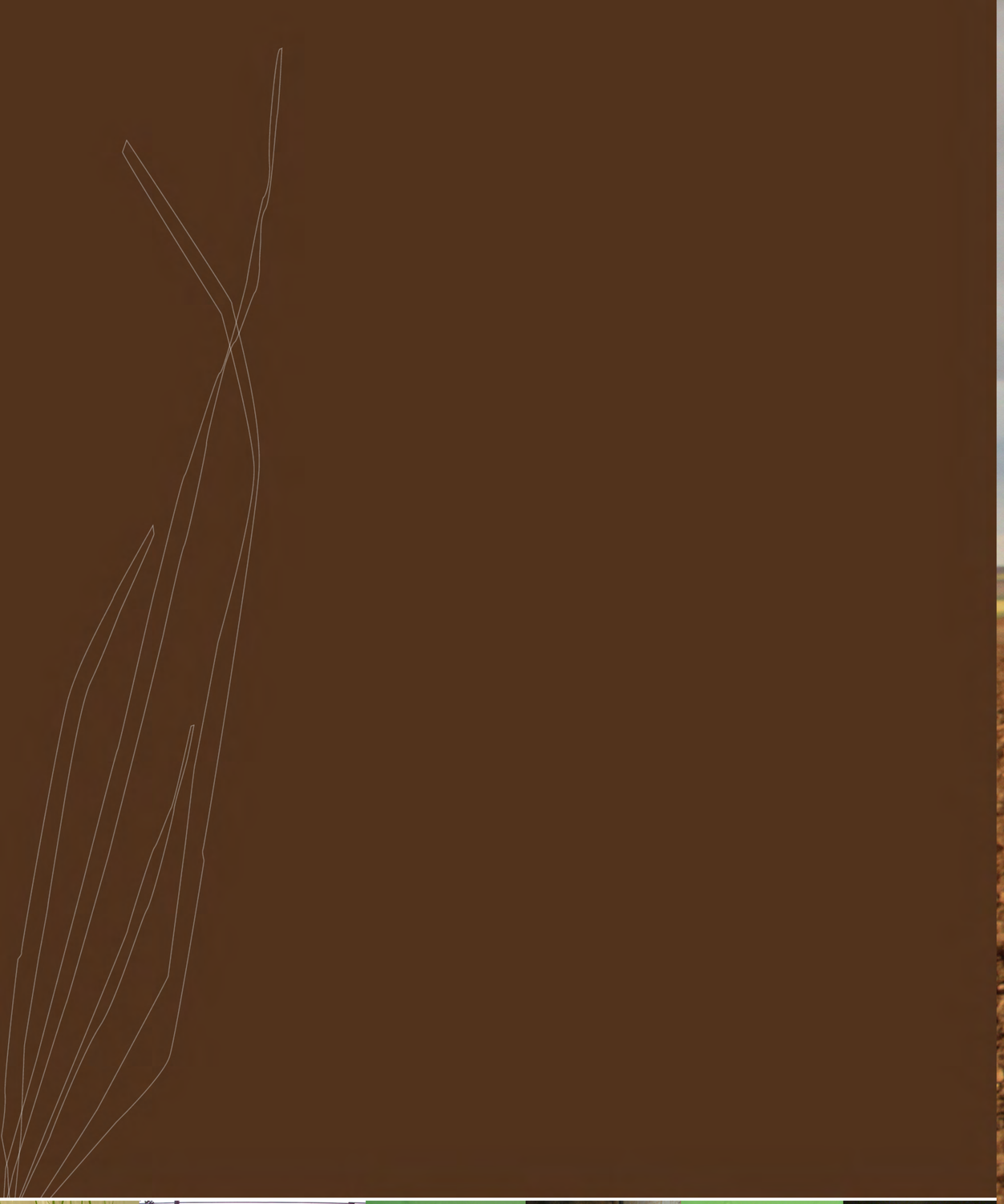

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Departamento de Ciências da Vida

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Dissertação apresentada à Universidade de Coimbra para a obtenção do grau de Doutor em Biologia, especialidade em Ecologia, realizada sob a orientação científica do Professor Doutor José Paulo Sousa, Professor Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra e co-orientação do Professor Doutor Evaldo Luiz Gaeta Espíndola, Professor Associado do Departamento de Hidráulica e Saneamento da Escola de Engenharia de São Carlos da Universidade de São Paulo, Brasil.

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Resumo

O incremento da produção agrícola está a provocar um aumento constante do consumo de pesticidas um pouco por todo o mundo, colocando em risco a saúde Humana e dos ecossistemas.

Em esquemas de Avaliação de Risco Ecológico (ARE) de pesticidas, é recolhida informação acerca do destino final do pesticida aplicado (e dos seus metabolitos) assim como sobre os potenciais perigos para os organismos de solo e aquáticos, tendo como objectivo final o uso sustentável destes produtos.

Em ARE de pesticidas, o reforço da ligação entre dados laboratoriais e os cenários de contaminação real constitui um grande desafio. Para obter dados mais realistas, são necessários aperfeiçoamentos quer ao nível das estratégias quer das ferramentas-teste. O trabalho de investigação levado a cabo na presente tese pretende ser uma contribuição para preencher estas lacunas e promover o aperfeiçoamento da avaliação de risco ecológico de pesticidas em áreas agrícolas, particularmente em áreas com clima temperado quente e tropical.

Mais especificamente os objectivos foram: (1) desenvolver e validar um simulador para aplicações de pesticidas em laboratório, possibilitando a simulação de diferentes cenários de contaminação e também a avaliação dos riscos potenciais para os organismos de solo mas também dos efeitos tóxicos indirectos para os organismos de água (devido a fenómenos de escorrência superficial e lixiviação); (2) avaliar a eficácia de uma ferramenta de bioremediação de solos contaminados com atrazina à escala de microcosmos e de semi-campo, nos compartimentos solo e água, utilizando testes ecotoxicológicos; (3) implementar o uso de comunidades de organismos de solo (nemátodes e microartrópodes) em ensios ecotoxicológicos como ensaios “higher-tier”; (4) comparar a toxicidade de carbofurão em ambientes tropicais/sub-tropicais e de clima temperado quente.

No capítulo 2, uma ferramenta de bioremediação desenvolvida para solos contaminados com atrazina, consistindo na bio-augmentação (utilizando *Pseudomonas sp.* ADP) e bioestimulação (utilizando citrato) do solo foi optimizada para a escala de microcosmos. A comunidade de organismos de

solo foi simulada através da adição de invertebrados de solo (*Eisenia andrei* e *Folsomia candida*) e plantas (*Avena sativa*). A eficácia da ferramenta de bioremediação foi avaliada nos compartimentos solo e água (no caso deste último, mediante a recolha de amostras de eluatos e lixiviados) através da realização de ensaios ecotoxicológicos com *A. sativa* e *E. andrei* (avaliação da função habitat do solo) e com *Daphnia magna* e *Pseudokirchneriella subcapitata* (avaliação da função de retenção do solo). Os resultados evidenciam claramente a eficácia da ferramenta após 10 dias de bioremediação, uma vez que aparentemente, a atrazina e eventuais metabolitos foram reduzidos para níveis não tóxicos para plantas e organismos aquáticos.

No capítulo 3, um protótipo do simulador da aplicação de pesticidas foi desenvolvido e testado em condições tropicais, através da comparação dos resultados de análises químicas (ao solo) e testes ecotoxicológicos realizados com amostras recolhidas no ensaio com o simulador e também num ensaio paralelo realizado em campo. Os efeitos da aplicação de carbofurão em organismos de solo assim como os potenciais efeitos indirectos nos organismos aquáticos devido à escorrência superficial e/ou lixiviação do pesticida foram avaliados através da realização de ensaios ecotoxicológicos padronizados com invertebrados de solo (*Enchytraeus crypticus*, *E. andrei* e *F. candida*) e cladóceros aquáticos (*Ceriodaphnia silvestrii*), respectivamente. Os resultados demonstraram um aumento de toxicidade na seguinte ordem: *E. crypticus* < *E. andrei* < *F. candida* < *C. silvestrii*. Assim, é de esperar um risco particularmente grande para os organismos aquáticos uma vez que as amostras de eluatos, lixiviados e águas de escorrência superficial foram altamente tóxicas para a espécie aquática testada. Não obstante, os riscos parecem ser mais elevados em caso de exposição dos organismos aquáticos a águas de escorrência contaminadas, reforçando a importância desta via de entrada de pesticidas em águas superficiais.

Os parâmetros de toxicidade calculados para os ensaios realizados com o simulador e em campo, tal como as análises químicas às amostras de solo, são semelhantes e indicam que o simulador é uma ferramenta válida para realizar avaliações integradas dos efeitos da aplicação de pesticidas.

No capítulo 4, o simulador desenvolvido anteriormente (capítulo 3) foi aperfeiçoado e utilizado na transposição da ferramenta de bioremediação testada no capítulo 2 para uma escala de semi-campo. Neste processo foi seguida uma abordagem semelhante à descrita no capítulo 2, embora adaptada a condições de "semi-ar livre", que incluiu, entre outras adaptações, uma aplicação dupla da ferramenta de bioremediação. Para além da avaliação da eficácia da bioremediação em amostras de solo, eluatos e lixiviados, também foram testadas amostras de águas de escorrência superficial. A bateria de testes incluiu duas espécies de plantas e uma microalga. Após sete dias de bioremediação, não foi observada toxicidade da atrazina (ou observou-se uma toxicidade ligeira) para *A. sativa* e para *P. subcapitata* nas amostras de eluatos e de águas de escorrência superficial (no caso dos lixiviados não foi observada qualquer toxicidade nos solos não bioremediados). Os resultados dos testes com *Brassica napus* apontam para uma bioremediação incompleta. Apesar disso, os dados das análises químicas e dos ensaios ecotoxicológicos deixam antever a aplicação com êxito desta ferramenta em campo.

Nos capítulos 5 e 6, respectivamente, os efeitos da aplicação de carbofurão em comunidades de nemátodes e microartrópodes (amostrados em áreas tropicais/sub-tropicais e de clima temperado quente), foram avaliados aplicando duas abordagens distintas: uma taxonómica e outra baseada em traços particulares dos organismos (i.e. hábitos alimentares no caso dos nemátodes e características relacionadas com a capacidade de dispersão no caso dos microartrópodes). Em ambos os ensaios, a metodologia seguida foi semelhante e consistiu na extracção dos organismos de solo fresco para solo previamente contaminado com carbofurão, seguida de incubação em laboratório.

A toxicidade de carbofurão nas comunidades de nemátodes (capítulo 5) foi mais evidente no ensaio realizado com solo Português, com uma redução significativa na abundância total e no número de famílias, com consequentes alterações na estrutura da comunidade em termos de famílias. No caso do ensaio realizado com solo recolhido no Brasil, não foram encontradas diferenças estatisticamente significativas (excepto para a estrutura da comunidade em termos de famílias), apesar dos padrões de resposta terem

sido semelhantes. A estrutura trófica da comunidade, reflectida pela abundância relativa dos diferentes grupos alimentares de nemátodes não foi afectada pela contaminação com carbofurão, indicando que estes traços em particular não foram sensíveis para detectar as mudanças induzidas pelo insecticida.

A exposição das comunidades de microartrópodes (provenientes de solo recolhido em Portugal e no Brasil) à contaminação por carbofurão (capítulo 6) provocou uma diminuição da diversidade taxonómica, tendo sido detectadas mudanças significativas na estrutura da comunidade, nos diferentes tratamentos com insecticida. A abundância de colêmbolos diminuiu ao longo do gradiente de contaminação enquanto, de um modo geral, a resposta oposta ocorreu para os ácaros (particularmente para os Oribatida). Apesar da maior sensibilidade encontrada para os indivíduos das comunidades do solo recolhido no Brasil, foram encontradas respostas análogas entre as comunidades do solo recolhido em Portugal. A contaminação do solo por carbofurão também induziu alterações ao nível da composição funcional das comunidades de colêmbolos, traduzidas no favorecimento de espécies adaptadas a camadas mais superficiais do solo, como foi revelado pela análise baseada nos traços de dispersão destes organismos. Os ensaios com comunidades e a descrição dos efeitos de pesticidas com base em traços morfo-ecológicos revelaram-se promissores para o estabelecimento de semelhanças nos padrões de resposta ecotoxicológica de comunidades de diferentes regiões geográficas.

Palavras-chave: ensaios ecotoxicológicos; pesticidas; ecotoxicologia tropical; atrazina; carbofurão; bioremediação; função habitat do solo; função de retenção do solo; simulador; águas de escorrência superficial; lixiviação; ecotoxicologia de comunidades; nemátodes; microartrópodes.

Summary

The worldwide demand for increased agricultural yields is leading to a constant increase in pesticide consumption, endangering Human health and ecosystems.

In pesticide Ecological Risk Assessment (ERA) schemes, information on the final destination of the applied pesticide (and its metabolites) as well as the potential effects in soil and aquatic biota are assessed, having as ultimate goal the sustainable use of pesticides.

A big challenge in ERA of pesticides is strengthen the link between laboratory data and real contamination scenarios. To achieve more realistic data, improvements in the test tools and/or strategies are necessary. The research carried out in the present thesis intended to be a contribution to fill these gaps and improve the ecological risk assessment of pesticide applications on agricultural areas, particularly from warm temperate and tropical areas.

More specifically, the objectives were: (1) to develop and validate a cost-effective laboratory simulator of pesticide sprayings, enabling the simulation of different contamination scenarios and the evaluation of potential risks for soil organisms but also the indirect toxic effects on aquatic organisms due to surface runoff and leaching; (2) to evaluate the efficacy of a bioremediation cleanup tool for atrazine contaminated soils at microcosm and semi-field scales, on both soil and water compartments, using ecotoxicological tests; (3) to implement the use of soil organism communities (nematodes and microarthropods) in ecotoxicological testing as a higher tier test; (4) to compare the toxicity of carbofuran under tropical/sub-tropical and warm temperate conditions.

In chapter 2, a previously developed cleanup tool for atrazine contaminated soils, combining bioaugmentation (using *Pseudomonas sp.* ADP) and biostimulation (using citrate) was scaled-up to open soil microcosms. The soil community was mimicked by including invertebrate (*Eisenia andrei* and *Folsomia candida*) and plant (*Avena sativa*) species. The efficacy of the bioremediation tool was evaluated on both soil and water compartments (for the last, using soil eluates and leachates) performing ecotoxicological tests with *A. sativa* and *E. andrei* (evaluation of soil habitat function) plus *Daphnia*

magna and *Pseudokirchneriella subcapitata* (evaluation of soil retention function). Results clearly showed the efficacy of the cleanup tool after 10 d of bioremediation, since atrazine and eventual intermediary metabolites appear to be reduced to levels that were no longer toxic to both plants and aquatic organisms.

In chapter 3, a prototype of the pesticide simulator was developed and tested under tropical conditions by comparing the results of ecotoxicological tests and chemical analysis performed with samples collected from the simulator and from a parallel field trial. The effects of carbofuran applications for soil biota and also the potential indirect effects on aquatic biota due to surface runoff and/or leaching were evaluated with standardized tests with soil invertebrates (*Enchytraeus crypticus, E. andre*i and *F. candida*) and aquatic cladocerans (*Ceriodaphnia silvestrii*), respectively. Results show increased toxicity in the following order: *E. crypticus* < *E. andrei* < *F. candida* < *C. silvestrii*. Aquatic organisms are expected to be particularly endangered due to carbofuran applications since eluate, leachate and runoff samples were highly toxic to the tested species. Notwithstanding, the risks seemed to be higher for exposure to pesticide runoff, strengthening the importance of this transport route as entrance of pesticides into surface waters. Derived toxicity parameters, as well as soil chemical analysis, were comparable between field and simulator trials, indicating that the simulator is a valuable tool to perform an integrated assessment of pesticide applications.

In chapter 4, the simulator previously developed (chapter 3) was improved and used in a further scaling up of the bioremediation tool tested in chapter 2, i.e., to a semi-field level. A similar approach to the one described in chapter 2 was used, though adapted to semi-outdoor conditions, which included, among other adaptations, a double application of the bioremediation tool, was performed. Besides soil, eluates and leachates, the bioremediation efficacy was also evaluated on runoff samples and the test battery included two plant and one microalgae species. After 7 d of soil bioremediation, no or slight atrazine toxicity was observed for *A. sativa* and for *P. subcapitata* in runoff or eluate samples (no atrazine toxicity was observed for leachates in non-bioremediated soils). Results from tests with *Brassica napus* point for an incomplete bioremediation. Despite this, both chemical and ecotoxicological

data gave promising indications of a successful field application of this cleanup tool.

In chapters 5 and 6, the effects of carbofuran applications on nematode and microarthropod communities (from tropical/sub-tropical and warm temperate areas), respectively, were evaluated using trait-based (i.e. feeding habits in case of nematodes and dispersion abilities in case of microarthropods) and taxonomic approaches. The parallel methodology consisted in the extraction of organisms from fresh soil to previously carbofuran contaminated soil, followed by laboratory incubation.

The toxicity of carbofuran on soil nematode communities (chapter 5) was stronger within the Portuguese assay, with a significant reduction in total abundance and number of families with consequent shifts in the family structure. For the assay with the Brazilian communities no statistically significant differences were found (except for the family structure), although the patterns of responses were similar. The trophic structure, reflected by the relative abundance of nematode feeding-groups, was not affected due to carbofuran contamination, indicating that feeding traits were not sensitive to detect insecticide induced changes.

The exposure of Portuguese and Brazilian microarthropod communities to carbofuran contamination (chapter 6) induced a decrease in the taxonomic diversity, with significant shifts detected in the microarthropod community structure, in the different insecticide treatments. The abundance of collembolans decreased along the contamination gradient while the opposite response generally occurred for mites (particularly for Oribatida). Despite the higher sensitivity found for the individuals from Brazilian communities, analogous responses were observed within the Portuguese test. Carbofuran soil contamination also induced changes in the functional composition of the Collembola communities, reflected by the favoring of species adapted to higher soil layers, as revealed by trait based analysis. The community assays and the description of pesticide effects based on traits showed to be promising approaches to establish similarities in ecotoxicological response trends of communities from different geographical regions.

Key words: ecotoxicological tests; pesticides; tropical ecotoxicology; atrazine; carbofuran; bioremediation; soil habitat function; soil retention function; simulator; runoff; leaching; community ecotoxicology; nematodes; microarthropods.

Chapter 1

General introduction

I. Trends in the use of pesticides and legal framework regulating their application

World population growth and the global aim of eradicating poverty and hunger act as major drivers for the increase in food production. This is usually achieved not only with an improvement of soil and water management, but also with the increment in arable land and, consequently, in the use of agrochemicals, organic fertilizers and biological control agents (Carvalho, 2006).

Recent estimates point that the largest increases in world population will occur in developing countries (Cohen, 2005; UN, 2011), where the financial resources are scarce and the legislation regulating the safe use of pesticides and their environmental monitoring does not exist or is incipient (DeSilva et al, 2009; Ecobichon, 2001; Abhilash and Singh, 2009; Wesseling et al, 2005). Hence, pesticides that have been banned in developed countries due to their proven environmental hazards (e.g. DDT, HCH, lindane), constitute cheaper alternatives to the less persistent and more environmental friendly (but more expensive) pesticides (Carvalho, 2006).

At the global scale, pesticide usage is continuously increasing and data from 2007 points for approximately 2.4 million tones, with the largest proportion of total use being occupied by herbicides (40%), others (33%) and insecticides (17%) (Grube et al, 2011). The top one in the world consumption belongs, since 2008, to Brazil. In 2009, more than 260 000 tones were sold, among herbicides, fungicides and insecticides/acaricides (Rebelo et al, 2010).

Within the European Union (EU), the consumption of pesticides continues to growth (Eurostat, 2009). Projections for the year 2050 show an increase from 1.9- to 4.8-fold comparatively to the present time (Turbé et al, 2010). In Portugal, the total volume of plant protection products (PPPs) used more than doubled between 1992 and 2003 (from ≈ 6000 to 14.000 tones of active substances; Muthmann, 2007). In 2010, sales reached 21.057 tones, with larger dominance of fungicides (≈ 56%; ANIPLA, 2011).

The first European legislation on pesticides was introduced in 1979 (Council Directive 79/117/EEC; European Economic Community, 1979). Progresses have been made with the adoption of Directive 91/414/EEC

(European Economic Community, 1991), and related guidance documents on aquatic and terrestrial ecotoxicology (SANCO, 2001, 2002), regulating the commercial release of plant protection products and presenting the framework for the environmental risk assessment of these compounds. More recently, further advances were achieved with directives 98/8/EC (European Commission, 1998) and 2009/128/EC (European Commission, 2009a), respectively, regulating the use of biocides and the sustainable use of pesticides. Starting from 14 June 2011, Regulation (EC) No 1107/2009 replaced the first two directives mentioned above, and reinforced the need for an effective evaluation on pesticide environmental fate and effects (European Commission, 2009b). Defining more restrictive protection goals and broadening the spatial scale where effects have to be evaluated, this new directive points on the direction of a more ecologically sound risk assessment based on the available scientific and technical knowledge. Under this context, the European Food Safety Authority (EFSA) is undertaking the mission to revise the technical guidance documents on ecotoxicology mentioned above, and produced a series of documents dealing with the definition of specific protection goals for pesticide registration (Nienstedt et al, *in press*) and new developments in exposure assessment in soil (EFSA, 2010a, b).

References to pesticide contamination can be found in other legislative documents. For example, the water framework directive established quality standards for chemical substances in European surface and groundwaters (European Commission, 2000). Regarding soil protection, although being a recent issue in regulatory decisions, compared to the aquatic compartment, the EU thematic strategy for soil protection highlighted soil local and diffuse contamination as one of the major threats to soil quality and recognized the need for protecting the soil functions (European Commission, 2006a); in addition, the proposal for a soil framework directive sets out common principles for the protection of European soils (European Commission, 2006b).

II. Ecological risk assessment of pesticides in brief

In Ecological Risk Assessment (ERA) of pesticides, performed for their registration and regulation, the likelihood that adverse effects may occur in ecosystems inhabitants as a result of pesticide exposure is evaluated (European Commission, 2003). This is done separately for the aquatic and the terrestrial compartments and considering representatives of key ecological receptors (microorganisms, plants, invertebrates and vertebrates) (European Economic Community, 1991).

Giving the terrestrial compartment as an example, risk assessment involves the analysis of the fate of the compound (and the estimation of exposure concentrations) and the evaluation of effects in soil microorganisms, soil invertebrates, plants, bees, other non-target foliage arthropods and vertebrates. This is done independently for each ecological receptor via the calculation of risk quotients [Toxicity Exposure Ration (TER) or Hazard Quotient (HQ) values, the last being the reciprocal of the first] and their comparison with trigger values. The process follows a tiered approach, where TER or HQ values (the last are set for bees and non-target arthropods) may indicate no risk (no further action is required) or may trigger (when combined to persistence data of the compound) the need for further information to refine the risk. This could be done by refining both exposure and effects assessment, i.e., applying more robust fate models and performing longer term and/or higher tier (semi-field / field) tests. Risk mitigation measures, like reduction of application rate, drift reduction techniques, maintenance of ecological structures off-field, can also be considered. If refined risk does not met the criteria for authorization, than it is not granted (SANCO, 2002b).

II.1 Assessment of pesticide fate

The fate of pesticides comprises their release and transport trough soil as well as potential transfer to air, water and food (Ariaz-Estevez et al, 2008). The application of pesticides to targeted biological receptors inevitably results in the transport of a significant portion of these chemicals and their degradation products to surrounding non-target areas and organisms.

Problems may start even before the application of a pesticide to a specific biological target, with the deficient storage conditions at industrial units, dealerships or at mix-load sites and the careless handling and protection of people that will prepare and spray the chemicals (Ecobichon, 2001; Abhilash and Singh, 2009). Once the pesticide is applied, only a percentage reaches its target (less than 0.1%, according to Pimentel, 1995), and the remaining is lost by several processes illustrated in Figure 1.1.

The pesticide spraying is concomitantly followed by losses to the atmosphere by spray drift and volatilization (Schulz, 2004). Non-target plants, soil organisms and microorganisms are also biological receptors trough absorption or ingestion processes. At the soil surface, the pesticide may be washed trough runoff related-events due to rain or irrigation and contaminate the surrounding areas and surface waters (Schulz, 2004; Rice et al, 2007). Along the soil profile, the pesticide may adsorb to soil particles, and is also subjected to both chemical and microbial degradation. Besides that, it may be also leached into deeper soil layers, eventually reaching groundwater resources (Rice et al, 2007).

Several factors like soil properties, weather conditions but especially the pesticide physico-chemical properties, determine the extent of pesticide degradation trough all of the above referred pathways (for a review see Ariaz-Estevez et al, 2008).

Often, natural pesticide degradation is not effective enough to reduce its concentrations to non-hazard levels and, consequently, pesticides and/or their degradation products contaminate soil, food but especially water resources (e.g. Dasgupta et al, 2002; Cerejeira et al, 2003; Abhilash and Singh, 2009). Ultimately, Human health, wildlife as well as terrestrial and aquatic ecosystems may be endangered due to pesticide exposure (Carvalho, 2006).

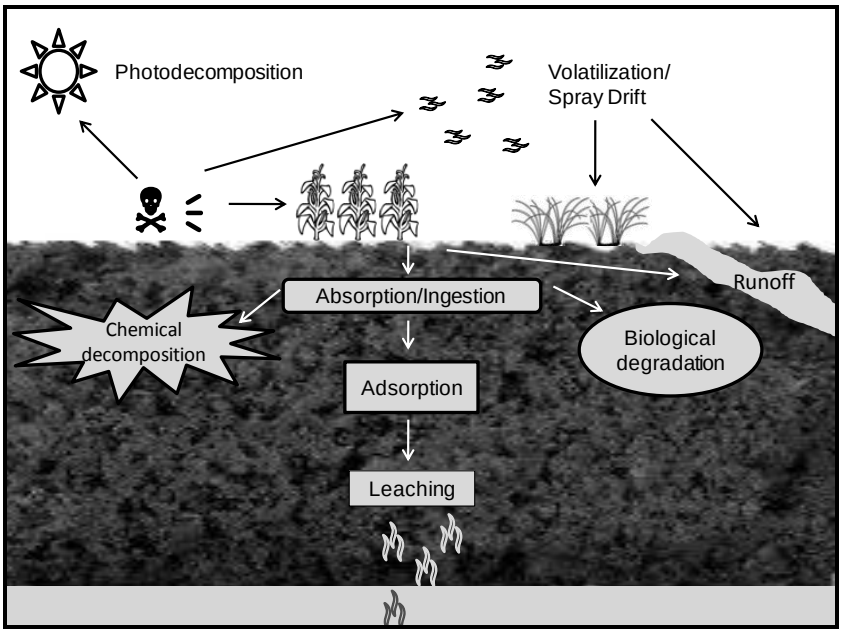

Figure 1.1. Pathways of pesticide loss. Adapted from Rao et al, 1983.

In ERA of pesticides, the exposure assessment is usually based in fate modeling for the different environmental compartments, using pesticide properties (e.g. solubility, vapour pressure, half-life, sorption coefficient, degradation), physico-chemical data from soil and water, information from the area under study (slope, catchment size, point and diffuse sources of pesticide release) plus climate information. A Predicted Environmental Concentration (PEC) is thus calculated for each compartment (Dubus and Surdyk, 2006).

In Europe, the FOCUS (FOrum for the Co-ordination of pesticide fate models and their Use) project has been carried out to harmonize the calculation of PECs of pesticides. The variability of European climate has been taken into account and standard scenarios of contamination were developed, considering leaching to groundwater, spray drift, dissipation drainage and run-off events (more information available at: http://focus.jrc.ec.europa.eu/; Assessed 13th October 2011).

More recently, within the European Union, the definition of soil exposure scenarios has gained importance and the EFSA Panel on Plant Protection Products is developing tiered exposure-assessment approaches for soil organisms and realistic worst-case pesticide exposure scenarios for the North/ Centre/South regulatory zones (EFSA, 2010a). Also, a modeling approach has been used to define soil ecoregions within Europe, based on life forms of earthworms, enchytraeids, collembolans and isopods, aiming to define realistic worst-case soil depth profiles for pesticide short term exposure (EFSA, 2010b).

II. 2 Assessment of pesticide effects

The characterization of pesticide effects is traditionally performed by exposing test-organisms to a gradient of concentrations under laboratory conditions (Van Straalen, 2002). After a certain period of time, effects on distinct endpoints such as mortality, behavior, growth, reproduction or bioaccumulation are evaluated (ISO, 2003). If results point for unacceptable risks, higher tier tests (e.g. Terrestrial Model Ecossystems - TMEs- or field studies for the terrestrial environment and mesocosms for the aquatic systems) are performed (Jänsch et al, 2006). The data obtained in the ecotoxicological tests is then subjected to statistical analysis to calculate ecotoxicological parameters (e.g. LC50, EC50, NOEC, LOEC) and, after the application of extrapolation methods (e.g. safety factors, Species Sensitivity Distributions) the Predicted No Effect Concentration (PNEC) (UNEP/IPCS, 1999) is derived. The risk characterization can be performed based on the evaluation of ratio between PEC/PNEC or other indicator quotients (TER and/or HQ; SANCO, 2002a,b).

Considering the soil compartment, several standardized ecotoxicological tests are available to evaluate the risks of pesticides (Römbke and Knäcker, 2003; ISO, 2003), covering different trophic levels (producers, decomposers, grazers, predators) and routes of exposure (via soil pore water, food and soil particles ingestion and inhalation of air present in the soil pores; EFSA, 2009).

Among lower tier tests, the most commonly performed are single species tests with soil invertebrates and plants, which are summarized in Table 1.1, although not all are required for the authorization of the pesticide release to the market (SANCO, 2002b). Microbial tests include the determination of soil microbial biomass (ISO, 1997a, b), diversity (Nielsen and Winding, 2002) plus soil nitrification and carbon mineralization (OECD, 2000a, b), being the last two required for pesticide registration (SANCO, 2002b).

Single species tests are widely accepted as tools to gain consistent and inter-comparable data, but they do not take into account the interactions between species from different trophic and community levels (Kuperman et al, 2009). Semi-field (higher tier) tests like micro and mesocosms (e.g. Terrestrial Model Ecossystems - TMEs) make the bridge between laboratory and field. They try to simulate the processes and interactions between organisms occurring in the environmental compartment under study (for a recent overview on the available semi-field approaches for soil, see Schäffer et al, 2010), thus addressing a more realistic exposure. Field tests (e.g. earthworm field test; ISO, 1999b) and functional tests (e.g. litter decomposition, bait lamina; see Römbke et al, 2003) represent even higher levels of complexity and ecological realism.

Standardized guidelines for aquatic systems have been developed earlier than for soil and thus a larger data set on pesticide toxicity is available (Van Straalen, 2002). Some of the available single species protocols required for the authorization of the pesticide release to the market (SANCO, 2002a) are shown in Table 1.2. The use of higher tier outdoor aquatic systems (e.g. mesocosms) is well established in aquatic ecotoxicology as they have been used in the assessment of pesticide effects for more than 20 years (for a review see Caquet et al, 2000).

Table 1.1. Overview on soil single species ecotoxicological tests. Those required for the authorization of the pesticide release to the market (SANCO, 2002b) are marked in bold italics.

Organism	Species	Endpoint	Guideline/reference
Earthworms	*Eisenia andrei* *Eisenia fetida*	Avoidance Behavior	ISO 17512-1 (ISO, 2008)
		Mortality	***ISO 11268-1 (ISO, 1998a)*** ***OECD 207 (OECD, 1984a)***
		Reproduction	***ISO 11268-2 (ISO, 1998b)*** ***OECD 222 (OECD, 2004a)***
	Eisenia fetida	Bioaccumulation	ASTM E1676 - 04 (ASTM, 2004) OECD 317 (OECD, 2010)
Collembolans	*Folsomia candida*	Avoidance Behavior	ISO 17512-2 (ISO, 2011)
		Reproduction	***ISO 11267 (ISO, 1999a)***

Table 1.1. (continued)

Organism	Species	Endpoint	Guideline/reference
Enchytraeids	*Enchytraeus albidus* *Enchytraeus crypticus*	Avoidance Behavior	Amorim et al (2005) Chelinho et al (2011)
		Reproduction	ISO 16387 (ISO, 2004)
	Enchytraeus albidus	Bioaccumulation	ASTM E1676 - 04 (ASTM, 2004)
Nematodes	*Caenorhabditis elegans*	Mortality	ASTM E 2172-01 (ASTM, 2008)
		Reproduction	ISO 10872 (ISO, 2009)
Mites	*Hypoaspis aculeifer*	***Reproduction***	***OECD draft (OECD, 2007)***
Isopods	*Porcellio scaber*	Growth	Lokke and Van Gestel, 1998
		Reproduction	Lokke and Van Gestel, 1998
Plants	Several species of Mono and Dicotyledonous	Plant Root Growth	ISO 11269-1 (ISO, 1993a)
		Seed Emergence	***ISO 11269-2 (ISO, 1993b)*** ***OECD 208 (OECD, 2003)***
		Plant Growth	***ISO 11269-2 (ISO, 1993b)*** ***OECD 208 (OECD, 2003)***

Table 1.2. Overview on aquatic single species ecotoxicological tests required for the authorization of the pesticide release to the market (SANCO, 2002a).

Organism	Species (e.g.)	Endpoint	Guideline/reference
Insects	*Chironomus riparius*	Immobilisation	OECD 235 (OECD, 2011)
		Emergence	OECD 218 (OECD, 2004b)
		Development Rate	OECD 219 (OECD, 2004c)
Cladocerans	*Daphnia magna*	Mortality	OECD 202 (OECD, 2004d)
		Reproduction	OECD 211 (OECD, 1998)
Fishes	*Oncorhynchus mykiss*	Mortality	OECD 203 (OECD, 1992)
		Juvenile Growth	OECD 215 (OECD, 2000c)
		Reproduction	OECD 229 (OECD, 2009)
		Bioaccumulation	OECD 305 (OECD, 1996a)
Algae	*Pseudokirchneriella subcapitata*	Growth Inhibition	OECD 201 (OECD, 1996b)
Macrophytes	*Lemna minor*	Growth Inhibition	OECD 221 (OECD, 2006)

III. New challenges in risk assessment of pesticides – placing this thesis into context

III. 1 Assessing relevant exposure routes

Soils are quite heterogeneous and complex matrices and, therefore, the consequences of pesticide applications on soil but also on aquatic systems due to the displacement from their target areas, need to be further investigated. Indeed, ecotoxicology and ERA of pesticides face several challenges/research needs to improve test methods (especially the exposure conditions) and testing strategies and increase the ecological relevance of the data obtained (Kuperman et al, 2009; Breitholtz et al, 2006).

With respect to testing strategies, a more straight connection between ERA of water and soil, which are usually performed separately, is desirable. As stated before, the final destination of pesticides and/or of their metabolites sprayed into the soil is frequently the adjacent or nearby water resources.
International guidelines for ecotoxicological characterization of contaminated soils (ISO, 2003; 2005) established a series of bioassays to be performed, focusing on soil habitat and retention functions (determined by tests with the soil matrix and with the soil extracts, respectively). By assessing the potential of substances to be moved via water pathway and affect aquatic organisms, the evaluation of soil retention function is an expression of soil leaching potential. However, besides leaching, pesticides can be transported into surface waters via surface runoff, which seems to be the major source of nonpoint-source pesticide contamination of aquatic systems (Schulz, 2004). Therefore, when assessing the effects of pesticides in aquatic systems, realistic exposure scenarios of surface water (and also groundwater) contamination should be included (Moreira et al, 2010; Lopes et al, 2007). Runoff of pesticides is a particular problem not only in tropical regions, where severe and unpredictable heavy rainfalls often occur immediately after large pesticide applications (Henriques et al, 1997; Lacher and Goldstein, 1997; Castillo et al, 1997; Carvalho, 2006), but also on Mediterranean regions, where soils are often eroded and rainwater is poorly retained (Yaloon, 1997;

Voltz et al, 2003), and where erratic but intense rain events are becoming more common (Alcamo et al, 2007). Yet, no standard procedures and/or cost effective tools enabling the joint collection of soil and aqueous samples to assess the ecotoxicological effects of pesticide sprayings on both soil and water compartments are available.

Overall, simultaneous assessment of the risks associated with pesticide use for soil and aquatic organisms is hardly investigated, and often an underestimation either of the pesticide concentration that may appear in one of two target compartments or their toxic effects occurs. Consequently, using an integrated approach, simultaneously combining information from ecotoxicological characterization of soil system and of the potential effects due to surface runoff and leaching, would be a valuable contribution for ERA of pesticide applications on agricultural areas. Thus, the evaluation of effects under more realistic exposure routes (e.g. runoff and leaching) may also help to obtain more relevant data.

III. 2 Considering different exposure scenarios for different regions

In a global world, the potential risks of pesticide applications cannot be ignored, even if we are thousands of kilometers apart. In fact, in many parts of the world, the lack of scientific knowledge on pesticide fate and effects is a threat to the implementation of their sustainable and safe use (Ecobichon, 2001). In the tropics, risk assessment schemes rely mostly on the extrapolation of temperate data (Römbke et al, 2008; Kwok et al, 2007) overlooking the impact that the different physical, chemical and biological conditions of the tropics might exert in the overall hazards (Castillo et al, 1997; Lacher and Goldstein, 1997). Contradictory information is given when comparing the pesticide toxicity under temperate and tropical conditions. For the aquatic compartment, and depending on the pesticides, the sensitivity of tropical species can be higher, similar or lower than temperate species from the same taxonomic group (Maltby et al, 2005; Kwok et al, 2007). For the soil ecosystems, despite the scarcity of information if compared with the aquatic

ones, the overall sensitivity seems to be similar (De Silva et al, 2009, 2010; Garcia et al, 2008; 2011).
Therefore, the development of specific scenarios for risk assessment for these regions is recommended (Römbke et al, 2008; De Silva et al, 2009). The same recommendation is valid for the warm temperate regions (e.g. Mediterranean region), with particular climatic conditions, as well as soils and agricultural practices (Ramos et al, 2000).

III.3 Evaluating the efficacy of remediation measures

When is not possible to prevent the contamination of environmental compartments by pesticides, the challenge is to develop and employ mitigation technologies but also to prove its efficacy. Indeed, to enhance the degradation of pesticides, research has been conducted on the development of remediation and bioremediation technologies either to reduce soil concentrations to non hazard levels and/or to prevent the contamination of the surrounding environmental compartments (Rice et al, 2007). Bioremediation takes advantages from microorganisms or plants that are able to promote a faster and/or higher degradation of a certain pollutant. Traditional physico-chemical remediation processes are more expensive and can have collateral effects over the soil matrix itself as well as on its native flora and fauna (Dua et al, 2002; Philp and Atlas, 2005; Timmis and Pieper, 1999).
For the assessment of the efficacy of remediation and bioremediation strategies, toxicity bioassays are useful tools to estimate the bioavailable fraction of the contaminant (s) under study (Juvonen et al, 2000; Fernandez et al, 2011).

