Universidade de Aveiro Departamento de Biologia
Ano 2014

MARIA DOS PRAZERES RODRIGUES DE LIMA

EFEITO COMBINADO DE STRESSORES QUÍMICOS E NATURAIS EM ORGANISMOS DO SOLO E PLANTAS

COMBINED EFFECTS OF CHEMICAL AND NATURAL STRESSORS TO SOIL ORGANISMS AND PLANTS

Universidade de Aveiro Departamento de Biologia
Ano 2014

MARIA DOS PRAZERES RODRIGUES DE LIMA

EFEITO COMBINADO DE STRESSORES QUÍMICOS E NATURAIS EM ORGANISMOS DO SOLO E PLANTAS

COMBINED EFFECTS OF CHEMICAL AND NATURAL STRESSORS TO SOIL ORGANISMS AND PLANTS

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares (Professor Catedrático do Departamento de Biologia da Universidade de Aveiro) e co-orientação da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro).

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma bolsa de doutoramento (SFRH/BD/ 65274/ 2009) atribuída a Maria dos Prazeres Rodrigues de Lima. E do "Programa Alban" apoio da união européia para estudantes latino americanos - (E07D402936BR) E pelo projeto CLIMAFUN (FCOMP-01-0124-FEDER-008656).

Ao Único, cujo Ser é a causa da minha existência, cuja alegria
é a minha fôrça, cujo amor é a causa de minha redenção, dedico.

o júri

Presidente	Mário Guerreiro Silva Ferreira Professor catedrático do Departamento de Engenharia de Materiais e Cerâmica
	Prof. Doutor José Paulo Filipe Afonso de Sousa Professor Auxiliar daFaculdade de Ciências e Tecnologia da Universidade de Coimbra
	Doutora Sónia Alexandra Paiva dos Santos Investigadora auxiliar do Instituto Politécnico de Bragança
	Professor Doutor José Manuel Moutinho Pereira Professor auxiliar da Universidade de Trás-os-Montes e Alto Douro
	Doutora Glória Catarina Cintra da Costa Pinto Investigador em Pós-doutoramento CESAM & Departamento de Biologia da Universidade de Aveiro
	Professor Doutor Amadeu Mortágua Velho da Maia Soares Professor Catedrático do Departamento de Biologia da Universidade de Aveiro (orientador)

Agradecimentos

A Deus por tudo.

Ao meu Brasil que me proporcionou as bases para que eu pudesse hoje estar aqui.

Ào meu professor Dr. José Roberto Botelho de Souza da UFPE-Brasil que deu o pontapé inicial para que eu pudesse realizar este doutoramento.

Ao professor Dr. Fernando Morgado que foi intermediário de muitas coisas, ainda que eu nao soubesse, mas agradeço e peço desculpas pelo meu "silêncio".

Ao professor Dr. Amadeu Soares por dar-me esta oportunidade e proporcionar tudo o que foi preciso para o bom desenvolvimento do meu trabalho.

À Susana Loureiro, mais que professora, mais que co-orientadora, sempre com um sorriso no rosto e com uma solução para problemas "impossíveis".

Ao Abel pela sua valiosa ajuda e disponibilidade.

Aos colegas de Lab, que de uma forma ou outra fizeram diferença em minha vida.

Ao Darlan pela ajuda com as minhocas e ao Diogo pela parceria com os collembola.

À Vanessa sempre sorrindo, sempre disposta a ajudar, mesmo distante.

A minha amiga "Raquelita" por fazer com que minha vida na UA fosse mais doce, pelos almoços, pela companhia, pelas conversas.

Aos meus pais que com muito pesar abençoaram para que eu pudesse alçar vôos, mesmo sabendo que eu poderia não voltar. Amo-vos!

Aos meus amigos brasileiros que nos apoiaram em nossa vinda, aos quais me lembro a cada dia.

Ao marido, amigo, irmão, companheiro, filho, pai e pastor que embarcou nesta viagem sem saber ao certo o que nos aguardava. Superou barreiras, choques culturais, saudades, mas que permaneceu fiel ao que nos foi proposto. Te amo, Tiago!

A Ana Júlia, pedaço de Portugal que levarei para sempre em minha vida.

Ao meu irmão João Paulo Lima, pelas palavras fortes e decisivas em momentos de fraqueza, pela ajuda com a parte técnica da formatação da tese. Pelas belíssimas figuras que enriqueceram minha tese.

À Camilla Lima e Mikael pela ajuda na reta final.

