.

Crop	Sampling date	Type of action [a]	Active ingredient (a.i.)	Concentration (mg kg^{-1})
Potato	T0 – March 16th	I	chlorpyriphos	< LOQ (0.015)
		F	cymoxanil	< LOQ (0.05)
		F	dimethomorph	< LOQ (0.05)
		F	fluazinam	< LOQ (0.03)
		H	flufenacet	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	metribuzin	< LOQ (0.05)
		I	thiamethoxam	< LOQ (0.05)
	T1 – June 25th	I	chlorpyriphos	< LOQ (0.015)
		F	cymoxanil	< LOQ (0.05)
		F	dimethomorph	< LOQ (0.05)
		F	fluazinam	< LOQ (0.03)
		H	flufenacet	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	metribuzin	< LOQ (0.05)
		I	thiamethoxam	< LOQ (0.05)
	T2 – September 3rd	I	chlorpyriphos	0.063
		F	cymoxanil	< LOQ (0.05)
		F	dimethomorph	0.067
		F	fluazinam	< LOQ (0.03)
		H	flufenacet	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	metribuzin	< LOQ (0.05)
		I	thiamethoxam	< LOQ (0.05)
Onion	T0 – March 16th	F	azoxystrobin	< LOQ (0.06)
		F	copper	< LOQ (1.0)
		H	ioxynil	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	oxyfluorfen	< LOQ (0.06)
		H	pendimethalin	< LOQ (0.05)
	T1 – June 25th	F	azoxystrobin	< LOQ (0.06)
		F	copper	5.4
		H	ioxynil	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	oxyfluorfen	< LOQ (0.06)
		H	pendimethalin	< LOQ (0.05)
	T2 – September 3rd	F	azoxystrobin	< LOQ (0.06)
		F	copper	5.4
		H	ioxynil	< LOQ (0.05)
		F	mancozeb	< LOQ (0.05)
		H	oxyfluorfen	< LOQ (0.06)
		H	pendimethalin	0.14

[a] I – insecticide; H – herbicide; F – fungicide.

ANNEX VI - Pesticides active ingredient concentrations in soil from potato, onion and maize (areas A and B) crops field during the 2010 agricultural season (cont.).

Crop	Sampling date	Type of action [a]	Active ingredient (a.i.)	Concentration (mg kg^{-1})	
Maize				**A**	**B**
	T0 – March 16th	I	cypermethrin	< LOQ (0.015)	< LOQ (0.015)
		H	s-metolachlor	< LOQ (0.05)	< LOQ (0.05)
		H	terbuthylazin	< LOQ (0.05)	< LOQ (0.05)
	T1 – June 25th	I	cypermethrin	< LOQ (0.015)	< LOQ (0.015)
		H	s-metolachlor	< LOQ (0.05)	< LOQ (0.05)
		H	terbuthylazin	< LOQ (0.05)	< LOQ (0.05)
	T2 – September 3rd	I	cypermethrin	< LOQ (0.015)	< LOQ (0.015)
		H	s-metolachlor	0.16	0.13
		H	terbuthylazin	< LOQ (0.05)	< LOQ (0.05)
	T3 – October 8th	I	cypermethrin	< LOQ (0.015)	< LOQ (0.015)
		H	s-metolachlor	0.11	0.11
		H	terbuthylazin	< LOQ (0.05)	< LOQ (0.05)

[a] I – insecticide; H – herbicide; F – fungicide.