III. 4 Higher tier testing in soil

The biggest challenge of all in ERA and ecotoxicological testing can be probably resumed to the word extrapolation (Vignati et al, 2007; Van den Brink, 2008). For example, extrapolating from data obtained from laboratory

single species tests to the effects on natural communities (Van den Brink, 2008; Kuperman et al, 2009), from single exposure to multiple exposure, from single compounds to effects of mixtures (Lydy et al, 2004), from artificial to natural field soils (Römbke and Amorim, 2004) and across different geographical regions (Van den Brink, 2008). Summarizing, a central problem of ecotoxicology is to get data, usually obtained in the laboratory, which are relevant to the real conditions of an ecosystem (Römbke and Moltmann, 1996).

The down side of obtaining more reliable and ecological relevant data from higher tier tests is frequently more experimental effort and associated costs, as well as higher data variability *(*Breitholtz et al, 2006; Van den Brink et al, 2005; Schäffer et al, 2008). Notwithstanding, in terrestrial ecotoxicology, the need to develop new testing strategies using the communities of organisms, covering higher levels of biological organization has been highlighted by several authors (Jänsch et al, 2006; Kuperman et al, 2009; Römbke et al, 2009; Turbé et al, 2010; Van Straalen, 2002).

A current innovative trend in community testing is to express the effects of chemicals and other stress factors using the morphological/physiological/ecological characteristics of the organisms belonging to that community, the so called Trait-Based Risk Assessment (TERA; Baird et al, 2008; see the special series published in Integrated Environmental Assessment and Management (IEAM) journal, Volume 7, Issue 2, April 2011). This approach can provide a more functional and complete description of structure and function of ecosystems as well as to extrapolate those effects to a broader range of species and communities from different geographic areas (Baird et al, 2008).

IV. Outline of the Thesis

The main objective of the research presented in this thesis was to improve the ecological risk assessment of pesticide applications on agricultural areas, particularly from warm temperate and tropical regions, by tackling some of the above mentioned challenges/needs. Innovative methodologies and/or tools were developed and/or tested, namely: the integrated evaluation of pesticide effects using a cost-effective laboratory simulator, where different pathways of exposure were evaluated gaining information from both soil and aquatic ecotoxicological lines of evidence; the reinforcement of the use of community studies with soil organisms in ERA schemes; and the use ecotoxicological evaluations to assess the efficacy of bioremediation strategies.
More specifically, the objectives were:

- To develop and validate a cost-effective and ready amenable to standardization laboratory simulator of pesticide sprayings and pesticide mobilization via the water pathway by both leaching and surface runoff;

- To evaluate the efficacy of a bioremediation cleanup tool for atrazine contaminated soils and scaled up at a microcosm level, on both soil and water compartments, using ecotoxicological tests.

- To apply the developed and validated laboratory simulator to assess the efficacy of the bioremediation cleanup tool for atrazine contaminated soils referred above, at a semi-field scale.

- To test new complementary approaches to classical standardized single species toxicity tests, using the natural soil microarthropod and nematode communities, adopting conventional test strategies and use taxonomic and trait-based approaches to describe the effects of carbofuran applications in soil.

- To compare the toxicity of carbofuran under tropical/sub-tropical and warm temperate conditions.

Therefore, the studies presented in this thesis may be grouped into three main branches of innovation: i) the development, testing and validation of a laboratory simulator of pesticide applications (Chapters 3 and 4); ii) the scaling up and ecotoxicological evaluation of a bioremediation tool for atrazine contaminated soils (Chapters 2 and 4); iii) the development and testing of a soil community approach using conventional laboratory methodologies, to evaluate the effects of pesticide applications on soil organisms; besides the description of effects at taxonomic level, a special focus was given on TERA, by using functional traits of soil organisms (Chapters 5 and 6).

Thus, the second chapter refers to the scaling up of a previous developed bioremediation tool for atrazine contaminated soils to a microcosm level. An integrated monitoring of the bioremediation efficacy on both soil habitat and retention functions was performed using ecotoxicological tests with plants plus soil and aquatic invertebrates.

In the third chapter, a prototype of the laboratory simulator was developed, enabling the simulation of different scenarios of pesticide contamination and the collection of soil and aqueous samples. These features allowed evaluating the potential risks for soil organisms but also the indirect toxic effects on aquatic organisms due to the pesticide mobilization via the water pathway. Water compartments might be endangered not only due to the loss of the soil retention function and, consequently, due to leaching to groundwater reservoirs and/or due to surface runoff from contaminated soils. The testing strategy comprised the performance of two parallel trials with a tropical soil, under laboratory (using the simulator) and field conditions. The validation of this laboratory simulator was based in the comparison of the toxicity data obtained in ecotoxicological tests performed with samples collected from both simulator and field trials, where soil and aquatic standard test-species were exposed to carbofuran contamination.

In the fourth chapter, based on the experience gathered in the assays conducted in Brazil (and described in chapter 3) some functional aspects of the laboratory simulator prototype developed before were improved. The final version of this device was validated under a semi-field scenario where, at the same time, the efficacy of the bioremediation tool for atrazine contaminated soils (previously demonstrated at the microcosm level as described in chapter

2) was again evaluated, now at a higher level of complexity, foreseeing its potential routine use under real field scenarios.

The two last chapters (5 and 6) aimed to implement the use of native communities of soil organisms in ecotoxicological tests and contributing to the application of TERA based approaches in soil ecotoxicology as a way of improving ecological relevance of toxicity data. A common testing strategy was adopted for both studies. Basically, it consisted in the extraction of organisms from fresh clean soil to previously contaminated (and defaunated) soil with a model pesticide (carbofuran), with further incubation under laboratory conditions. After the incubation period, the organisms were again extracted and effects were assessed at the taxonomic level (e.g. changes in the number of taxonomic entities and total abundance) but also at the ecomorphological trait level.

Thus, in Chapter 5, this approach was followed using two soil nematode communities (from sub-tropical and Mediterranean climates) and their feeding habits (as well as abundance, number of families and family structure) were used to describe the responses of the communities to insecticide contamination.

In chapter 6, two geographically distinct soil microarthropod communities (from Portugal and Brazil) were selected as the test-group. Effects on the taxonomic composition (besides changes in richness and abundance) of both communities along the contamination gradient focused on the two most abundant groups, Acari and Collembola. In addition, collembolans were used as a case-study of TERA in soil and the extracted organisms were assorted according to functional traits (related with their dispersion abilities).

On chapter 7, a general discussion of the results showed in chapters 2 - 6 is presented.

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Chapter 2

Cleanup of atrazine-contaminated soils: ecotoxicological study on the efficacy of a bioremediation tool with Pseudomonas sp. ADP

Based on the following manuscript:

Chelinho S, Moreira-Santos M, Lima D, Silva C, Viana P, André S, Lopes I, Ribeiro R, Fialho AM, Viegas CA, Sousa JP. 2010. Cleanup of atrazine-contaminated soils: ecotoxicological study on the efficacy of a bioremediation tool with *Pseudomonas* sp. ADP. *Journal of Soils and Sediments* 10: 568-578.

Abstract

To mitigate the environmental effects of atrazine one of the cleanup strategies available is based on the use of atrazine degrading bacteria. This work aimed to evaluate the efficacy of a previously developed bioremediation tool for atrazine contaminated soils (combining bioaugmentation with *Pseudomonas* sp. ADP, hereafter designated as *P.* ADP, and biostimulation with citrate) on both soil habitat and retention functions, by performing ecotoxicological tests with standard soil and aquatic species. Soil microcosms (incorporating earthworms, collembolans and plants) were spiked with three doses of Atrazerba FL, an atrazine commercial formulation: the recommended dose (RD; 2L/ha), 10×RD and 20×RD to simulate over-use / accidental spills scenarios. The experiment included two main groups of treatments: (i) microcosms sprayed solely with Atrazerba, i.e., non-bioremediated soils (NB) and (ii) microcosms sprayed with both Atrazerba and the bioremediation tool (addition of *P.* ADP plus citrate), i.e., bioremediated soils (B). Control microcosms with no herbicide or *P.* ADP plus citrate addition were also set up. Besides soil chemical analysis, the following ecotoxicological endpoints were assessed to monitor bioremediation: plant biomass production, earthworm reproduction, microalgae growth (in eluates—collected five and ten days after the bioremediation treatment – and leachates—collected on day seven) and cladoceran reproduction (in soil eluates).

In NB soils, all Atrazerba doses induced a severe reduction in plant biomass production and no effects were found for earthworm's reproduction. Eluates and leachates obtained from the NB soils caused deleterious effects on both microalgae growth and cladoceran reproduction. Chemical analysis showed that atrazine degradation was faster in B soils than in the correspondent NB soils. Data from toxicity tests indicated that test organisms performance was enhanced in B soils and respective eluates and leachates, compared to the NB samples. In fact, for soils contaminated with 10× and 20×RD Atrazerba doses, plant biomass production was significantly higher in the B soils than in the correspondent NB soils. Regarding the effects of soil bioremediation on the toxicity of soil eluates and leachates, for the soil contaminated with 10×RD of Atrazerba, over a five days treatment period, both microalgae growth and

cladoceran reproduction were significantly higher in water extracts obtained from the B soils when compared with the NB extracts and also similar to the control. By the contrary, for the highest Atrazerba dose tested (20× RD), no significant differences were found on the toxicity of B and NB eluates towards both aquatic test organisms. However, for this same dose, after seven days, microalgae growth was higher in B than in the NB leachates and similar to the control. Yet, after a longer bioremediation period of ten days, eluates were also no longer toxic to both aquatic organisms.

Based on atrazine soil chemical analysis, one can state that the addition of *P.* ADP plus citrate to the atrazine-contaminated soils was clearly effective in promoting atrazine biodegradation. In addition, ecotoxicological data support the efficacy of this cleanup tool. Indeed, results showed that the bioremediation treatment resulted in a relevant reduction on soil toxicity to a plant (approximately 100 and 72% of control, respectively, for 10×RD and 20×RD contaminated soils). In addition, five days of *P.* ADP activity were enough to annul atrazine toxic effects towards microalgae and cladocerans in eluates obtained from the soil contaminated with 10×RD of Atrazerba. For 20×RD, an effective detoxification of eluates was achieved only after a longer bioremediation period of ten days.

The ecotoxicity tests proved not only the effective detoxification of bioremediated soils in 10 days, but also the potential ability to concurrently reduce atrazine contamination of water compartments due to leaching and/or run-off events, to levels that may no longer be hazardous to ecosystems.

Due to the worldwide continued use of atrazine/triazine based herbicidal formulations, further studies viewing the optimization of this cost-effective cleanup tool at larger scales (mesocosm and real field scenarios) and testing of other commercial formulations containing mixtures of atrazine/triazine and other active ingredient are still needed so that bioremediation can be used as a valuable tool to reduce herbicide toxicity in contaminated land.

Keywords: Atrazine; Bioremediation; Ecotoxicology; Pesticides; Soil contamination.

I. Introduction

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-1-s-triazine) has been widely used in pre- and post-emergence weed control in several crops (Solomon et al, 1996). Because atrazine is somewhat persistent in soil, with a half-life of 41 to 231 days (Kruger et al, 1993), and moderately soluble in water, it can move through soil during irrigation and/or rainfall events and contaminate sediments, surface and groundwaters (Solomon et al, 1996; Schwab et al, 2006). A summary of atrazine concentrations in aquatic systems (water/sediments) worldwide reported values ranging from less than 0.001 to 1,000 µg/L (Graymore et al, 2001), clearly exceeding legislation limits (e.g., 0.1 µg/L in the EU; European Union, 1998). Likewise, in Portugal, atrazine was the most frequently detected herbicide in surface and groundwaters, with maximum concentrations of 0.6 and 30 µg/L, respectively (Cerejeira et al, 2003). Also, it has been detected in soil at concentrations highly above the recommended application rates (1-1.5kg/ha or ~ 0.65-1mg/kg in Atrazerba FL, the commercial formulation used in this study) due to accidental spills (e.g. maximum concentrations of 29000 mg/kg were reported by Strong et al 2000), negligent handling in mix-load sites (e.g. Chirnside et al, 2007 reported maximum concentrations of 205 mg/kg) or overuse (concentrations of 500 mg/kg were measured in a cereals growing field – Aresta et al, 2004).

The risks for human health and the environment posed by atrazine applications have long been demonstrated (Solomon et al, 1996; Graymore et al, 2001; Sass and Colangelo, 2006)**.** As a result, and confronted with the reported levels of environmental contamination, this herbicide was recently banned from EU countries (European Commission, 2004), though, it is still intensively used worldwide, namely in USA, Africa, Latin America, Asia and Australia (Sass and Colangelo, 2006; Correia et al, 2007; Kadian et al, 2008; Lewis et al, 2009).

To mitigate the environmental effects of atrazine, several atrazine-degrading bacteria have been isolated and tested with atrazine contaminated soils (Topp et al, 2000; Chirnside et al, 2007; Wackett et al, 2002). *Pseudomonas* sp. ADP is the best characterized atrazine-mineralizing

bacteria (Wackett et al, 2002). Recently, a cleanup strategy combining bioaugmentation using *Pseudomonas* sp. ADP and biostimulation with citrate (Silva et al, 2004) was examined in soil microcosms and proved to be effective in diminishing atrazine concentrations from soils spiked with an atrazine commercial formulation (Lima et al, 2009). However, to fully evaluate this technology, besides data on the fate of atrazine and its metabolites, it is essential to investigate the responses of representative organisms of the threatened ecosystems (effect assessment). According to the ISO guidelines on soil quality (ISO, 2003), the ecotoxicological assessment of contaminated soils comprises the evaluation of i) their capacity to function as habitat for organisms (soil habitat function), and ii) their ability to impede the release of contaminants to ground and surface water (soil retention function).

The objective of the present study was to evaluate the efficacy of the application of a bioremediation tool for soils contaminated with an atrazine commercial formulation, at the ecotoxicological level. The effects on both soil habitat and retention functions were evaluated by performing toxicity tests with standard soil and aquatic species.

II. Materials and methods

II.1 Experimental design

A summary of the experimental design/sampling scheme is available in Table 2.1. The experiment included two main groups of treatments: (i) microcosms sprayed with Atrazerba FL (an atrazine commercial formulation from Sapec, Lisboa, Portugal; ~500 g a.i./L); and (ii) microcosms sprayed with both Atrazerba and the bioremediation tool, i.e., the addition of the bioaugmentation (*Pseudomonas* sp. strain ADP, hereafter designated as *P.* ADP; see section 2.2) and biostimulation (citrate) agents. The treatments of group (i), corresponding to non-bioremediated soils, will be designated as NB, while those of group (ii), i.e., bioremediated soils, will be designated as B. A control treatment (microcosms with no herbicide or *P.* ADP plus citrate addition) was also set up. At the beginning of the experiment (day 0), the soil

surface was sprayed with the following doses of Atrazerba: the recommended dose (RD) for weeds in corn plantations (2 L/ha), 10×RD (20 L/ha) and 20×RD (40 L/ha) for NB treatments and only the latter two for B treatments (Table 2.1). The two highest Atrazerba doses intended to mimic worst-case scenarios for pesticide applications, such as intensive use (e.g. Correia et al, 2007), overuse (e.g. Aresta et al, 2004), careless disposal (e.g. Chirnside et al, 2007, Dasgupta et al, 2007), as well as accidental spills (e.g. Strong et al, 2000).

After the herbicide application, the 3 cm topsoil were mixed and a rainfall was simulated using artificial rain (Velthorst, 1993), to incorporate the herbicide (corresponding to 12 hours/day of rain during the rainiest month in Coimbra; Geophysical Institute of the University of Coimbra 2007). For B treatments, prior to herbicide spraying, a concentrated solution of trisodium citrate was added to each Atrazerba suspension to amend soil with 1.2 and 2.4 mg citrate/g of soil DM (dry mass) for the 10 and 20×RD, respectively (Lima et al, 2009). These concentrations were used to achieve a C_s:N_{atz} ratio of 50 (Silva et al, 2004). After Atrazerba (plus citrate) incorporation, the soil was bioaugmented by distributing the inoculum suspension ($2.8 \pm 0.5 \times 10^{10}$ CFU of *P*. ADP/ml) at the soil surface, and was again mixed and watered. In the control microcosms, the herbicide, *P.* ADP and citrate spraying were replaced by the same volume of artificial rain. The total amount of liquid added per microcosm was adjusted to obtain initial soil moisture of about 50% of the soil WHC (water holding capacity).

The experiment was conducted at controlled temperature (21ºC), photoperiod (16-h:8-h light:dark; 8000 lx light intensity) and relative humidity (70%), for six weeks. Microcosms were weighted daily, during the first 10 days, and thereafter twice a week. The water lost by evaporation was adjusted using artificial rain. For determination of soil atrazine concentrations, soil was collected at days 0, 5 and 42 (Table 2.1). For microbiological analysis, B microcosms were sampled daily from day 0 to 5 (Table 2.1).

Table 2.1. Sampling scheme to evaluate the efficacy of a bioremediation tool to clean up soils contaminated with different doses of Atrazerba (0, 1, 10 and 20 times the Recommended Dose-RD), through chemical analysis on atrazine concentrations, microbial analysis on *Pseudomonas* sp. ADP viability and toxicity tests with aquatic (on leachates and soil eluates) and soil organisms. [a] Daily from day 0 to 5.

Actions taken during the experiment	**Treatment**		**Sampling time (days)**					
	Bioremediation	**Atrazerba dose (×RD)**	0	5	7	10	24	42
SCA - Soil sampling for Chemical Analysis	No (NB treatments)	0	SCA	PAs	SL	SE	CAs	ER
				SE				
		1		PAs	SL		CAs	ER
SMA - Soil sampling for Microbiological Analysis				SE				
		10	SCA	PAs	SL	SE	CAs	SCA
PAs - Planting *Avena sativa*				SE				ER
SE - Preparing soil eluates	Yes (B treatments)	10	SMA[a]	SCA	SL		CAs	SCA
				SMA[a]				ER
SL - Collecting soil leachates				PAs				
				SE				
CAs - Cutting *A. Sativa*		20	SMA[a]	SCA	SL	SE	CAs	SCA
				SMA[a]				ER
ER - Evaluating Earthworm Reproduction				PAs				
				SE				

To evaluate the bioremediation efficacy on the soil retention function soil eluates for aquatic toxicity tests were prepared at days 5 and 10 (Table 2.1). Based on a previous work (Lima et al, 2009), 5 days was viewed as adequate for most of the atrazine to be biodegraded. A second sampling at day 10 was also scheduled to evaluate the bioremediation efficacy for the highest dose (20×RD). Artificial rain representing the rainiest month in Coimbra (5 mm/day; Geophysical Institute of the University of Coimbra, 2007) was simulated daily from days 0 to 6. This procedure allowed the soil to achieve its WHC and the water in excess to drain into the leachate collectors, to be collected at day 7 (Table 2.1). To investigate the bioremediation efficacy on soil habitat function, at day 5 two *Avena sativa* seeds were buried (about 2 cm deep) in each microcosm and plant biomass production was evaluated after 24 days (Table 2.1).

The number of replicated microcosms per treatment was as follows (Table 2.1): six for soil chemical analyses (3 for day 0 and 3 for day 5), two for microbial analysis (up to day 5), six to prepare soil eluates (3 for day 5 and 3 for day 10), eight to assess plant biomass (day 24) and earthworm´s reproduction (day 42), and within the latter, three were also used to collect leachates at day 7 and for chemical analysis at day 42.

II.2 Bioaugmentation agent

A spontaneous rifampicin-resistant mutant of the *P.* ADP was used. This mutant can mineralize atrazine with equal efficiency than the wild-type (García-González et al, 2003). The cell suspension used here as inoculum was prepared from a late-exponential culture of *P.* ADP grown as previously described (Lima et al, 2009).

II.3 Test organisms

Earthworms (*Eisenia andrei*; Lumbricidae) and Collembola (*Folsomia candida*; Isotomidae) were used as soil invertebrate models. Both earthworms

and springtails play a crucial role in the soil ecosystem (Lavelle et al, 1997; Hopkin, 1997) and due to pratical reasons have since long been proposed and widely used in ecotoxicological studies (Jänsch et al, 2005), so that standardized procedures are already available (ISO 1996, 1999) and are recommended to evaluate the habitat function of soils (ISO, 2003). Animals were obtained from laboratory cultures maintained as described by Natal-da-Luz et al (2009). Oat (*Avena sativa*) was selected as the model crop because it is integrated in a list of non target species for use in standardized plant toxicity tests (ISO, 1994; OECD, 2006), including tests on plant protection products, and is known to be sensitive to atrazine (Crommentuijn et al, 1997); seeds were obtained from a commercial supplier (Hortícola, Coimbra, Portugal).

As model aquatic organisms, the microalgae *Pseudokirchneriella subcapitata* (strain Nr. WW 15-2521; Carolina Biological Supply Company, Burlington, NC, USA) and the cladoceran *Daphnia magna* (clone IRCHA, University of Sheffield, UK) were chosen, and their culturing procedures were as previously outlined (Rosa et al, 2009). Both *P. subcapitata* and *D. magna* are key aquatic organisms in a wide range of habitats and are widely recommended for freshwater toxicity studies for which standard guidelines have been established (Environment Canada, 1992; OECD 1984, 1998).

II.4 Soil microcosms

The microcosms consisted of a glass tube (15 × 5 cm Ø) with the bottom covered by Teflon mesh (1.5 × 0.7 mm nominal aperture). They were filled with 1 cm height Ø2 mm glass beads (to facilitate leachate collection) under 210 g (fresh mass) of natural soil, representative of a corn production field with no history of pesticide applications (Coimbra, Central Portugal), as previously described (Lima et al, 2009). Soil properties are shown in Table 2.2; handling procedures are available elsewhere (Lima et al, 2009). To simulate the soil community, three clitellated earthworms (weighting 250-600 mg) and 30 adult collembolans were added to the soil surface in each microcosm (the organisms went naturally deep into the soil) before herbicide

spraying. Microcosms were coupled to a glass leachate collector wrapped with black paper to avoid atrazine photo-degradation.

II.5 Ecotoxicological tests

Seventy-two hour *P. subcapitata* growth tests and 9-days *D. magna* reproduction tests were carried out on soil eluates. Eluates were prepared following standard methods (DIN, 1984). The soil was mixed with water (1:10 ratio, w/v, based on the soil DM), magnetically stirred during 12 hours, centrifuged at room temperature, and the supernatant collected as eluate and stored at 4°C in the dark until use (within 48 hours). Eluates for cladoceran tests were prepared using reconstituted hard water (ASTM, 2002) and were centrifuged at 3,370 *g* for 20 minutes, whereas for microalgae tests eluates were prepared using deionized water and centrifuged 2×20 min also at 3,370 *g*.

Table 2.2. Main pedological properties of the tested soil. OM – Organic matter; CEC – Cation exchange capacity; WHC – Water holding capacity.

pH	**OM**	**Sand**	**Silt**	**Clay**	**Total N**	**CEC**	**WHC**
KCl 1M	%	%	%	%	mg/g	cmol/g	%
6.14 ± 0.05	3.10	62.4	21.2	16.4	0.83	0.0125	32.8 ± 2.89

Growth tests with the microalgae were also performed on the leachates, which were centrifuged at 8500*g* for 3 min prior to use.

Microalgae tests were carried out following standard guidelines (OECD, 1984; Environment Canada, 1992), on 24-well sterile microplates, at 21 to 23°C and under continuous cool-white fluorescent illumination (100 µE/m^2/s). Three 900 µl sub-replicate cultures per replicated eluate/leachate and a

standard control with six replicates were set up and inoculated with 100 µl of algal inoculum. For further details on testing procedures see (Rosa et al, 2009). At the end of the 72-hours exposure, algal growth was estimated as the mean specific growth rate per day.

The cladoceran tests were conducted according to the OECD guidelines for a 21 days reproduction test (OECD, 1998), and were based on a previous work (Guilhermino et al, 1999) in which the shortening of the conventional reproduction test to a first-brood test was proposed, without losses in sensitivity. Twelve replicates were set up for the standard control and four sub-replicates per replicated eluate, each with 50 ml of test solution and one juvenile (less than 24-hours old). A detailed description of the testing procedures is available (Rosa et al, 2009). After the 9 days exposure period, reproduction was determined both as the time to release the first brood and the total number of juveniles released per female.

For the *P. subcapitata* tests, pH and conductivity were measured at the start of the test, whereas for the *D. magna* tests, pH, conductivity and dissolved oxygen were measured in old and fresh medium at all medium renewals. Measured levels were comparable across treatments and not expected to have deleterious effects on the test organisms (Rosa et al, 2009; Environment Canada, 1992; OECD, 1998; Guilhermino et al, 1999).

In case of the plant tests, two weeks after the emergence of more than 50% of the seeds in the controls (day 24), the aerial part of the plants was cut and biomass production was evaluated following standard guidelines (ISO, 1994). Concerning the soil organisms, the earthworm's reproduction was evaluated at the end of the experiment (day 42). The soil was transferred to plastic vessels placed in a water-bath at 60ºC to force adults and juveniles to reach the surface. Then, the soil was sieved (1 mm mesh) to collect the cocoons. The collembolans reproduction could not be accurately assessed since some of the organisms escaped from the microcosms.

II.6 Microbiological analysis

To enumerate *P*. ADP viable cells (as CFU/g of soil DM), soil samples (mean ± SD of 1.3 ± 0.3 g) were diluted in saline solution and serial dilutions were spread plated onto LB agar supplemented with rifampicin (50 mg/L) and cycloheximide (100 mg/L). Colonies were counted after 72 hours incubation at 30°C.

II.7 Chemical analysis

Soil samples (20 g DM) were collected from the microcosms (top 3 cm) and were stored at -20ºC to be processed and analyzed for atrazine and metabolites deethylatrazine (DEA) and deisopropylatrazine (DIA) by GC - Electron Ionization (EI)-MS and for hydroxiatrazine (HA) by LC-EI-MS, as previously described (Lima et al, 2009). Recovery ranged between 75 and 90%. The limits of quantification were 25 ng/g of soil DM. The commercial formulation used was also analysed for atrazine concentration either by GC-EI-MS and by the absorbance at 225 nm (García-González et al, 2003), based on diluted solutions of Atrazerba and calibration curves from standard solutions of pure atrazine; the concentration value of 475 ± 40 g of atrazine / L was consistent with the label information.

II.8 Data analysis

Statistical analysis was carried out to respond to three major questions: (i) Were the three Atrazerba doses toxic to the tested organisms?; (ii) Was there bioremediation for the 10 and 20×RD of Atrazerba, i.e., did the addition of *P.* ADP plus citrate to the B treatments cause decreased toxicity compared to the correspondent NB treatments?; and (iii) What was the efficacy of the bioremediation tool, i.e., what was the performance of the organisms in the B treatment compared to the control, for each Atrazerba dose?

To answer question (i), microalgae growth (on eluates and leachates) and cladoceran reproduction (on eluates) responses, and those of plant growth, in the NB treatments and control, were tested for significant differences through one-way nested analysis of variance (ANOVA) and one-way ANOVA, respectively, followed by the Dunnet's test to determine significant differences between the control and each Atrazerba dose. To examine question (ii), the organism responses in the NB and B treatments were compared by one-way nested ANOVA (microalgae growth and cladoceran reproduction) and students *t*-test (plant growth), for the 10 and 20×RD. Finally, question (iii) was evaluated by comparing the latter organism responses in the control and B treatments through similar statistical tests. The violations of normality and homoscedasticy were checked using Shapiro-Wilk's and Bartlett's tests, respectively. Whenever theses assumptions were violated, even after data transformation, equivalent nonparametric tests were used, Kruskall-Wallis followed by a one-tailed comparison of the control to the other groups, or a one-tailed Mann-Whitney test. When such violations occurred for toxicity tests with a nested design (only for cladocerans), the Kruskall-Wallis test was first used to verify that the 12 sub-replicates of the three replicates of each treatment could be pooled.

III. Results

III.1 Biodegradation of atrazine by *P. ADP* in soil microcosms

Results from atrazine concentrations in soil microcosms show a faster degradation in B soils (bioaugmentated with $6.3 \pm 2.0 \times 10^8$ CFU *of P.* ADP / g of soil and biostimulated with citrate at day 0) contrasting with the correspondent NB soils (Table 2.3).

Indeed, despite the progressive drop observed on the survival of *P.* ADP over the initial 5 days (by approximately one order of magnitude, to mean ± SD $6.4 \pm 1.2 \times 10^7$ CFU/g) (data not shown), during this period the initial atrazine concentration for the 10×RD in B soils was reduced by 83% (from 2.31 to 0.413 µg/g soil, table 2.3), while a 45% reduction was observed for the

20×RD (from 11.6 to 6.39 µg/g soil, table 2.3). Moreover, by the end of the experiment (day 42), in B soils, atrazine levels had declined to merely 2 and 1% of the initial content, respectively for 10×RD and 20×R (0.05 and 0.15 µg/g soil, table 2.3), whereas in NB soils, for the same doses, 36% and 19% of the initial atrazine remained in the soil (0.827 and 2.16 µg/g soil; table 2.3).

During the course of the bioremediation treatment, the levels of the atrazine metabolites DEA, DIA and HA were not significative (< 0.1, 0.05 and 1 µg/g soil, respectively) and they did not show detectable accumulation (results not shown).

III.2 Efficacy of the bioremediation tool on soil habitat function

All Atrazerba doses provoked a severe reduction in *A. sativa* shoot dry mass ($p < 0.001$) (Figure 2.1).

Concerning the earthworm reproduction in NB soils, none of the three Atrazerba doses tested caused a significant reduction in both *E. andrei* juvenile (mean ± SD of 5.4 ± 2.1, 8.3 ± 4.1, 6.8 ± 2.6 and 9.6 ± 4.4, respectively for ct, RD, 10 and 20×RD) and cocoon production (corresponding means ± SD of 11.2 ± 5.4, 8.0 ± 2.6, 10.5 ± 2.2 and 10.6 ± 2.6).

With the addition of *P.* ADP plus citrate to the 10× and 20×RD Atrazerba treated microcosms 5 days before planting the seeds (Table 2.1), shoot dry mass in B soils significantly increased compared to the corresponding NB soils ($p < 0.001$ for both doses). Furthermore, for the 10×RD, the aerial biomass produced in the B soils was similar to the one obtained in the non-contaminated control soil ($p = 0.078$). On the other hand, for the highest dose tested (20×RD), shoot dry mass in the B treatment was lower than in the control ($p = 0.003$; Figure 2.1).

Table 2.3. Mean (n=3) atrazine (Atz) concentrations (as µg/g of soil dry mass and as the percentage of the initial concentration) in the microcosms contaminated with two doses of Atrazerba (10 and 20 times the Recommended Dose-RD) and subsequently sprayed with (B) and without (NB) the bioremediation tool, at days 0, 5 and 42.

Atrazerba Dose (×RD)	Atz - Day 0 µg/g	Soil treatment	Atz - Day 5 µg/g	Atz - Day 5 % of initial	Atz - Day 42 µg/g	Atz - Day 42 % of initial
10	2.31 ± 0.497	B	0.413 ± 0.286	17	0.0500 ± 0.0350	2
		NB	n.d.[a]	n.d.[a]	0.827 ± 0.512	36
20	11.6 ± 1.95	B	6.39 ± 5.16	55	0.150 ± 0.156	1
		NB	n.d.[a]	n.d.[a]	2.16 ± 1.41	19

[a] not determined

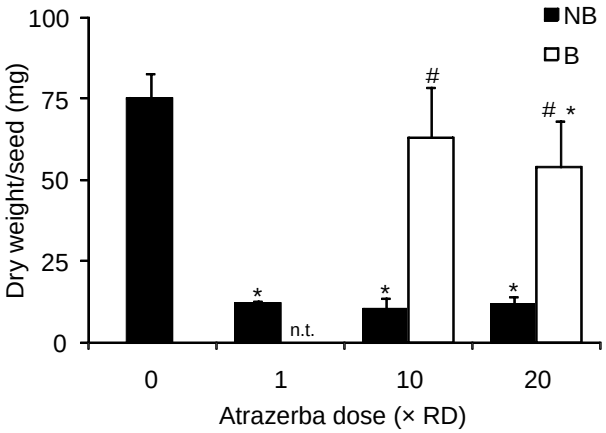

Figure 2.1. Mean (n=8) shoot dry mass per emerged seed of *Avena sativa* in soil microcosms contaminated with Atrazerba (0, 1, 10 and 20 times the Recommended Dose-RD) and subsequently sprayed with (B) and without (NB) the bioremediation tool. Error bars indicate + 1 standard deviation; * - indicates mean statistically different from control; # - indicates mean statistically different from the NB treatment within the same Atrazerba dose; n.t. denotes treatment not tested.

III.3 Efficacy of the bioremediation tool on soil retention function/leaching potential

In all tests performed with the aquatic organisms, the guideline's validity criteria for the standard control were fulfilled. Although there are no available guidelines for a first-brood *D. magna* reproduction test, according to Guilhermino et al (1999) and Moreira-Santos (personal communication), the fulfilment of the control validity criteria in a 21-days reproduction test implies that: i) first brood is laid before day eight, ii) mean number of juveniles is equal to or higher than 11 and iii) coefficient of variation (CV) is equal to or lower than 25%. In the present study the first brood was always laid on day eight with a mean number of juveniles equal to or higher than 13 and a CV between 11 and 24%.

Results on the aquatic test species performance in water extracts obtained

from NB treatments show that all eluates (days 5 and 10) and leachates (day 7) caused deleterious effects on both organisms (Table 2.4). Indeed, microalgae growth rates in NB eluates and leachates were significantly reduced compared to the control ($p < 0.001$; Table 4). Analogous results were found for the cladoceran reproduction in eluates obtained from NB treatments contaminated with the three Atrazerba doses: the first brood release was significantly delayed and the number of juveniles was significantly reduced ($p \leq 0.003$; Table 2.4).

Regarding the bioremediation of soil contaminated with the 10×RD of Atrazerba, in the water extracts obtained at days 5 (eluates) and 7 (leachates), the organisms performance was significantly improved in B compared to NB eluates/leachates ($p \leq 0.001$ for microalgae growth and $p \leq 0.003$ for cladoceran reproduction; Table 2.4).

Moreover, for this same dose, no significant differences were found in organisms performance between B and control water extracts ($p = 0.99$ and $p = 0.25$, for microalgae growth in eluates and leachates, respectively, and $p \geq 0.12$ for cladoceran reproduction in eluates) (Table 2.4).

A different scenario was observed in eluates obtained from B soils contaminated with the highest Atrazerba dose (20×RD) over a five-days treatment period: when comparing B versus NB eluates, no significant differences were found for microalgae growth ($p = 0.29$; Table 2.4) or cladoceran reproduction ($p \geq 0.17$; Table 2.4), meaning that B eluates were still causing adverse effects on the organisms.

However, for this dose, after seven days, microalgae growth was significantly higher for B than for NB leachates ($p = 0.018$) and similar to that in the control ($p = 0.084$; Table 2.4).

In addition, after a longer bioremediation period (10 days) of the soils contaminated with the 20×RD, no toxic effects of eluates were registered. Microalgae growth in B eluates was significantly higher than in NB eluates ($p = 0.006$) and similar to that in control eluates ($p = 0.99$; Table 2.4). Likewise, *D. magna* laid the first brood significantly earlier and with significantly more juveniles in B than in NB eluates ($p \leq 0.03$), and both reproductive endpoints were similar in B and control eluates ($p \geq 0.47$; Table 2.4).

Table 2.4. Mean (± standard deviation; n=3) 72-hours growth rate of *Pseudokirchneriella subcapitata* and 9-days reproduction of *Daphnia magna*, (in terms of time to release the first brood and number of juveniles per female), on eluates (prepared at days 5 and 10) and leachates (prepared at day 7) from microcosms contaminated with Atrazerba (0, 1, 10 and 20 times the Recommended Dose-RD) and subsequently sprayed with (B) and without (NB) the bioremediation tool. * indicates mean statistically different from control; # - indicates mean statistically different from the NB treatment within the same Atrazerba dose; n.t. denotes treatment not tested.

	Microcosms	***P. subcapitata*** **Growth rate (/day)**		***D. magna*** **Time to 1st brood (days)**	***D. magna*** **Nr. juveniles/female**
Treatment period (days)		Eluates	Leachates	Eluates	
5 (eluates)	0	1.4 ± 0.03	1.6 ± 0.06	8 ± 0	18 ± 1
	RD – NB	0.10 ± 0.03*	0.29 ± 0.06*	9 ± 0 *	9 ± 1 *
	10×RD – NB	0.14 ± 0.01*	0.21 ± 0.06*	14 ± 2 *	2 ± 1 *
	10×RD – B	1.5 ± 0.03 #	1.4 ± 0.28 #	8 ± 0 #	12 ± 4 #
7 (leachates)	20×RD – NB	0.12 ± 0.05*	0.13 ± 0.07*	11 ± 4 *	7 ± 7 *
	20×RD – B	0.23± 0.16	1.1 ± 0.40 #	14 ± 4	3 ± 3

Table 2.4. (Continued)

	Microcosms	***P. subcapitata*** **Growth rate (/day)**		***D. magna*** **Time to 1st brood (days)**	**Nr. juveniles/female**
Treatment period (days)		Eluates	Leachates	Eluates	
10	0	0.96 ± 0.35	n.t.	11 ± 4	9 ± 9
	20×RD – NB	0.18 ± 0.04	n.t.	15 ± 1	3 ± 3
	20×RD – B	0.96 ± 0.26 #	n.t.	9 ± 1#	10 ± 3 #

IV. Discussion

IV.1 Biodegradation of atrazine by *P.* ADP in soil microcosms

Based on atrazine soil chemical analysis, one can point that the addition of *P.* ADP plus citrate to the B soils was clearly effective in promoting higher atrazine biodegradation. Indeed, an almost complete atrazine removal took place during the six weeks of the experiment since 98% and 99% of the initial herbicide concentrations were biodegraded, respectively for 10×RD and 20×RD (table 2.3).

Furthermore, effective atrazine biodegradation was supported by the ecotoxicological monitoring of the B and NB treatments reported above. Since the soil used was not likely to contain indigenous microorganisms that could support atrazine degradation (Lima et al, 2009), the decrease in atrazine levels observed in the NB soils after 42 days (64% for 10×RD and 81% for 20×RD) may be associated with a combination of abiotic degradation of the herbicide (Shin and Cheney, 2005) and bound (non-extractable) residue formation (Barriuso et al, 2004; Blume et al, 2004) in the sandy loam soil during the six weeks of the experiment.