À Igreja Plenitude da Palavra, pela torcida, pela constante oração para que tudo vá sempre bem, pelo acolhimento total em terra lusitana.

palavras-chave

Carbaril, organismos do solo, umidade do solo, temperatura, radiação UV, mudanças climáticas, pesticida, stress de seca, stress de alagamento, *Triticum aestivum, Brassica rapa, Folsomia candida, Eisenia andrei.*

Resumo

Nas últimas décadas, a Terra tem experimentado um aquecimento global e mudanças nos padrões de precipitação. Muitos estudos sobre a avaliação de risco de agrotóxicos em organismos não-alvo foram realizados com base em protocolos padronizados, com condições abióticas controladas. Mas, em campo, os organismos são expostos a flutuações de vários fatores ambientais, bem como a poluentes, que podem alterar os limites de tolerância dos organismos aos stressores naturais, bem como alterar a toxicidade ou biodisponibilidade do químico em causa. Considerando isso, o principal objetivo deste trabalho foi o de avaliar de que modo e em que medida os fatores ambientais (temperatura, humidade do solo e radiação UV) podem interagir uns com os outros ou afetar a toxicidade do carbaril para invertebrados do solo e plantas. Para isso, foram utilizadas quatro espécies padrão: *Folsomia candida, Eisenia andrei, Triticum aestivum e Brassica rapa*, e simulados diferentes cenários climáticos, com vários parâmetros letais e subletais analisados. A exposição combinada foi analisada utilizando, quando possível, a ferramenta MIXTOX, com base no modelo de referência de acção independente (IA) e possíveis desvios, assim como rácios sinergísticos/antagonísticos (a partir de valores de EC_{50}/LC_{50}), quando a dose-resposta de um dos stressores não foi obtida. Todos os fatores de stress aplicados isoladamente causaram efeitos significativos sobre as espécies testadas e sua exposição combinada com carbaril, apresentaram respostas diferenciadas: para as minhocas, a seca e temperaturas elevadas aumentaram os efeitos deletérios do carbaril (sinergismo), enquanto o alagamento e temperaturas baixas diminuíram sua toxicidade (antagonismo). Para os colêmbolos, o modelo IA mostrou ser uma boa ferramenta para prever a toxicidade do carbaril tanto para temperaturas altas como para as baixas. Para as duas espécies de plantas foram encontradas diferenças significativas entre elas: em termos gerais, as interações entre carbaril e os stressores naturais foram observadas, com sinergismo aparecendo como o padrão principal relacionado com a radiação UV, solos secos e temperaturas elevadas, enquanto o padrão principal relacionado com temperaturas baixas e stress de alagamento foi o antagonismo. Quando os efeitos de dois stressores naturais (radiação UV e humidade do solo) em plantas foram avaliados, uma interação significativa foi encontrada: a seca aliviou o efeito deletério da radiação UV em *T. aestivum* e o alagamento aumentou os seus efeitos, mas para *B. rapa* a adição de ambos os stresses de água causou um aumento (sinergismo) dos efeitos deletérios da radiação UV para todos os parâmetros avaliados. Portanto é necessário que as diferenças sazonais e latitudinais, bem como as mudanças climáticas globais, sejam integradas na avaliação de risco de contaminantes do solo.

keywords

Folsomia candida, Eisenia andrei, Triticum aestivum, Brassica rapa, climatic changes, carbaryl, ultraviolet radiation, temperature, soil moisture, Independent Action.

Abstract

In the last decades the Earth has been experiencing a global warming and changes in rainfall patterns. Many studies on risk assessment of pesticides on non target organisms have been performed based on standardized protocols, with controlled abiotic conditions, but, in field organisms are exposed to several environmental factors as well as pollutants, that may change the tolerance limits of organisms to natural stressors, as well as alter their toxicity or its bioavailability to organisms. Considering this, the main objective of this work, focused on how and to what extent environmental factors (temperature, soil moisture and UV radiation) may interact with one another or affect the toxicity of carbaryl to soil organisms and plants. For that, four standard species were used: *Folsomia candida, Eisenia andrei, Triticum aestivum and Brassica rapa*, different climatic scenarios were simulated and several endpoints were analyzed. The combined exposure was analyzed using, when possible, the MIXTOX tool, based on the reference model of independent action (IA) and possible deviations, but when the dose-response was not achieved; synergistic/antagonistic ratios were calculated from EC/LC$_{50}$ values. All stressors applied alone caused significant effects on species tested and its combined exposure with carbaryl showed different responses: for earthworms, the drought and high temperature increased the deleterious effects of carbaryl (synergism), while flood and low temperature decreased its toxicity (antagonism). For collembola the IA model showed to be a good tool for predicting the toxicity of carbaryl both for low and high temperatures. For the two plant species significant differences between their responses were found. In general terms, the interactions between carbaryl and natural stressors in plants occurred with synergism showing up as the main pattern related to UV irradiation, dry soils and high temperature, while antagonism, the main pattern related with low temperature and flood stress. When the effect of two natural stressors was evaluated (UV radiation and soil moisture), a significant interaction was found: the drought alleviated the deleterious effect of UV radiation on *T. aestivum* and the flood increased its effects, but for *B. rapa* the addition of both water stresses caused an increase (synergism) of deleterious effects of UV radiation for all endpoints evaluated. Therefore it is necessary that the seasonal and latitudinal differences, as well as global climate change, are integrated into risk assessment of soil contaminants.