The present study included a higher level of complexity in the bioremediation experiments, compared to a previous study using a somewhat similar apparatus (Lima et al, 2009). The more realistic conditions adopted here may have contributed for the relatively slower activity of the biodegradative bacteria observed in the microcosms spiked with 20×RD where atrazine initial concentration decreased by 55% after 5 days (from 11.6 to 6.39 µg/g of soil), as compared with a degradation rate of more than 97%, to 0.07 µg/g of soil (within the same period) for a soil initially contaminated with 7.2 µg/g of atrazine, reported before (Lima et al, 2009). Firstly, the addition of soil invertebrates to the microcosms may have contributed to decline the numbers of *P.* ADP and hence its atrazine-degradation ability (Kersanté et al, 2006).

In addition, the mode of application of the commercial formulation and of the bioremediation agents was different from that used before where both Atrazerba and bioremediation agents were thoroughly mixed with all the soil in

the microcosms (Lima et al, 2009). Indeed, in the present study, they were applied to the soil surface at each microcosm, and the soil was subsequently showered with artificial rain and mixed to simulate incorporation of the chemical and the bioremediation agents. This apparently less efficient mixing, that may however be closer to the application of the bioremediation tool under field situations, may have contributed to create spatial heterogeneities and diminish the atrazine-bacteria contact area, reducing the herbicide degradation rate (Sturman et al, 1995).

IV.2 Efficacy of the bioremediation tool in soil habitat function

The observed plant toxicity in NB soils was expectable for an herbicide that inhibits photosynthesis and is in accordance with a previous study where a 50% inhibition in *A. sativa* growth in a sandy soil for atrazine concentrations of 0.3 mg/kg was reported (Crommentuijn et al, 1997).

In opposition, the bioremediation tool herein examined showed to be highly effective in reducing atrazine deleterious effects to this plant species in B soils. Indeed, bioremediation of the 10×RD proved to be complete during the period that the plants remained in the soil (from days 5 to 24), since plant biomass production levels were similar to the ones obtained in non contaminated soil (control) (Figure 2.1); this is consistent with the removal of most of the initial atrazine (down to 0.41 µg/g soil) during the first 5 days (Table 2.3). Results from B soils contaminated with 20×RD indicate that bioremediation took place (since plant biomass production was higher than in the corresponding NB soils) and was almost complete (approximately 72% effective compared to the control).

In the present study, soil atrazine contamination did not affect the reproduction of earthworms, indicating that effects for *E. andrei* seem to occur at higher concentrations than the ones used here. Indeed, the highest measured atrazine concentration was 11.6 ug/g of soil, almost six times lower than the reported atrazine median lethal concentration for *E. fetida* (LC_{50} = 64 µg/g), a close relative to *E. andrei* (Frampton et al, 2006).

IV.3 Efficacy of the bioremediation tool in soil retention function/leaching potential

Atrazine deleterious effects on nontarget terrestrial organisms are considered to be of lower concern when compared with aquatic systems (Solomon et al, 1996). Accordingly, in the present study, the water extracts (eluates and leachates) obtained from soil contaminated with the three Atrazerba doses (NB soils) were highly toxic to both microalgae and cladoceran species.

For the same reason reported above for plants, the observed algae toxicity is consistent with the herbicidal properties of Atrazerba. According to literature, among the aquatic trophic groups, phytoplankton is the most sensitive to atrazine (Solomon et al, 1996; Graymore et al, 2001), while zooplanktonic species like *D. magna* are expected to be much less affected. In the present study, however, the toxicity of Atrazerba towards *D. magna* was similar to that observed for the microalgae; toxicity was observed even at the lowest dose, although the extent of effects were lower for the cladoceran than for the microalgae (Table 2.4). This similarity may be due to the possible presence of other than atrazine toxic ingredients in the commercial formulation, which could also be detrimental for cladocerans.

Thus, our results corroborate the low soil retention function and the high leaching potential (ISO, 2003) of atrazine contaminated soils referred by several authors (e.g. Solomon et al, 1996; Graymore at al, 2001; Fava et al, 2007). They also support the notion that the indirect effects of atrazine agricultural applications via surface runoff to adjacent streams and leaching to groundwater reservoirs constitute a serious risk for aquatic systems (Detenbeck et al, 1996; Graymore et al, 2001).

Concerning the efficacy for the aquatic compartment of the bioremediation tool herein examined, results for the water extracts obtained from soil contaminated with the 10×RD, showed that during the first five (eluates) and seven days (leachates) of bioremediation treatment there was an effective soil cleanup, as atrazine (and possible metabolites) were reduced to non toxic levels for both aquatic organisms.

By the contrary, for soils contaminated with 20×RD of Atrazerba, a bioremediation period of five days was not enough to annul toxicity of eluates since both microalgae growth and cladoceran reproduction in B eluates were similar to those obtained in NB eluates (Table 2.4), consistent with the high atrazine concentration still present in the soil after 5 days of bioremediation treatment (Table 2.3).

Nevertheless, for this dose, examination of the toxicity for the microalgae of the leachates taken at day 7 points for a complete cleanup of the B soils. An effective detoxification of the eluates obtained from the soils contaminated with the 20×RD was proved to be eventually achieved after a bioremediation treatment period of 10 days.

The higher toxicity toward microalgae observed when using soil eluates compared to soil leachates may be related with the timing of sampling preparation/collection. The leachates were collected two days after the eluates preparation, a time gap which may have allowed a higher (bio)degradation of atrazine in leachates. Moreover, the preparation of the eluates involving the vigorous shaking of soil with water during 12 hours could have contributed to the release of a more significant part of the atrazine in the soil porewater, compared to that released in soil leaching.

V. Conclusions

With the multiparametric approach used in this work, i.e., the monitoring of atrazine bioremediation using chemical analysis and ecotoxicological assays with standard organisms, it was demonstrated that an effective detoxification of the soil and respective water compartments was achieved for both doses of Atrazerba in a relatively short period of time (up to 10 days) due to the application of a cleanup tool combining bioaugmentation with *P.* ADP and biostimulation with citrate. The ecotoxicological endpoints used to assess the bioremediation efficacy proved to be very powerful and when complemented by chemical analysis, may provide information on the bioavailable and hence the potentially toxic fraction of the contaminant and/or its metabolites to different ecologically relevant organisms as well as on synergistic and

antagonistic interactions.

Particularly important was the ecotoxicological assessment of soil leachates and eluates over the bioremediation process, as these methods evaluate the potential risks for aquatic ecosystems of the mobilization of atrazine (and possible toxic metabolites) extractable and bounded fractions via water, due to runoff and leaching. Moreover, it may help in obtaining a clearer and more realistic view of the ecological impact associated with soil bioremediation.

VI. Recommendations and perspectives

Although the use of the cleanup tool presented here under larger scale (field) conditions (in situations of accidental spills, overuse, intensive atrazine applications or deliberate disposal) would require improvements, the present results demonstrated its potential to reduce the presence of atrazine and its toxic metabolites in soil, and also to prevent ground and surface water contamination due to leaching and runoff. Such results were partly confirmed by the soil chemical analysis on atrazine, indicating that the inclusion of chemical and ecotoxicological data is of major significance to monitor and manage bioremediation programmes. Moreover, besides soil chemical analysis, information on contaminant concentrations on eluate/leachate/runoff samples would be useful to evaluate the indirect effects of soil contamination for the aquatic compartment. Thus, the evaluation of soil retention function should comprise the performance of ecotoxicological tests on these three different types of water samples, to gain a more comprehensive insight on the cleanup efficacy. As test-battery, besides phytoplanktonic (*P. Subcapitata*) and zooplanktonic (*D. magna*) species, higher trophic levels should also be represented with the inclusion, for example, of a freshwater fish, also known to be sensitive to atrazine (Graymore et al, 2001).Concerning the evaluation of soil habitat function, and since the present results pointed out for negligible effects on soil organisms, even at the highest dose, it is advisable to include non only monocotyledons plant species but also dicotyledons species, since atrazine is recommended for the control of both grasses and broadleaf plants.

Moreover, due to the worldwide continued use of atrazine/triazine based herbicidal formulations, testing of other commercial formulations containing mixtures of atrazine/triazine and other active ingredient are still needed so that this bioremediation approach can be used as a valuable tool to reduce herbicide toxicity in contaminated land.

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Chapter 3

Integrated Ecological Risk Assessment of Pesticides in Tropical Ecosystems: A Case Study with Carbofuran in Brazil

Based on the following manuscript:

Chelinho S, Lopes I, Natal-da-Luz T, Domene X, Nunes MET, Espíndola ELG, Ribeiro R, Sousa JP. Integrated Ecological Risk Assessment of Pesticides in Tropical Ecosystems: A Case Study with Carbofuran in Brazil. *Environmental Toxicology and Chemistry* (*Accepted*).

Abstract

The present study aimed at contributing for an ecologically relevant assessment of the ecotoxicological effects of pesticide applications in agricultural areas in the Tropics using an integrated approach with gathered information from soil and aquatic compartments. Carbofuran, a widely used insecticide/nematicide in sugarcane crops, was selected as model substance. To evaluate the toxic effects of pesticide spraying for soil biota and also the potential indirect effects on aquatic biota due to surface runoff and/or leaching, field and laboratory (using a cost effective simulator of pesticide applications) trials were carried out. Standard ecotoxicological tests were performed with soil (*Eisenia andrei*, *Folsomia candida* and *Enchytraeus crypticus*) and aquatic organisms (*Ceriodaphnia silvestrii*) using serial dilutions of soil, eluate, leachate and runnoff samples. Among soil organisms, sensitivity was found to be *E. crypticus* < *E. andrei* < *F. candida.* Among the aqueous extracts, mortality of *C. silvestrii* was extreme in runoff samples while eluates were by far the least toxic samples. A generally higher toxicity was found in the bioassays performed with samples from the field trial indicating the need for some improvements in the laboratory simulator. However, the developed tool proved to be valuable to evaluate toxic effects of pesticide sprayings in soils and the potential risks for aquatic compartments.

Keywords: Soil retention function; Soil habitat function; Laboratory simulator; Flume; Runoff.

I. Introduction

Tropical ecosystems constitute major reservoirs of biodiversity which are subjected to several threats including agricultural expansion (Lacher and Goldstein, 1997). This represents a great menace to biodiversity and ecosystem stability. The intensification of agriculture has lead to an exponential growth of the demand for pesticides, especially in the tropics (Carvalho, 2006; Henriques et al, 1997) and Brazil has become, since 2008, the world top consumer (Rebelo et al, 2010). However, in most of the tropical countries, this demand for agrochemicals was not properly accompanied by the development of national legislation regulating their safe use as well as the monitoring of their environmental hazards (Lacher and Goldstein, 1997; Castillo et al, 1997).

The knowledge on the impact of pesticides in the tropics is small when compared with temperate systems and risk assessment schemes mostly rely on the extrapolation of data from temperate regions (Lacher and Goldstein, 1997; De Silva et al, 2009; Kwok et al, 2007; Römbke et al, 2008). This approach can lead to biased evaluations of the fate and effects of pesticides as physical, chemical and biological conditions in the tropics differ from those in temperate systems (Lacher and Goldstein, 1997). Indeed, the risk of pesticide contamination of environmental compartments might be higher in the tropics, namely the nonpoint-source contamination of both soil and water due to spray drift, volatilization and mobilization via water through edge of field runoff and leaching (Rice et al, 2007; Schulz, 2004). Particularly, the current severe and unpredictable rainfalls (Lacher and Goldstein, 1997, Henriques et al, 1997) plus the existence of extensive systems of irrigation and drainage channels (Henriques et al, 1997; Castillo et al, 2006), allied to an intensive and/or careless use of pesticides (Castillo et al, 1997; Adhilash and Singh, 2009), can enhance environmental risks. Once in the environment, pesticide toxicity can be magnified since the number of species affected is usually higher in such highly biodiverse ecosystems (Lacher and Goldstein, 1997).

The available data on pesticide fate and effects in the tropics, using relevant local scenarios and test species, are mainly focused on the aquatic

compartment (Lacher and Goldstein, 1997; Castillo et al, 1997; Kwok et al, 2007). Several studies concentrate on the rainfall induced runoff as the main route of pesticide entries in surface waters (Schulz, 2004; Castillo et al, 2006, Moreira et al, 2010). Concerning the soil compartment, notwithstanding the scarcity of data (especially effect data), the overall picture is improving with new inputs from recent studies, using standardized methods and/or local species (De Silva et al, 2009, 2010; Römbke et al, 2008; De Silva and Van Gestel, 2009; Garcia et al, 2008, 2011). Nevertheless, as emphasized by several authors, more research in tropical ecotoxicology is needed to provide clearer insights on the potential hazards of pesticides in these particular environments (Lacher and Goldstein, 1997; De Silva et al, 2009; Kwok et al, 2007; Römbke et al, 2008; Garcia et al, 2008).

Further improvements in the ecological risk assessment of pesticides, both in tropical and temperate ecosystems, also include the integration of fate and effect data from both soil and aquatic compartments. Specifically, the risk assessment strategy should compass the evaluation not only of the pesticide toxic potential of soils as sink of contaminants for soil organisms but also of the consequences to aquatic organisms of its mobilization via the water pathway by both leaching and surface runoff. Moreover, the water-mediated transport of pesticides will ultimately determine the quality of the water resources for Human consumption (Rice et al, 2007). Therefore, coordinated approaches comprising research on both soil and water protection and using, whenever possible, cost-effective tools under realistic exposure conditions, are needed (Halm and Grathwohl, 2006; Lopes et al, 2007).

The present study aimed at contributing for an ecologically relevant assessment of ecotoxicological effects of pesticide applications in agricultural areas in the tropics on both soil and aquatic biota. To achieve the main goal, three specific objectives were defined: to evaluate the habitat and retention functions of a tropical soil after carbofuran spraying; to evaluate the potential effects of the pesticide spraying on aquatic biota due to surface runoff and leaching; and to develop cost-effective and ready amenable to standardization tools to perform laboratory simulations of pesticide sprayings, leaching and

surface runoff, and to evaluate these tools through the comparison of field and laboratory trial results.

II. Materials and Methods

II.1 Field Trial

An area located in the Brazilian sugarcane belt (São Carlos, -22° 10' 13.53", -47° 53' 58.12"; state of São Paulo, hereafter designated as SP) was chosen. The carbamate insecticide carbofuran, widely used in sugarcane plantations (Sparokev et al, 2001) was used as a model pesticide.

To simulate realistic exposure scenarios of soil and water contamination, a field with no history of pesticide contamination was selected. The loamy sand soil (5.33 pH, 13.5% organic matter, 79.5% sand, 18.6 % silt, 2.17% clay, 0.24% total organic N, 0.687 µg/g total P and 67.1% water holding capacity) was analyzed by CRHEA (Centro de Recursos Hídricos e Ecologia Aplicada; São Carlos, SP, Brazil) according to methods described elsewhere (Nunes, 2010).

Three days after the soil tillage, three realistic scenarios were simulated as described below and illustrated in Figure 3.1.

Scenario F1 - soil contamination through pesticide spraying

Three parallel strips of land from a flat area (3 × 1m each and separated by a buffer area of 2 m, to avoid cross-contamination) were selected (Figure 3.1). Afterwards, two strips were sprayed with the insecticide Furadan 350SC (a commercial formulation of carbofuran; FMC, SP, Brazil; 350 g a.i./L). First, the recommended dose (RD) for sugarcane plantations (5L/ha; 1.167mg a.i./kg soil dry weight (DW) considering an average soil density of 1.5g/cm3 and an incorporation depth of 10 cm) was applied. Second, another strip was

sprayed with two times the recommended dose, hereafter designated as Highest Dose - HD, to mimic pesticide overuse, a common practice among local farmers (Dasgupta et al, 2001). Per dose, the required amount of pesticide was diluted in 5L of water collected at a nearby reference lagoon (Represa do Broa, São Carlos, SP, Brazil; -22º 10' 29.98'', -47º 54' 5.45'').

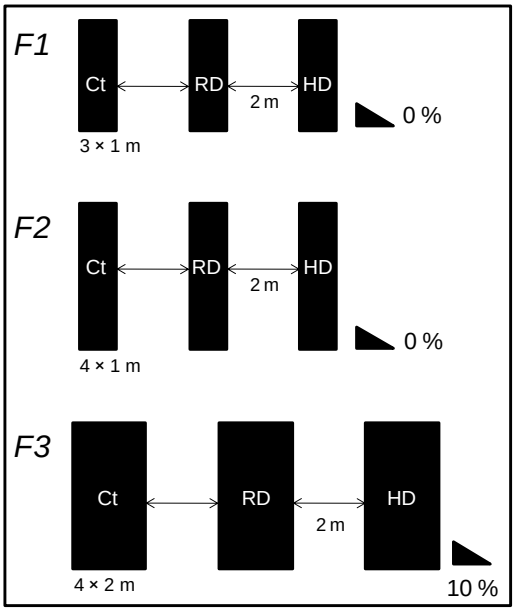

Figure 3.1. Plan of the field trial where three worst case scenarios of soil contamination with carbofuran were simulated: contamination toward soil habitat and retention functions (F1), aquatic organisms due to leaching (F2) and aquatic organisms due to surface runoff (F3). For each scenario, the land stripes, separated by a 2m buffer zone, were sprayed with the Recommended Dose (RD - 5L/ha) and two times the RD (HD - 10L/ha) of Furadan (350g a.i. /L of carbofuran) and lagoon water (Ct). See section II.1 for details. ◣ - slope (%).

The third strip of land, the control, was sprayed with the same volume of the lagoon water but without the pesticide. After the pesticide or lagoon water spraying, further 10L of lagoon water were sprayed to promote insecticide incorporation.
After approximately 18 h, soil samples from the top 10cm were collected for both ecotoxicological tests and chemical analysis.

Scenario F2 - leaching of the pesticide-sprayed soil

Similarly to scenario F1, three strips of land (4 × 1m each, located in a flat area and separated by a buffer zone) were also used (Figure 3.1). For each strip of land, a 30cm layer of soil was removed and a plastic lid was laid to create an impermeable stratum to allow the leachate collection. This hole was filled with a 10cm layer of gravel and topped with the initially removed soil (20cm layer). Pesticide application was performed as described above for scenario F1. One hour after the soil spraying, a continuous rain was simulated, provoking the soil to achieve its water holding capacity and the water to leach. Approximately 240L of lagoon water were applied, corresponding to the highest daily precipitation registered in February 2007 (63.9mm) by the meteorological station of CRHEA, in the surroundings of the field assay. Leachate drained along the plastic lid into a hole dug at the end of the strip and samples were collected and stored at 14ºC until further processing.

Scenario F3 - surface runoff of the pesticide-sprayed soil

For this particular case, the three strips of land (4 x 2m, separated by the 2m buffer zone) were located in a 10% slope area. Pesticide application was also performed as described above for scenario F1. One hour after the soil contamination, an heavy rainfall (using ~190L of the lagoon water; corresponding to half of the above mentioned highest daily precipitation) was

simulated and the runoff water plus the removed soil were collected at a hole dug at the end of the strip and stored at 14ºC until further processing.

II. 2 Simulator Trial

A parallel laboratory experimental scheme, with the same soil and the three scenarios described for the field trial, was set up but at a smaller scale. Our aim was to develop and validate a system to perform laboratory simulations of soil contamination and pesticide mobilization via the water pathway. Field scenarios F1, F2, and F3 thus corresponded to the laboratory scenarios S1, S2, and S3. Nine simulators, corresponding to 3 doses × 3 scenarios, were built with plastic trays (1.10 × 0.49 × 0.17m of length × width × depth). All the procedures regarding pesticide applications and rainfall simulations mimicked the field trial. Notwithstanding, for the leachate experiment (scenario S2) there was a 7cm layer of gravel plus 10cm layer of soil. For the runoff experiment (scenario S3), approximately 9L of the lagoon water were added while for the leachate experiment the amount of water was approximately 14L. The perforation of the bottom of trays facilitated the collection of leachate. For the runoff experiment, plastic containers were adapted to the sloping tray and received the transported soil and water. Slope was similar to that used in scenario F3.

II. 3 Test-organisms

Earthworms (*Eisenia andrei*; Lumbricidae), enchytraeids (*Enchytraeus crypticus*; Enchytraeidae) and collembolans (*Folsomia candida*; Isotomidae) were used as soil test species. They play a crucial role in the soil ecosystem and are widely used in ecotoxicological studies (Jänsch et al, 2005). Standardized procedures to evaluate the habitat function of soils due to chemical contamination are available (ISO, 1998, 2004, 1999).

The soil invertebrates were obtained from laboratory cultures (Laboratory of Soils, University of Coimbra, Coimbra, Portugal). Earthworms and collembolans were maintained as described by Natal-da-Luz et al (2008). The culture of *E. crypticus* was gently provided by ECT GmbH (Flörsheim, Germany). In the laboratories of origin, these organisms were maintained in an uncontaminated natural soil at 20 ± 2 ºC with a photoperiod of 16:8 h - light:dark, and fed weekly with rolled oats. Upon arrival to the CHREA, all organisms were acclimated at 22 ± 1 ºC; 12:12 h light:dark during at least one week before the start of the tests. Originally, it was intended to acclimate the organisms and perform the ecotoxicological tests at 24 º C, the average of maximum temperatures occurring in the winter season in this area (available at: http://www.cpa.unicamp.br/outras-informacoes/clima_muni_549.html), as the assays were performed in July 2007. However, due to logistic constrains in the laboratory at the time we had to run the tests, the desired temperature conditions could not be met. Therefore acclimation was done under a different temperature (1 ºC lower) than the one used for the tests (23 ± 1 ºC, see below).

The selected aquatic test organism was a native tropical cladoceran (*Ceriodaphnia silvestrii*; Daphnidae). It was chosen due to its wide geographical distribution through South America and since it belongs to one of the most sensitive groups of organisms that occupy a central position within lentic aquatic food chains and that are commonly used to determine toxicity of chemicals and set environmental health standards (Hazanato, 2001). Also, the Brazilian Association of Technique Norms established a standardized protocol for chronic assays with this species (ABNT, 2005). To allow a direct comparison between lethal and sublethal effects, acute assays were also performed with *C. silvestrii*.

The organisms were continuously reared in CRHEA laboratories in ASTM softwater (hardness between 40 to 48 mg/L CaCO3; (ABNT, 2005) supplemented with Vitormonio® and Sera Morena (1ml per 9 ml of ASTM), under controlled temperature and photoperiod (24 ± 2°C and 16:8 h light:dark, respectively). Medium was changed every 2 d and organisms were fed daily with *Pseudokirchneriella subcapitata* (10^5 cells/ ml).

II. 4 Ecotoxicological tests

Soil

The soil used for both field and simulator trials was previously defaunated by a freezing (F) - thawing (T) cycle (48hF- 8hT-24hF) and sieved (5mm). Control soil was mixed with soil sprayed with HD in different proportions to obtain the following dilution series: 0, 1.25, 2.5, 5, 10, 25, 50, and 100% of HD. The soil organisms were exposed to the different dilutions, except for *E. crypticus* which was not exposed to the three lowest dilutions, and both mortality and reproduction were assessed. Soil contaminated with the RD of Furadan was also used to compare the performance of organisms with the 50%HD dilution. For all tests the moisture was adjusted to about 50% of the maximum water holding capacity with deionised water.
E. andrei and *F. candida* tests were performed following ISO guidelines (ISO, 1998, 1999, respectively). *E. crypticus* tests were also based on ISO guidelines (ISO, 2004), although using test duration of 28 d and maintaining the adults in the vessels until the end of the test. The tests were carried out at 23 ± 1ºC to simulate conditions occurring in the winter season in the study area, as stated before, and with a 16:8 (light:dark) photoperiod. The standard control OECD soil (OECD, 1984) was used to guarantee test validity.

Water

Lethal and sublethal toxicity assays were carried out by exposing 6 to 24-h old juveniles of *C. silvestrii* to a serial dilution of water samples collected from the runoff and leachate simulations and eluates prepared from soil samples collected from both trials. All dilutions were carried out with the culture medium, ASTM softwater, also used as the control for all toxicity assays.

Soil eluates were prepared following DIN 38 414 – S4 guideline (DIN, 1984) and stored at 4°C in the dark until use for toxicity testing (within 12 to 48 h).

Lethal assays followed ABNT guidelines (ABNT, 2004). Four replicates were set up per dilution (runoff and leachates: 0.05, 0.07, 0.10, 0.14, 0.20, 0.28, and 0.39% of RD - the latter was only tested in the samples obtained from the simulator trial; eluates of soil samples: 0.07, 0.10, 0.14, 0.20, 0.28, 0.39, 0.55, 0.76, and 1.07% of RD - the latter was only tested in the samples obtained from the simulator trial). Each replicate contained 10 ml of test solution and five neonates of *C. silvestrii*. Mortality was recorded after 24 and 48h. Assays were carried out under controlled temperature and photoperiod (22 ± 1ºC and 12 h light:12 h dark, respectively). Also in this case, it was intended to perform the tests at 24 º C, but due to similar logistic problems in the laboratory it was not possible to attain this temperature.

Sublethal assays, carried out within one week after the start of the lethal assays, followed ABNT guidelines (ABNT, 2005). Ten replicates were set up per dilution. Based on the results obtained in lethal assays, for both laboratory and field simulations, the following range was used in runoff, leachate and eluate samples: 0.0031, 0.00625, 0.0125, 0.025, 0.05, and 0.1% of RD. Each replicate contained 15 ml of test solution and one neonate of *C. silvestrii*. After 9-d of exposure under controlled temperature and photoperiod (22 ± 1ºC and 12 h light :12 h dark, respectively), the time to release the first brood and the total number of juveniles released per female were recorded. Conductivity, pH and dissolved oxygen were measured before and after solution renewal and ranged between 7.0 to 8.0 and 163 to 181 µS/cm, respectively for the first two parameters; dissolved oxygen was always above (> 6.9 mg/L) the critical value for cladocerans (2 mg/L; OECD, 1998).

II. 5 Chemical analysis

Soil samples (~150 g DW) from each dilution were frozen until further processing by the Laboratory of Environmental Chemistry, IQSC, University of São Paulo, Brazil. Carbofuran quantifications were performed by liquid chromatography (Model SCL-10A-Shimadzu, with UV detector SPD-10A); confirmation was performed by GC-MS (model QP2010-Shimadzu). The analytes were extracted by SPE method, using C18 columns (EPA 3500 and 8270C; methods available at: http://www.caslab.com/EPA-Methods/) and ultra-sound (EPA 3550B; method available at: http://www.trincoll.edu/~henderso/textfi~1/416%20notes/3550b.pdf). Recovery range varied between 78 and 86%. The limit of detection was 5.0 µg/kg DW.

II. 6 Statistical analysis

Data were analyzed for normality using Kolmogorov-Smirnov test and for variance homogeneity using Hartley, Cochran and Bartlett's tests. To evaluate the differences between the organisms performance in the control and in the contaminated samples, a one-way analysis of variance (ANOVA) followed by post-hoc comparisons with the control (Dunnett's test) was performed using Statistica 7.0 (Available at: http://www.statsoft.com/; Assessed 26th July 2011). The lethal concentrations causing 50 and 20% of mortality (LC50s and LC20s; the latter only for tests with *C. silvestrii*), and the respective 95% confidence intervals were calculated by probit analysis with the logarithm of the tested concentrations, using the PriProbit software (Available at: http://www.ars.usda.gov/Services/docs.htm?docid=11284; Assessed 26th July 2011).

The EC50s for reproduction and 95% confidence intervals were calculated using non-linear regressions (Environment Canada, 2007).

III. Results

III.1 Carbofuran Concentrations

Carbofuran concentrations in soil samples are shown in Table 3.1. A soil dilution gradient was effectively created. With two exceptions, the concentrations in the soil samples from the laboratory trial were slightly lower than those in the field trial (Table 3.1).

Table 3.1. Carbofuran concentrations (as milligrams per kilogram of soil DW) in the soil samples collected from the field and simulator trials where the soil has been sprayed with two doses of Furadan [the Recommended Dose (RD - 5L/ha) and two times the RD (HD - 10L/ha); 350g a.i. /L)]. The soils samples of HD treatment were diluted with uncontaminated soil to obtain the dilutions mentioned below. Ct - Control.

Dilutions (% of HD)	Carbofuran (mg/kg DW)	
	Field	Simulator
Ct	<0.010	<0.010
1.25	0.027	0.017
2.5	0.039	0.035
5	0.079	0.084
10	0.460	0.191
25	0.400	0.429
50	1.520	1.031
75	1.540	1.170
100	2.460	1.765
RD	1.290	1.178

III. 2 Carbofuran toxicity to soil invertebrates

The effects of carbofuran in the survival and reproduction of the three tested species of soil invertebrates, both in field and simulator trials, are presented in Figure 3.2. Derived toxicity data for the same assays are summarized in Table 3.2. The validity criteria defined by the ISO guidelines (ISO, 1998, 2004, 1999) were achieved in all toxicity tests. The performance of organisms in OECD artificial soil also fulfilled the validity criteria (adult survival: 100, 93 and 98%; average number of juveniles ± standard deviation: 41 ± 10, 584 ± 107 and 494 ± 55; respectively for *E. andrei*, *E. crypticus* and *F. candida*; data not shown).

Among the three test species, collembolans were the most affected by carbofuran soil contamination. At very low concentrations, 0.460 and 0.429 mg/kg for field and simulator samples, respectively, no adults or juveniles were recorded (Figure 3.2 F3 and S3). Reproduction followed a similar pattern to mortality since LC50s and EC50s calculated for each assay are almost similar: respectively 0.057 and 0.073 mg/kg DW for the field trial; 0.09 and 0.12 mg/kg DW, for the simulator trial (Table 3.2), suggesting that beyond a critical concentration, both survival and reproduction are concurrently impaired.

In contrast, enchytraeids were the least sensitive species. Although reproduction significantly diminished in contaminated soils when compared with controls (one-way Anova, $p << 0.001$; Figure 3.2 F2 and S2), the effect was similar in all concentrations tested (Figure 3.2 F2 and S2). Despite this, for both field and simulator trials, it was possible to derive the EC50 values of 0.750 and 0.739 mg/kg DW for the reproduction of enchytraeids exposed to the range of carbofuran concentrations. The effects on adult survival were above the highest concentrations tested (Table 3.2).

Concerning earthworms, mortality was observed at the three highest carbofuran concentrations, ranging from 25 to 47% and 52 to 77% in the contaminated soil collected from field (F1) and simulator (S1) trials, respectively (Figure 3.2).

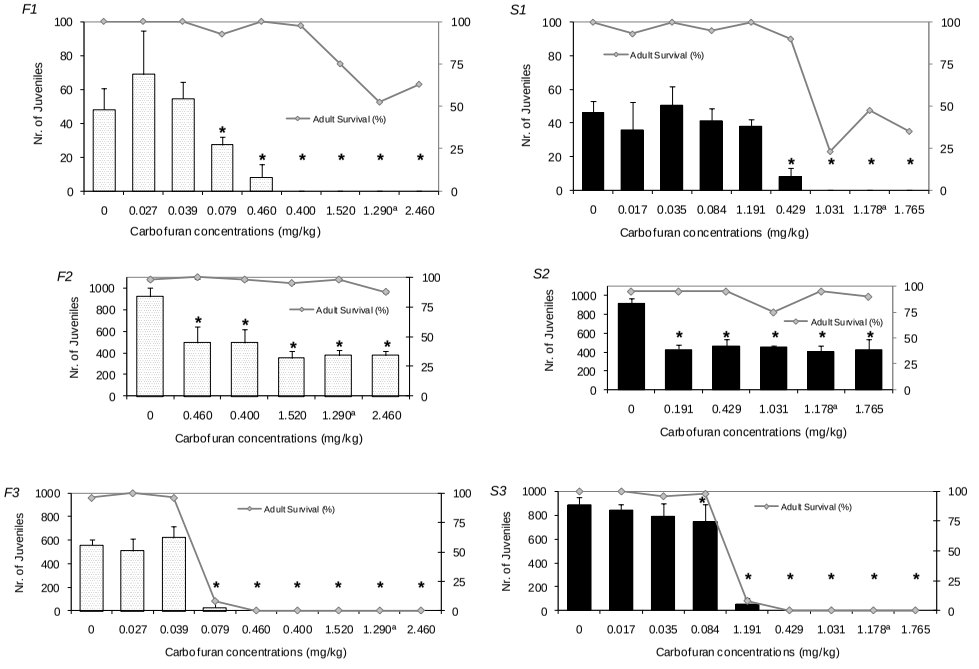

Figure 3.2. Adult survival (grey lines) and juvenile production (bars; average ± standard deviation) of three soil invertebrate species - (1) *Eisenia andrei*, (2) *Enchytraeus crypticus* and (3) *Folsomia candida* - exposed to soil sprayed with carbofuran [the Recommended Dose (RD - 5L/ha) and two times the RD (HD - 10L/ha) of Furadan; 350g a.i. /L)] in the Field (F) and Simulator (S) trials (see section II for details and Table 3.1 for correspondences with Furadan dilutions). ª - concentration corresponding to RD; * - Nr of juveniles significantly lower than control (One Way Anova, Dunnet Test, $p < 0.05$).

Table 3.2. Toxicity of carbofuran to *Eisenia andrei*, *Enchytraeus crypticus* and *Folsomia candida* exposed to soil contaminated with the Recommended Dose (RD - 5L/ha) and two times the RD (HD - 10L/ha) of Furadan (350g a.i. /L), in the Field and Simulator trials (see section II for details). Toxicity data include LC50 (effects on survival) and EC50 (effects on reproduction) values, as well as the 95% confidence intervals (inside brackets). Data are expressed in terms of carbofuran concentrations (as milligrams per kilogram of soil DW; see Table 3.1 for correspondences with Furadan dilutions).

Organism	**Carbofuran Toxicity (mg/kg DW)**			
	Survival (LC50)		**Reproduction (EC50)**	
	Field	**Simulator**	**Field**	**Simulator**
E. andrei	3.13 (2.32 - 5.69)	0.75 (0.64 - 0.87)	0.08 (0.06-0.11)	0.30 (0.22 - 0.41)
E. crypticus	> 2.46	> 1.77	0.75 (0.41 - 1.36)	0.74 (0.38 - 1.44)
F. candida	0.057 (0.053 - 0.063)	0.09 (0.07 - 0.10)	0.073 (0.069 - 0.078)	0.12 (0.10 - 0.14)

Abnormal behavior like coiling, secretion of mucus and inability in burrowing into the soil was also observed. The reproduction seemed to be stimulated at the two lowest concentrations, especially in the case of dilutions prepared from the field (F1) samples (Figure 3.2 F1). Despite the higher mortality observed in the test performed with samples from the simulator trial, the effects on reproduction were more pronounced in the test performed with samples from the field trial. In the latter, the EC50 value was 0.08 mg/kg DW, while for the simulator it was 0.30 mg/kg DW (Table 3.2).

Although the same carbofuran dilutions were used in both assays, toxicity was somehow higher in soil samples from the field trial. For instance, the EC50 values for reproduction in soil dilutions from the field assay were approximately 3.8 and 1.6 fold lower, respectively for *E. andrei* and *F. candida* (Table 3.2).

III. 3 Carbofuran toxicity to cladocerans

The mortality of *C. silvestrii* in the ASTM controls and in the non-contaminated water samples was lower than 10%, fulfilling the validity criteria defined by the guidelines for lethal assays (ABNT, 2004). In the sub-lethal tests, the average number of neonates produced by female at the end of the 9-d assay was slightly below the value indicated by the ABNT guideline (15 neonates per female) (14.6 ± 4 and 12.8 ± 3 for the tests with simulator and field samples, respectively; data not shown). These results were most probably due to the lower temperature (22 ± 1 ºC) of the room where tests were conducted comparatively to that indicated in the guideline (24 ± 1 ºC). In fact, temperature is a factor that strongly influences the age at first reproduction (namely for *C. silvestrii*; please see Fonseca and Rocha, 2004 and references therein).

The lethal effects of carbofuran on *C. silvestrii* in eluate, leachate and runoff samples collected from contaminated soil are shown in Figure 3.3 and the derived toxicity values for lethal and sub-lethal tests are presented in Table 3.3. Results revealed that this cladoceran species is extremely sensitive to carbofuran contamination.

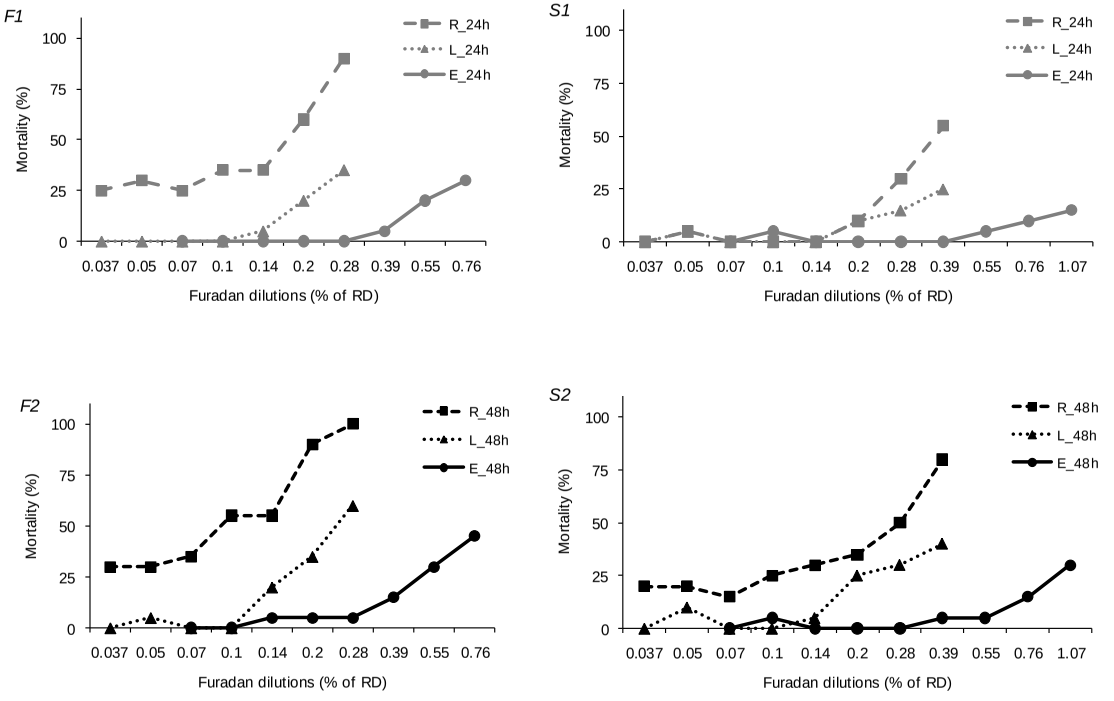

Figure 3.3. Mean neonate mortality of *Ceriodaphnia silvestrii* exposed 24 (1) and 48h (2) to several dilutions of runoff (R), leachate (L) and eluate (E) samples collected following the soil spraying with the Recommended Dose (RD - 5L/ha) of Furadan (a.i. carbofuran, 350 g/L), in the Field (F) and Simulator (S) trials (see section II for details).

Table 3.3. Toxicity of Furadan (a.i. carbofuran, 350 g/L) to *Ceriodaphnia silvestrii* exposed to eluate, runoff and leachate samples collected following the soil spraying with the Recommended Dose (RD-5L/ha) from the Field and Simulator trials (see section II for details). Toxicity data include LC20, LC50 (effects on survival) and EC50 (effects on reproduction) values, as well as the 95% confidence intervals (inside brackets). Data are expressed in terms of percentage of RD.

	Furadan Toxicity (% RD)					
	LC20 (48h)		**LC50 (48h)**		**EC50 (9d)**	
Sample	**Field**	**Simulator**	**Field**	**Simulator**	**Field**	**Simulator**
Runoff	0.04 (0.05 – 0.02)	0.06 (0.09 – 0.03)	0.08 (0.10-0.07)	0.24 (0.44-0.17)	> 0.05 < 0.1	> 0.1
Leachate	0.15 (0.18 – 0.12)	0.21 (0.30 – 0.15)	0.25 (0.36-0.21)	0.54 * (1.44-0.35)	> 0.1	> 0.1
Eluate	0.43 (0.57 – 0.33)	0.96 (2.65 – 0.64)	0.89 * (1.80-0.65)	2.76* (30.17-1.40)	> 0.1	> 0.1

* - Extrapolated values.

From the three types of aqueous samples, high lethality was observed for runoff followed by leachates and eluates (Figure 3.3). The LC20s derived from runoff dilutions were, for both trials, below 0.07%RD (Table 3.3) and at the highest dilutions (0.28% and 0.39%RD), after 48h of exposure, 80% or more of the cladocerans died (Figure 3.3 F2 and S2).

The LC20s and LC50s computed for the runoff samples indicated that these samples were twice as toxic as the leachate ones (Table 3.3). Despite this, median lethal effects occurred at dilutions of only 0.25 and 0.53%RD for the field and simulator trials, respectively (note that the latter value was extrapolated; Table 3.3).

In the assays performed with eluate samples, toxicity was much lower since less diluted samples induced lower mortality (at the highest dilutions, 0.76 and 1.07% of RD for the field and simulator trials, respectively, the percentage of dead cladocerans were ≤ 30% after 24h - Figure 3.3 F1 and S1- and ≤ 45% after 48h of exposure - Figure 3.3 F2 and S2-). Although for these tests the LC50s were extrapolated from the regression model and thus comparisons should be cautious (Table 3.3), the LC20s point to a significant toxicity at dilutions lower than 1%RD (Table 3.3).

As with the tests with soil organisms, a higher lethal toxicity was observed for samples obtained from the field trial. This was more evident in the derived LC50s that varied by a factor of 2.9, 2 and 3 for the tests with runoff, leachate and eluate samples, respectively (Table 3.3).

In the chronic toxicity tests, the calculation of EC50s was not possible since, excepting for 0.1%RD of the field trial, none of the dilutions caused reproduction impairment equal or above 50%, except for 0.1%. Despite this, for runoff samples, the highest dilution tested (0.1%of RD) caused a significant decrease in reproduction, compared to the control in both field and simulator trials (one-way Anova; Dunnett's Test, $p \leq 0.001$; data not shown). Indeed, the reproduction of cladocerans was in average 60 and 38% lower at this dilution relatively to the control for the field and simulator trials, respectively (data not shown).

IV. Discussion

IV.1 Carbofuran concentrations

The measured soil carbofuran concentrations in RD and HD treatments at the beginning of the trials were close to the expected values (1.167 and 2.33 mg/kg DW for RD and HD, respectively; see section II and Table 3.1). The higher concentrations found in the samples from the field trial are unexpected. Indeed, pesticide losses in the field are expectable to be higher than in a laboratory context. Nevertheless, in both trials the pesticide spraying was done in the late afternoon and very close to the soil surface, to minimize pesticide dissipation by volatilization and/or spray drift.

IV. 2 Carbofuran toxicity to soil organisms

The three soil invertebrate species used in the present study showed a markedly different sensitivity to carbofuran. The highest toxicity registered in *F. candida* tests was probably related with the insecticidal properties of carbofuran. The derived toxicity parameters were clearly below the recommended doses, for both field and simulator trials. Indeed, EC50s and LC50s corresponded, at most, to 9% and 7% of the concentration found in the RD, respectively (Tables 3.1 and 3.2), anticipating a serious risk of carbofuran applications for non-target arthropods, if a significant amount of the applied pesticide reaches the soil surface. The LC50s derived in the present study (Table 3.2) are within the range of the values -0.06 to 0.15 mg/kg- reported by Van de Plassche et al (1994).

Most of the data available from literature on the effects of carbofuran on soil organisms respect to earthworms, which were the second most affected group in the present study. As an inhibitor of AChE activity, this carbamate provokes neurotoxic effects, more or less reversible, depending on the amount and time of exposure (Panda and Sahu, 2004). Toxicity was higher in the field trial, except for earthworm survival which was less effected than in

the tests with samples from the simulator trial (Figure 3.2 F1). Notwithstanding this difference, the abnormal behavior observed in earthworms recovered from the highest carbofuran concentrations indicate that, for both assays, these organisms would probably die within a short period of time. The acute effects of carbofuran to earthworms in field and laboratory tests were reviewed by Van Gestel (1992). The author reported 14d LC50s in artificial soil for *E. andrei* between 5 and 10 mg/kg, which are higher than those obtained in our study. Indeed, LC50s varied between 3.13 and 0.75 mg/kg DW for the field and simulator assays, respectively (Table 3.2). This difference can be related with different pedological properties of the test-soils since they can strongly modify chemical toxicity (Lanno et al, 2004). In field studies, Van Gestel (1992) reported that (estimated) soil carbofuran concentrations of 1.4 to 16mg/kg caused a reduction in earthworm population equal or higher than 50%. These results are slightly above the range of concentrations measured for the RD of the commercial formulation used in the present study: 1.29 and 1.18 mg/kg DW for the field and simulator trials, respectively (Table 3.1).

Recently, aiming to generate more data on tropical soil toxicity, a series of tests with tropical and temperate earthworm species, including *E. andrei*, in pesticide contaminated soils (OECD artificial and modified soil plus tropical and temperate natural soils) were conducted by De Silva et al (2009, 2010) and De Silva and Van Gestel (2009). When exposing *E. andrei* to standard OECD and two modified standard soils (alternatives for the tropics) plus two natural soils (representing temperate and tropical soils) contaminated with carbofuran, both LC50s and EC50s were higher than the ones obtained in the present study. Indeed, the lowest LC50 and EC50 were respectively 8.46 and 0.6 mg/kg (De Silva et al, 2009; De Silva and Van Gestel, 2009) while in our study the same toxicity parameters for the field (F) and simulator (S) assays were 3.13 (F) – 0.75 (S) and 0.08 (F) - 0.30 (S) mg/kg DW (Table 3.2). Again, the different soil properties of the test substrates (Lanno et al, 2004), namely the higher pH, as well as the pesticide formulation used (De Silva et al, 2009) could explain these variations. The latter study used the pure carbofuran while in our study a commercial formulation was applied. In another work, the same authors found higher toxicity of the formulated carbofuran, if compared with the pure substance, for the tropical earthworm *Perionyx excavatus* (De Silva

et al, 2010). The avoidance behavior of *E. andrei* was also found to be stronger towards a soil contaminated with a commercial formulation of the herbicide penoxsulam when compared with the pure chemical (Marques et al, 2009). Commercial formulations probably contain other chemical agents that can either potentiate the pesticide toxicity or be themselves noxious. The higher toxicity of pesticide formulations relatively to pure substances has been documented for other test-species (see references cited by De Silva et al, 2010).

Data from *E. crypticus* tests suggest that the application of the carbofuran formulation within the recommended doses does not seem to be lethal since, with two exceptions, adult survival was always higher than 90% in all concentrations tested (Figure 3.2, F2 and S2). Despite this, the reproductive potential of the population may be endangered since the reproduction in the lowest concentration tested, corresponding to 10% HD, was about 45 and 30% less than in the controls for the tests with field and simulator samples, respectively (Figure 3.2, F2 and S2; Table 3.1). Moreover, in the tests with samples from both the field and simulator trials, carbofuran concentrations of about 0.7 mg/kg DW caused a decrease of 50% in reproduction (Table 3.2). Data from field and semi-field tests on carbamate toxicity to enchytraeids suggest that these organisms are sensitive to some carbamates and indifferent to others (Didden and Römbke, 2001).

IV. 3 Carbofuran toxicity to cladocerans

Carbamate insecticides are known to be highly toxic to aquatic organisms and aquatic crustaceans are particularly endangered (Iesce et al, 2006; Werner et al, 2000). Indeed, carbofuran was much more toxic for cladocerans than for soil organisms. For instance, in the present study, median lethal values for *F. candida*, the most affected soil species, corresponded to approximately 5%RD (Table 3.1 and 3.2) while for *C. silvestrii*, in tests with runoff samples the calculated values were below 0.25%RD (Table 3.3).

Among the three tested water samples, runoff was clearly the most toxic. According to the literature, pesticide losses by runoff seem to be the most

important exposure route of nonpoint-source pollution of surface waters (Schulz, 2004) and peaks of concentration and toxicity have been reported to occur after applications and/or rainfalls (Schulz, 2004; Castillo et al, 2006; Werner et al, 2000; Brady et al, 2006). For example, high concentrations of carbofuran, ranging from 0.010 from 1.823 mg/L and its metabolites have recently been detected in water samples from Kenya during the rainy season (Otieno et al, 2010). Schulz (2004) estimated that 1 to 10% of the total amount of applied pesticide, or even more if severe rainstorms follow pesticide application, is lost by edge of field runoff.

The higher toxicity observed in cladocerans exposed to leachate dilutions is consistent with the high leaching potential of carbofuran (Martins et al, 2007; Singh and Srivastava, 2009). This behavior is due to its high water solubility (322 mg/L), low sorption (K_{oc} ~23.3 ml/g) and moderate persistence (soil DT50 ~29d; aqueous hydrolysis DT50 at 20^{o}C and pH 7 ~37d; data available at; http://sitem.herts.ac.uk/aeru/iupac/118.htm; Accessed 20th August 2011). Martins et al (2007) calculated that approximately 6% of the total amount of carbofuran applied to the surface of a tropical soil is leached below 50 cm. The lower toxicity of leachate dilutions, when compared with runoff, may be related with the adsorption of some carbofuran to the soil organic matter during its percolation trough the soil profile. Despite this contribution of soil organic matter to pesticide sequestration, the sandy texture seems to have mitigated this effect and increased carbofuran mobility (Singh and Srivastava, 2009).

Eluates were by far the least toxic samples: the calculated LC20s and LC50s were at least 11 times higher than those derived for tests with runoff dilutions (the most lethal) (Table 3.3). The time involved in the eluate preparation, consisting of 12h shaking plus 12h of resting, might have contributed to some carbofuran degradation. Also, the basic conditions (average pH values of 7.8 for field and 7.7 for laboratorydilutions; data not shown) seem to favor carbofuran hydrolysis (Iesce et al, 2006). The soil eluates are recommended by ISO Guidelines (ISO, 2003) as a useful method to evaluate the soil retention function and leaching potential and thus, the risks for organisms exposed to pesticide mobilization via water. However, results from the present study clearly showed that eluates are not good

indicators of the possible contamination of both ground and surface waters. Therefore, it is strongly recommended that, on the assessment of the potential hazard effects of pesticide contaminated soils on aquatic systems, the testing strategy should include the performance of tests with runoff and leachate samples.

Generally, the range of dilutions tested did not cause effects on reproduction of *C. silvestrii*. Since they were defined taking into account the results from lethal tests, these unexpected results are much probably due to the rapid carbofuran degradation in the water samples during the gap of time between the beginning of lethal and sub-lethal tests.

Summarizing, results from the present study clearly show that the application of this insecticide at the recommended doses poses a serious risk for the aquatic ecosystem since, in the runoff and leachate samples collected from a worst-case scenario simulation, dilutions of less than 1%RD were clearly toxic to a cladoceran species. Notwithstanding, in a real contamination scenario, several variables may increase or decrease the threats posed by carbofuran. Indeed, on the one hand, when entering a water system, the carbofuran concentration in runoff (and leachate) would be more or less reduced depending on factors like the size of the waterway, the flow or other physical conditions that affect bioavailability to aquatic organisms (e.g., amount of dissolved particles, dissolved organic carbon, temperature, pH) and, thus, the exposure level (Brady et al, 2006). But, on the other hand, intensive and/or excessive applications of the pesticide, as well as the severe precipitation occurring in the tropics, might amplify the risk (Castillo et al, 2006).

The hazardous carbofuran concentration affecting 5% of the aquatic species (HC5) in single species acute tests estimated by Maltby et al (2005) was 0.2µg/L. With exception of the European Union, that established a maximum level of 0.1µg/L for each pesticide (European Union, 1998), this value is clearly below the guidelines for carbofuran concentrations in drinking waters established by several countries, that vary from 5µg/L in Australia to 90µg/L in Canada (WHO, 2004), thus pointing a hazard potential of legally accepted carbofuran concentrations at least for some aquatic organisms. In addition, the disappearance of natural grazers such as zooplankton (due to

lethal insecticide concentrations) may indirectly alter the abundance and composition of phytoplankton community, eventually leading to algal blooms (De Lorenzo et al, 2001). Moreover, the lack of food for zooplankton predators, such as fish larvae, may also disturb the local food-webs.

IV. 4 Toxic balance of field and simulator trials

The higher carbofuran toxicity observed in the field versus the simulator trial is consistent with the higher measured carbofuran concentrations in the former assay (Tables 3.1 and 3.2). Moreover, the toxicity parameters traducing the responses of the organisms, both for soil and aquatic species, in the two sets of field and simulator trials, varied from 1 to 3.8 times (Tables 3.2 and 3.3). Despite the parallel methodologies followed in both trials, these deviations seem to be acceptable as uncertainties associated with, for example, the different application-scales and physical conditions. Römbke and Moltmann (1996) referred that results from the same test-system, using the same chemical but performed in different laboratories could have results with a deviation factor of up to 10. Thus, the system developed to simulate pesticide applications in laboratory, allowing the further evaluation of toxic effects to both soil and aquatic organisms, showed to be a good surrogate of expensive and complex field studies.

Notwithstanding, to go on with a standardization process, some improvements like changing from plastic to steel to avoid possible pesticide adsorption or adding features to facilitate either the testing under variable slopes or the collection of aqueous samples are most advisable.

V. Conclusions

The field application rates of the carbofuran formulation were hazardous to soil organisms, indicating deleterious effects on habitat function. The aquatic cladocerans were the most affected by the carbofuran applications, suggesting that the soil retention function is low. Among the three tested soil species, the most affected group were collembolans, followed by earthworms and enchytraeids. The present study also showed that, in the aquatic compartment, major risks of carbofuran contamination and toxicity arise from surface runoff inputs to adjacent water systems due to heavy rainfalls after pesticide applications. In fact, from the three aqueous samples tested, the highest lethal toxicity to *C. silvestrii* was found with runoff dilutions. Although two times less toxic than runoff samples, the high mortality observed in leachate samples pointed to increased risks of groundwater contamination. Soil eluates were by far the least toxic samples showing the need to include tests with runoff and leachate samples in the test strategy. Moreover, as they represent realistic routes of exposure of aquatic organisms to pesticide contamination, the ecological relevance of the gathered data would be favored.

A good consistency was found between the toxicity results of tests performed with samples collected from field and simulator trials. Thus, even though some improvements are still needed, the laboratory simulator proved to be a valuable and useful tool to evaluate the toxic effects of pesticide sprayings in soils and the potential risk for aquatic organisms due to runoff and leaching.

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Chapter 4

Semi-field testing of a bioremediation tool for atrazine contaminated soils: evaluating the efficacy on soil and aquatic compartments

Based on the following manuscript:

Chelinho S, Moreira-Santos M, Silva C, Costa C, Viana P, Viegas CA, Fialho AM, Ribeiro R, Sousa JP. Semi-field testing of a bioremediation tool for atrazine contaminated soils: evaluating the efficacy on soil and aquatic compartments. (*Submitted to Environmental Toxicology and Chemistry*).

Abstract

This study aimed at evaluating, at a semi-field scale, the bioremediation efficacy of a previously developed cleanup tool for atrazine contaminated soils (*Pseudomonas* sp. ADP plus citrate; *P.* ADP+CIT), by combining information from chemical analysis and soil and aquatic ecotoxicological tests.

Three experiments representing worst-case scenarios of atrazine contamination for soil, surface water (due to runoff) and groundwater (due to leaching) were performed, in laboratory simulators (100×40×20cm). For each experiment, three simulator/treatments were set up: a bioremediated (soil sprayed with 10× the Recommended Dose (RD) for corn of Atrazerba FL and with *P.* ADP+CIT at day 0 and a similar amount of *P.* ADP at day 2), a non-bioremediated (soil sprayed only with 10×RD of Atrazerba FL at day 0) plus a control simulator. After 7 days of treatment, samples of soil (and respective eluates), runoff and leachate were collected for ecotoxicological tests with two plants (*Avena sativa* and *Brassica napus*) and one microalgae species (*Pseudokirchneriella subcapitata)*.

In the non-bioremediated soils, atrazine was very toxic to both plant species, with more pronounced effects on plant growth than on seed emergence. The bioremediation tool annulled atrazine toxicity to *A. sativa* (86 and 100% efficacy, for seed emergence and plant growth, respectively), but *B. napus* performance in bioremediated soil was still lower than in the control, pointing for incomplete bioremediation. For the microalgae, eluate and runoff samples from the non-bioremediated soils were extremely toxic but a slight toxicity was registered for leachates. In just 7 days, the ecotoxicological risk for the aquatic compartments seemed to be significantly diminished with the application of *P.* ADP+CIT. Indeed, in aqueous samples obtained from the bioremediated soils, the microalgae growth was similar to the control for runoff samples and slighlty lower than control (by 11%) for eluates.

Keywords: herbicides; *Pseudomonas* sp. ADP; simulator; runoff; leaching; soil habitat function; soil retention function.

I. Introduction

Atrazine is one of the most intensively used herbicides worldwide and due to its moderate to high persistence in the environment, it has been detected above legislation limits and its toxic effects (especially for aquatic organisms) have been extensively reported (for a recent review on environmental fate and effects of atrazine and other *s*-triazine herbicides see Viegas et al, in press). As a consequence, atrazine has been banned in Europe (European Commission, 2004). However, its continued use is allowed in several parts of the world like Africa (Getenga et al, 2009), Asia (Srivastava and Mishra, 2009; Yang et al, 2010), Latin America (Correia et al, 2007), USA (Sass and Colangelo, 2006) and Australia (APVMA, 2008).

Severe atrazine contamination problems have been reported due to intensive applications, accidental spills and/or deficient storage conditions at industrial units, dealerships or at mix-load sites (Aresta et al, 2004; Chirnside et al, 2007, 2009; Jin and Ke, 2002; Strong et al, 2000). These are serious threats to soil ecosystems but particularly to adjacent or nearby water resources due to edge-of-field runoff and/or leaching (Rice et al, 2007; Schulz, 2004). Indeed, the most important cause of nonpoint-source pollution of surface waters is associated to pesticide losses due to runoff (Flurry et al, 1996; Schulz, 2004; Schulz and Liess 2001), with peaks of concentration (and toxicity) registered after applications and/or rainfalls (Brady et al, 2006; Castillo et al, 2006; Leonard et al, 1992; Schulz, 2004).

To mitigate and/or prevent the hazard effects of atrazine, research has been undertaken towards the development of bioremediation methodologies, based on the ability of some microorganisms to convert atrazine into less toxic or non-toxic substances (Rice et al, 2007; Sene et al, 2010; Viegas et al, in press). A bioremediation tool for atrazine contaminated soils, consisting of soil bioaugmentation with the atrazine-mineralizing bacterium *Pseudomonas* sp. ADP (hereafter designated *P.* ADP) and bioestimulation with trisodium citrate (CIT) (Silva et al, 2004) was recently tested at a microcosm scale under different worst-case scenarios of soil contamination, yielding very promising results. Indeed, a strong and rapid decline of atrazine concentration in soil as well as the effective reduction of the soil toxicity to plants, cladocerans and

microalgae within 5 or 10 days were reported (Chelinho et al, 2010; Lima et al, 2009).

Aiming at evaluating the potential of the atrazine cleanup tool (Silva et al, 2004, Lima et al, 2009) for routine use under real field scenarios, a scaling up of previous microcosms experiments was considered a crucial step. A further evaluation of the cleanup tool efficacy on both soil and water compartments was carried out at a semi-field scale, using a novel cost-effective and standardizable simulator for pesticide applications. In accordance with previous studies on the potential of this bioremediation tool to reduce atrazine toxicity in soil and aquatic environments (Chelinho et al, 2010), in the present work an integrated approach was followed to assess the soil habitat function and the soil retention function (ISO, 2003), as well as the atrazine removal from soil and water (through chemical analysis), but at a larger semi-field scale. In addition, the simulator was developed so that the indirect toxic effects on aquatic organisms due to the mobilization of atrazine via the water pathway in soils were evaluated by assessing not only the soil retention function, but also toxic effects due to leaching and surface runoff from contaminated soils. Based on the work of Chelinho et al (2010), where results pointed out for negligible effects on soil invertebrates, in the present study no soil animals were used. Instead, two plant species (a mono- and a dicotyledonous, to investigate a possible different sensitivity to atrazine) and an aquatic microalgae species were selected as organisms representative of the soil and aquatic compartments, respectively.

II. Materials and Methods

II. 1 Laboratory simulators

Laboratory simulators (100×40×20 cm; length, width and high, respectively) were used to test the fate and effects of pesticide applications, mimicking different worst-case scenarios of soil and water contamination (see below) while allowing the collection of soil and aqueous samples (runoff and leachate) (Figure 4.1). They consisted of two adjoining stainless steel trays of

the same size, movable relatively to each other allowing the independent regulation of slopes. The bottom of each tray was slightly funnel-shapped and equipped with a tap at the bottom of the funnel to collect leachate samples. One of the trays was also equipped with a U shaped channel with an opening at the centre to drive and collect the runoff samples.

II.2 Experimental design

The bioremediation efficacy of the atrazine cleanup tool was evaluated by performing three experiments with the simulators set up to represent three different worst-case scenarios of atrazine contamination for soil and aquatic organisms. To assess soil toxicity, i.e., the soil habitat and retention functions (ISO, 2003), a first experiment (A) was carried out with the two trays of the simulators in the horizontal position (slope of 0%), to maximize the amount of pesticide remaining in the soil (see Figure 4.1 A and scheme A of Table 4.1). A second experiment (B) was carried out with both trays of the simulators at a slope of 42% (see Figure 4.1 B and scheme B of Table 4.1), to assess the maximum risk for aquatic organisms due to surface runoff originating from severe and unpredictable rainfalls. Finally, to assess the highest risk for aquatic organisms due to leaching, a third experiment (C) was carried out with one tray of the simulator at a slope of 42% and the other in the horizontal, to mimic locations where leaching to groundwater is maximized by the occurrence of a field with an inclination adjacent to a flat area, which may receive runoff inputs (see Figure 4.1 C and scheme C of Table 4.1).

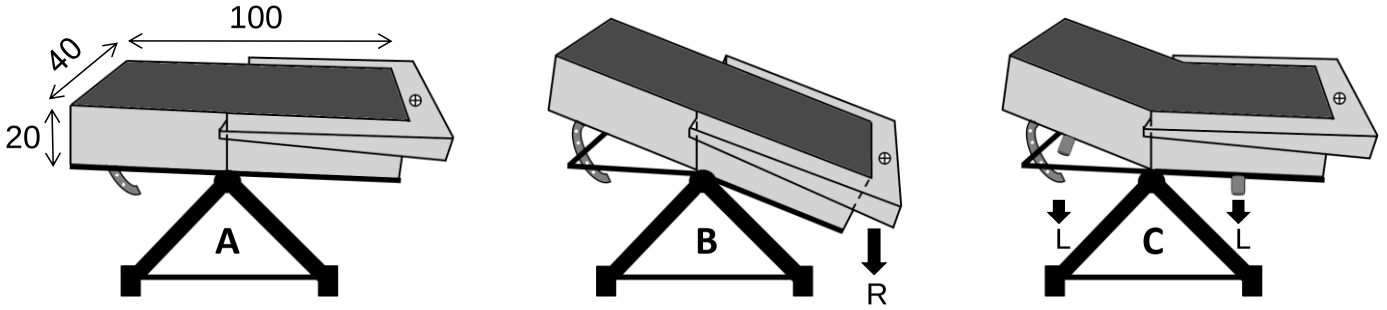

Figure 4.1. Scheme of the laboratory simulator developed to evaluate the bioremediation efficacy of an atrazine clean up tool under three different worst-case scenarios of pesticide contamination toward (A) soil habitat and retention functions, (B) aquatic organisms due to surface runoff and (C) aquatic organisms due to leaching. The dimensions are expressed in cm. The black arrows represent the points of collection of runoff (R) and leachate (L) samples.

Table 4.1. Summary of the experimental design to evaluate the efficacy of a bioremediation tool to cleanup soils contaminated with 10 times the Recommended Dose (RD) of Atrazerba under three different worst-case scenarios of pesticide contamination towards soil habitat and retention functions (Experiment A), aquatic organisms due to surface runoff (Experiment B) and aquatic organisms due to leaching (Experiment C). Evaluation included atrazine analysis (on soil, eluates, runoff and leachates), microbial analysis (on *Pseudomonas* sp. ADP viability) and toxicity tests (on soil with plants and on eluates, runoff and leachates with microalgae).

Actions taken during experiment	Experiment (simulator scheme)	Treatment	Sampling time (days)					
		Atrazerba dose + Bioremediation tool	0	1	2	3	4	7
AS - Atrazine Spraying	A	0× RD + NO (Ct)	SCA					SCA SC
BS – Bioremediation tool (*P.* ADP+CIT) Spraying	B							SCA RS
SCA - Soil sampling for Chemical Analysis	C			LS [b]			LC [c]	SCA
SMA - Soil sampling for Microbiological Analysis	A	10× RD + NO (NB)	AS SCA					SCA SC
SC - Soil Collection (for eluates [a] preparation and soil toxicity tests)	B							SCA RS
RS - Runoff Simulation [a]	C			LS [b]			LC [c]	SCA
LS - Leachate Simulation [b]	A	10× RD + YES (B)	AS BS SCA SMA					SCA SMA SC
LC – Leachate Collection [a,c]	B			SMA	BS[d] SMA	SMA		SCA SMA RS
	C			LS [b]			LC [c]	SCA SMA

[a] samples used for chemical analysis and microalgae tests; [b] daily from days 1 to 6; [c] daily from days 4 to 7; [d] - *P.* ADP spraying.

For each experiment three simulators/treatments were set up: (1) control (Ct), with no herbicide or bioremediation tool sprayed onto the soil; (2) soil sprayed with 10× the Recommended Dose (RD) of Atrazerba FL (~500 g atrazine/L; Sapec, Lisboa, Portugal) for weed control in corn plantations (10×2 L Atrazerba/ha; equivalent to ~13.3 mg atrazine/kg of soil dry weight [DW], assuming a soil density of 1.5g/dm^3 and a herbicide incorporation up to 5 cm depth) and with the bioremediation tool (*P.* ADP+CIT; see below), hereafter designated as B (bioremediated) treatment; (3) soil sprayed solely with 10×RD Atrazerba, hereafter designated as NB (non bioremediated) treatment (Table 4.1).

Each simulator was filled with a 5 cm layer of Ø1cm glass beads (to facilitate leachate percolation) and a 15 cm layer of a sandy loam soil (6.14 pH, 3.10% organic matter, 62.4% sand, 21.2% silt, 16.4% clay, 0.83 mg/kg total N, 0.0125 cmol/g CEC and 32.8% water holding capacity (WHC); analysed according to methods referred in Lima et al, 2009). Soil was representative of a corn production field with no history of pesticide applications (Coimbra, Central Portugal). The soil (top 10 cm) was collected one day before the start of each experiment and major stones and vegetation were manually removed. During all experiments the simulators were placed under a 7 m^2 (3.5×2 m) semi-open space, protected from direct sunlight and rain but exposed to outside temperature and humidity.

At the beginning of each experiment (day 0), the soil surface was sprayed with 10×RD of Atrazerba for both NB and B treatments (Table 4.1). This high Atrazerba dose intended to represent worst-case scenarios of atrazine contamination, like accidental spills (e.g. Strong et al, 2000), careless disposal (e.g. Chirnside et al, 2007; Dasgupta et al, 2007) and intensive use (e.g. Correia et al, 2007) or overuse (e.g. Aresta et al, 2004). After spraying, a rainfall was simulated using artificial rain (Velthorst, 1993) and the top 5 cm of soil was mixed, to facilitate the herbicide incorporation into the soil. For B treatments, a mixture of *P.* ADP+CIT (bioremediation tool) was sprayed onto the soil surface (see section II.3 for details on the preparation of the inoculum), approximately 2 h after the incorporation of Atrazerba, and its incorporation was as described for the herbicide (Table 4.1). In the NB treatments, the application of the bioremediation tool was replaced by the

same volume of artificial rain, while in the controls both the spraying of herbicide and bioremediation tool were replaced by that of artificial rain. The total amount of artificial rain added to each treatment at day 0 (for herbicide and *P.* ADP+CIT incorporation) corresponded to 1.8 h of rain during the rainiest day of October 2005 in Coimbra (26.2 mm; Geophysical Institute of the University of Coimbra, 2010). The total amount of aqueous solutions added per simulator was adjusted to obtain initial soil moisture of ~60% of WHC.

At day 2, a second application of the same amount of *P.* APD (no CIT incorporation) was performed, following the same procedures of day 0. Although a single soil application of *P.* ADP at day 0 proved to be effective as a bioremediation strategy for treatment of a soil contaminated with this same dose of Atrazerba, at a microcosms scale under laboratory controlled conditions (Chelinho et al, 2010), in the present study a second application of the bacteria intended to minimize a possible decrease in its efficacy under semi-field exposure conditions. The natural fluctuations of environmental variables (e.g. temperature, rainfall, wetting-drying cycles) as well as the presence of indigenous communities of soil microorganisms (that may act as competitors) and soil micro and mesofauna (that may act as predators/grazers) may diminish the number of viable cells of the biodegradative bacteria and/or the atrazine-bacteria contact area and hence its atrazine degradation activity (Issa and Wood, 2005; Kersanté et al, 2006). Also, for these reasons, in the present study, the experimental period selected to evaluate the efficacy of the bioremediation tool was 7 d, instead of the 5 d reported in Chelinho et al (2010). Composite soil samples (3 per treatment; top 5cm) were collected at the beginning and end of each experiment to determine soil atrazine concentrations, whereas in aqueous samples, concentrations were determined only at day 7 (Table 4.1). For microbial analysis, B treatments were sampled on days 0, 1, 2, 3, and 7 (Table 4.1).

After 7 days, soil and aqueous samples were collected from each treatment to assess the efficacy of the bioremediation tool towards soil and aquatic organisms, specifically two terrestrial plants and one aquatic microalgae species, respectively (see section II.5). For experiment (A), the top 3 cm of soil were sampled to evaluate the soil habitat and retention functions,

the latter through the preparation of soil eluates (Table 4.1). For experiment (B), surface runoff was obtained by simulating a heavy rainfall with artificial rain (Table 4.1). The precipitation values used were those of a tropical country (Brazil) where atrazine is extensively used (Correia et al, 2007) and where the ecological risks of pesticide runoff into adjacent water basins are especially high (Castillo et al, 2006; Correia et al, 2007). Ten litters of artificial rain per simulator were used corresponding to 9 h of rain in the rainiest day of October 2007 (66 mm) in an intensive agricultural region in central S. Paulo state (Meteorological station of CRHEA/SHS/EESC/USP, Itirapina, SP, Brazil; 22° 10' 13.53", -47° 53' 58.12"). In experiment C, the collection of leachates comprised the simulation of a continuous period of rainfall, daily from days 1 to 6 (Table 4.1), using artificial rain and corresponding to 1.2 h of rain during the rainiest day of October 2005 in Coimbra (26.2 mm; Geophysical Institute of the University of Coimbra, 2010). Since the soil achieved its maximum WHC, leachate could be collected into glass vials attached to the taps of the simulators. Although leachate samples were obtained already at day 2, only samples collected on the last 4 d (days 4 to 7) were used for toxicity testing, to give time for the bacteria to work, since results from a previous microcosm study (Chelinho et al, 2010) pointed out for a period of 5 to 7 d to obtain an effective cleanup of the dose of Atrazerba used in the present study. All samples were stored either at 4ºC in the dark until use (within 24 h or 15 d, for the plant and microalgae tests, respectively) or at -20°C for chemical analysis of atrazine.

The mean temperature values were 22 ± 3.7, 18 ± 4.7 and 18 ± 2.7 ºC during the performance of experiments A, B and C, respectively (mean ± standard deviation; n= 336), while the correspondent humidity values were 68 ± 16, 52 ± 20 and 85 ± 6.6% (n=336). Water losses by evaporation were estimated every two days, by weighing a vessel containing a 15 cm layer of moist soil (~5 kg DW), extrapolated to the amount of soil in the simulators and replenished using artificial rain.

II.3 Bioaugmentation agent

A spontaneous rifampicin-resistant (Rif^r) mutant of *P.* ADP was used. This mutant can mineralize atrazine with equal efficiency than the wild-type (García-González et al, 2003). The cell suspension used as inoculum was prepared from a late-exponential culture of *P.* ADP Rif^r grown as previously described (Lima et al, 2009). The mixture sprayed onto soil consisted of a concentrated solution of CIT, to amend the soil with 1.2 mg trisodium citrate/g of soil DW corresponding to a C_s:N_{atz} ratio of 50 (Lima et al, 2009; Silva et al, 2004), mixed with a concentrated inoculum suspension ($2.8 \pm 0.5 \times 10^{10}$ Colony Forming Units (CFU) of *P.* ADP/mL).

II.4 Test organisms

Oat (*Avena sativa*) and rape (*Brassica napus*) were selected as model plants. They are part of a list of non target species for use in standardized plant toxicity tests (ISO, 1994) and are known to be sensitive to atrazine (Crommentuijn et al, 1997); seeds were obtained from a commercial supplier (Hortícola, Coimbra, Portugal). The microalgae *Pseudokirchneriella subcapitata* (strain Nr. WW 15-2521; Carolina Biological Supply Company, Burlington, NC, USA) was chosen as model aquatic organism, as it has for long been recommended for freshwater toxicity studies and standard guidelines are available (Environment Canada, 1992; OECD, 1984). Cultures were maintained under non-axenic conditions as previously outlined (Rosa et al, 2010).

II.5 Ecotoxicological tests

To evaluate the impacts of atrazine on the aquatic compartment, toxicity tests with the microalgae *P. subcapitata* were carried out on soil eluate, runoff and leachate samples originating from the simulators (Table 4.1). Eluates

were prepared following standard methods (DIN, 1984) as previously described (Chelinho et al, 2010). The runoff samples were centrifuged following the same procedures used for the eluates preparation (at 3370*g* during 20 min) to remove excess of suspended particles. Whereas for the NB treatments a series of five dilutions was prepared and tested (100, 50, 25, 12.5 and 6.25%), for the control and B treatments only the original sample (100% dilution) was tested. The 72-hours *P. subcapitata* growth tests were performed according to standard guidelines (Environment Canada, 1992; OECD, 1984), on 24-well sterile microplates, at 21 to 23°C and under continuous cool-white fluorescent light (100 μE/m^2/s). Three 900 μL replicate cultures per each eluate, runoff and leachate sample and six control (standard medium; also used to prepare NB dilutions) replicates were set up and inoculated with 100 μL of the algal inoculum. For further details on testing procedures see Rosa et al (2010). At the end of the 72-h exposure, algal growth was estimated as the mean specific growth rate per day. Conductivity and pH were measured at the start of the test. Measured levels were comparable across treatments and not expected to have deleterious effects on the test organisms (Environment Canada 1992; OECD 1984).

To evaluate the bioremediation efficacy on soil habitat function, plant germination and growth were evaluated following the ISO guideline (ISO, 1994). For each treatment and each plant species, the soil was carefully mixed and distributed among four replicates (six for the control) that consisted of plastic boxes (12 × 9 × 6 cm; width × length × height) filled with 250 g (DW) of soil. After this, 10 seeds of *A. sativa* or *B. napus* were buried into the soil (~1 cm deep). The tests ran at controlled temperature (21ºC), photoperiod (16-h:8-h light:dark; 100 μE/m^2/s) and relative humidity (70%). To provide suitable soil moisture during the tests, each box was perforated at the bottom and connected to another box by a glass fiber wick, filled with deionized water, which functioned as water reservoir. Fourteen days after the emergence of more than 50% of the seeds in the controls, the aerial part of the plants was cut, dried (for 16 h at 80ºC) and weighted, to estimate growth as shoot dry weight per emerged seed.

II.6 Microbiological analysis

To determine the number of *P.* ADP Rif^r viable cells (expressed as CFU/g of soil DW), soil samples (mean ± SD of 1.3 ± 0.3 g Wet Weight) were diluted in saline solution (0.9% w/v NaCl) and serial dilutions were spread plated onto selective medium (agarized Lennox Broth supplemented with rifampicin (50 mg/L) and cycloheximide (100 mg/L) (Silva et al, 2004). Colonies were counted after 72 h of incubation at 30°C.

II.7 Chemical analysis

Soil samples (~20 g DW) were extracted with ethylacetate (3 × 10 mL) using a Liarre 60 ultrasonic apparatus (20 min; frequency 28–34 kHz), centrifuged (15 min; 2500 pm) and analyzed for atrazine by GC-EI-MS, as previously described (Lima et al, 2009). Atrazine from eluate, runoff and leachate samples (~250 mL each), was extracted with the automated system ASPEC XL (Gilson, Villiers-le-Bel, France) at neutral pH. Oasis 60 mg cartridges HLB (Waters, USA) were conditioned with 6 mL of dichloromethane, 6 mL of acetonitrile and 6 mL of HPLC water. Samples were percolated through the cartridge (flow rate of 6 mL/min) that was subsequently rinsed with 1 mL of HPLC water (flow rate of 30 mL/min) and after that the adsorvent was dried with nitrogen for 30 min. The elution was performed with 2.5 mL acetonitrile- dichloromethane (1:1) and 3 mL of dichloromethane. The final extract was concentrated to 200 µL with a gentle nitrogen flow.