Index

Chapter 1. General Introduction

1.1 Global Climatic Changes 1

1.1.1. The effects of climate change on soil ecosystem 2

1.1.2 Natural stressors 5

1.1.3 Effects of climate change on chemical fate and behavior in the environment. 9

1.1.4 Predictions of joint effects of natural and chemical stressors 11

1.2. Objective and thesis structure 13

1.3 General procedures 13

1.3.1 Test Organisms 13

1.3.2 Test chemical 14

References 18

Chapter 2. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios

Abstract 30

2.1 Introductionn 31

2.2 Materials and methods 33

2.2.1. Test substance and test species 33

2.2.2. Single exposure - chemical stressor 33

2.2.3. Single exposure - natural stressor 34

2.2.4. Combined exposure 35

2.2.5. Statistical analysis 35

2.3. Results 36

2.3.1. Single-exposure 36

2.3.2. Combined exposure 41

2.4. Discussion 44

2.4.1. Single stressor exposures 44

2.4.2. Combined exposures 46

2.5. Conclusions 48

Acknowledgements 49

2.6 Supplementary Data 50

References 54

Chapter 3 Carbaryl toxicity prediction to soil organisms under high and low temperature regimes

Abstract 58

Keywords: Temperature, Carbaryl, *Folsomia candida*, *Eisenia andrei*, *Triticum aestivum*, *Brassica rapa* 58

3.1 Introductionn 59

3.2 Materials and methods 61

3.2.1. Test species 61

3.2.2 Test soil and test chemical 62

3. 2.3. Single exposure to carbaryl 62

3.2.5. Combined temperature and carbaryl exposure 64

3.2.5. Statistics 65

3.3. Results 67

3.3.1. Effects of single exposure to carbaryl 67

3.3.2. Effects of single temperature on test species 70

3.3.3. Effects of combined exposure of carbaryl and temperature 71

3.4. Discussion 73

3.4.1. Effects of single carbaryl exposure 73

3.4.2. Effect of single temperature exposure 74

3.4.3 Combined exposure of Carbaryl and temperature. 76

3.5. Conclusions 79

Acknowledgments 80

3.6 Supplementary Data 81

References 82

Chapter 4. Responses of wheat (*Triticum aestivum*) and turnip (*Brassica rapa*) to the combined exposure of carbaryl and ultraviolet radiation

Abstract 94

4.1 Introduction 95

4.2 Material And Methods 96

4.2.1 Plants growth bioassay 96

4.2.2 Stressors: test chemical and UV radiation 97

4.2.3 Joint toxicity of carbaryl and UV radiation 98

4.2.4 Statistical analysis 99

4.3 Results 100

4.3.1 Single exposure to carbaryl and UV radiation. 101
4.3.2 Combined exposure to carbaryl and UV radiation...................................... 105
4.4 Discussion 109
4.4.1 Single exposure 109
4.4.2 Combined exposure 112
Acknowledgments 113
4.5 Supplementary Data 114
References 114

Chapter 5. Drought and flood conditions in soils affect the responses of crop plants to ultraviolet radiation

Abstract 124
5.1 Introduction 125
5.2. Material and Methods 126
5.2.1 Plants and growth conditions 126
5.2.2.Single exposure 127
UV radiation treatments 127
Drought and flood treatments 128
5.2.3 Exposure to combined treatments 128
5.2.4 Endpoints 128
5.2.5 Statistical analysis 129
5.3. Results 129
5.3.1 Single exposure 129
5.3.2 Combined exposure 130
5.4 Discussion 137
5.4.1 Single exposure 137
5.4.2 Combined exposure 138
5.5. Conclusions 140
Acknowledgments 140
Reference 141

Chapter 6. General discussion and final considerations

General discussion and final considerations 147
References 157

Figure index

Fig. 1.1. Chemical structure of carbaryl. 15

Fig. 2.1 Effect of carbaryl on *Eisenia andrei* survival after 7 and 14 days of exposure (A) and weight loss after 14 days of exposure (B). * and + - P≤0,05 Dunn's test, for 7 and 14 days, respectively, compared to the control 38

Fig. 2.2 Effect of carbaryl on the dry weight (DW) and fresh weight (FW) of *Brassica rapa* (A) and *Triticum aestivum* (B) after 14 days of exposure. * - P≤0,05 Dunn's test, comparison to the control (0 mg/Kg). 39

Fig. 2.3 Effect of carbaryl on *Brassica rapa* (A) and *Triticum aestivum* (B) length after 14 days of exposure. * - P≤0,05 Dunn's test, comparison to the control 40