The GC-EI-MS analyses were performed with Perkin-Elmer Model Clarus 500 (USA). The mass spectrometer was operated in the electron impact ionization mode with an ionizing energy of 70 eV. A FV (Varian)-5MS (30 m x 0.25 mm i.d. with 0.25 µm film thickness) programmed from 50ºC (1min) to 150ºC at 10ºC/min, 150 to 240ºC at 4ºC/min and to 270ºC at 15ºC/min, keeping this temperature for 2 min. Helium was used as the carrier gas at 30 mL/min, under the splitless mode and using 1 µL of injection volume. Chromatograms were recorded under time-scheduled selected ion monitoring (SIM). Full scan conditions (from 50 to 450 amu) were also used. All extracts

were injected in SIM, for quantitative purposes and by scan mode, to confirm the presence of the analyte. The quality control comprised the use of control standards and the performance of recovery tests. Recovery ranged between 85 and 100%. The limits of quantification were 0.1 µg/g of soil DW or 0.1 µg/L.

II.8 Data analysis

In accordance with the study previously conducted at a microcosm scale (Chelinho et al, 2010), statistical analysis was carried out to answer three major questions: (1) Was the 10×RD dose of Atrazerba toxic to the test organisms? (2) Was there bioremediation, i.e., did the addition of *P.* ADP+CIT to the B treatments cause a decrease in toxicity compared to the correspondent NB treatments? and (3) What was the efficacy of the bioremediation tool, i.e., what was the performance of the organisms in the B treatment compared to that in the control? All three questions were answered by comparing organism responses through a two-sample one-tailed *t*-test between NB versus control, NB versus B, and B versus control treatments, for question 1, 2 and 3, respectively. Normality and homoscedasticy were checked using Shapiro-Wilk's and Levene's tests, respectively. Whenever these assumptions were violated, even after data transformation, the equivalent nonparametric one-tailed Mann-Whitney test was used.

Additionally, to fully characterize the ecotoxicological potential of the aqueous samples from NB soils, microalgae growth responses in the control and tested dilutions (100, 50, 25, 12.5, 6.25 and 0 %) were fitted to a logistic model using the least squares method (Stephenson et al, 2000), to estimate the effective concentrations inducing 20% (EC20) and 50% (EC50) of growth inhibition relatively to the control, and respective 95% confidence limits.The growth inhibition caused by a 10% dilution of the aqueous extracts was also quantified, as this corresponded roughly to the spraying of the RD of Atrazerba.

III. Results

III.1 Survival of *P.* ADP and atrazine biodegradation

The quantification of the viable cell numbers of the bioaugmentation bacteria during the course of the three experiments carried out representing worst-case scenarios of pesticide contamination is shown in Figure 4.2. After a decline in bacterial survival during the first two days upon soil bioaugmentation with *P.* ADP Rif^r and biostimulation with CIT, the second inoculation with the atrazine-mineralizing bacteria allowed to achieve viable cell numbers higher than 3x10^8 CFU/g of soil during at least one day more, after which a progressive drop on bacterial survival occurred (Figure 4.2).

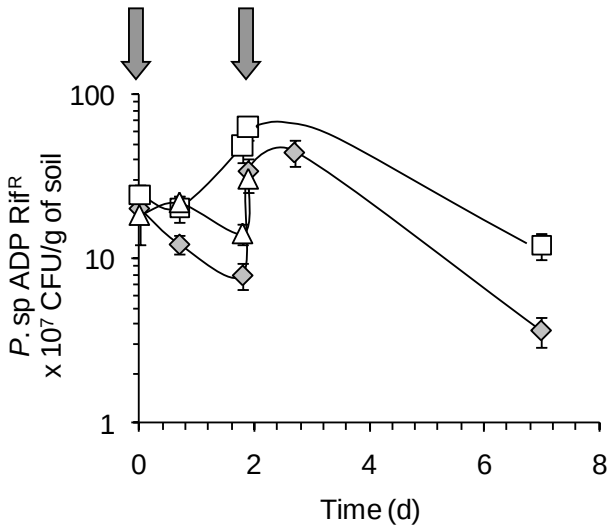

Figure 4.2. Time - course variation of the concentration of viable cells of *P.* ADP Rif^r in the simulators contaminated with 10× the Recommended Dose (RD) of Atrazerba and subsequently sprayed with the bioremediation tool *P.* ADP + CIT at day 0 plus the same amount of *P.* ADP at day 2 (signalled by the grey arrows) during the 7 d experiments representing worst-case scenarios of pesticide contamination (see section II.2 and Table 4.1 for details). Symbols are as follows: Experiment A (◆), B (□) and C (△). Error bars indicate ± standard deviation.

Initial atrazine concentrations (overall values between 7.60 and 15.7 μg/g of soil DW) were strongly reduced in the B soils (by 98%, declining to less than 0.17 μg/g of soil DW) but not in the NB soils (approximately 32 to 100% of the initial atrazine remained in the soil) after the 7 d treatments (Table 4.2). In the control soils (without application of Atrazerba), atrazine was always below the detection limits (data not shown). Consistent with the decrease in atrazine concentrations in the B soils, concentrations in all the aqueous extracts (eluates, runoff and leachates) was considerably lower than in the correspondent extracts from NB soils after the 7 d bioremediation period (by at least 98%), even though atrazine concentration in NB leachate was ~100 fold lower than in NB eluate or runoff (Table 4.2).

III.2 Efficacy of the bioremediation tool on soil

In both plant tests, the validity criterion of more than 70% seed emergence in the controls (ISO, 1994) was surpassed. A highly significant inhibition ($p < 0.001$) in shoot dry weight due to Atrazerba spraying in NB soil relatively to the control was observed for both *A. sativa* (by 70%) and *B. napus* (by 88%) (Figures 4.3 A and B); evident signals of leaf chlorosis and necrosis, especially in *B. napus*, were also noted. Regarding seed emergence, no effects were observed for *B. napus* ($p > 0.05$), while for *A. sativa* the percentage of emerged seed was lower than in the control ($p < 0.001$) (Figures 4.3 A and B).

The bioremediation of the atrazine contaminated soil during 7 d resulted in an increase in shoot dry mass relatively to the respective NB soil, for both plant species ($p < 0.001$; Figures 4.3 A and B); seed emergence of *A. sativa* was also higher in the B than in the NB soil ($p < 0.003$; Figure 4.3 A). Comparisons between results from the B soil relatively to those of the control showed that germination of *A. sativa* was lower in the former treatment ($p < 0.05$), while its aerial biomass was enhanced ($p < 0.001$) (Figure 4.3 A). For *B. napus*, plant biomass produced in B soil was lower than in the control ($p < 0.05$) and no significant effects on seed germination were observed ($p > 0.05$; Figure 4.3 B).

Table 4.2. Mean atrazine (ATZ) concentrations in soil (µg/g of soil DM) and in the respective aqueous extracts (eluates, runoff and leachates; µg/L) at the beginning and the end of 7 days treatment of soil contaminated with 10× the Recommended Dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool for the three experiments representing worst-case scenarios of pesticide contamination (see section II.2 and Table 4.1 for details). Soil and eluate concentrations represent mean plus standard deviation values of 3 sub-replicates while runoff and leachate concentrations respect to a single sub-replicate.

Experiment	Day	Treatment			
		Soil (µg ATZ/g soil DW)		Aqueous extract (µg ATZ/L)	
		B	NB	B	NB
A - Soil +	**0**	15.7 ± 3.05	18.0 ± 6.24	n.a.	n.a.
eluates	**7**	0.120 [a]	8.23 ± 2.64	10.1 ± 3.52	652 [a]
B - Runoff	**0**	14.7 ± 4.72	10.1 ± 1.66	n.a.	n.a.
	7	0.165± 0.0919 [b]	10.5 ± 0.923	1.20	445
C - Leaching	**0**	7.60 ± 0.854	10.4 ± 1.91	n.a.	n.a.
	7	<0.1	3.30 ± 0.100	<0.1	4.70

[a] two sub-replicates with concentrations below detection limit; [b] one sub-replicate with concentration below detection limit; n.a. - not applicable.

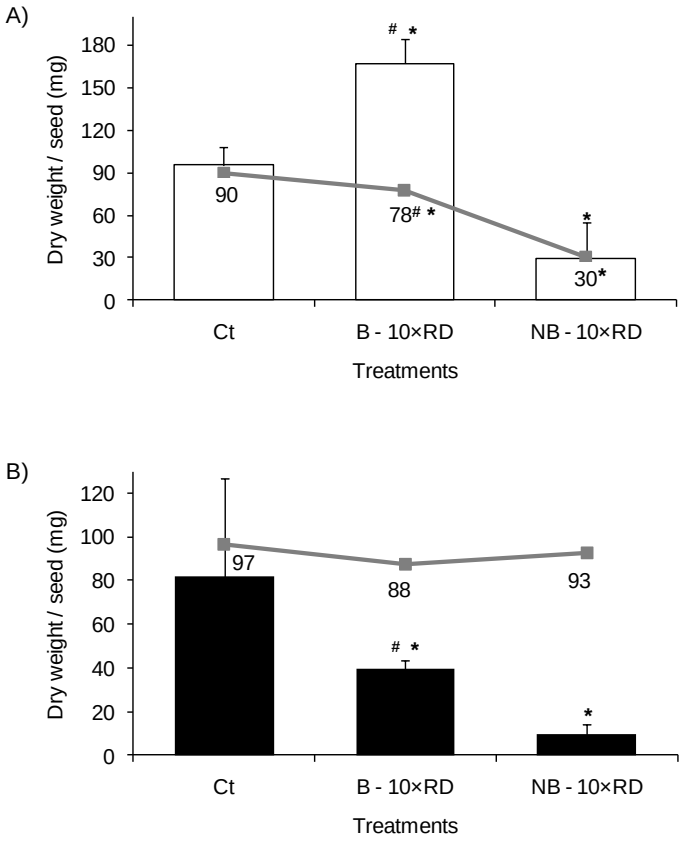

Figure 4.3. Mean seed germination (in %; grey line) and growth (shoot dry weight/emerged seed; bars) of *Avena sativa* (A) and *Brassica napus* (B) in soil collected from the simulators contaminated with 10× the Recommended Dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool. Error bars indicate ± standard deviation; * indicate mean statistically different from control; # indicate mean statistically different from the NB treatment.

III.3 Efficacy of the bioremediation tool for soil aqueous extracts

The validity criteria established for the algae growth in the standard control (cell density increase of at least 16 fold and coefficient of variation of mean growth rate ≤ 20%) were fulfilled in the three assays (Environment Canada, 1992; OECD, 1984). Microalgae growth was inhibited by at least 90% in eluate and runoff obtained from NB soils when compared to the respective control ($p < 0.001$; Figure 4.4). Similar results were obtained for the leachate samples, though growth was inhibited by merely 18% ($p < 0.01$) (Figure 4.4). In accordance, EC20 and EC50 values (in %; and respective 95% CL) for eluate (3.3 [2.2 - 4.4] and 7.6 [6.3 – 8.9]) and runoff (3.2 [2.9 - 4.3] and 9.8 [8.8 – 10.9]) were very close, whereas respective values for leachate could not be determined due to a low growth inhibition. Furthermore, exposing *P. subcapitata* to aqueous extracts resulting from the hypothetical soil application of the RD of Atrazerba (10% of 10×RD) would cause a growth inhibition of 61, 51 and 0%, for eluate, runoff and leachate samples, respectively.

Growth rate of *P. subcapitata* in eluate and runoff was higher in samples obtained from B than from NB soils ($p < 0.001$) (Figure 4.4). On the contrary, no differences were found between NB and B leachates ($p > 0.05$) (Figure 4.4). Microalgae growth in runoff samples collected from B soil was similar to that in control soil ($p > 0.05$). On the other hand, growth in eluate samples from B soil was still lower than that from control soil ($p < 0.001$), even though such difference in growth was of merely 11% (Figure 4.4), and thus considered not to represent a toxic effect.

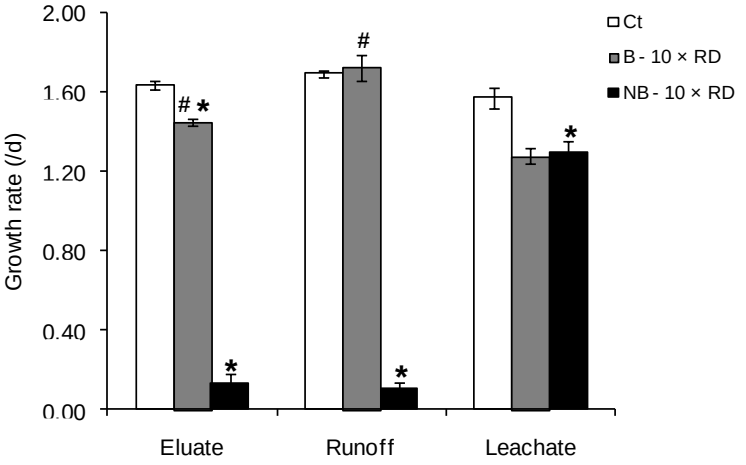

Figure 4.4. Mean 72-h growth rate of *Pseudokirchneriella subcapitata*, on eluates, runoff and leachates collected after 7 d from the simulators contaminated with 10 × the Recommended Dose (RD) of Atrazerba and subsequently sprayed with (B) and without (NB) the bioremediation tool, for the three experiments representing worst-case scenarios of pesticide contamination (see section II.2 and Table 4.1 for details). Error bars indicate ± standard deviation; * indicates mean statistically different from control; # indicates mean statistically different from the NB treatment.

IV. Discussion

IV.1 Biodegradative performance of *P.* ADP + CIT at semi-field scale

In the present work, carried out at a semi-field scale and under less controlled and presumably less favourable conditions to bioremediation than the ones tested before in laboratory microcosms experiments (Chelinho et al, 2010; Lima et al, 2009), still an effective removal of atrazine was achieved within a period of just 7 d. Indeed, a treatment comprising double bioaugmentation with *P.* ADP, within a 2 d interval, and a single initial

amendment with CIT of a soil previously contaminated with 10×RD of an atrazine commercial formulation, promoted the biodegradation of more than 98% of the initially applied atrazine. The two successive inoculations of soils with *P.* ADP apparently contributed to delay the tendency for a decline in bacterial survival that occured in a natural soil 1 to 2 d upon soil bioaugmentation (Chelinho et al, 2010; Lima et al, 2009). The high numbers of physiologically active *P.* ADP maintained in the soil (> 10^8 CFU/g of soil DW in average) may have contributed for the extent and fast degradation of atrazine at the semi-field scale. Nevertheless, in the soils not sprayed with the bioremediation tool, a moderate decrease of atrazine was also observed during the 7 days: 50% in experiment A, 68% in experiment C and no decrease in experiment B. Despite this difference, and since intrinsic biodegradation is not likely to occur in this soil (Lima et al, 2009), it seems conceivable that abiotic degradation (Shin and Cheney, 2005), probably accompanied by the formation of non-extractable residues (Barriuso et al, 2004; Blume et al, 2004), also occurred in the sandy loam soil herein used (Chelinho et al, 2010).

Because atrazine biodegradation was equally effective for the three independent experiments carried out, it can be suggested that, with the amendment of the soil with the cleanup tool, small variations in the natural environmental conditions (e.g. temperature, humidity, light) may not significantly affect the rate and extent of atrazine biodegradation under field conditions.

IV.2 Ecotoxicological monitoring of the efficacy of the bioremediation tool

Soil

The herbicidal properties of atrazine caused, as expected, severe toxic effects on both *A. sativa* and *B. napus*, though different sensitivities were observed depending on the endpoint measured. For *A. sativa*, phytotoxicity was observed during seed germination and plant growth while for *B. napus*

only the aerial biomass production was negatively affected. Despite this, *B. napus* seemed to be most sensitive since shoot dry mass in NB soils was reduced by 88%, when compared to the control, while for *A. sativa*, the same parameter was reduced by 70%. The present results are in agreement with the notion that dicotyledonous appear to be more sensitive to atrazine than monocotyledonous species (USEPA, 2006; White and Boutin, 2007). They also highlight the potential risks of atrazine applications on non-target plants that may exist in the surrounding fields and that may be exposed to herbicide by spray drift or accidental spillage (Viegas et al, in press).

According to the chemical analysis, at the time of the collection of soil samples in the plant tests (day 7), at least 99% of the initial atrazine on the bioremediated soil was removed, and thus, theoretically, atrazine toxicity would be strongly diminished. However, results of plant tests in this soil showed that bioremediation was not 100% effective, especially in the case of *B. napus*, since plant growth was reduced by 48% compared to control soil. This fact suggests that this plant species is highly susceptible to atrazine, even at low concentrations in soil. The observed toxicity in the present study may have been due to the presence of atrazine soil bound residues, which might still be bioavailable (Gevao et al, 2001).

The efficacy of the bioremediation tool in reducing atrazine toxic effects to plants was clearly highest in the case of *A. sativa*: it was 86% effective for seed germination and 146 % for plant growth, the last value traducing a boosting effect probably due to the addition of *P.* ADP+CIT to the soil. Some strains of *Pseudomonas* seem to act as plant-growth promoters while others play the opposite role (Preston, 2004), but this feature has not been reported for the strain used in the present study. Wenk et al (1997) also reported successful atrazine biodegradation and restoration of normal plant growth (*Nasturtium officinale* and *Solanum nigrum)* due to the amendment of soil contaminated with the herbicide (0.06 to 4 ppm) with an atrazine-degrading *Pseudomonas* strain different from the one herein used, under both laboratory and greenhouse conditions.

Soil aqueous extracts

As expected for an herbicide, atrazine significantly inhibited the growth of *P. subcapitata* exposed to eluates and runoff, though a slight toxicity was observed for leachates collected from NB soils. Moreover, according to the results of the present study, not only misapplications of the herbicide atrazine, but also recommended label rates are potentially toxic to phytoplankton, as shown by the EC20 and EC50 values for both eluates and runoff always below 10% of 10×RD of Atrazerba and by the inhibition of at least 50% in microalgae growth in eluates and runoff estimated to have such RD. Indeed, according to literature, among aquatic organisms, phytoplanktonic species like *P. subcapitata* are more susceptible to atrazine contamination than other aquatic organisms from higher trophic levels (Graymore et al, 2001; Solomon et al, 1996). The lower toxicity observed for leachates in NB soils was most probably related with their the time of collection, only towards the end of the experiments (from days 4 to 7, as the aim of the present study was to evaluate the efficacy of the cleanup tool upon a period of at least a 4 d bioremediation treatment). Thus, during the first days of artificial rain (days 0 to 3), a great amount of atrazine was most likely leached in advance from the top soil layer. Despite this, concentrations of 4.7 µg/L of atrazine (like those found in the leachates collected from in the present study from non bioremediated soils and that caused an 18% inhibition in microalgae growth) might even though have deleterious effects on phytoplankton (single species tests with *P. subcapitata*; for a review see Solomon et al, 1996) and corroborate the high leaching potential of atrazine reported in previous works (Correia et al, 2007; Fava et al, 2007).

Thus, according to the results of the present study, both misapplications of the herbicide atrazine and recommended label rates may pose a risk to aquatic producers when soils have low ability to retain atrazine and its metabolites, preventing its mobilization into groundwater (ISO, 2003) and/or when their mobilization by surface runoff from adjacent fields takes place (Correia et al, 2007; Giddings et al, 2005; Solomon et al, 1996). The addition of the bioremediation tool to atrazine contaminated soils significantly enhanced the removal of the herbicide in the B soils. Consistently, the

respective eluates and runoff water were significantly less toxic to the microalgae than those obtained from NB soils. In comparison with the control soil, no toxicity was even observed for the runoff obtained from B soils, while for eluates, the divergence of only 12% between B treatment and control suggest an almost complete bioremediation in soil.

Therefore, these results point for an effective detoxification of the water compartment as a result of the bioremediation treatment of soil, as atrazine (and possible metabolites) in eluates and runoff were presumably reduced to essentially nontoxic levels for the microalgae.

V. Conclusions

Under semi-field conditions, the application of the bioremediation tool comprising soil amendment with *P.* ADP + CIT was clearly effective in reducing the potential environmental risks of atrazine misuse applications for both soil and aquatic compartments in just 7 days. Indeed, besides the improved extent of herbicide removal from soil (> 98% of the initial concentration), an effective cleanup of soil was long-established by the results from the ecotoxicological monitoring of the bioremediation treatment. This decontamination of the bioremediated soil and of the aqueous extracts collected from it (namely runoff and eluates) was revealed by the decreased or annulled toxicity of atrazine to two plant (*A. sativa* and *B. napus*) and one microalgae (*P. subcapitata*) species, when compared to that observed in samples collected from non-bioremediated soils. Thus, the present work also indicates that the application of this technology in real field scenarios of atrazine contamination might be feasible in a short time span, although its performance in different soil types as well as with other atrazine formulations needs to be evaluated.

Also, the integrated approach used here, namely the evaluation of the bioremediation efficacy under different worst-case scenarios of atrazine contamination, gathering chemical and ecotoxicological information, proved to be a robust and relevant method that somehow may be transposable to other situations of bioremediation of contaminated soils.

In addition, a cost effective laboratory simulator of pesticide applications, enabling runoff and leaching scenarios, is now available.

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Chapter 5

Carbofuran effects in soil nematode communities: Using trait and taxonomic based approaches

Based on the following manuscript:

Chelinho S, Sautter KD, Cachada A, Abrantes I, Brow G, Duarte AC, Sousa JP. 2011. Carbofuran effects in soil nematode communities: Using trait and taxonomic based approaches. *Ecotoxicology and Environmental Safety* 74: 2002-2012.

Abstract

This work intends to implement the use of native soil nematode communities in ecotoxicological tests using a model pesticide and two geographically nematode communities (mediterranean and sub-tropical) in order to obtain new perspectives on the evaluation of the toxic potential of chemical substances.

The environmental condition of the nematode communities was described using a trait-based approach (grouping the organisms according to their feeding traits) and a traditional taxonomic method (identification to family level). Effects on total nematode abundance, number of families and abundance of nematode feeding groups as well as potential shifts in both trophic and family structure were assessed.

Agricultural soils from Curitiba (Brazil) and Coimbra (Portugal) were sampled and the corresponding nematode communities were extracted. Part of the collected soil was defaunated and spiked with four doses of a carbofuran commercial formulation. Afterwards each of the replicates was inoculated with a nematode suspension containing ≈ 200 or 300 nematodes. After 14 and 28 d of exposure the nematodes were extracted, counted and identified at family level and separately classified according to their feeding traits. The patterns of nematode responses revealed a decrease in the total abundance and a reduction in the number of families. Despite the similar effects observed for both communities, statistically significant toxic effects were only found within the Portuguese community. The total nematode abundance was significantly reduced at the highest carbofuran concentrations and significant shifts in the family structure were detected. However, the trophic structure, i.e., the contribution of each feeding group for the overall community structure, did not significantly change along the contamination gradient. Results showed that using such a trait-based approach may increase the ecological relevance of toxicity data, by establishing communalities in the response to a chemical from two different taxonomic communities, although with potential loss of information on biodiversity of the communities.

Keywords: feeding traits; nematode community; pesticides; tropical soil; Mediterranean soil; toxicity.

I. Introduction

Ecological Risk Assessment (ERA) based on ecological/functional traits (TERA – Trait-Based Risk Assessment; Baird et al, 2008) advocates the use of morphological/physiological/ecological characteristics of organisms to describe the effects of toxic substances or other stress factors at the community level, in terms of species abundance, diversity, distribution and interactions with other species and the environment (Baird et al, 2008; Clements and Rohr, 2009; Van den Brink, 2008). Scientists working in ERA and/or biomonitoring realized that the description of community responses to stress based only in taxonomic data was a limited approach (Baird et al, 2011). Hence, a good way to enhance a more complete characterization of structure and function of ecosystems would be also to express the status and/or responses of the communities as a combination of traits (Baird et al, 2011*;* De Bello et al, 2010; Vandewalle et al, 2010).

For the soil system, the use of nematodes, a sensitive group to chemical contamination, can increase the accuracy of predictions on the community responses to several forms of pollution induced threats (Bongers and Bongers, 1998; Sochová et al, 2006; Yates and Bongers, 1999). Indeed, nematodes are ubiquitous in all environments and play a crucial role in the soil system as they intervene in many soil processes and interact with other soil organisms by feeding on them or being their food (Bongers and Ferris, 1999). Moreover, the structure of their mouthparts and pharynx is closely related with their feeding habits and a grouping system based on this particular biological trait – the feeding habit -, has been developed (please see the synthesis by Yeates et al, 1993 and references therein). Since they live in the soil interstitial water and have a permeable cuticle, they can be exposed (and adversely affected) to dissolved chemicals (Bongers, 1999; Sochová et al, 2006).

The use of nematodes as test-organisms in soil ecotoxicology has significantly increased over the past two decades. Some laboratory single species tests, assessing the effects of chemicals on nematode reproduction, growth and/or lethality in soil have been proposed and two of them have been

standardized (ASTM, 2008; Donkin, 1993; ISO, 2009; Kammenga et al, 1996; Van Kessel, 1989). Laboratory studies on the effects of pollutants on soil nematodes have been mainly focused on heavy metal toxicity in temperate systems (for a review see Sochová et al, 2006), using a reduced number of nematode species (mostly bacterivorous species like *Caenorhabditis elegans* - Rhabditidae and *Plectus acuminatus* - Plectidae) (e.g Höss et al, 2002; Kammenga et al, 1996; Sochová et al, 2007). On the other hand, in field (or semi-field, i.e, microcosms) studies, the effects of chemicals on nematode communities are described in terms of changes in feeding and/or life strategies and also by using classical structural endpoints like abundance and diversity (Sochová et al, 2006). However, in such experiments, researchers have to struggle with the high spatial and temporal variability and with the influence of several abiotic parameters affecting the responses of organisms (e.g. Bongers, 1990; Lazarova et al, 2004; Moser et al, 2004).

The present study intends to introduce an innovative approach on testing nematodes in soil ecotoxicology. The effects of soil contamination with a carbofuran commercial formulation were evaluated using a community approach under laboratory conditions. Aiming to improve ecological relevance of the testing strategy, soil native nematode communities, instead of the classical (single) standardized species, were previously extracted from clean soil and exposed to a gradient of contaminated (but defaunated) soil.

Moreover, effects were described using two distinct approaches, comprising functional and structural endpoints. In the trait-based approach, the organisms were grouped according to their feeding traits and changes in the trophic structure (the percentage contribution of each feeding group to the global community structure) and total abundance of each feeding group were assessed. A traditional taxonomic methodology was also used and consisted in the identification of nematodes at family level. Hence, effects on abundance, number of families and family structure were reported.

Our main goal was to contribute to the adoption of the community test approach in soil ecotoxicological testing by comparing the effects of a model pesticide (carbofuran) towards two geographically distinct (Mediterranean and sub-tropical) nematode communities. More specifically, the objectives of the

present study were to evaluate and compare the effects of carbofuran applications on the total abundance, total abundance of feeding groups, trophic structure and number of families of two soil nematode communities from different biogeographic regions and, to implement the use of nematode feeding traits as an alternative to taxonomic descriptions when evaluating disturbances at community level.

II. Materials and Methods

II.1 Soil Sampling and handling

In Brazil, soil samples were collected in Pinhais (Curitiba, Brazil; -25.391667, -49.125000), from an agricultural field with no history of pesticide application or chemical fertilization over the last 6 years. In Portugal, samples were taken from an agricultural soil, located in Carapinheira (Coimbra; 40.209528, -8.657913), with no application of pesticide or chemical fertilizers at least for the last 2 years. A parcel of fallow land was selected at each country (≈ 15 × 15 m in Brazil and ≈ 4 × 7m in Portugal). The vegetation layer was removed and several soil cores, with approximately 10 cm Ø × 10cm height, were collected in parallel lines. They were mixed, sieved (5mm) and stored at 4ºC until further processing (two days for the Portuguese soil and one week for Brazilian soil, which was sent to Portugal for testing). Two weeks before the test, part of each soil was defaunated trough two freeze-thawing cycles (48h freeze at -20ºC and 24h heating at 65ºC) (Viketoft, 2008).

The soil properties (Table 5.1) from Portugal were analyzed by DRAPN (Porto, Portugal) as described by Chelinho et al (2011) and the Brazilian soil by the Department of Soils of UFPR (Curitiba, Brazil) according to Marques et al (2003) and EMBRAPA (1997).

Table 5.1. Pedological properties of the test soils.

Soil	pH KCl 1M	OM %	Sand %	Silt %	Clay %	N_{tot} %	C/N	WHC %	CEC Meq/100g	Soil type
Portugal	6.3	3.7	75.9	14.7	9.4	0.13	16.5	42.4	10.4	Loamy sand
Brazil	5.3	4.8	22.2	15.3	62.5	0.26	10.8	76.8	14.3	Clay

Codes: **OM** - Organic Matter, **C/N** - Organic carbon/Total nitrogen; **WHC** - Water Holding Capacity; **CEC** - Cation Exchange Capacity.

II.2 Experimental procedure

As the test with the Brazilian nematode community ran before the Portuguese and in the former a low nematode recovery was detected, it was decided to change some procedures aiming to improve test performance. The incubation period for the microflora and the number of inoculated organisms were increased and the soil moisture level was diminished (see sections II.2.1 and II.2.3).

II.2.1 Soil microflora inoculation

To ensure a rapid recolonization of the soil by the original microflora and to provide food to some groups of nematodes, a soil suspension was prepared with 1000 g of fresh soil and 2000 mL of water, centrifuged twice at 600 *g* during 5 min (Viketoft, 2008). The suspension was passed through a 20 µm sieve, to ensure the absence of nematodes. The inoculation of soil microflora (consisting of Protozoa, Fungi, Bacteria, Actinomycetes and Algae according to Coyne, 1999; although not subjected to confirmation by microbiological analysis) was made by mixing 100 mL of soil suspension to 3000 g (Dry Weight, DW) of defaunated soil. This mixture was incubated at 23±1 ºC in the dark for 3 or 7 days, respectively for the Brazilian and Portuguese assays.

II.2.2 Nematode extraction

Fresh soil was used to extract the original nematode community using the tray method (Abrantes et al, 1976) that allows the extraction of large soil samples without the decrease in extraction efficiency of other methods such as Baermann funnel (van Bezooijen, 2006). Based on preliminary assays, several trays were set up (36 × 26 × 5 cm), each one containing 250 cm^3 of soil (≈ 220g) spread over paper tissue, supported by a plastic mesh and moist

with ≈ 1000 mL of tap water. After 60 h at 22 ± 2ºC, the meshes were removed, briefly drained into the tray and discharged. The content of each tray was passed into a 20 µm sieve to collect the nematodes.

II.2.3 Test performance

The soil previously reinoculated with the original microflora was spiked with an aqueous solution of the insecticide/nematicide Furadan 350SC (a carbofuran commercial formulation from FMC, SP, Brazil; 350 g a.i./L). Different proportions of the stock solution were mixed in the pre-moistened soils to create the following range of dilutions: 25 %, 50 %, 100 % and 200% of the recommended dose (RD) for sugar cane plantations (5 l/ha; ~1.167 mg a.i./kg soil DW considering a soil density of 1.5 g/cm^3 and 10 cm of incorporation depth). To the control (uncontaminated) soils only water was added.

After soil contamination, plastic boxes (7 cm Ø × 6 cm height; with perforated lids) were filled with 20 g (DW) of soil. Then, each one of the 12 or 16 replicates (6 × 2 exposure periods for treated samples or 8 × 2 exposure periods for the controls, respectively) was inoculated with 2 mL of the nematode suspension (an analogous procedure to the introduction of the test organisms in a standardized single species test), containing about 200 or 300 nematodes respectively, for the test with the Brazilian or Portuguese communities, which is within the expected range for agroecosystems (Yeates and Bongers, 1999).

During the inoculation of control and spiked samples, 9 aliquots (2 mL) from each original nematode suspension (Brazilian or Portuguese) were collected at randomly chosen intervals (e.g. the first aliquot was taken after 5 successive inoculations, the second after 20 and so on) into 5 mL glass vessels and kept at 4º C for further characterization of the initial nematode community.

The amount of solutions added per treatment (for the inoculation of microflora, moistening of the soil before pesticide spiking, carbofuran

contamination and nematode inoculation) was adjusted to achieve initial moisture content of 70% or 60% of the WHC, respectively, for the Brazilian or Portuguese assays.

An additional vessel, without nematodes, was prepared for controlling pH and moisture during the test. The replicates were incubated at 23 ± 1 Cº or 21 ± 1 (respectively, for Brazilian and Portuguese tests) in the dark. After 14 and 28 days of exposure (hereafter designated as 14 d or 28 d), for each treatment, 6 replicates (8 for the control) were processed, i.e., the soil was removed from the vessels to the extracting trays with a spatula; the vessels were slightly rinsed with water and the solution was added to the soil surface above the trays. The nematodes were then extracted as described in section 2.2.2 (although smaller trays, consisting of cylindrical boxes of 10 cm Ø × 4 cm, were used), and kept at 4ºC until further nematode counting and identification (performed, at most, within 10 d).

II.2.4 Nematode identification and quantification

The first 100 individuals (or the total number, in samples containing less than 100 individuals) were spread into Doncaster plates (Doncaster, 1962) and identified to family level under an inverted microscope (100 and 200× magnification) according to Goodey (1963) and to an Interactive Nematode Identification Key (Available at: http://nematode.unl.edu/key/nemakey.htm; Assessed 28th September 2008). The main organs/structures used for taxonomic identification were the body cuticle, head/mouthparts, oesophagus, reproductive system and tail.

Whenever doubts about the taxonomic group of a specific organism persisted, it was transferred to a glass slide, killed by heat and observed under an optical microscope (100 and 400× magnification). Each nematode was also assigned to a trophic group (plant parasites/feeders - PLF, fungal feeders - FGF, bacterial feeders - BTF and predators/omnivores – PD-OM) according to Yeates et al. (1993). According to this author, for most soil

nematode families, the organisms belonging to a specific family share the same feeding habit.

The trophic composition of the 100 identified organisms was used to extrapolate the total trophic and taxonomic composition of the respective sample.

The remaining nematodes were counted under a low magnification (40 and 100× magnification) under an inverted microscope.

II.3 Chemical analysis

Soil samples (400 g Wet Weight/treatment) were collected and frozen for further determination of carbofuran concentrations in soil and in soil eluates.

Eluates were prepared in duplicate, following standard methods (DIN, 1984). The soil (50 g DW) was mixed with deionized water (1:10 ratio, w/v), magnetically stirred during 12 hours, centrifuged (3370*g*) at room temperature, the supernatant collected and stored (4°C; dark) until use (within 48 hours). Eluates were filtered through a 0.45 µm nylon membrane (Millipore) and submitted to solid phase extraction (Discovery® DSC-18); Carbofuran was eluted with 5 mL of acetonitrile and the extracts were dried. The volume of extracts was reduced in a rotavapor (40°C), transferred to a vial and evaporated to dryness under a gentle stream of nitrogen. Extracts were kept frozen (-18ºC) and re-dissolved in mobile phase just before analysis.

Soil carbofuran extraction and analysis was based on the USEPA method 8318A (USEPA, 2007). About 2 g of soil (Wet Weight; WW) were extracted with 5×3 mL of acetonitrile in an ultrasonic bath and centrifuged (2000 *g*). Extracts were processed as described for eluates.

The analytical instrumentation included an HPLC Jasco model with a Rheodyne 7125 injector and a loop size of 50 µL coupled to an UV detector UV Chrom-A-Scope (BarSpec) operating between 190 and 370 nm. Acquisition was performed at 210 nm and started at 5 min until 15 min. For peak confirmation, the existence of a peak at 278 nm was checked at the specific retention time and the UV spectrum of the sample compared with the

standard (Carbofuran standard, CIL Inc.) spectrum. The analytical column used was a Luna C-18 (250 x 4.6 m; 5 µm; 100 Å), with a guard column of the same material. The mobile phase selected consisted of Milli-Q water and 60% of methanol (flow rate of 0.8 mL/min).

Carbofuran standard and a stock standard solution (10 mg/L) were prepared in acetonitrile. Calibration standards were prepared by dilution of the stock solution with the mobile phase (from 0.25 to 3 mg/L). External calibration was used for quantification.

The limit of quantification (LOQ), calculated based upon an S/N ratio of 10:1, was 20 µg/kg for soil samples and 0.7 µg/L for eluates. Mean recovery was 81±7% for soil samples and 94 ± 8% for eluates.

II.4 Statistical evaluation

To investigate the effects of carbofuran on total abundance (total number of organisms recovered per replicate), total abundance of each feeding group, trophic structure (relative abundance of each feeding type) and number of families, a one-way ANOVA followed by post-hoc comparisons with the control (Dunnets' test) to derive NOEC (No Observed Effect Concentrations) values. Prior to ANOVA data on the above mentioned endpoints were analysed for normality (Kolmogorov-Smirnov test) and for variance homogeneity (Levene Test). When violations of normality and/or homogeneity occurred, a log (x+1) transformation was applied. Effects were considered statistically significant for p levels ≤ 0.05.

Whenever feasible, the eluate concentrations causing 50% of decline (EC50 and respective 95% confidence intervals) in the total abundance or abundance of nematode feeding types (only feasible for the Portuguese data) were calculated (as the main exposure route of nematodes to chemicals in soil is expected to be via interstitial water; Sochová et al, 2006) using non-linear regressions (Environment Canada, 2004). To eliminate the higher inter-replicate variability, the average number of nematodes per treatment was

used. All analyses were performed in Statistica 7.0 (Available at: http://www.statsoft.com/, Assessed 23th October).

Potential effects of carbofuran on the community structure were analysed by Analysis of Similarity (ANOSIM), by comparing the trophic structure and family composition of the carbofuran treated samples with those of the control. Whenever significant differences were found, the Similarity of percentages (SIMPER) analysis was used to identify the families or feeding groups responsible for the observed change and their individual contribution (in terms of percentage) for the overall shift. Both ANOSIM and SIMPER analysis were ran in Primer 5.2.6 (Clarke and Gorley, 2001) using log(x+1) transformed data.

III. Results

III.1 Carbofuran concentrations

The carbofuran concentrations obtained in soil samples were within the expected values for the RD used (about 1.167 mg a.i./kg soil DW, see section II.2.3). Soil concentrations were quite similar for Brazilian and Portuguese samples (Table 5.2). Based on the lower eluate concentrations determined for the Brazilian samples, the carbofuran exposure of nematodes extracted from this soil was also expected to be lower (Table 5.2).

Table 5.2. Carbofuran concentrations (mg /kilogram of soil DM) in the soil samples and eluates (prepared from soil and using a dilution factor of 10×; see section II.3) contaminated with four doses of Furadan, collected at the beginning of the experiment (d=0). RD – Recommended Dose.

Furadan dose (%RD)	Portugal		Brazil	
	Soil (mg/kg)	Eluates (mg/L)	Soil (mg/kg)	Eluates (mg/L)
0	bLOQ	-	bLOQ	-
25	0.315 ± 0.020	0.026 ± 0.001	0.193 ± 0.004	0.016 ± 0.002
50	0.755 ± 0.015	0.050 ± 0.002	0.514 ± 0.033	0.037 ± 0.006
100	1.302 ± 0.079	0.105 ± 0.003	1.023 ± 0.021	0.070 ± 0.002
200	2.977 ± 0.354	0.203 ± 0.006	2.432 ± 0.162	0.123 ± 0.014

bLOQ – below limit of quantification.

III.3 Effects of carbofuran in total nematode abundance

The nematode recovery rate in the controls was approximately 25 and 30% of the initial abundance (see Table 5.3 for the estimated abundance of the inoculated communities), respectively for the Brazilian and Portuguese communities, and did not varied greatly between the two exposure periods (Figure 5.1). In general, a high variability was found between replicates. The results indicate a markedly different response of the two nematode communities to carbofuran contamination. At the Portuguese community, total abundance significantly decreased at the two highest concentrations, already within 14 d of exposure (One Way Anova, Dunnet test; NOEC = 0.050 mg/L; $p < 0.05$; Figure 5.1 PT) when compared to the control). The estimated EC50s for the decline in total nematode abundance indicate a higher toxicity after 28d of exposure (Table 5.4).

At the Brazilian community, although a decrease in the average number of nematodes was found at the highest insecticide concentrations when comparing with the controls (14 d - from 45 to 25, 28d - from 52 to 37, respectively for the controls and at 0.123 mg/L of carbofuran), these differences were not statistically significant (One Way Anova, Dunnet test; $p > 0.05$; Figure 5.1 BR).

Table 5.3. Composition of the inoculated nematode communities of Portugal and Brazil, expressed as relative abundance of families, allocated in four feeding groups and total abundance (sum of the organisms extracted and inoculated per replicate). Values express mean ± standard deviation and are based in the counting of 9 samples (see section II.2.3 for details). F – Individuals found in the treated samples but not in the initial inocula.

	Portugal	Brazil
Relative abundance (%)		
Bacterial feeders (BTF)	**59.7 ± 4.4**	**13.6 ± 3.4**
Cephalobidae (Acrobelinae)	0.4 ± 0.5	3.0 ± 1.7
Cylindrocorporidae	0.5 ± 1.3	0.3 ± 0.5
Diplogasteridae	5.6 ± 2.6	0.7 ± 1.0
Camacolaimidae (Halaphanolaiminae)	0.7 ± 0.9	F
Monhysteridae (Prismatolaimus sp)	-	0.9 ± 1.3
Panagrolaimidae	39.3 ± 3.6	3.1 ± 3.8
Plectidae	0.2 ± 0.6	-
Rhabditidae	13.0 ± 3.1	5.6 ± 4.5
Plant parasites/feeders (PLF)	**28.3 ± 4.0**	**77.1 ± 3.7**
Criconematidae	0.1 ± 0.3	F
Heteroderidae	2.1 ± 1.0	-
Hoplolaimidae	7.9 ± 4.1	72.6 ± 4.5
Pratylenchidae	2.3 ± 1.6	0.1 ± 0.3
Tylenchidae	15.9 ± 4.1	4.4 ± 1.3

Table 5.3. (continued)

	Portugal	Brazil
Relative abundance (%)		
Fungal feeders (FGF)	**5.7 ± 2.8**	**2.2 ± 1.5**
Aphelenchoididae	5.5 ± 2.9	2.2 ± 1.5
Diphtherophoridae	0.2 ± 0.4	F
Predators/omnivorous (PD/OM)	**6.3 ± 1.8**	**7.1 ± 2.1**
Dorylaimidae	5.1 ± 1.7	5.3 ± 2.3
Mononchidae	1.2 ± 0.6	1.8 ± 1.2
Trichodoridae	F	-
Total abundance	303.8 ± 19.8	219.1 ± 21.5

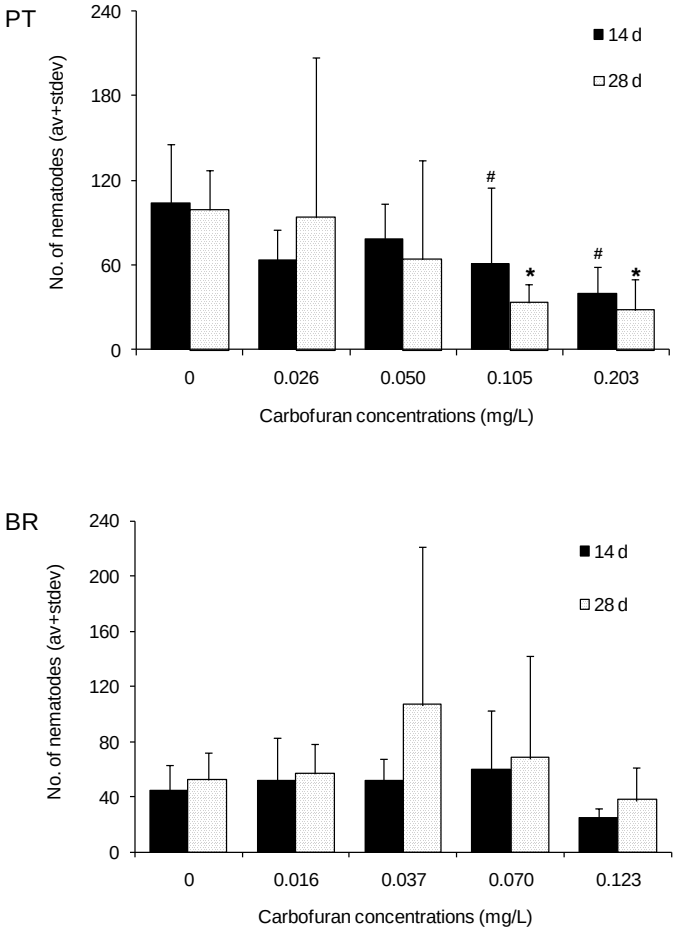

Figure 5.1. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 5.2 for correspondences between Furadan doses and carbofuran concentrations) on total abundance (number of organisms recovered per treatment) of nematodes from Portugal (PT) and Brazil (BR), 14 and 28 days after the test start (black and white pointed bars, respectively). Values express mean (n=6 or n=8, respectively for treated and control samples) ± standard deviation. *, # - Statistically different from the respective controls (One Way Anova, Dunnet Test, $p < 0.05$).

Table 5.4. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 5.2 for correspondences with Furadan doses and soil concentrations) on the decline of total abundance and abundance of feeding types of a Portuguese nematode community, exposed during 14 and 28 days. Values represent EC50 (and 95% confidence intervals). BTF - bacterial feeders; PLF - Plant parasites/feeders; FGF - Fungal feeders; n.d. - not determined.

	Carbofuran toxicity (mg/L)	
	14d - EC50	*28d - EC50*
Total abundance	0.153 (0.091-0.257)	0.064 (0.034-0.119)
BTF	n.d.	0.078 (0.068-0.089)
PLF	0.069 (0.043-0.112)	0.068 (0.046-0.099)
FGF	0.051 (0.029-0.089)	n.d.

III.4 Effects of carbofuran in the nematode family composition

As it was observed with nematode abundance, stronger toxic effects in the family composition were observed for the Portuguese nematode community (Figure 5.2 PT). After 14 days of exposure to carbofuran contamination, a significant decrease in the number of families was detected at eluate concentrations equal to or higher than 0.105 mg/L, in comparison to the control (One Way Anova, Dunnet test; $p < 0.05$; NOEC = 0.050 mg/L; Figure

5.2 PT-14 d). This toxic effect increased after 28 days as the exposure to carbofuran concentrations of 0.050 mg/L or higher reduced significantly the number of families found (One Way Anova, Dunnet test; $p < 0.05$; NOEC = 0.026 mg/L; Figure 5.2 PT-28 d). For both exposure periods, the carbofuran soil contamination caused a significant change in the family structure in all concentrations tested (ANOSIM; $p< 0.05$). Diplogasteridae, Rhabditidae, Aphelenchoididae and Tylenchidae had the major contribution to the community shifts (Figure 5.3).

For the Brazilian nematode community, as observed for total abundance data, none of the carbofuran concentrations caused a significant reduction in the number of families relative to the control (One Way Anova, Dunnet test, $p > 0.05$; NOEC ≥ 0.123 mg/L; Figure 5.2 BR). Despite this, a decrease in the maximum number of families was found (from 11 in the control to 6 for carbofuran concentrations of 0.123 mg/L; Figure 5.2 BR) after 28 days of exposure (Figure 5.2 BR-28 d).

Similarly, after this period, ANOSIM detected significant differences in the family structure of the Brazilian nematode community at this concentration, when compared to the control (ANOSIM, $p < 0.05$). The families that contributed most to this dissimilarity were Rhabditidae, Panagrolaimidae (their abundance decreased at the highest carbofuran concentration, in comparison to the control) and Aphelenchoididae (with higher abundance at the highest carbofuran concentration than in the control), respectively with 27, 12 and 19 % of contribution (SIMPER, $p < 0.05$; data not shown).

Also, focusing on the total abundance of each nematode family in both assays, there were some families that disappeared (generally the less abundant ones) or their abundance was strongly reduced along the contamination gradient (see table 5.A of appendix).

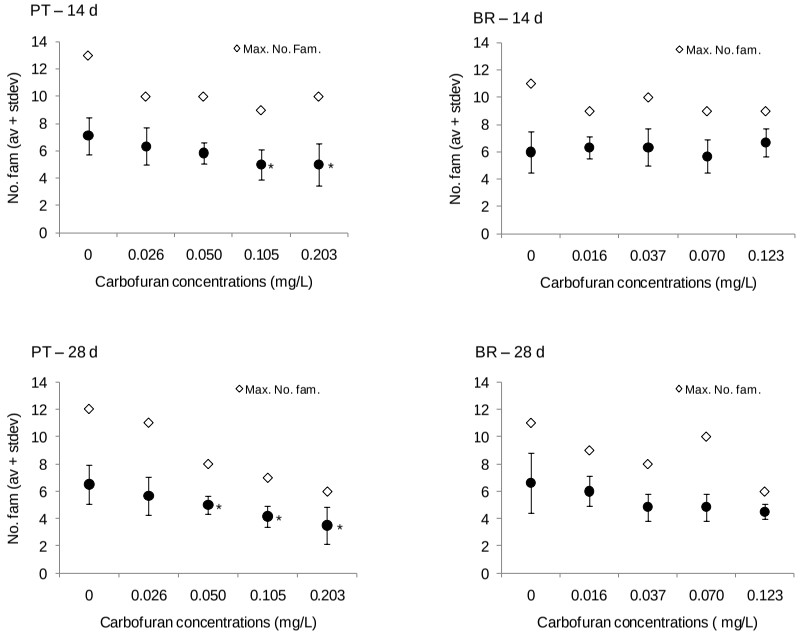

Figure 5.2. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 5.2 for correspondences between Furadan doses and carbofuran concentrations), on the number of families found in two nematode communities from Portugal (PT) and Brazil (BR), after 14 and 28 days of exposure. Black points represent mean (n=6 or n=8, respectively for treated and control samples ± standard deviation) values and diamonds represent the maximum number of families (Max. No. fam.) found within treatments. * – Mean number of families statistically different from control (One Way Anova, Dunnet Test, $p < 0.05$).

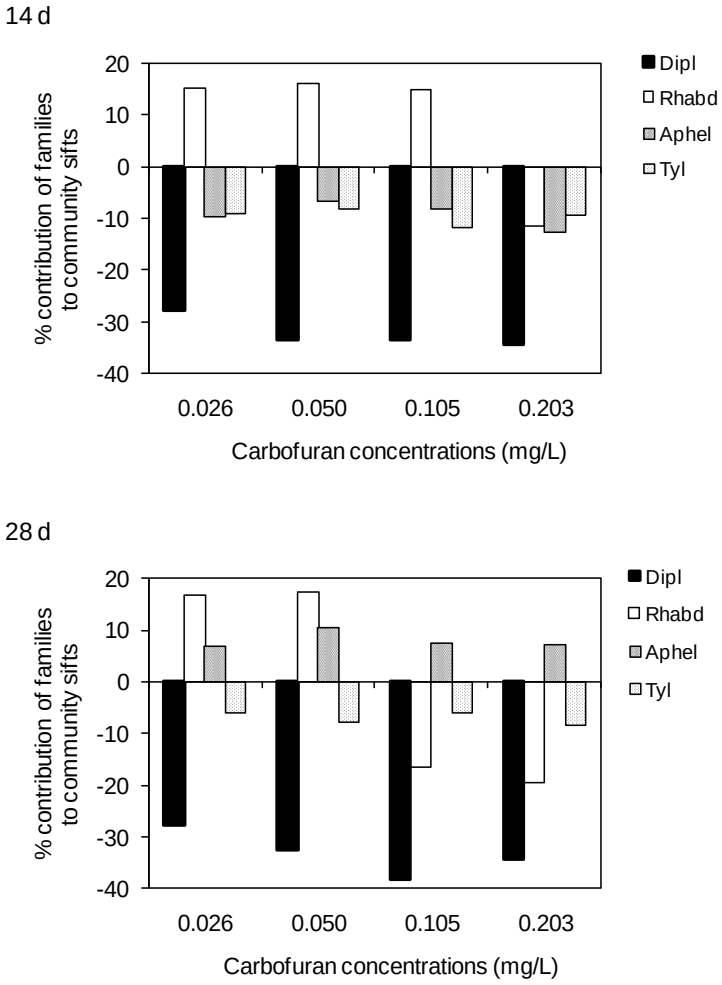

Figure 5.3. Shifts in the family structure of a Portuguese nematode community induced by carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 5.2 for correspondences between Furadan doses and carbofuran concentrations): representation of the four families with the greatest contribution to the overall changes. Deviations above the zero line (representing the control) represent an increase in abundance along the concentration gradient while deviations below the line represent a decrease in abundance in comparison with the control. Dipl - Diplogasteridae; Rhabd - Rhabditidae; Aphel - Aphelenchoididae; Tyl - Tylenchidae.

III.5 Effects of carbofuran in the total abundance of nematode feeding groups and global trophic structure

In both assays, an “incubation effect” was observed since the trophic structure in the controls (after 14 and 28 d; Figure 5.4) was different from the inoculated community (IC) (Table 5.3). In the Portuguese nematode community there was a strong increase in the bacterial feeders (from about 60% in the IC - Table 5.3 to more than 80% in the controls - Figure 5.4 PT) accompanied by the decline of the other three trophic groups, specially the plant feeders (from about 28% in the IC to 9% and 6% in the controls, after 14 and 28 d, respectively; Table 5.3 and Figure 5.4 PT). The same trend was observed in the controls inoculated with the Brazilian community: the proportion of plant feeders was much lower than in the IC (77% in the IC and 45% and 20% respectively after 14 and 28 d; Table 5.3 and Figure 5.4 BR), while the bacterial and fungal feeders increased their relative abundance (from about 2% and 14% in the IC to about 13% and 50% in the controls, after 14 and 28 d, respectively for BTF and FGF; Table 5.3 and Figure 5.4 BR).

The high variability of data on the absolute abundance of each feeding group often impaired the detection of significant differences between the different carbofuran concentrations and the control. This was especially true for the data with the Brazilian nematode community (all NOECs ≥ 0.123 mg/L). However, significant effects were found within the Portuguese nematode community (graphs are available in figure 5A of the appendix). After 14 d, the total abundance of plant and fungal feeders was significantly lower for carbofuran concentrations of 0.105 and 0.203 mg/L, if compared to the control (One Way ANOVA, Dunnet test; $p < 0.05$; NOEC = 0.050 mg/L). Estimated EC50s were lower for fungal feeders if compared to plant and bacterial feeders (Table 5.4). After 28d, the same significant effect was found but for bacterial feeders (One Way ANOVA, $p < 0.05$; Dunnet test; NOEC = 0.050 mg/L); the abundance of plant feeders was also significantly decreased at concentrations of 0.050 and 0.203 mg/L (One Way ANOVA, Dunnet test; $p < 0.05$; NOEC = 0.026 mg/L).

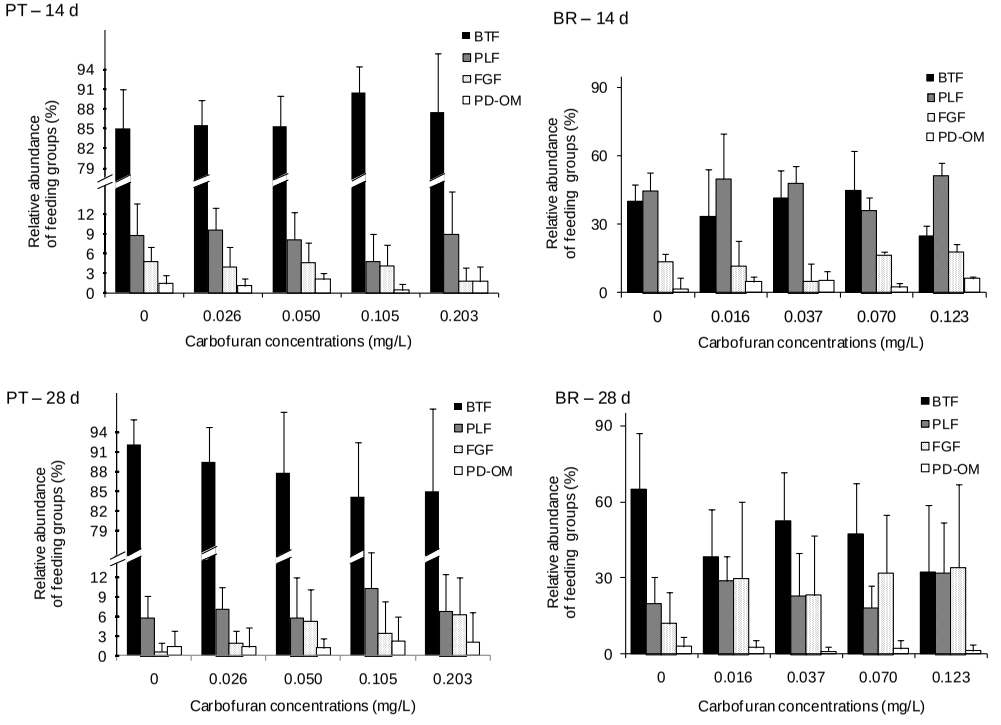

Figure 5.4. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 5.2 for correspondences between Furadan doses and carbofuran concentrations) on the trophic structure of two nematode communities from Portugal (PT) and Brazil (BR), after 14 and 28 days of exposure. Vertical bars represent mean (n=6 or n=8, respectively for treated or control samples ± standard deviation) values of relative abundance of four feeding groups. BTF – Bacterial feeders; PLF – Plant parasites/feeders; FGF – Fungal feeders; PD/OM – Predators/omnivores.

Carbofuran (eluate) concentrations of 0.068 and 0.078 mg/L were expected to cause a 50% decline in the populations of plant and bacterial feeders, respectively (Table 5.4).

No significant effects of carbofuran treatment in the relative abundance of each feeding group were found for both countries (One Way Anova; $p > 0.05$). Accordingly, analysis of similarity (ANOSIM) detected no significant differences in the global trophic structure neither for Portuguese nor Brazilian nematode communities. Despite this, the increase of predators-omnivores and fungal feeders in the contaminated soil from Brazil was observed, respectively, after 14 days or at both exposure periods (Figure 5.4 BR); fungal feeders also increased their relative abundance in the Portuguese contaminated samples after 28 days (Figure 5.4 PT-28 d).

IV. Discussion

IV.1 General considerations on the testing strategy

The low nematode recovery rate, obtained in the controls and in the treated soil samples, was found to be the main difficulty of the testing strategy used in the present work. Moreover, the procedural changes taken for the Portuguese assay did not produce satisfactory results given the slight increase in the nematode recovery rate (5%). This problem has been reported in previous studies involving soil inoculation with nematodes and/or extraction from uncontaminated (control) soil after short periods of time (Djigal et al, 2004; Kammenga et al, 1996; Parmelee et al, 1997; Viketoft, 2008).

Unfortunately, in soil nematode testing, one of the major challenges seems to be to find a more effective method for recovering nematodes and, at the same time, obtain an accurate assessment of the composition of nematode community as biased estimations occur independently of the method used (McSorley and Frederick, 2004; Sochova et al, 2006 and references therein). Notwithstanding, active extraction methods based on the motion capacity of live nematodes (like the one used in the present work),

seem to be preferable above others (Bell and Watson, 2001; Kammenga et al, 1996).

The extraction procedures for all samples and the inoculation of the nematode initial community (IC) probably worked as a stress factor that killed some organisms and impaired reproduction of the majority.

The low recovery rate in the inoculated samples was also followed by a shift in the community trophic structure of the controls from both countries, when compared to the IC. These facts suggest that these shifts were somehow more pronounced than the ones induced by carbofuran contamination, for the same endpoint.

Considering the total abundance of each one of the feeding groups in the control and in carbofuran treated samples, after both 14 and 28 d of exposure, the BTF nematodes generally increased while a decline in the PLF was observed (Figure 5.4; Figure 5A of appendix). This was somehow expected since there was no particular food supply to plant feeders, contrasting with the pre-inoculation of soil with the original microflora that probably led to increased food availability for bacterial feeders. The rate of BTF increase after 14 d, when compared to the IC, was approximately of 30% in both Portuguese and Brazilian assays, suggesting that the extension of the incubation period for the microflora in the Portuguese assay did not greatly influence the results.

Furthermore, it is likely that the defined exposure periods would only allow the reproduction of some opportunistic bacterial feeding nematodes while for the other groups only lethal effects could be observed. Indeed, life span of nematodes can be as short as one week to opportunistic bacterial feeders, 4-8 weeks for plant feeders until several years for large plant feeders and predators/omnivores under undisturbed conditions (Ferris, 2004). Thus, for further validation of the testing strategy, improvements are needed, namely extending the exposure periods (to, at least, two months), provide food to plant feeders (e.g. by sowing a seed in each replicate) and extending the final extraction period to 96 h (McSorley and Frederick, 2004), besides testing of other nematode communities.

However, the reported drawbacks were expected and much probably constitute associated risks of introducing ecological realism in the testing strategy. The use of nematode communities, consisting of several unidentified

species, for which optimal ecological requirements (e.g. food, temperature and moisture) are unknown, is surely associated with increased uncertainty/variability of data, if compared with traditional standardized single species testing (ASTM, 2008; ISO, 2009).

Summarizing, despite the reported drawbacks and the improvements needed, the testing strategy showed to be valid as a starting point to promote the testing of nematode communities under laboratory conditions in ecotoxicology related-studies. Moreover, some of the problems reported in section I for field and semi-field studies could presumably be diminished trough the laboratorial exposure of nematodes, generating data with lower variability associated and requiring, at the same time, less space, time and costs.

IV.2 Toxicity of carbofuran to the nematode communities

The two nematode communities responded similarly to carbofuran contamination, although the Portuguese community was clearly the most negatively affected. Some possible explanations could be the moderately higher carbofuran concentrations in the Portuguese samples (in average, ≈ 27% higher for soil and 34% for eluate samples; Table 5.2) or a higher intrinsic sensitivity of Portuguese nematodes to pesticides (not investigated). However, the critical factors determining the different responses of the organisms have, much probably, been the soil properties, namely the clay and organic matter contents. Carbofuran soil adsorption positively correlates with both clay and organic matter contents (Singh and Srivastava, 2009; Weber et al, 2004). Since the last two were clearly higher in the Brazilian soil (62.5 % and 4.8 %; Table 5.1), a stronger adsorption of carbofuran was expected, resulting in a lower available fraction in the soil pore water (main exposure route for soil nematodes). Accordingly, the decreased efficacy of nematicides in clayed soil, when compared with sandy soils, has been previously reported (Araya, 2003, Bond et al, 2000). Also, a higher moisture level (as in the Brazilian soil) seems to favor the carbofuran biodegradation (Shelton and Parkin, 1991) lowering the possible toxic effects.

IV.2.1 Effects on the total nematode abundance

Toxic effects of carbofuran on the total abundance of nematodes were only registered within the Portuguese community, at the two highest carbofuran concentrations, with more pronounced effects after 28 d of exposure (Figure 5.1).

Comparisons with other data are difficult since the available information on the effects of pesticide pollution (mainly nematicides and insecticides) on total nematode abundance respects to field or semi-field studies. Even though, in some cases, decreased abundance was observed in treated soils (Pen-Mouratov and Steingerger, 2005; Yardim and Edwards, 1998) while for the majority, no negative effects were found (Coleman et al, 1994; Griffiths et al, 2006; Moser et al, 2004; Parmelee et al, 1997; Wada and Toyota, 2008). This might be related with the larger variance associated to experiments carried out of the laboratory context (Sochova et al, 2006). Moreover, nematicides are not expected to have direct lethal effects on target nematodes, but to limit their mobility and thus the ability to infect the plant hosts (Wright and Womack, 1981) as it is the case for carbofuran, an acetylcholine esterase (AChE) inhibitor (IUPAC, 2010). Despite the low persistency of carbofuran in soils (typical DT50 of 29d; IUPAC, 2010), it is possible that the chronic exposure of nematodes might have boosted its toxicity and delayed the recovery of AChE activity, as it was reported for earthworms (Panda and Sahu, 2004). Also, the presence of metabolites resultant of carbofuran degradation (e.g. 3-hydroxy-carbofuran and 3-keto-carbofuran) might have caused toxicity, like it was reported for *Meloidogyne incognita* (Nordmeyer and Dickson, 1990).

Data from single species tests with other soil invertebrates and carbofuran suggest that the sensitivity of nematode communities to this insecticide might be similar to that of earthworms. The comparable sensitivity of nematode single species tests with enchytraeids, earthworms or springtails tests has been previously suggested (see Sochová et al, 2006 and references therein). For instance, in the present study, carbofuran soil concentrations of 1.3 and 2.9 mg/kg (0.105 and 0.203 mg/L were found in soil eluates, respectively; Table 5.2) had significant hazard effects on nematode abundance in the

Portuguese assay (Figure 5.1 PT). These are within the range of lethal concentrations causing 50% mortality (LC50s) in *Eisenia andrei* in artificial soil (5-10 mg/kg), reported by Van Gestel (1992). Recently, exposing the same earthworm species to the same pesticide in three artificial soils, De Silva and Van Gestel (2009) estimated slightly higher LC50 values (≈ 12 mg/kg) but median effects on reproduction (EC50s) were one order of magnitude lower (≈ 1mg/kg). The results obtained in the present study are somehow in agreement given that the estimated EC50s ranged between 0.064 (28d) and 0.153 (14d) mg/L (Table 5.4); these roughly correspond to soil concentrations of 0.6 to 1.5 mg/kg (only considering the dilution factor of 10× used for eluate preparation; see section II.3).

IV.2.2 Effects on the nematode family composition

The decrease in the number of families found in the carbofuran treated samples (Figure 5.2), especially within the Portuguese community, showed that there was a loss of diversity due to insecticide contamination. The significant shifts detected in the family structure, at all carbofuran treatments in the Portuguese nematode community, and at the highest carbofuran concentration after 28 d, in the Brazilian community, were due mainly to the general decrease in relative abundance of most families along the treatments, that, in some cases, reached zero values (table 5A of appendix). The reduction in the number of nematode taxa after exposure to the fungicide carbendazim has already been reported (Moser et al, 2004).

However, for both Portuguese and Brazilian assays, there was a strong increase in the number of Rhabditidae and Panagrolaimidae in some replicates, which contributed to the high variability found in the nematode abundance (especially at the doses 25%, 50% and 100%RD; see table 5A of appendix and Table 5.2 for correspondences with carbofuran concentrations). Members of these families are classified as extreme opportunists and rapid colonizers, having explosive growth patterns under high microbial activity (Bongers, 1999). One cannot exclude that these observations might have been an artifact of the test system. Indeed, previous inoculation of soil with the

native microflora together with the disappearance or decrease in abundance of other families most vulnerable to carbofuran contamination might have increased food availability for these opportunistic families that rapidly increased their numbers.

IV.2.3 Effects on the total abundance of nematode feeding groups and global trophic structure

The soil contamination by carbofuran caused statistically significant shifts in the total abundance of nematode feeding groups. This occurred on the Portuguese nematode community only, where a general decrease in the abundance of most feeding groups was observed.

The abundance of the dominant group, the bacterial feeders (BTF), significantly decreased after four weeks of exposure only at the two highest carbofuran concentrations. The existence of higher carbofuran concentrations during the first two weeks may have stimulated the growth of microbial populations (Lo, 2010), leading to more food availability and reducing the potential hazard effects of carbofuran to this group of organisms. However, a significant reduction of bacterial feeding nematodes following the in situ contamination of a semi-arid grassland soil with carbofuran was reported by Ingham et al (1986). The application of other nematicides and insecticides under field conditions also caused a significant decrease in BTF nematodes (Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998).

A lower number of plant feeding nematodes (PLF) was recovered at the highest carbofuran concentrations in both exposure periods indicating a toxic effect of this insecticide. However, contradictory information has been reported since there were cases where insecticide applications had stimulatory effects to this feeding group (Yardim and Edwards, 1998) and others where the opposite response occurred (Parmelee et al, 1997; Pen-Mouratov and Steinberger, 2005).

The fungal feeders (FGF) were affected by carbofuran contamination but opposite responses were observed at the two exposure periods. If 14 d of exposure to carbofuran caused significant toxic effects at the two highest

carbofuran concentrations, the longer exposure caused a slight increment in the abundance of these organisms (although not significant). These observations might indicate direct toxic effects of the insecticide over the nematodes or lower food availability during the first period. Our results somehow conflict with other data since short-term studies (one week) on the effects of the insecticide malathion on FGF nematodes in a semi-field microcosm system did not cause toxic effects (Parmelee et al, 1997), while in longer studies (from one month to one year), a lower abundance of FGF was found in insecticide treated samples (Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998).

Predators-omnivores, the less abundant group on both Portuguese and Brazilian assays (table 5A of appendix), are known to be indicators of soil disturbance (Bongers and Bongers, 1998; Moser et al, 2004) and sensitive to nematicides (Smolik, 1983) and other insecticides (Yardim and Edwards, 1998). However, in the present study no significant effects were found perhaps due to the low number of organisms recovered. Indeed, in the cited experiments, performed under field conditions, the number of recovered predators-omnivores was substantially higher (Smolik, 1983; Yardim and Edwards, 1998).

Despite the decrease in the number of families and in the abundance of most feeding groups, the relative contribution (percentage) of each feeding group to the global trophic structure was not significantly affected by the insecticide contamination in any carbofuran treatment, in both Portuguese and Brazilian assays. This last endpoint seemed to be a conservative trait, i.e., the disappearance (or strong decline in abundance) of some nematode families (frequently, the less abundant), with consequent shifts in the community composition, was not closely followed by a functional shift.

However, the observed functional stability does not necessarily indicate that these nematode communities are highly resilient to pesticide disturbances as significant shifts in other endpoints (reported above) were detected.

The lack of effects also means that the disappearance (or decrease in abundance) of nematodes from a certain family was either replaced by an increase of another family within the same feeding group or accompanied by a general decrease in abundance of all feeding groups. This functional

redundancy (the replacement of lost species by others with similar traits) was pointed as a weakness of trait-based approaches (Van den Brink et al, 2011). Although gaining in ecological realism, the resolution of the trait-based approach used in the present study was somehow lower than the traditional taxonomic approach. Indeed, as one nematode family usually represents a single feeding type, changes in the feeding structure can be also detected besides the ("traditional") changes in the family structure. However, the opposite scenario is not true since changes in the feeding structure do not necessarily represent changes in the family structure, e.g. if the effects of a certain chemical mainly comprise the decrease in the species numbers of the most abundant families. Notwithstanding, confirmation is needed with further tests with other pesticides/chemicals and nematode communities.

It is difficult to compare our results with literature data, even restricting comparisons to studies where the effects of pesticides and metals on nematode communities were evaluated. All of them were performed under field or semi-field conditions, with different soil types/land-uses and, consequently, with dissimilar nematode communities. Therefore, direct comparisons have always a high degree of uncertainty associated. For example, in most cases, the percentage of each feeding group in the whole community is not presented and the analyses were performed using the absolute abundance of each group. Indeed, significant changes in the absolute abundance of at least one nematode feeding group, as a consequence of soil contamination, have been described (Korthals et al, 1996; Parmelee et al, 1997; Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998). The experiments conducted by Moser et al (2004) constitute an exception given that the relative abundance of nematodes was used to describe the hazard effects of the fungicide carbendazim and a significant decrease in the percentage of predators and omnivores was reported.

V. Conclusions

Although some methodological aspects need to be improved (especially the extraction efficacy), our results revealed that the use of soil nematode communities as sensitive indicators of toxic effects of pesticides is promising. A higher toxicity was observed for the Portuguese nematode community but the patterns of response of both communities were similar. The lack of significant effects for most parameters within the Brazilian nematode community was probably due to the higher clay and organic matter contents of soil that caused a lower carbofuran bioavailability but also to the high variability found among replicates.

The two major response patterns of nematode communities to carbofuran soil contamination were the decrease in the total nematode abundance and the reduction in the number of families. Significant shifts in the family composition were detected mainly within the Portuguese community (for the Brazilian, only significant shifts in the family composition were detected after 28 days, at the highest carbofuran concentration). However, the proportion of each feeding group in the trophic structure of the community did not significantly change with the different insecticide dosages. Thus, for this particular study, the trait-based approach used was not powerful enough to reveal the hazard effects of carbofuran in the trophic structure. Most probably, for this specific pesticide nematode community responses do not comprise changes in this endpoint but rather a general decrease in the abundance of all feeding groups.

The use of such a (feeding) trait-based approach is advantageous since effects can be evaluated without the need of identification at the species level. Moreover, response patterns of communities/populations are much more ecologically relevant than information from single species testing. However, an important drawback of using only this approach is the fact that possible losses of biodiversity (disappearance of some taxa, like it was observed in the present study using the taxonomic approach) cannot be observed or predicted. Thus, in nematode community ecotoxicological testing (as well as in other community related-studies), the implementation of feeding trait based approaches as an alternative to the taxonomic characterization requires

further evaluation, and depending on the objectives defined, it is recommended to consider the advantages of integrate both approaches (Van den Brink et al, 2011).

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Table 5A. Family composition of the nematode communities of Portugal (PT) and Brazil (BR) (expressed as total abundance of the nematode families, i.e., the sum of organisms extracted per family per treatment) exposed to soil contaminated with four doses of Furadan during 14 and 28 days. BTF – Bacterial feeders; PLF – Plant feeders; FGF – Fungal feeders; PD-OM – Predators/omnivores; RD – Recommended Dose. IC - Initial Community. Pan - Panagrolaimidae, Rha - Rhabditidae; Dip - Diplogasteridae; Cep - Cephalobidae (Acrobelinae); Cyl - Cylindrocorporidae; Cam - Camacolaimidae (Halaphanolaiminae); Mon - Monhysteridae (Prismatolaimus sp); Ple - Plectidae; Hop - Hoplolaimidae; Tyl - Tylenchidae ; Pra - Pratylenchidae; Het - Heteroderidae; Cric - Criconematidae; Aph - Aphelenchoididae; Dip - Diphtherophoridae; Dor - Dorylaimidae, Mon - Mononchidae; Tri - Trichodoridae.

	BTF								**PLF**				**FGF**			**PD-OM**		
Treatments (%RD)	**Pan**	**Rha**	**Dip**	**Cep**	**Cyl**	**Cam**	**Mon**	**Ple**	**Hop**	**Tyl**	**Pra**	**Het**	**Cric**	**Aph**	**Dip**	**Dor**	**Mon**	**Tri**
PT																		
IC	1187	393	169	12	15	21	0	6	239	480	70	63	3	166	6	154	36	0
14 d																		
0	402	94	305	1	0	1	0	0	3	55	13	6	0	41	1	12	3	0
25	192	108	27	0	0	0	0	0	4	23	6	3	0	15	0	3	1	0
50	254	142	6	0	0	0	0	0	2	30	3	3	0	23	0	3	4	0
100	146	189	2	0	0	0	0	0	1	10	3	2	0	12	0	2	0	0
200	170	41	1	0	0	0	0	0	1	14	1	2	0	4	0	1	2	0
28 d																		
0	305	112	309	1	0	6	0	0	0	30	6	5	0	5	0	7	3	1
25	205	150	113	0	0	59	0	0	1	22	2	3	0	5	0	4	1	0
50	188	158	4	0	0	0	0	0	0	8	1	1	0	19	0	4	0	0
100	156	16	1	0	0	0	0	0	0	17	0	1	0	7	0	2	0	0
200	118	29	0	0	0	0	0	0	0	8	0	0	0	8	1	3	0	0

Table 5A. (Continued)

	BTF								PLF				FGF			PD-OM		
Treatments (%RD)	**Pan**	**Rha**	**Dip**	**Cep**	**Cyl**	**Cam**	**Mon**	**Ple**	**Hop**	**Tyl**	**Pra**	**Het**	**Cric**	**Aph**	**Dip**	**Dor**	**Mon**	**Tri**
BR																		
IC	61	109	13	59	7	0	18	0	1430	88	2	0	0	44	0	105	35	0
14d																		
0	46	105	5	2	1	0	1	0	119	22	0	0	0	52	0	6	1	0
25	54	83	0	1	0	0	0	0	113	17	1	0	0	33	0	6	5	0
50	46	64	12	1	0	22	0	0	130	9	0	0	0	15	0	12	3	0
100	31	194	2	2	0	0	0	0	82	11	0	0	0	34	0	3	1	0
200	21	14	0	3	0	0	0	0	69	8	1	0	0	28	0	4	4	0
28d																		
0	95	116	1	8	0	0	10	0	55	19	0	0	1	102	0	6	3	0
25	97	12	0	0	0	0	2	0	75	9	5	0	0	132	0	4	3	0
50	95	261	0	1	0	0	0	0	66	2	0	0	0	206	1	8	0	0
100	83	82	6	1	0	0	0	0	50	1	0	0	1	177	0	3	1	0
200	50	7	0	0	0	0	0	0	45	9	0	0	0	112	0	2	0	0

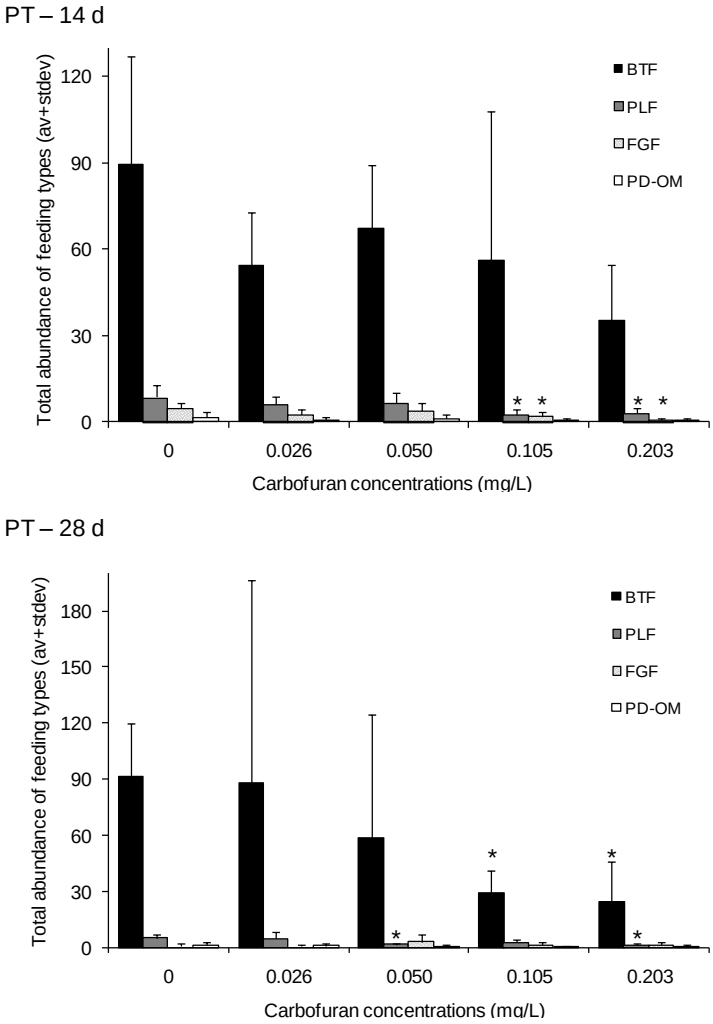

Figure 5A. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil on the total abundance of four feeding groups of a nematode community from Portugal (PT), after 14 and 28 days of exposure. Vertical bars represent mean (n=6 or n=8, respectively for treated or control samples ± standard deviation) values. BTF – Bacterial feeders; PLF – Plant feeders; FGF – Fungal feeders; PD/OM – Predators/omnivores. See Table 5.2 for correspondences between Furadan doses and carbofuran concentrations. * - Statistically different from the respective feeding group in the control (One Way Anova, Dunnet Test, $p < 0.05$).