Fig. 2. 4. Effect of carbaryl on the hydric content of *Brassica rapa* (A) and *Triticum aestivum* (B) after 14 days of exposure. * - P≤0,05 Dunn's test, comparison to the control (0 mg/Kg). 41

Fig**.** S 2.1. Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Brassica rapa* exposed to carbaryl under several drought regimes [10, 20, and 40% Water Holding Capacity (WHC)]: independent action (IA) model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3. 50

Fig. S 2.2 Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Brassica rapa* exposed to carbaryl under several flood regimes [80, 100, and 120% Water Holding Capacity (WHC)]: dose level (DL) deviation (A), antagonism deviation (B and C) and after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3. 51

Fig. S 2.3 Concentration–response for length (A), fresh weight (B) and dry weight (mg) of *Triticum aestivum* exposed to carbaryl under several drought regimes [10, 20, and 40% Water Holding Capacity (WHC)]: dose ratio (DR) deviation (A), antagonism deviation (B and C) after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3. 52

Fig. S 2.4. Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Triticum aestivum* exposed to carbaryl under several flood regimes [80, 100, and 120% Water Holding Capacity (WHC)]: dose level (DL) deviation (A and B) and antagonism (DL) deviation (C) after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3. 53

Fig. 3.1. *Folsomia candida* survival and reproduction. Effect of single carbaryl exposure (a) and single temperature exposure (b). Data is shown as mean value ± std. error. */+$p \leq 0.05$. Dunn's test: when temperatures are employed the 20 °C was used as control and for the carbaryl treatments the solvent control was used for comparison................69

Fig. 3.2. Effects of single temperature exposure on *Brassica rapa* (■) and *Triticum aestivum* (□) length (a), water content (b), fresh weight (c) and dry weight (d). Data is shown as mean value ± std. error. */ $p \leq 0.05$. Dunn's test, compared to the control (20ºC)..72

Fig. 4.1. The effects of UV radiation after 14 days of exposure on *Triticum aestivum* shoot length (A), fresh weight (B) and water content (C),and *Brassica rapa* shoot length (D), fresh weight (E) and water content (F). Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test)..104

Fig. 4.2 Dose-response relationship for the combination of carbaryl and UV- radiation on T. aestivum, showing an additive effect (Independent Action) for the parameter length (A) and dose- level dependent deviations for the parameters fresh weight (B) and dry weight (C). 107

Fig. 4.3 Dose-response relationship for the combination of carbaryl and UV- radiation on *B.rapa*, showing a dose-ratio dependent deviation from the IA model for the parameter length (A) and dry weight (C) and an additive effect (Independent Action) for the parameter fresh weight (B). 107

Fig. 5.1. Effects of UV radiation on *Triticum aestivum* shoot length (A, E, I) and water content (B, F, J) and *B.rapa* shoot length (C, G, K) and water content (D, H, L) subjet to different soil moisture regimes. Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test). 132

Fig. 5.2. Effects of UV radiation on *Triticum aestivum* fresh weight and dry weight in drought stress (A); control conditions (B) and flood stress (C). Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test). 133

Fig. 5.3. Effects of UV radiation on *B. rapa* fresh weight and dry weight in drought stress (A); control conditions (B) and flood stress (C). Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test). 134

Fig. 6.1. Climate model projections of rainfall changes and soil moisture by end of 21st Century in A1B scenario (based on average over all IPCC models). Dots represent areas where at least five of the nine models agree in determining that the change is statistically significant. Source: IPCC (2007). 148

Table index

Table 2.1 EC50, LC50 and NOEC values obtained after single exposures of *Eisenia andrei*, *Brassica rapa* and *Triticum aestivum* to carbaryl (mg/kg) and soil moisture (%WHC). Soil moisture is depicted as percentage of soil water holding capacity (WHC). 37

Table 2.2 Toxicity of carbaryl to *Eisenia andrei*, *Brassica rapa* and *Triticum aestivum* in different soil moisture regimes. Data is presented as EC_{50} or LC_{50} (mg/Kg) with standard error (SE). Moisture regimes are depicted as percentage of soil water holding capacity. 42

Table 2.3. Summary of the analysis done for the effects on length, fresh weight (FW) and dry weight (DW) of *Brassica rapa* and *Triticum aestivum* exposed to the combination of carbaryl and drought or flood regimes. IA is independent action; SS is the objective function used for continuous data; r^2 is the coefficient of determination; a and b are parameters of the deviation functions; S/A is synergism/antagonism; DR and DL are “dose ratio” and “dose level” deviation from the reference model....................43

Table 3.1 Summary of single and combined experiments for each test-species. In the combined exposures, the carbaryl ranges were used under each temperature...............65

Table 3.2 EC/LC_{50} values (mg/kg) and synergistic ratios (SR) calculated for carbaryl toxicity co-occurring at each temperature in tested species. Data from 20 ºC was used as the control to calculate the SR. Gray areas represent conditions that were not present in the experimental setup..68

Table 3.3 Median lethal temperature (LT_{50}) and median effective temperature (ET_{50}) values for the exposures of *Folsomia candida* and *Eisenia andrei* to low (8, 12,16 ºC) and high temperatures (22, 24, 26 ºC). 71

Table S 3.1 - Carbaryl concentrations (mg/kg) in Lufa 2.2 soil after soil spiking and after 15 days of exposure under 8ºC, 20ºC and 28ºC. Data is presented as mean values and standard values are in brackets. 81

Table S 3.2 - Seed germination (days to germinate and % of success) and plant survival of *Brassica rapa* and *Triticum aestivum* after exposure to carbaryl at different temperatures. 81

Table 4.1. Concentrations of carbaryl in soil after spiking (time zero-T0), after 15 days of exposure without UV-radiation (15d without UV) and exposed to UV-radiation (15d with UV), and carbaryl degradation (%) compared to T0. Data is presented as mean values and standard error in brackets.* $p<0.05$, t-test with T0. 100

Table 4.2. EC_{50}/ED_{50} values with standard error values (under brackets) and LOECs for the exposures of *Triticum aestivum* and *Brassica rapa* to carbaryl and UV radiation alone and upon a combined exposure. 103

Table 4 3 Sensitivity of *Brassica rapa* and *Triticum aestivum* to UV radiation, using the percentage of changes in DW and plants length and the biomass accumulation (dry weight) for all UV radiation doses used. 105

Table 4.4 Summary of carbaryl and ultraviolet radiation effect on *Triticum aestivum and Brassica rapa*. IA is independent action; SS is the objective function used for continuous data; r2 is the coefficient of determination; a and b are parameters of the deviation functions; S is synergism; DR and DL are “dose ratio” and “dose level” deviation from the reference model. 106

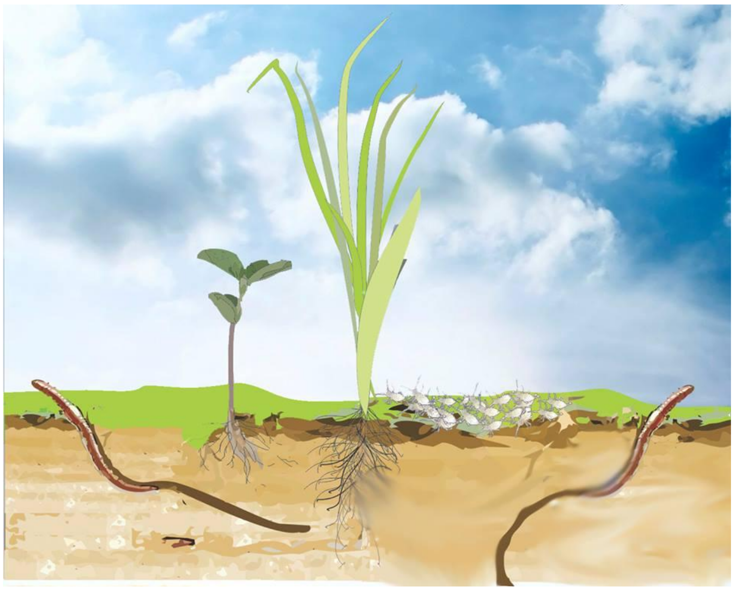
Table. S 4.1 - Comparative summary of the biologically effective UV radiation doses using different biological spectral weighting functions (BSWFs) commonly used for terrestrial plants in each time of exposure used in the present work.............................114

Table 5.1. ED50 (SE) and Sinergistic Ratios (SRs) for the exposures of *Triticum aestivum* and *Brassica rapa* to UV radiation under different soil moisture. 136

Table 6.1. Summary of the main responses of tested species to carbaryl under different natural stressors 154

CHAPTER 1

General Introduction



Chapter 1. General Introduction

1.1 Global Climatic Changes

Climate change is one of the greatest challenges and an increasing problem of the last decades, which has caused visible changes in environmental factors as temperature, UV radiation and rainfall around the world. These phenomena have generated different impacts and bring serious, far-reaching consequences for life on Earth.

The main factor contributing to climatic change is undoubtedly human activity which has caused large modifications on Earth's surface, where fossil fuel combustion, CO2 emissions, deforestation, afforestation, cultivation and mineral extraction are considered some examples to highlight.

The Intergovernmental Panel on Climate Change (IPCC) published four assessments about the impacts of climate change (IPCC, 2007). They report that in future decades the Earth will experience a global warming with an increase on the global mean air and ocean temperatures, occurrence of ice melts, and rising global sea level. Several scenarios have been modeled considering a substantial increased precipitation in Northern and Central Europe, North and South America, and Northern and Central Asia; on the other hand Southern Africa and Asia and the Mediterranean region are projected to experience severe drought conditions.