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Chapter 6

Soil microarthropod community testing: a new approach to increase the ecological relevance of effect data for pesticide risk assessment.

Based on the following manuscript under preparation:

Chelinho S, Domene X, Natal-da-Luz T, Andrés P, Norte P, Rufino C, Lopes I, Cachada A, Espindola ELG, Ribeiro R, Duarte AC, Sousa JP. Soil microarthropod community testing: a new approach to increase the ecological relevance of effect data for pesticide risk assessment.

Abstract

In the present study, a new complementary approach combining the use of the natural soil microarthropod community and conventional test methods was used. The effects of soil contamination with the insecticide carbofuran on two geographically microarthropod communities (warm temperate and Tropical) were evaluated in their soils of origin under controlled laboratory conditions.

After contamination of two agricultural soils from Portugal and Brazil, a gradient of concentrations was prepared. Soil cores were taken from the respective uncontaminated surrounding areas and the mesofauna of three cores was extracted directly to the test soil. After extracting the microarthropod communities to the test soil, these were incubated under laboratory conditions for 4 weeks, after which the mesofauna was extracted again. The organisms were assorted into higher taxonomic groups and Acari and Collembola were respectively assorted into order/sub-order/cohort and family. Collembolans were still classified according to morphological traits and used as a case-study of trait based risk assessment (TERA, Baird et al, 2008) of pesticides.

The exposure to insecticide contamination caused the impoverishment of the taxonomic diversity in both communities. Significant shifts in the microarthropod community structure in the different carbofuran treatments were found for both soils, although effects were more pronounced in the assay performed with the soil from Brazil. Collembolans were the most affected group with a strong decline in their abundance. A dose response relationship was observed, showing a consistent decline on the relative abundance of Isotomidae, closely followed by an increase of Entomobryidae individuals. Contrastingly, Acari (especially Oribatida) tended to increase their numbers with higher concentrations.

Trait based analysis of Collembola data suggested that a shift in the functional composition of the communities occurred due to carbofuran soil contamination and that species adapted to deeper soil layers were more vulnerable to insecticide toxicity.

Keywords: community ecotoxicology; carbofuran; diversity; life-traits.

I. Introduction

The toxicity of pesticides to soil fauna is usually evaluated through laboratory assays exposing single standard species to a series of concentrations of the pesticide of concern and measuring their acute and/or chronic effects (Van straalen, 2002; ISO, 2003; Van den Brink, 2008). However, such approach does not take into account the interactions between species within a community, as well as possible differences in the responses of communities from different ecoregions (Van straalen, 2002; Van den Brink, 2008; Kuperman et al, 2009; Clements and Rohr, 2009).

Higher tier methods include semi-field tests like micro and mesocosms, attempting to combine the controlled laboratory conditions with the complex network of organisms' interactions that naturally occur in the field (Burrows and Edwards, 2002; Knacker et al, 2004; Scott-Fordsmand et al, 2008). Several tools have been developed (for a recent review see Shäffer et al, 2010) but only Terrestrial Model Ecosystems (TME) have been standardized (ASTM, 1993). Although with higher ecological realism, the semi-field tests are usually associated to increasing variability, higher experimental effort and costs (Van den Brink et al, 2005; Schäffer et al, 2010).

Despite this, the introduction of more ecological information in ecotoxicology, like using species abundance and community composition to predict responses and recoveries of communities towards anthropogenic disturbances, is a challenge for many ecotoxicologists (Filser et al, 2008; Clements and Rohr, 2009).

A step forward was the proposal of an innovative approach, called Trait-Based Risk Assessment (TERA; Baird et al, 2008), advocating that morphological/physiological/ecological characteristics of organisms can be used to describe the effects of toxic substances or other stress factors at the community level. Several papers have been published, supplying not only the theoretical background but also proposing frameworks and identifying research needs (e.g. Baird et al, 2008; Van den Brink, 2008, Clements and Rohr, 2009; De Lange et al, 2010; see also the special series on TERA published in IEAM journal, 2011).

Following the history of ecotoxicology, trait based ecotoxicological studies are being implemented earlier in the aquatic field (e.g. Relyea and Hoverman, 2006; Baird and Van den Brink, 2007; Liess and Beketov, 2011). However, the validation and further consolidation of this approach requires its transposition to the assessment of soil contamination (De Lange et al, 2009).

In the present study, a new complementary approach using the natural soil microarthropod community (that play a key role in the decomposition processes and nutrient cycling; Seasted, 1984) was tested, adopting conventional test strategies and using a specific taxonomic group as a case-study of TERA in soil.

Specifically, the objectives of the present study were (i) to assess the effects of an insecticide (carbofuran) application on two geographically distinct soil microarthropod communities (warm temperate and tropical); and (ii) to describe the potential changes in the composition of soil Collembola communities using functional traits of organisms.

Thus, two microarthropod communities from Portugal and Brazil were extracted to two distinct soils, previously contaminated with carbofuran, a carbamate insecticide, also with nematicidal and acaricidal properties. The exposure to pesticide contamination took place under laboratory controlled conditions, less demanding in terms of space, time and costs, when compared to field and semi-field studies, and with presumably lower variability associated.

Effects were assessed based on traditional taxonomic approaches (describing changes in richness and abundance of the different taxonomic groups) and on an innovative trait-based approach in which the individuals of the second most abundant group - Collembola - were classified according to functional traits (related with their dispersion abilities).

II. Materials and Methods

II.1 Areas of soil sampling

In Brazil, an agricultural area with no history of pesticide application, located in São Carlos (SP; -22° 10' 13.53", - 47° 53' 58.12") was chosen. In Portugal, a parcel of fallow land, not cultivated at least during the last 5 years, located in the surroundings of Coimbra (40° 14' 46.5066", - 8° 20' 23.9964") was selected. The study took place in the autumn of 2007 (Brazil) and 2009 (Portugal).

The soils from Brazil and Portugal were respectively analyzed by CHREA, ESESC, University of São Paulo (Brazil) and Direcção Regional de Agricultura e Pescas do Norte (DRAPN, Porto, Portugal) as described in Chelinho et al (accepted) and Chelinho et al (2011a). The pedological properties of the test soils are shown in Table 6.1.

II. 2 Soil contamination

In Brazil, the contamination of soil took place under field conditions and was integrated in a broader project (Chelinho et al, accepted). Briefly, the soil was tilled and after three days, two parallel strips of land (3 × 1m), separated by a buffer area of 2 m (to avoid cross-contamination) were used to simulate a pesticide spraying over an agricultural field.

One of the strips was sprayed with the insecticide Furadan 350 SC (a carbofuran commercial formulation from FMC, SP, Brazil; 350 g a.i./L) at two times the recommended dose (2×RD) for sugar cane plantations (10 l/ha; ~2.334 mg a.i./kg soil Dry Weight (DW), taking into account an average soil density of 1.5 g/cm3 and an incorporation depth of 10 cm). This dose mimicked pesticide overuse, a very common practice among local farmers (Dasgupta et al, 2001). The insecticide was diluted in 5L of water collected at a nearby reference lagoon.

Table 6.1. Pedological properties of the tested soil. BR - Brazil; PT - Portugal; OM – Organic matter; CEC – Cation exchange capacity; WHC – Water holding capacity.

	pH	OM	Sand	Silt	Clay	Total N	WHC	Soil Type
	KCl 1M	%	%	%	%	%	%	
BR	5.33 ± 0.107	13.5	79.5	18.6	2.17	0.62	67.1± 3.48	Loamy sand
PT	3.9 ± 0.03	9.89	40.0	44.5	15.5	0.48	74.7 ± 4.23	Loam

To facilitate the pesticide incorporation, the top 5 cm of soil were mixed and another 10L of lagoon water were sprayed. The second strip of land, which acted as control, was previously sprayed with the same amount of the lagoon water (5+10L).

In the early morning of next day, soils from both strips were collected (top 10cm) for ecotoxicological evaluations and chemical analysis. The contaminated soil samples (as well uncontaminated (control) soil) were sieved (5mm) and defaunated by a freezing (F) - thawing (T) cycle (48h F - 8h T - 24h F). The control soil was mixed with soil sprayed with 2×RD of Furadan in different proportions to obtain the following dilution series: 0, 2.5, 5, 10, 25, 50 and 100% of 2×RD.

For the assay conducted in Portugal, several samples of soil (top 10cm) were randomly collected in an area of 40 m^2, mixed, sieved (5mm) and defaunated trough one freezing (F) - thawing (T) cycle (48h F - 8h T - 24h F). Afterwards, the soil was spiked in the laboratory with different proportions of a stock solution of Furadan 350SC (the same commercial formulation of carbofuran referred above) diluted in deionised water to create the following range of concentrations: 0, 2.5, 5, 10, 25, 50 and 100% of 2×RD.

In both assays, the amount of solutions added per treatment was adjusted to achieve initial moisture content of 50 % of the WHC. Afterwards, the contaminated soil was distributed by plastic boxes with perforated lids (~300g DW × 7 replicates per treatment).

II. 3 Sampling, extraction and incubation of soil microarthropods

For the assay performed in Brazil, in the uncontaminated surroundings of the area where the test soil was collected, the upper vegetation layer was removed and soil cores (7cm Ø × 10cm) were taken along chosen transects randomly outlined and placed in plastic bags. A similar procedure was undertaken in the fallow land used as study area in Portugal.

In both assays, the content of 3 soil cores (randomly selected) was mixed and used in each test replicate as a source of microarthropods. The microarthropod communities were extracted using Berlese funnels (Brazil) or

a Macfadyen high-gradient extractor (Portugal). Organisms were extracted directly to the treated (contaminated with carbofuran) and control soils , during 14 or 7 d (respectively in the Brazilian and Portuguese assay).
In parallel, the same method was used to extract the microarthropods into 8 smaller vessels (for each assay) containing 80% ethyl alcohol instead of contaminated soil, to further characterize the initial communities (ICs) of both countries.

After this extraction period, the vessels containing the treated soil and the microarthropods were incubated under laboratory conditions (23 ± 1ºC or 20 ± 1ºC, respectively for Brazilian and Portuguese assays; 16:8 - light : dark photoperiod) for 4 weeks.

Following the incubation period, microarthropods were extracted again (during the same period reported above for the first extraction) and preserved in 80% ethyl alcohol.

II. 4 Microarthropod sorting and identification

The extracted organisms, preserved in 80% ethyl alcohol, were initially counted and sorted into higher taxonomic entities under a stereomicroscope (40x magnification) according to Barrientos (1988) and Minor and Robertson (2006).

In a second phase, mites were sorted into four main groups: (suborder) Oribatida, (order) Mesostigmata, (suborder) Prostigmata, and (cohort) Astigmata, according to Lindquist et al (2009).

For the identification of collembolans, taxonomic and trait-based approaches were followed. These organisms were identified and assorted into five families (Entomobryidae, Isotomidae, Onychiuridae, Poduridae and Sminthuridae) according to Gisin (1960), but also according to different morphological traits related to dispersion features of collembolans, namely the ocelli, furca, antenna, pigmentation and the presence of hairs and scales (see table 6A of appendix).
Within each Collembola family, the organisms exhibiting a different combination of trait scores were considered as representing different

morphospecies (see table 6B of appendix). Thus, for the Portuguese and Brazilian assays, 19 and 18 morphospecies were considered, respectively.

II. 5 Chemical analysis

Samples (~ 400g Wet Weight) from each dilution/concentration were stored at -20ºC for further analysis of carbofuran concentrations. The analysis of the Brazilian and Portuguese samples were performed by IQSC, University of São Paulo (Brazil) and CESAM - Department of Chemistry, University of Aveiro (Portugal), respectively, as described before (Chelinho et al, accepted; Chelinho et al, 2011b).

II. 6 Statistical analysis

To investigate the effects of carbofuran on the total or relative abundance of Acarina and Collembola (the two most abundant groups; see section III.1), a one-way ANOVA followed by post-hoc comparisons with the control (Dunnets' test) or a Kruskall Wallis test followed by multiple comparisons with the control (if assumptions were violated, even after data transformation) were used. The same analyses were performed for the Collembola families and the four groups of mites (see section II.4).

The data were previously analysed for normality (Kolmogorov-Smirnov test) and for variance homogeneity (Levene Test). If violations of normality and/or homogeneity occurred, a log (x+1) transformation was applied. If after data transformation, those assumptions were not fulfilled, a Kruskall Wallis test followed by multiple comparisons with the control was used. All analyses were performed in Statistica 7.0 (Available at: http://www.statsoft.com/; assessed 28th May 2011). The relative abundance of the four groups of Acari and five families of Collembola was calculated as a function of the total numbers of each group found per treatment.

Potential effects of carbofuran on microarthropods community composition and in the Collembola and mite groups (for the last two endpoints, only

relative abundance data was used) were evaluated by Analysis of Similarity (ANOSIM), comparing the community composition of the carbofuran contaminated samples with those of the control. Whenever significant differences were found, the Similarity of Percentages (SIMPER) analysis was used to identify the families or groups responsible for the observed change and their contribution (in terms of percentage) for the overall shift. Both ANOSIM and SIMPER analysis were performed in Primer 5.2.6 (Clarke and Gorley, 2001) using log(x+1) transformed data.

Regarding the morphological traits of Collembola, data were pooled per treatment and used to calculate two functional trait indices per treatment: the mean trait per community (mT) and the Functional Diversity (FD), following a similar approach to that carried out by Vanderwalle et al (2010). For each morphospecies, the scores of individual traits were sum to determine the "Life-form" trait that was used for the calculation of indices indicated above (see Vanderwalle et al, 2010). It ranged between 5 (minimum, indicative of euedaphic species) and 25 (maximum, indicative of epigeic species).

The mT index consisted in the trait average of each treatment (considered as a different community) taking into account the relative abundance of each morphospecies. The values calculated for each carbofuran treatment were compared with the respective control using a *t* - Test.

The FD index reflected the range of trait values within each treatment (or community; Díaz et al, 2007) and was calculated according to Lepš et al (2006).

Simpson (Simpson, 1949) and Shannon diversity indexes (Shannon and Weaver 1949) were also calculated.

III. Results

III. 1 Composition of the Microarthropod Community

The initial communities (ICs) of Portugal and Brazil were composed by 12 and 16 groups of microarthropods, respectively, with clear dominance of mites (74 and 78% of the total of individuals, respectively; Table 6.2).

Oribatid and mesostigmatid mites dominated the Brazilian microarthropod community (51 and 20%, respectively; data not shown) while in the Portuguese assay, prostigmatid and oribatid mites were the most abundant (46 and 16%, respectively; data not shown). Collembolans were the second most abundant group and, together with mites, represented approximately 92 and 87 % of the total of individuals found, respectively for the Portuguese and Brazilian assays (Table 6.2).

The dominance of collembolan families was also different between the two countries. Indeed, in Portugal, the majority of collembolans was assorted in the Onychiuridae and Isotomidade families (12 and 6% of the total microarthropods, 66 and 30% of the total Collembola, respectively; data not shown) while in Brazil, Entomobryidae and Isotomidae represented approximately 3% of the total of microarthropods (39 and 47% of the total Collembola, respectively; data not shown).

Among the less abundant microarthropod groups, some were exclusively found within one of the communities. For example, Aphididae and Pseudoscorpionidae were only found within the Portuguese (IC, control and/or treated) samples while Coleoptera (other than Staphylinidae) Pauropoda, Thysanoptera, Isoptera and Diplopoda were specific of communities from Brazilian (IC, control and/or treated) samples (Table 6.2). The abundance of Protura and Larvae contrasted between the initial communities of both countries. The former group was the third more abundant within the Brazilian samples, while the last were clearly more abundant in the Portuguese ones (Table 6.2).

Table 6.2. Group composition of soil microarthropod communities from Portugal (PT) and Brazil (BR) (expressed as total abundance of major taxonomic groups) exposed to soil contaminated with Furadan (see section II. 2 for details). RD – Recommended Dose; IC - Initial Community; Aca - Acari; Col - Collembola; Prt - Protura; Sym - Symphyla; Pau - Pauropoda; Iso - Isopoda; Chil - Chilopoda; Dip - Diplopoda; Isopt - Isoptera; Pso - Psocoptera; Thy - Thysanoptera; Ara - Araneae; Pse - Pseudoscorpionidae; Sta - Staphylinidae; Cole - other Coleoptera; Aph - Aphididae; For - Formicidae; Lar - Larvae.

Doses (% 2×RD)	**Aca**	**Col**	**Prt**	**Sym**	**Pau**	**Iso**	**Chil**	**Dip**	**Isopt**	**Pso**	**Thy**	**Ara**	**Pse**	**Sta**	**Cole**	**Aph**	**For**	**Lar**
PT																		
IC	2317	568	4	24	0	0	5	0	0	0	0	0	1	0	3	12	170	27
0	709	199	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	2
2.5	845	97	0	1	0	0	1	0	0	0	0	1	0	0	0	1	2	3
5	1032	108	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	1
12.5	828	133	0	1	0	1	1	0	0	0	0	0	2	1	0	0	0	3
25	862	79	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	3
50	550	12	1	0	0	0	2	0	0	0	0	0	0	0	0	0	3	6
100	622	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3

Table 6.2. (Continued)

Doses (% 2×RD)	Aca	Col	Prt	Sym	Pau	Iso	Chil	Dip	Isopt	Pso	Thy	Ara	Pse	Sta	Cole	Aph	For	Lar
BR																		
IC	4301	487	460	13	8	1	2	6	2	1	4	1	0	4	40	0	175	5
0	1371	1214	24	1	1	0	1	0	0	0	0	1	0	5	7	0	15	3
2.5	1514	651	89	0	2	0	0	0	0	0	0	3	0	6	21	0	42	4
5	1657	658	217	1	0	0	0	1	0	0	0	3	0	4	16	0	19	2
10	1028	382	0	1	0	0	1	0	0	0	0	2	0	1	8	0	67	1
25	1528	279	3	0	0	0	1	0	0	0	0	1	0	1	10	0	60	0
50	2049	133	0	0	0	0	1	0	0	0	0	6	0	0	9	0	25	0
100	2294	27	0	0	0	0	0	0	0	2	0	0	0	0	7	0	0	1

III. 2 Effects of Carbofuran in the community composition and abundance of microarthropod groups

For the two assays, the total number of microarthropods recovered in the controls, when compared with the IC, decreased strongly (70 % and 52%, respectively for the Portuguese and Brazilian experiments; Table 6.2), reflecting effects of incubation. Also, in general, a high variability was found between replicates in the total number of individuals and in the relative abundance of the microarthropod goups.

The exposure of both soil communities to a gradient of carbofuran concentrations (measured values are available in Table 6.3) caused a decrease in the community richness since the number of microarthropod groups has progressively decreased (Figure 6.1 PT and 6.1 BR; Table 6.2). Significant differences in the microarthropod community composition were also detected by ANOSIM. In the Portuguese experiment, the community at the two highest doses (50 and 100% of 2 × RD) was significantly different from the control (ANOSIM, $p < 0.05$); Collembola and Acarina (both with decreased abundance at the two highest doses if compared to the control) were the groups that most contributed to this dissimilarity (SIMPER analysis; see Table 6C of appendix).

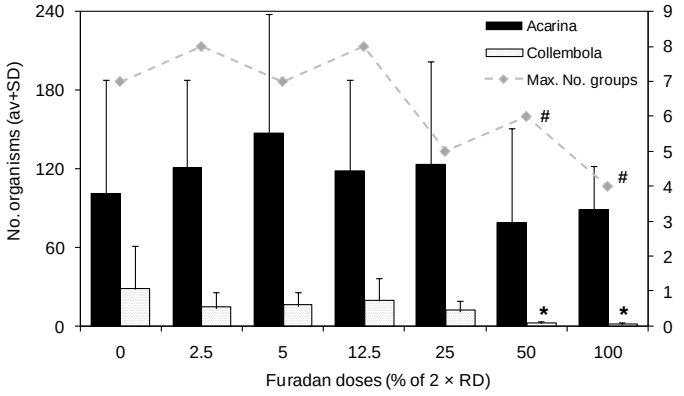
Stronger effects of carbofuran were observed within the communities extracted from the Brazilian soil since, with one exception (5% of 2 × RD), all the doses caused significant changes in the community structure when compared with the control (ANOSIM, $p < 0.05$); the SIMPER analysis highlighted Protura, Collembolla (in both cases, their abundance was negatively affected by the treatments) and Formicidae (more abundant in the treated doses than in the control) as the groups that mostly contributed to the dissimilarity detected (SIMPER analysis; see Table 6C of appendix).

Focusing on the two most abundant groups, a common pattern of response was found for the collembolans: the average number of individuals decreased along the contamination gradient, with significant conditioning effects found for the highest doses (50 and 100% of 2 × RD or 25, 50 and 100% of 2 × RD, respectively for the Portuguese and Brazilian experiments; One Way Anova, Dunnet test; $p < 0.05$; Figure 6.1 PT and 6.1 BR).

Table 6.3. Carbofuran concentrations (expressed as milligrams/kilogram of soil DM) in the laboratory spiked soil (Portuguese experiment) or in the field contaminated soil (Brazilian experiment) with 2× the Recommended Dose (2×RD) of Furadan. n.a. - not applicable.

Doses (% of 2×RD)	**Carbofuran Concentrations (mg/kg)**	
	Portugal	**Brazil**
0	<0.020	<0.010
2.5	0.054	0.039
5	0.113	0.079
10	n.a.	0.460
12.5	0.509	n.a.
25	0.964	0.400
50 (RD)	2.025	1.520
100	3.438	2.460

PT



BR

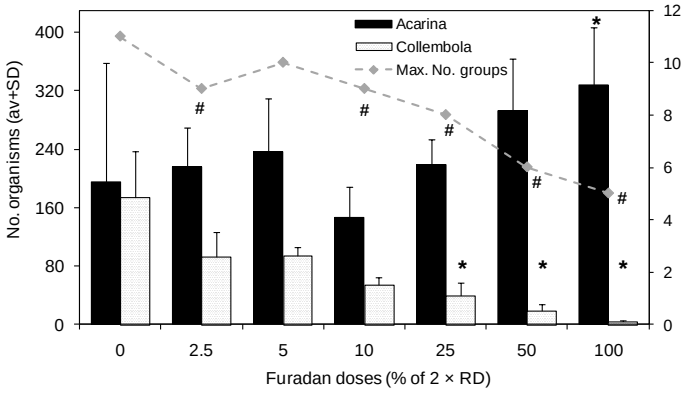


Figure 6.1. Effects of Furadan (a.i. carbofuran) contaminated soil on the total abundance of Acarina (black bars), Collembola (white dotted bars, left axis) and maximum number of soil microarthropod groups (dotted line, right axis) found in two soil communities from Portugal (PT) and Brazil (BR). Values express average (± standard deviation - SD) values. RD - Recommended Dose; * – Statistically different from the respective control (One Way Anova, Dunnet Test, $p < 0.05$); # - Microarthropod community statistically different from the control (ANOSIM, $p < 0.05$).

With respect to the community of mites, on the Portuguese experiment, although the variability among replicates impaired the establishment of statistically significant effects (One Way Anova, Dunnet test; $p > 0.05$), the average number of organisms tended to increase at the low and intermediate doses and decreased at the two highest doses (Figure 6.1 PT).
The increase in carbofuran concentrations was generally linked with a higher number of mites in the Brazilian experiment with significant effects found for the highest dose (One Way Anova, Dunnet test, $p < 0.05$; Figure 6.1 BR).

III. 3 Effects of Carbofuran in the community composition of mites and collembolans

The shifts in the overall community structure of Collembola in the different carbofuran treatments were somehow similar for the two geographical communities.
Thus, in the Portuguese experiment, the relative abundance of Entomobryidae tented to increase, while the opposite happened for the Isotomidae (Figure 6.2 PT). Onychiuridae also followed the same tendency of the former family, excepting at the two highest doses, where a sharp decline in the relative abundance was observed (Figure 6.2 PT). Despite this, no significant differences were found for any family (One Way ANOVA, Dunnet test, $p> 0.05$; Figure 6.2 PT).

A significant increase in the relative abundance of Entomobryidae was also observed in the Brazilian experiment, which contrasted with the significant decline of Isotomidae, in all Furadan doses, except at the highest dose, where a high variability was found (significant differences found for the doses 10, 25 and 50 % of 2 × RD, for both families; Kruskall Wallis Test and multiple comparisons with the control, $p< 0.05$; Figure 6.2 BR).

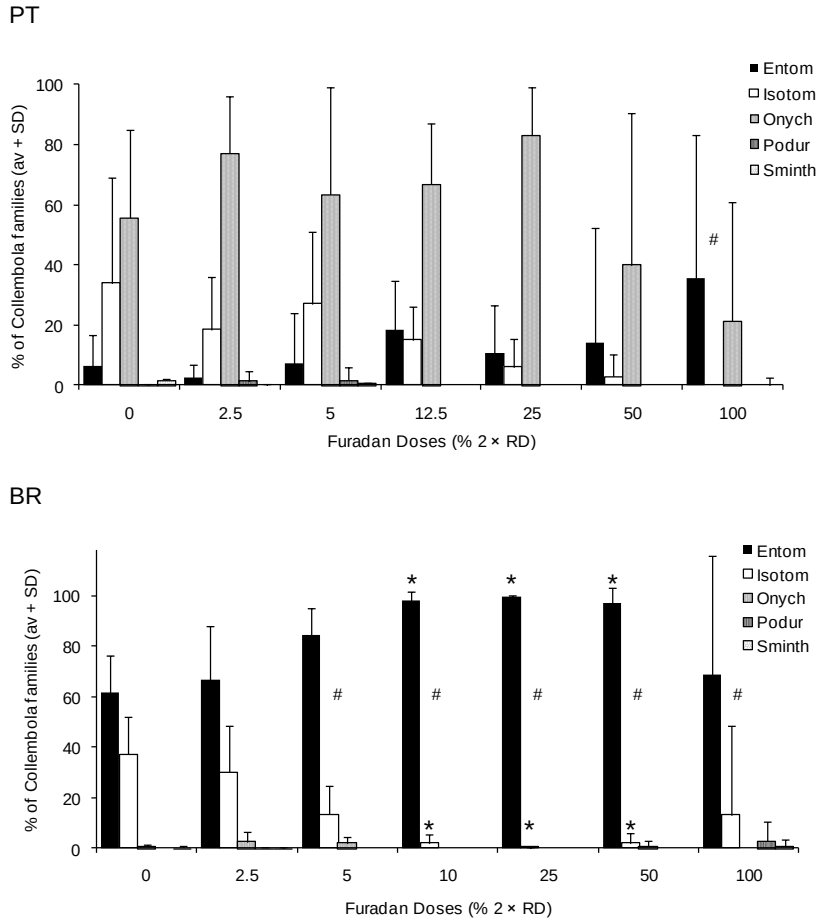


Figure 6.2. Effects of Furadan (a.i. carbofuran) contaminated soil on the relative abundance of the families of Collembola found in two soil microarthropod communities from Portugal (PT) and Brazil (BR). Values express average (± standard deviation - SD) values. RD - Recommended Dose; * – Statistically different from the respective control (Kuskall Wallys test and multiple comparisons with the control; $p< 0.05$); # - Collembolan community statistically different from the control (ANOSIM, $p< 0.05$). Entom - Entomobryidae; Isotom - Isotomidae; Onych - Onychiuridae; Podur - Poduridae; Sminth - Sminthuridae.

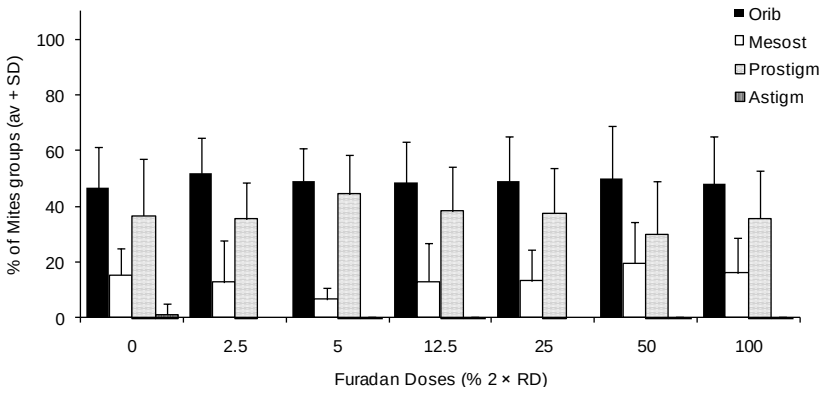
In terms of the global community composition of Collembola, in the Portuguese experiment, significant differences were found only at the highest dose (100% of 2 × RD) when compared to control (ANOSIM, $p < 0.05$; Figure 6.2 PT). Entomobryidae, Isotomidae and Onychiuridae families explained 95% of the observed dissimilarity (SIMPER analysis; see Table 6C of appendix and Figure 6.2 PT). The effects of the insecticide were more clear within the Brazilian experiment, since ANOSIM detected significant differences at all the doses, except the lowest (2.5% of × RD), relatively to the control (ANOSIM, $p < 0.05$). Again, the most abundant families, Isotomidae, Entomobryidae and Onychiuridae contributed most to these differences (SIMPER analysis, see Table 6C of appendix and Figure 6.2 BR).

Mite community from the Portuguese soil revealed to be more resistant to carbofuran contamination, compared to Collembola communities, since no significant differences were found between the relative abundance of any of the four groups individually and that of the control (One Way ANOVA, $p > 0.05$; Figure 6.3 PT) nor for the global community structure (ANOSIM, $p > 0.05$).

Contrastingly, in the Brazilian experiment, a significant drop in the relative abundance of Mesostigmata and Prostigmata (doses 10, 25, 50 and 100% of 2 × RD lower than the control; One-Way ANOVA, Dunnet Test, $p < 0.05$; Figure 6.3 BR) was observed. In parallel, the relative abundance of oribatid mites consistently increased (all doses, excepting 2.5 % of 2 × RD, higher than the control; One-Way ANOVA, Dunnet Test, $p < 0.05$; Figure 6.3 BR).
The community of mites was different from the control at all the doses, except at the lowest (ANOSIM, $p< 0.05$); these differences were mainly influenced by the decrease in relative abundance of Mesostigmata and Prostigmatid mites (SIMPER, see Table 6C of appendix).

The effects of carbofuran on the total abundance of Collembola and Acarina groups were quite similar to the ones described above for the relative abundance data and are available in Figure 6A and 6B of the appendix, respectively.

PT



BR

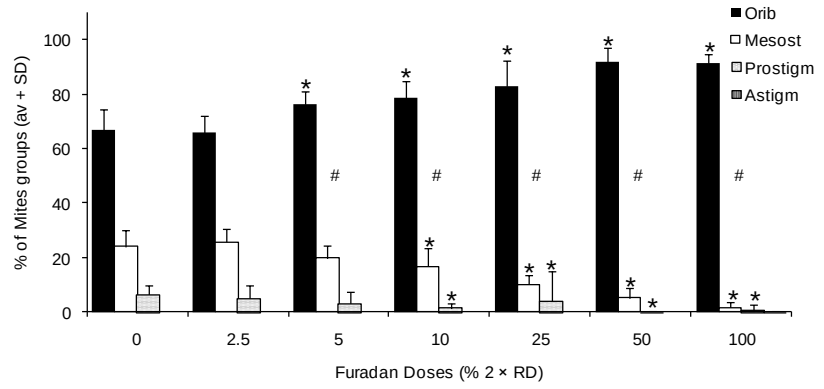


Figure 6.3. Effects of Furadan (a.i. carbofuran) contaminated soil on the relative abundance of the four groups of Acarina found in two soil microarhopod communities from Portugal (PT) and Brazil (BR). Values express average (± standard deviation - SD) values. RD - Recommended Dose; * – Statistically different from the respective control (One Way Anova, Dunnet Test, $p < 0.05$); [a] - Mites community statistically different from the control (ANOSIM, $p < 0.05$). Orib - Oribatida; Mesost - Mesostigmata; Prostigm - Prostigmata; Astigm - Astigmata.

III. 4 Effects of carbofuran on functional traits of collembolans

In both assays, the soil contamination generally decreased diversity of morphospecies, as evidenced by the decreases of both Simpson and Shannon diversity indices (Table 6.4). The exception was the dose “50% of 2 × RD” in the Brazilian experiment, for which a higher value for the Shannon index was calculated, as a consequence of the higher number of species found (and their equitable distribution) in comparison with the earlier and latter Furadan doses (Table 6.4).

Among the two indexes used to describe changes in functional traits of collembolans along the gradient of insecticide treatments (mT and FD), the tendencies were not always similar. The mT values tended to increase in both assays, reflecting the higher representation of morphospecies adapted to surface soil layers (Table 6.4). Relatively to the controls, this variation was statistically significant at the two highest Furadan doses for the Portuguese experiment (*t*-test, $p < 0.05$; Table 6.4) and at the highest dose for the Brazilian experiment (*t*-test, $p < 0.05$; Table 6.4).

On the other hand, for the Portuguese experiment, there was a tendency for FD values to increase along the contamination gradient (Table 6.4), which were not correlated with the decrease in the diversity indices reported above. An opposite scenario was observed for the Brazilian experiment, since lower FD values were obtained for the high Furadan treatments and this trend was strongly correlated with the variation of both Simpson and Shannon indices ($r = 0.90$ and 0.82 and $p < 0.001$ and < 0.005, respectively for FD vs Simpson and FD vs Shannon).

Table 6.4. Summary of the responses of two soil communities of Collembola, from Portugal (PT) and Brazil (BR) to increasing Furadan treatments. Results are expressed in terms of the mean trait per community (mT), functional diversity (FD) and diversity of morphospecies (Simpson and Shannon indices). RD - Recommended Dose; IC - Initial Community. n - Number of morphospecies (organisms exhibiting a different combination of trait scores; see section II.4).

Indexes	**Furadan doses (% of 2 × RD)**							
PT	**IC**	**0**	**2.5**	**5**	**12.5**	**25**	**50**	**100**
mT	0.637	0.805	1.374	0.975	1.164	1.166	3.944*	7.250*
FD	0.129	0.142	0.120	0.150	0.244	0.135	0.375	0.256
Simpson	0.616	0.669	0.622	0.711	0.590	0.468	0.542	0.375
Shannon	1.251	1.387	1.165	1.434	0.995	0.985	0.888	0.562
n	13	11	6	9	8	7	3	2
BR	**IC**	**Ct**	**2.5**	**5**	**10**	**25**	**50**	**100**
mT	1.097	1.111	1.508	1.457	1.706	2.830	1.665	5.198*
FD	0.321	0.270	0.262	0.240	0.125	0.099	0.203	0.108
Simpson	0.735	0.704	0.710	0.743	0.622	0.538	0.710	0.524
Shannon	1.680	1.435	1.399	1.584	1.184	0.967	1.578	0.814
n	12	13	10	11	10	6	10	3

* - statistically different from Ct (*t*-Test; $p < 0.05$).

IV. Discussion

IV.1 Testing strategy

The testing strategy adopted in the present study aimed to combine the advantages of both community studies and ecotoxicological conventional tests (performed under a laboratory context during a relatively short period of time, compared for example, with monitoring and biodiversity studies).

From a methodological point of view, one of the major limitations of this approach was the low recovery rate of the microarthropods in the controls and treated samples, comparatively to the initial community. This might be related with the fact that organisms were confined to a small area from which it was not possible to escape, either from potential predators (e.g. Staphylinidae and/or Araneae; Bohac, 1999; Marc et al, 1999) or from unfavorable environmental conditions (e.g. temperature, humidity and/or light). In fact, under laboratory conditions, it was unfeasible to simulate exactly the environment of a real scenario. In addition, the possibility that the extraction procedures might have worked as a stress factor to some of the organisms cannot be discharged. Improvements can be further adopted namely extending the exposure period for at least four more weeks (possibly allowing the reproduction of some species and thus, the assessment of sub-lethal effects) and consider the supply of food during the test.

Despite the methodological constrains, results showed that this testing approach was valid and sensitive enough to detect the effects of a pesticide over two soil microarthropod communities. Moreover, its further use as a tool to introduce more ecological realism in the data gathered from ecotoxicology-related studies seems to be promising.

IV. 2 Effect of carbofuran contamination on the microarthropod communities

Both soil microarthropod communities from Portugal and Brazil were negatively affected by the insecticide contamination, although stronger effects were registered within the Brazilian assay, where even low dosages of carbofuran induced significant shifts when compared to the control.