For the next few decades it is predicted extreme weather events, such as heat waves, heavy precipitation and thunderstorms (Cecchi et al., 2010). Therefore, a concern about the response of terrestrial ecosystems to this global climate change has increased substantially in recent years. Efforts have been concentrated on the effect of natural stressors on soil organisms, as well as the pollutants to which these organisms are normally exposed in the environment. Results from these studies have contributed to improve our understanding of the responses of soil organisms and plants to several factors as warming, UV radiation and changes in water availability. Nevertheless they are still scarce in order to derive accurate conclusions and therefore further investigation should be carried out.

1.1.1. The effects of climate change on soil ecosystem

The main factors that have been altered by global climate change are soil moisture and temperature regimes, which are closely related to the soil ecosystem functioning. The soil as substrate and source of recourses affects vegetation and edaphic organisms due it directs influence on water availability and soil temperature (Cheddadi et al., 2001), affecting species biology, biomass, composition and distribution in the ecosystem, i.e. the overall biodiversity functioning.

The effects of climate change may be different in different latitudinal areas (Lal, 2004). Regions projected to experience increasing temperatures and decreasing precipitation levels may reflect on a decrease of the net primary production; this can be the case of tropical regions, southern Europe and eastern USA (White et al., 1999).

The ecology, biology and distribution of soil organisms and plant fitness (especially for crops) depend critically on the degree of precipitation and evaporation, which is related to several abiotic factors such as temperature, soil moisture and UV radiation.

Many studies have clearly indicated significant indirect effects resulting from warming on soil organisms, that can be depicted in moist soils as an increase on fauna biomass or the opposite with a decrease in dry soils (Harte et al., 1996). Warming is also known to cause dispersal of plant parasitic nematodes, earthworms and other soil organisms (Boag, 1991; Ghini et al., 2008), increasing therefore the dissemination of plagues and the increase needs of pesticide application.

Having a different perspective, in some areas of the world, climate change can induce positive effects on agricultural production. This is the case in areas where plants are currently growing in suboptimal temperatures and those changes in abiotic factors (e.g. warming) can possibly promote their growth and increase their development (McMichael and Burke, 1998). On the other hand, tropical and temperate regions where plants are currently growing within optimal or slight above optimal temperatures tend to be adversely affected and negative impacts on plants will be observed on crop productivity (Gregory et al., 2005; Morison and Morecroft, 2008; Parry et al., 2004). In dry regions as arid and semi-arid environments where crop production is mainly determined by precipitation, climate change has a profound influence on crop production and sustainability (Bannayan and Eyshi Rezaei, 2014).

An important component of soil ecosystems is soil invertebrates, which play important functions on soils, providing as the end optimal growth conditions to plants and soil structure improvement (Holmstrup, 2002). Among them, there are the so called "soil engineers", the earthworms, which due to their burrow activities can change soil structure, creating pores through which oxygen and water can enter and provide the way out to carbon dioxide (Edwards, 1996.). Another important functional group is the decomposers, from which the collembolan can be highlighted. They live on the litter or pore space of soil, preferring soils rich in organic matter, feeding on fungi, bacteria and leaf litter, and actively participating on decomposition and nutrient cycling processes (Lavelle and Spain, 2001).

Both earthworms and collembolans are mediators of soil function through their activities by stimulating microbial decomposition rates and nutrient recycling processes (Seastedt and Crossley, 1984), organic matter decomposition/turnover and increasing soil fertility. In addition, arthropod interactions with plants and microbes influence the shifts between living and dead organic matter and transfers of nutrients in terrestrial ecosystems (Seastedt and Crossley, 1984).

Soil organisms and plants are continuously affected by a variety of environmental factors, which play an important role in the evolution, biology, physiology and geographical distribution of species, agricultural crop productivity and microbial activity.

The effects that each abiotic factor exerts on an organism depends on its quantity or intensity (Ahmad and Prasad, 2011), and each species will response specifically as they are quite different physiologically, with distinct optimum ranges of environmental conditions such as temperature, moisture content, or light (Coleman and Crossley Jr, 2004). These conditions provide an optimal growth, development, emergence and reproduction, and any deviation from such optimum become a natural stress and may cause injuries to soil organisms. Therefore populations can be affected, inferring to communities and finally to ecosystem functioning.

Typically, organisms show a behavioral response to seasonal environmental changes, either by migration or becoming inactive, until favorable conditions are restored. However, climate change has caused unforeseen unfavorable conditions, sometimes convened as daily variations, but undoubtedly causing profound changes in organisms' tolerance to other stress sources. And these factors will, separately or combined, affect the functioning of soil ecosystems (Swift et al., 1998) and also alter the response of soil organisms to chemical contaminants. So it has become crucial to study how they will cope with these challenges to which they are commonly exposed in field.

Earthworm survival, distribution and physiology is affected by soil moisture, temperature, and pH (Peijnenburg and Vijver, 2009). In prolonged drought events earthworms tend to burrow to deeper lays or stay in diapauses. During rainy periods earthworms tend to rise to the surface, escaping from possible drowning (Peijnenburg and Vijver, 2009). Soil temperature influences their metabolism, limiting earthworms' activities during warm and cold periods. Regarding soil pH, the majority of earthworm species does not tolerate acid soils. Although it is known that earthworms are not expected to be exposed to UV radiation, because of their photosensitivity, heavy rains may induce their movement to the above ground which can signify an extra stressor (UV radiation) or a combination of stressors (UV and flood conditions).

Collembola are tolerant of a wide range of environmental conditions, but the sensitivity to environmental stress differ strongly between species (Gudleifsson and Bjarnadottir, 2008). It has been reported that they have the ability to change their body physiology when exposed to severe summer drought or cold stress (Bayley and Holmstrup, 1999; Holmstrup, 2002). Few studies were performed evaluating the effects of UV radiation on collembola, because they live in soil pores, where radiation hardly reaches. But this improbable scenario may change in cases of flooding, or soils compaction where interstitial spaces are reduced and therefore prone their exposure to UV radiation. But, in a study developed by Cardoso et al., (2014) collembola were exposed to UV radiation under natural soil Lufa 2.2, simulating natural conditions, and exhibited an avoidance behavior towards UV radiation. In addition, and after exposure, organisms exposed to UV radiation increased their reproduction effort.

Terrestrial plants play a very important role in soil ecosystem, as they represent an important source of organic matter, act in nutrient cycling and soil respiration, being the base of terrestrial food webs. Because plants are sessile organisms, they are more prone to be exposed to environmental and chemical stress mainly by root uptake or air deposition, and are generally good indicators of the soil ecosystem health. Some plants have developed numerous adaptive responses to cope with environmental stress as stomatal closure, reduction of leaf area, changes in membranes, among others that will be further described below.

1.1.2 Natural stressors

Temperature

The drastic increase on atmosphere concentration of carbon dioxide (CO_2) and others greenhouse gases mainly due to anthropogenic activities, has led to an increase in the average global surface temperature (IPCC, 2001). It has been predicted that global mean temperature will arise in the future, if greenhouse gas emissions are kept to actual levels or are increased.

As consequence of global warming, land-surface precipitation trend to increase in some regions of the world and decrease in others, which may cause an imbalance on soil ecosystems. These changes may lead to a disequilibrium on the soil organic carbon pool and alter soil physical properties, destabilizing its structure, with a possible increase in soil erosion or compaction.

Temperature greatly influences the rates of biological, physical, and chemical processes in soil. High temperature stimulates to some extent soil microbial activity, net nitrification rates, P and N mineralization rates, and total respiration (Andresen et al., 2010), also increasing root turnover and providing more rapid mineralization rates (Gill and Jackson, 2000), leading to a decrease in the soil organic carbon pool. Under lower temperatures soil organic matter can accumulate due a reduction in microbial activity and potential inactivity of macrodecomposers. In addition, high temperatures can also promote a faster decomposition of chemicals in soil compared to low temperature (Dalias et al., 2001), which can lead to less or higher toxicity to soil organisms, depending on the generated metabolites.

Temperature affects soil fauna in several ways. Vital metabolic processes are ruled by environmental temperature, which may affect enzymes involved in metabolic processes (Welch et al., 2010). The majority of soil organisms, in temperate regions, have their optimum temperature within (15 to 20 ºC), while in tropical regions, organisms live under an average temperature ranging around 25 to 30 ºC and special adaptations undergo on those organisms when extreme cold or hot temperatures occur.

Exposed to extreme cold temperatures (below 15 °C), some organisms developed cryophilic response to tolerate those conditions, as some nematodes (Clark et al., 2007), freeze-tolerant earthworms (Bindesbol et al., 2005; Holmstrup, 2007) and enchytraeids (Silva et al., 2013). These responses include accumulation of cryoprotectant compounds in their corporal fluid (Overgaard et al., 2007), changes in lipid chemistry (Holmstrup, 2007) or increase of oxidative stress as a defense response to provides cold acclimation (Silva et al., 2013) and also thermotactic behavior (Clark et al., 2007). Under cold temperatures, physiological/reaction rates slow down and may almost completely stop, thereat, some organisms, that cannot migrate, remain in a relatively inactive state (dormancy), until more moderate temperatures prevail (Cáceres, 1997), while others maintain some degree of activity despite reductions in their body temperature. Low temperature is also the most important limiting factor to the productivity of agricultural crops in cold areas, limiting energy for biochemical processes, decreasing membrane permeability, and increasing protoplasm viscosity (Aber et al., 2001). Freeze-tolerant plants have several strategies to reduce the formation of ice inside plant cells, that is the major cause for plant devastation (Allen and Ort, 2001) at cold temperatures. These strategies include reduction of freezing-point by production of solutes that increase intracellular osmolality (Kartha, 1985), alteration in the membrane lipid composition by increased fatty acid unsaturation (Sanghera et al., 2011) and production of antifreeze proteins (Griffith and Yaish, 2004).