These observations are not in agreement with the chemical analysis of the test soils that showed carbofuran concentrations generally higher in the laboratory spiked Portuguese soil than in the field contaminated Brazilian soil (Table 6.3). These different levels of contamination may be related with the high dispersion usually occurring for soil sprayings under field conditions (Schulz, 2004). On the other hand, the pedological properties of the two soils might have determinated a lower bioavailability of the pesticide for the organisms in the Portuguese soil, since higher adsorption of carbofuran seems to occur in silt loam and loam soils (such as the soil from Portugal) than in sandy loam soils (such as the soil from Brazil) (Singh and Srivastava (2009). In addition, carbofuran seems to degrade faster (and thus, causing lower toxicity) under moist conditions (Shelton and Parkin, 1991), which were higher for the Portuguese soil. An intrinsically higher sensitivity of the organisms from communities of Brazil to pesticides (not investigated) is also a possible explanation.

Most of literature data on the effects of insecticides on soil microarthropods refer to field or semi-field studies from temperate climate regions, focusing mostly on Acari and Collembola (e.g. Martikainen et al, 1998; Endlweber et al, 2006; Vig et al, 2006; Adamski et al, 2009). Notwithstanding, some information on this issue is available for tropical environments (e.g. Michereff-Filho et al, 2004; Joy and Chakravorty, 1991; Joy et al, 2005; Bambaradeniya and Edirisinghe, 2008).

Although direct comparisons with literature should be made with caution, due to the differences in experimental design and exposure conditions, the

impoverishment of taxonomic diversity as well as the general decrease in the overall abundance of microarthropods (more pronounced in the Brazilian assay; Table 6.2) observed in the present study is in agreement with data previously reported for carbofuran (Broadbent and Tomlin, 1982; Bambaradeniya and Edirisinghe, 2008) and other insecticides (Joy and Chakravorty, 1991; Frampton, 1999; Joy et al, 2005; Endlweber et al, 2006). However, other authors found low or no toxicity of insecticides (endosulfan, deltamethrin and diflubenzuron) for the same groups of organisms (Osler et al, 2001; Griffthis et al, 2006; Adamski et al, 2009).

As expected, Collembola, known to be particularly sensitive towards carbofuran (Frampton, 1994; Bambaradeniya and Edirisinghe, 2008) showed a dose-response pattern, with a lower abundance relatively to the control. This was most pronounced at the highest dose, with 5% or less of the abundance of control.

The opposite response occurred for mites, which was the dominant group since their abundance generally increased along the contamination gradient. However, in the Portuguese experiment, at the doses 5, 50 and 100% of 2 × RD, a slight decline (maximum 22%, compared with the control) was registered. Especially in the case of the Brazilian assay, the increase in the number of mites might be related with the concurrent decline of predators. Working with other insecticides with similar modes of action (Acetylcholinesterase inhibitors) like dimethoate and chlopyriphos, toxic effects were also observed for collembolans (Joy et al, 1991; Martikainen et al, 1998; Frampton, 1999, Endlweber et al, 2006; Frampton and Van den Brink, 2007) but not for mites (Joy et al, 1991). However, the absence of toxic effects of chlorpyrifos in tropical arthropod assemblages (including collembolans and mites) was also reported by Michereff-Filho et al (2004). In addition, Frampton (1999) and Frampton and Van den Brink (2007) did not found toxic effects of another carbamate insecticide, pirimicarb, on collembolan community.

IV. 3 Effects of carbofuran on the taxonomic groups and community structure of Acari

The response of mites over the increasing Furadan dosages was again more pronounced within the Brazilian assay. The continuous increase in the relative abundance of Oribatids, that are particle feeding saprophages and mycophages (Krantz, 2009), may be related with a reduction in the number of competitors (namely, collembolans) for the available organic matter (Filser, 2002). Moreover, their typically high body sclerotization (Norton and Behan-Pelletier, 2009), may work as a biological barrier to pesticide penetration conferring them higher resistance against carbofuran (Martin, 2007).

In parallel, the decline observed for predator mites (Mesostigmata), was probably a consequence of the strong decrease of collembolans, their potential preys (Koehler, 1997). Despite this, a direct toxic effect of the insecticide cannot be excluded (Koehler, 1997).

At the Portuguese assay, the taxonomic profile of the mite community was different, with a shared dominance of Oribatida and Prostigmata. The observed decrease of collembolans was also expected to cause the reduction of mesostigmatid mites. However, the relative abundance of the four taxonomic groups of mites remained more or less constant at all Furadan doses, causing no significant effects at the community level. The maintenance of the community structure might have been facilitated by prostigmatid mites, extremely diverse in their feeding habits (Walter et al, 2009). Thus, these mites might have competed with oribatids for organic detritus and with mesostigmatids for collembolans or could also be used as food for the latter predatory mites (Koehler, 1997). Despite this, their lower level of body sclerotization (Walter et al, 2009) should foreseen a higher sensitivity to carbofuran (Martin, 2007).

IV. 4 Taxonomic and morphological trait changes of communities of Collembola induced by carbofuran contamination

The most significant shifts in the relative abundance of collembolan families and, consequently, in the global community structure due to carbofuran contamination were observed for the Brazilian experiment. The two major trends observed were the significant increase of relative abundance of Entomobryidae and the decline in Isotomidae, observed at the four highest Furadan doses.

For the Portuguese experiment, the same tendencies were observed, although the low number of organisms found per treatment (if compared with the Brazilian assay) might have contributed to a higher variability and impaired the establishment of more reliable trends. Despite this, significant shifts in the global community structure (in relation to that of control) were detected at the highest furadan dose.

Similarly, in an 8 week study, the total abundance of Entomobryidae collected in pitfall traps of Brazilian cornfields subjected to chlorpyrifos spraying, increased 18%, while for Isotomidade a decline of 77% was registered (Michereff-Filho et al, 2004).However, under temperate conditions, the 44 d exposure of collembolans to the same insecticide caused a significant decline in the abundance of both Isotomidae and Entomobryidae (Frampton and Van den Brink, 2007).

Trait analysis also revealed significant changes in the community of collembolans induced by carbofuran. The diversity of combinations of trait scores, considered in the present study as morphospecies richness, decreased along the contamination gradient, as revealed by the two diversity indices (Table 6.4).

In both assays, the values of mT calculated for the communities of collembolans increased along the gradient of carbofuran concentrations (Table 6.4) and suggest that species adapted to deeper soil layers are more

vulnerable to toxic effects of this insecticide. Moreover, these results also indicate a shift in the functional composition of the communities, namely that epigeic species increased their representativeness along the contamination gradient. A consistent match is noticeable when linking these results with the life form traits assigned to the organisms of the most abundant families and with the variation in their relative abundances. Indeed, individuals from the family Entomobryidae, that are mostly epigeic species (Hopkin, 1997), presented the highest values of life form trait and increased their relative abundance and the opposite was registered for organisms assigned into the family Isotomidae, that are generally euedaphic species (Hopkin, 1997). Members of the family Onichiuridae, that have euedaphic life forms (Hopkin, 1997) and dominated the Portuguese communities, theoretically, could also disappear (or decrease their numbers) and be substituted by epigeic species. Although not significant, this tendency was observed for the two highest furadan doses. Despite this, much probably, hemiedaphic species also contributed to the observed increase in the mT.

Also, our results are in accordance with the assumptions of the biological quality index (QBS index) developed by Parisi et al (2005), where the impact on soil quality is evaluated by the loss of microarthropod morphospecies possessing ecomorphological traits that indicate a true edaphic life.

A possible explanation to these community shifts might be indirectly related with one of the Collembola adaptations against drought. Epigeic collembolans developed a low cuticular permeability, which provides them high resistance towards desiccation contrasting with the high cuticular permeability of euedaphic species (Kærsgaard et al, 2004). Since the cuticle also constitutes a biological barrier against the penetration of pesticides (Gillot, 1995; Martin, 2007) and carbofuran is highly soluble in water (320g/l at 25ºC; http://extoxnet.orst.edu/pips/carbofur.htm; Assessed 24th May 2011), the epedaphic collembolans, possessing a less permeable cuticle and less contact with soil pore water (Hopkin, 1997) would be less exposed to this insecticide. The higher mobility and lower contact with the soil pore water of epedaphic Collembola when compared with the euedaphic ones was the

explanation pointed by Fountain and Hopkin (2004) for the lower toxicity observed for epigeic springtails along a gradient of metal contamination.

FD index followed the decreasing of taxonomic diversity only for the Brazilian assay indicating that carbofuran contamination decreased the diversity in morphospecies traits within each community (Diaz et al, 2007). The opposite pattern found for the Portuguese assay is difficult to explain as the diversity of morphospecies drastically diminished at the highest carbofuran concentrations (e.g. from 11 in the control to 2 at the highest dose, Table 6.4). However, considering that FD index is a sum of the trait dissimilarity of all pairs of species, weighted by the their relative abundance (Vandewalle et al, 2010) and that along the contamination gradient the proportion (or relative abundance) of the morphospecies more dissimilar in terms of Life form trait increased (in comparison with lower Furadan doses), the final FD value would also be higher, which was observed for the Portuguese assay.

Despite it is not mandatory to find a correlation between species diversity indices and FD (Vandewalle et al, 2010), probably, the inclusion of more trait data (e.g. association to disturbed systems and stress tolerance) as well as the extension of the exposure period would allow to clarify these responses.

Thus, in the present study, the more sensitive descriptor of the community responses to the insecticide disturbance was the mT index rather than the global functional diversity as reported in other case studies presented before (Vandewalle et al, 2010).

Summarizing, in the case study presented, with the application of a trait based assessment of carbofuran effects on the community of collembolans, it was possible to identify which morphological characteristics make the organisms more vulnerable to insecticide contamination.

Ecological relevance was favored by using the original community of organisms as test-groups instead of laboratory introduced species or toxicity data collected from literature and subsequently grouped and analyzed (De Lange et al, 2009). However, further research, especially with other groups of

soil organisms are needed to gain clearer insights over the sensitivity of communities to pesticides and other toxic substances.

V. Conclusions

The present study showed the feasibility of assessing effects of pesticide applications at community level under a laboratory context. Moreover, it opened good perspectives of making reliable extrapolations among geographically distinct communities since the patterns of response of both microarthropod communities to carbofuran contamination were similar, although a higher toxicity was observed for the Brazilian assay.

Thus, significant shifts in the overall community structure of both microarthropod communities, reflected by a decrease in abundance and the impoverishment of taxonomic diversity were detected. Direct and strong negative effects were observed for Collembola while the abundance of Acari tended to increase with higher carbofuran concentrations.

Lowering the taxonomic level of assessment in the two most abundant groups, Acari and Collembola, the patterns of response were clarified. Thus, for mites, significant community shifts were only detected for the Brazilian organisms and were reflected by the increase of oribatids and the reduction of mesostigmatids.

Among collembolans, data revealed that individuals from Entomobryidae seem to have replaced Isotomidae along the contamination gradient.

Trait based assessment of effects showed to be sensitive in revealing the community responses of Collembola to insecticide contamination. Main trends comprised the decrease in species diversity (expressed as different combinations of trait scores) in treated soils accompanied by a major functional shift, the favoring of epedaphic species with loss of euedaphic species representativeness. This shift was consistent with the changes observed at the family level and may be a consequence of their

ecophysiological characteristics, namely the cuticular permeability. This is usually reduced in epigeic species (present in the family Entomobryidae) and higher in euedaphic ones (like Isotomids) and may confer increased protection against the insecticide penetration in the former organisms. Further methodological refinements are necessary to improve the information taken from this type of approach.

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Appendix

Table 6A. Collembola species traits and corresponding scores used to define the morphospecies.

Trait	Trait modality	Score
Ocelli	Absent	1
	Present	5
Antenna length	0 < X < 0.5 body length	1
	0.5 body length < X < 1 body lenght	3
	> 1 body lenght	5
Furca	Absent	1
	Reduced/short	3
	Fully developed	5
Hairs/scales	Absent	1
	Presence of hairs	3
	Presence of hairs and scales	5
Pigmentation	White	1
	Coloured, no patterns	3
	Coloured, patterns	5

Table 6B. Morphospecies (MPH) within Collembola families defined according to different combinations of trait scores for Portuguese (PT) and Brazilian (BR) assays. Entom - Entomobryidae; Isotom - Isotomidae; Onych - Onychiuridae; Podur - Poduridae; Sminth - Sminthuridae; Pigm - Pigmentation.

MPH	Family	Trait score				
		Ocelli	Antenna lenght	Furca	Hairs/Scales	Pigm
PT						
a	*Entom*	*5*	*1*	*5*	*5*	*3*
b	*Entom*	*1*	*1*	*5*	*5*	*1*
c	*Entom*	*5*	*1*	*5*	*5*	*1*
d	*Entom*	*5*	*1*	*5*	*1*	*3*
e	*Entom*	*5*	*3*	*5*	*5*	*3*
f	Isotom	1	1	3	3	1
g	Isotom	1	1	3	3	3
h	Isotom	5	1	3	3	3
i	Isotom	5	1	3	3	1
j	Isotom	1	1	5	3	1
l	Isotom	5	1	5	3	3
m	*Onych*	*1*	*1*	*1*	*3*	*1*
n	*Onych*	*1*	*1*	*1*	*3*	*3*
o	Podur	5	1	5	5	3
p	Podur	5	1	1	3	3
q	Podur	1	1	1	3	1
r	*Sminth*	*5*	*3*	*5*	*3*	*3*
s	*Sminth*	*1*	*3*	*5*	*3*	*1*
t	*Sminth*	*5*	*3*	*5*	*3*	*1*

Table 6B. (continued):

MPH	Family	Trait score				
		Ocelli	Antenna lenght	Furca	Hairs/Scales	Pigm
BR						
a_1	*Entom*	*5*	*1*	*5*	*5*	*1*
b_1	*Entom*	*5*	*1*	*5*	*5*	*3*
c_1	*Entom*	*5*	*1*	*5*	*3*	*3*
d_1	*Entom*	*1*	*1*	*5*	*5*	*3*
e_1	*Entom*	*5*	*1*	*5*	*3*	*1*
f_1	*Entom*	*5*	*3*	*5*	*5*	*5*
g_1	*Entom*	*5*	*1*	*5*	*5*	*5*
h_1	*Entom*	*5*	*3*	*5*	*5*	*3*
i_1	Isotom	1	1	5	3	1
j_1	Isotom	1	1	5	3	3
l_1	*Onych*	*1*	*1*	*1*	*3*	*1*
m_1	*Onych*	*1*	*1*	*1*	*3*	*3*
n_1	Isotom	1	1	3	3	1
o_1	*Entom*	*5*	*1*	*3*	*3*	*1*
p_1	*Entom*	*1*	*1*	*5*	*5*	*1*
q_1	*Entom*	*1*	*1*	*5*	*5*	*5*
r_1	Isotom	5	1	1	3	1
s_1	*Sminth*	*1*	*3*	*5*	*3*	*1*

Table 6C. Effects of to increasing Furadan doses in the responses of two soil microarthropod communities from Portugal (PT) and Brazil (BR): summary of Taxa contributing (expressed as percentage; % contrib.) most to the observed dissimilarity (Av. Diss.; expressed as percentage) between treated soils and the respective control, in which significant differences in community composition were detected (ANOSIM $p < 0.05$, followed by SIMPER analysis). Negative signs (-) represent a decrease in the relative abundance of the Taxa in Furadan treated soils in comparison to the control while positive signs (+) mean that abundance in the treated soils increased relatively to the control. Coll - Collembola; Acar - Acari; Form - Formicidae; Prot - Protura; Ento - Entomobryidae; Onyc - Onychiuridae; Isot - Isotomidae; Pros - Prostigmata; Meso - Mesostigmata.

Endpoint	Experiment /Country	Furadan Doses (% 2 × RD)	Av. Diss.	Taxa/Group (% contrib.)
Microarthropod community composition	PT	50	35.7	Coll (- 40.4) Acar (- 31.5)
		100	29.1	Coll (- 54.92) Acar (- 26.1)
	BR	2.5	22.7	Prot (-23.8) Form (+11.9) Coll (-15.9)
		10	23.7	Prot (-16.9) Form (+20.7) Coll (-22.9)
		25	23.2	Prot (-15.6) Form (+22.9) Coll (-27.4)
		50	27.5	Prot (-15.2) Form (+16.3) Coll (-34.1)
		100	36.6	Prot (-12.9) Form (+11.2) Coll (-50.5)

Table 6C. (Continued)

Endpoint	Experiment /Country	Furadan Doses (% 2 × RD)	Average Diss.	Taxa/Group (% contrib..)
Collembola community composition	PT	100	59.5	Ento (+35.3) Onyc (-30.5) Isot (-29.4)
	BR	5	18.8	Ento (+11.7) Onych (+25.5) Isot (-53.9)
		10	31.5	Ento (+11.5) Onyc (-10.9) Isot (-71.4)
		25	36	Ento (+10.7) Isot (-73.8)
		50	32.4	Ento (+10.9) Onyc (+12.9) Isot (-70.2)
		100	40.8	Ento (+18.3) Isot (-56.8)
Mite community composition	BR	5	8.7	Pros (-72.7) Meso (-18.0)
		10	10.8	Pros (-66.8) Meso (-24.2)
		25	17.3	Pros (-62.9) Meso (-29.0)
		50	23.1	Pros (-48.3) Meso (-42.9)
		100	29.9	Pros (-54.8) Meso (-37.2)

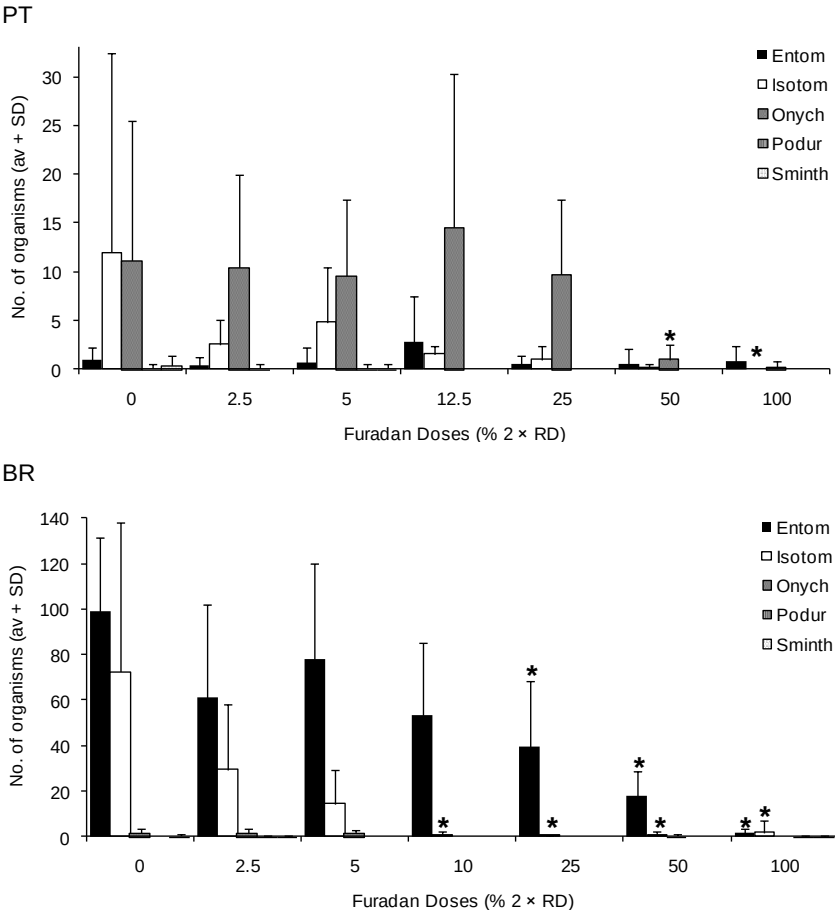
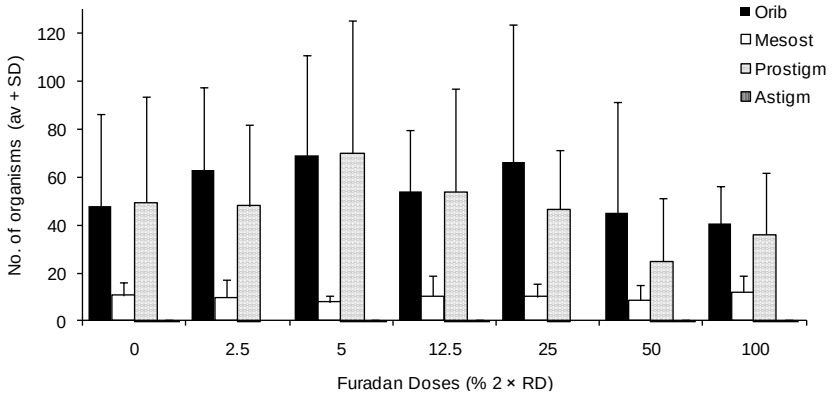


Figure 6A. Effects of Furadan (a.i. carbofuran) contaminated soil on the total abundance of the families of Collembola found in two soil microarthropod communities from Portugal (PT) and Brazil (BR). Values express average (± standard deviation - SD) values. RD - Recommended Dose; * – Statistically different from the respective control (One Way Anova and Dunnet Test or Kuskall Wallis test and multiple comparisons with the control; $p < 0.05$); Entom - Entomobryidae; Isotom - Isotomidae; Onych - Onychiuridae; Podur - Poduridae; Sminth - Sminthuridae

PT



BR

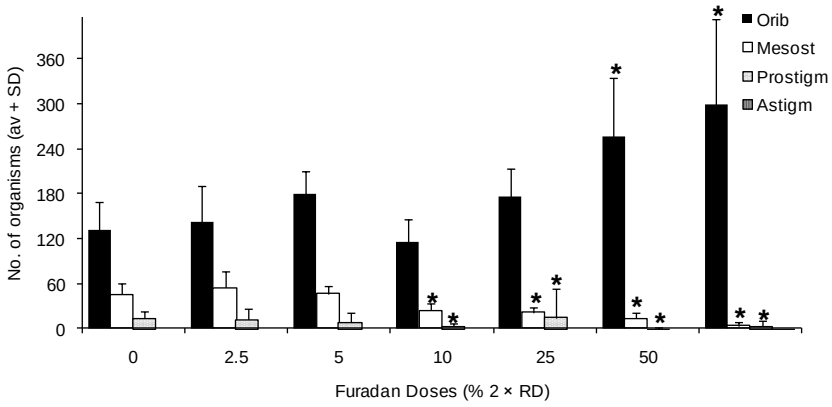


Figure 6B. Effects of Furadan (a.i. carbofuran) contaminated soil on the total abundance of the four groups of Acarina found in two soil microarhopod communities from Portugal (A) and Brazil (B). Values express average (± standard deviation - SD) values. RD - Recommended Dose; * – Statistically different from the respective control (One Way Anova, Dunnet Test, $p < 0.05$). Orib - Oribatida; Mesost - Mesostigmata; Prostigm - Prostigmata; Astigm - Astigmata.

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Chapter 7

General Discussion

I. Ecotoxicological evaluation of the efficacy of a bioremediation tool for atrazine contaminated soils

In Ecological Risk Assessment (ERA) of sites contaminated with pesticides or of remediated soils, the concentration of a certain chemical below the legislation limits in a soil sample does not necessarily mean that this is a clean soil and that habitat and retention functions (ISO, 2003) have been restored.

Since the bioavailability of a certain chemical is strongly dependent on soil properties and other environmental conditions (Lanno et al, 2004), ecotoxicological tests such as the ones used in the studies conducted in chapters 2 and 4, can contribute to a more proper monitoring of the cleanup process in soil as well as the potential consequences for the aquatic biota. Also, besides being simple and cheap tests, they proved to be sensitive indicators of chemical and biological degradation of the contaminant (in this case atrazine). Moreover, results were obtained in a relatively short period of time, i.e., at most 42 days (reproduction of *E. andrei*; see chapter 2) after the application of the bioremediation tool. Therefore, they showed to be valuable choices to include in the testing strategy in further schemes of (bio)remediation. Notwithstanding, to get a quick perception of the efficacy of the remediation process and on the restoration of soil functions, it is advisable to include other short-term tests, measuring other endpoints not covered in the present study, like behavior, lethality and functional parameters.

A higher degree of ecological realism was also achieved with the experiments performed in the laboratory simulator. Indeed, in the microcosms experiments (Chapter 2), the starting points were artificial assemblages consisting in columns of defaunated soil with introduced organisms, under controlled environmental conditions (temperature, light and humidity). In the experiments with the laboratory simulator, the native community of soil fauna was maintained and the bioremediation process took place under external temperature and humidity. Moreover, a third worst-case scenario of aquatic contamination due to runoff of atrazine was evaluated (Chapter 4).

Although atrazine has been banned in the European Union, it is still widely used worldwide (e.g. Getenga et al, 2009; Yang et al, 2010; Correia et al, 2007). Therefore, this cleanup strategy, comprising the monitoring of the

cleanup process combining chemical analysis and ecotoxicological tests may be useful to reduce and/or mitigate the ecological and human risks of atrazine misapplications in other parts of the world. Indeed, comparable results from microcosms (chapter 2) and semi-field (chapter 4) testing of the bioremediation tool prove both its efficacy in reducing soil concentrations of atrazine and its metabolites no non-hazard levels to standard test-species as well as the success of the scaling up to a higher tier level (Table 7.1). Thus, the use of this cleanup tool under field conditions is promising. However, prior to a full implementation in real contamination scenarios, a further scaling up and validation under field conditions and with other soil types is required.

Table 7.1. Cleanup of soils contaminated with 10×RD of Atrazerba (a.i. atrazine 350g a.i./L) and sprayed with a bioremediation tool (*P.* ADP+Citrate; only for bioremediated soils) when tested at microcosms (M) and semi-field (S-F) scale (data from chapters 2 and 4). Values express comparisons control vs bioremediated (B) and control vs non bioremediated (NB) soils. See Chapters 2 and 4 for details. n.d. - not determined.

Evaluated Endpoints (%)	**B**		**NB**	
	M	*S-F*	*M*	*S-F*
Atrazine remaining in soil*	17	1	n.d.	46
Maximum growth inhibition of *P. subcapitata* in leachates	12	18	87	17
Maximum growth inhibition of *P. subcapitata* in eluates	0	12	90	92
Maximum decrease in shoot dry weight per emerged seed of *A. sativa*	16	0	86	70

* - 5 or 7 days after the beginning of the experiment, respectively for the testing at microcosms (Chapter 2) or semi-field (Chapter 4) scale.

II. Development of a laboratory simulator for pesticide applications

In chapter 3, a prototype of the laboratory simulator was developed, tested and validated under three worst-case scenarios of pesticide contamination. In chapter 4, after some improvements in the simulator previously developed, a parallel trial was carried out with the herbicide atrazine. The system proved to be an efficient tool, allowing the mimicry of pesticide field applications under different field slopes, as well as the collection of three types of samples (soil, runoff and leachates) that can be used to obtain data both and fate and effects on soil and aquatic compartments.

In chapter 3, results from ecotoxicological tests performed with samples collected from the simulator trial (and also from soil chemical analysis) were comparable to the ones obtained in a parallel experiment comprising the field application of carbofuran (derived toxicity endpoints varied between 1 to 3.8 times; chapter 3). Thus, the developed simulator was validated as a cost-effective tool able to mimic the pesticide applications (and their ecotoxicological consequences) under field conditions.

Focusing on indirect effects for the aquatic compartment, a rank of toxicity can be established for the three types of aqueous samples collected in the simulator trials and tested for their toxicity to cladocerans (chapter 3) and microalgae (chapter 4). Thus, for cladoceran lethality, the rank of sensitivity was: runoff > leachates > eluates (chapter 3); while for microalgae growth inhibition was: runoff ≈ eluate > leachate (chapter 4).

These results show that, at least for some pesticides, the ecological risk might be underestimated if only eluates are used as surrogates of the potential toxic effect of pesticide applications for the aquatic systems. They confirm that runoff from agricultural fields, often neglected in effects assessment, is one of the most relevant routes of entry of pesticides and their degradation metabolites into surface waters. With the continuous release of new pesticide formulations, previously subjected to several tests before registration and authorization of use, to ensure its safety to Humans and ecosystems, as well as with the reviewing of older pesticides, an increase in the demand for ERA schemes is expectable. Therefore, the existence of cost-effective tools, able to mimic scenarios of field contamination and obtain

realistic data can be of enormous usefulness. Particularly innovative is the possibility of obtaining an integrated perspective on the risks of pesticide applications for both soil and water compartments.

Further improvements foreseeing the full validation and standardization of this tool comprise the testing with other soil types and chemicals. It is also recommended to increase the battery of ecotoxicological tests (e.g. including functional tests). It would also be interesting to evaluate the practicability of using soil monoliths instead of sieved soil. If feasible, it is theoretically possible to perform higher tier semi-field soil community studies.

III. Ecological Risk Assessment of pesticides under tropical and warm temperate conditions

The set of experiments presented in chapters 3, 5 and 6 contributed to increase the data on pesticide toxicity under tropical and Mediterranean conditions. Results showed that, in tropical environments, the application of the recommended doses of Furadan (the commercial formulation of carbofuran tested) may constitute a risk for both soil and aquatic organisms. Indeed, data from chapter 3 suggest that in field scenarios of carbofuran applications, populations of collembolans and earthworms may be subjected to lethal and sub-lethal effects. The aquatic biota can be particularly endangered if runoff inputs from agricultural fields, immediately after carbofuran application, can reach the nearby water basins. Groundwater resources might also be vulnerable due to leaching.

According to results from chapter 5, non-target nematode communities might undergo a decrease in total abundance as well as in diversity, i.e., a reduction of the number of families with consequent changes in the global family structure, as a result of carbofuran soil sprayings. Despite this, as discussed in this chapter, maybe due to the mode of action of this insecticide/nematicide, strong lethal effects are not expected, at least within the range of dosages tested.

By the contrary, results from chapter 6 highlight the risks of carbofuran applications to non-target microarthropods, reflected by the general decrease

in the abundance of organisms, impoverishment of taxonomic diversity and shifts in functional diversity (the last effect was observed in the communities of collembolans).

For both nematode and microarthropod communities, the patterns of responses to carbofuran contamination between tropical and warm temperate soils were very similar (Chapters 5 and 6).

The assessment of pesticide ecotoxicological effects under tropical conditions have been generally performed at higher temperatures than the ones used in the present studies (22 to 23 ºC versus 25 to 29 ºC; Garcia et al, 2004; De Silva et al, 2009; De Silva and Van Gestel, 2009a,b; De Silva et al, 2010; Nunes, 2010; Moreira et al, 2010). However, when comparing the output of the tests at different temperatures, Garcia (2004) found that for field soils, soil properties rather than temperature had more influence in the observed toxicity. Similar findings, for long-term tests, were reported by De Silva et al (2009). The results obtained in chapters 5 and 6 somehow corroborate these findings. Indeed, the different soil contents in organic matter, silt and clay much probably determined the higher sensitivity of the Portuguese nematode community (Chapter 5) and the Brazilian microarthropod community (Chapter 6) to carbofuran contamination, rather than the 2 and 3 degrees difference among tropical and temperate values for temperature, respectively for the nematode and microarthropod tests (Chapters 5 and 6, respectively).

A more accurate assessment of the risks of pesticide contamination under tropical and warm temperate conditions would also be achieved if standardized native and representative species of these two areas, from similar taxonomic groups to the ones used in the ecotoxicological tests performed in chapters 3 and 4, were available. Comparisons of species sensitivity from different eco-regions could thus be performed. Despite the indications of similar sensitivity of tropical and temperate earthworm species for pesticides (De Silva et al, 2009, 2010; Garcia et al, 2008; 2011), to obtain more consistent trends, other pesticides and species from different taxonomic groups (e.g. collembolans, enchytraeids, isopods) should be tested.

Notwithstanding, for tropical countries, the standardization of native test species has gained some developments with the works of Garcia (2004) and

Buch (2010), where some tropical species were used in ecotoxicological tests, including the earthworm *Pontoscolex corethrurus*. However, the tests were performed with populations collected in the field due to the reported problems of breeding these species in laboratorial mass cultures and thus, they are not likely to be standardized in the near future (Garcia, 2004). However, De Silva et al (2010) and De Silva and Van Gestel (2009b) used laboratory cultures of another tropical earthworm, *Perionyx excavatus* to investigate the effects of pesticide soil contamination, foreseeing the standardization of this species as representative of tropical systems.

Besides the above mentioned issue, the adaptation of the available test guidelines and/or the standardization of new protocols to the soil types and environmental conditions typical of tropical and conditions is also necessary (Garcia, 2004; Kuperman et al, 2009). These recommendations are also valid for warm temperate regions like the Mediterranean, although, realistically, due to its lower geographical representativeness, not all of them may be achievable, even within a long-term period of time. But undoubtedly, the incorporation of more data on pesticide toxicity within these two areas would be a great step towards the accomplishment of these proposals.

IV. Community Testing in Ecological Risk Assessment of Pesticides

To go further towards the implementation of community testing in ERA of pesticides in soil, a new community approach was presented and tested in chapters 5 and 6.

This approach somehow combined some of the advantages of both field/semi-field and laboratorial tests. Indeed, the natural variability and complexity of interactions between species is covered and effects can be described in terms of structural endpoints (e.g., trophic and taxonomic structure, abundance, number of families) but also with classical ecotoxicological endpoints like mortality, reproduction or growth. In fact, results show that the most powerful indicators of carbofuran toxicity were nematode abundance/mortality, number

of families and family structure (chapter 5) and, in case if microarthropods, taxonomic diversity plus overall abundance (Chapter 6).

As discussed in chapter 5, the sensitivity of nematodes to carbofuran seemed to be similar to that of earthworms. In case of microarthropods, the effects of carbofuran on the abundance of collembolans from Brazil (chapter 6) can be compared with the LC50s calculated for the tests with *F. candida* presented in chapter 3 (and performed in the same contaminated soil). Indeed, a correspondence can be found between the single-species and the community test: carbofuran concentrations ranging from 0.039 and 0.079 mg/kg caused a decrease of about 50% in the abundance of collembolans (chapter 6) while the derived LC50 for *F. candida* for the field assay was 0.057 mg/kg (chapter 3). Although more evident for the microarthropods, results from community testing suggest a similar sensitivity to single-species tests with other soil invertebrates.

Notwithstanding, the approaches presented here intended to be starting points for further studies. Moreover, a wide range of sensitivity of these and other communities of soil organisms to other pesticides and/or classes of chemicals is expected, as it happens with the different test-organisms from different groups usually used in ecotoxicological tests (Jänsch et al, 2006).

Some methodological problems were discussed in chapters 5 and 6. A major drawback was the incubation effect observed, i.e., the low recovery of organisms in the controls compared with the initial community and the changes in the community structure. This means that, either the extraction plus inoculation of the test-soils or the exposure under laboratorial controlled conditions to a batch of defaunated soil, worked as a stress factor to the organisms. Regarding the former hypothesis, both nematode extraction and inoculation as well as microarthropod extraction procedures were simple, quick and allowed the introduction of several species into the soil, also minimizing direct handling of organisms and thus, theoretically, diminishing handling related stress. The negative consequences of exposing several unknown species in laboratorial tests were somehow expected since it was not possible to fulfill the ecological requirements (e.g. temperature, humidity, food availability) of all the organisms. Also, the high variability among replicates often impaired the establishment of statistically significant

relationships, despite the homogenization procedures of samples in both assays to minimize spatial variability (Chapters 5 and 6). Further improvements include the increase of the exposure period, which possibly could help to obtain more consistent and clear trends, consider the supply of food at least for some organisms during the tests, to prevent mortality and facilitate reproduction.

Much of the above reported problems are common to other ecotoxicological tests with communities of organisms (Moser et al, 2004; Schäffer et al, 2010) and the number of standardized tests is fairly low. Only two are available for soil: the Terrestrial Model Ecosystem (TME) (a semi-field test; for a recent overview on other semi-field methods for ERA of pesticides in soil see Shäffer et al, 2010) and the earthworm field test (ISO, 1999). Recently, Römbke et al (2009) proposed a new field method to assess the effects of pesticides with soil mesofauna.

Regarding the assessment of effects based on species traits, in case of nematodes this approach was not sensitive enough to detect differences in the communities due to carbofuran contamination. This lack of sensitivity should be confirmed with other chemicals and nematode communities. Other traits (e.g. body size, life span; Mulder et al, 2011) might be more sensitive descriptors of pesticide contamination.

The case study of TERA carried out with the community of Collembola on chapter 6 constitutes, as far as it was possible to check in the literature, the first application in soil ecotoxicology. This approach was sensitive to detect significant shifts in the trait composition of communities of collembolans in carbofuran treated soils. A link was found between the increase of epigeic species (with concurrent loss of euedaphic species) and the increase in carbofuran contamination that could be connected not only to the higher sensitivity but also to a higher exposure of the later life-form group. However, more studies are needed to evaluate the relation between traits and ecotoxicological processes in soil, namely evaluating the sensitivity of different life-form groups or the probability of exposure according to compound mobility, to achieve a better understanding (and be able to make predictions) of population vulnerability.

Based on the vulnerability conceptual model of Van Straalen (1994), Rubach et al (2011) proposed three main categories for the use of traits in ecotoxicology: external exposure, intrinsic sensitivity and population sustainability. For each category, several traits were defined and the availability of data and the link between the trait and the affected process was evaluated.

One of the challenges to TERA is the scarcity of good quality data and comparable trait databases (Rubach et al, 2011). For aquatic organisms, the existence of several trait databases for specific taxa (some examples in Culp et al, 2011), allowed the use of soft traits (easy to measure but not necessarily related with a specific function like maximal size, aquatic stage, respiration, locomotion; Dubey et al, 2011) and hard traits (usually less easy to measure but with direct relationship with function; Dubey et al, 2011) such as dissemination, reproduction, aquatic stage, life duration. (e.g. Charvet et al, 2000; Ducrot et al, 2005; Baird and Van den Brink, 2007).

In TERA of soils, and taking into account the scarcity of trait data and databases when comparing with aquatic systems, it is advisable to use a reduced number of soft traits, easy to measure in several species and sites. If enough bibliographic information on the species/taxonomic groups under study is available, the use of hard traits should also be included. For example, measuring soft traits that reflect the vertical distribution in soil and dispersion abilities (such as the ones used for collembolans in Chapter 6), can give indications not only on the ability of organisms to avoid unfavorable conditions (thus decreasing external exposure), but also the potential recolonization of damaged habitats. Also, body size can give information about the bioconcentration of the pesticide and the mobility of organisms in soil. Moreover, feeding habits, that can be defined based on the morphology of the mouthparts and might be used to assess the exposure via food and the changes in the food sources.

Summarizing, the proposed community approaches showed to be valid and promising tools to be further tested and implemented in ERA of pesticides. Further validation and standardization should comprise the performance of parallel standardized single-species tests and field or semi-

field studies with soil invertebrates, to compare the sensitivity of both approaches.

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