On the other hand, at higher extreme temperature (above 40 °C), only thermophiles organisms can survive, and most of them are microorganisms (Lebedinsky et al., 2007). At extreme temperatures, it is expected that some vital functions of the cells, as enzymatic activity, are impaired or destroyed and the organism die. Thus, biological reactions and processes tend to occur more rapidly as temperature rises above the optimum temperature for each species. But the earthworm *Pontoscolex corethrurus* is one of the few metazoans that shows tolerance to extreme high temperatures, and its occurrence has been reported by Cunha et al. (2014) in active volcanic soils in the Azores islands. In those environments the maximum temperature can reach 100 ° C it is suggested by the authors that these tolerance may be related with micro-evolutionary process.

Soil moisture

Although soil moisture is usually related to the intensity of rainfall, it is, in fact, a result of temperature, precipitation, soil water-holding capacity (WHC) and texture. Soil moisture results from precipitation or irrigation exceeding evaporation (closely related to temperature) and soil drainage (closely related to soil texture and WHC), and it is exhausted by evaporation and drainage (Arnell, 1999).

Global warming have significant effects on the hydrological cycle, because increasing global surface temperatures is very likely to cause changes in rainfall and air humidity (Dore, 2005). In the last twentieth century, precipitation increased by approximately 10% in the mid to high latitudes of the Northern Hemisphere (Morison and Morecroft, 2008). These increases have caused increase on soil moisture and flood events in some areas within these latitudes (IPCC, 2001). Following an opposite trend, some parts of the world are reported to have significant reductions in precipitation (e.g. in Southwest Western Australia and North China). Therefore the Panel on Climate Change (IPCC) reported that global warming will cause extreme conditions of flood or droughts as potential future scenarios.

Soil water availability is one of most important limits on the distribution, abundance, life cycles and species dominance patterns of terrestrial organisms (Kennedy, 1993; Lindberg et al., 2002), considering water as essential for enzyme activity and metabolism, a solvent for biological nutrients and other chemicals. So extreme conditions in soil water content may become a natural stressor and cause a profound impact in soil organisms, altering soil process.

At the soil system level, soil moisture affects soil aeration, microbial population and its activity and soil organisms' movements. In general, dry soil hampers plants water uptake through the root system, due to the decrease of soil pores size, which interferes with water removal. Organisms exposed to long drought stress periods may increase their water loss through evapotranspiration, and impair their growth and development (Mahajan and Tuteja, 2005). It has been reported that some species of collembola have the ability to change their body physiology when exposed to severe summer drought or cold stress (Bayley and Holmstrup, 1999; Holmstrup, 2002). Drought stress prevents crop growth, development and productivity, especially in arid and semi-arid areas, where the input of fresh water is lower (Shao et al., 2009). On the other hand, higher levels of soil moisture contribute to higher decomposition rates, optimal plant root functioning and rapid microbial turnover rates compared to dry soils (Skopp et al., 1990; Wardle, 2002). But leading moisture to extremes, i.e. flooded soils, reduces the partial pressure of oxygen around the plant's root, reducing their water uptake capacity (Morison and Morecroft, 2008) and causing anoxic conditions that can lead to the death of other soil organisms, as oxygen does not diffuse through soil easily.

UV radiation

In addition to changes in temperature and precipitation, substantial changes in solar irradiance are also linked to global warming. The production of greenhouse gases has depleted the stratospheric ozone continuously, also resulting in increasing ultraviolet (UV) radiation reaching the Earth surface.

UV radiation comprises the wavelength UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (200-290 nm). The most dangerous UV-C is reflected by stratospheric ozone and therefore does not reach the Earth surface. The least harmful, UV-A, passes through the stratosphere and UV-B is the most significant biologically damaging radiation reaching the Earth's surface, but as most is absorbed by ozone in the troposphere and stratospheric, only part of UVB radiation crosses the Earth's surface.

Although organisms are adapted to UV-A, studies have reported important plant response to the UVA irradiation (Flint and Caldwell, 2003). Relative to UV-B radiation, the majority of the organisms are not adapted to this radiation and several studies have reported that, due the ozone depletion, levels of UV-B radiation have risen significantly in the tropics and in temperate regions (Kerr and McElroy, 1993). Increased solar UV-B radiation may exert effects on terrestrial ecosystems, affecting soil organisms and plants.

In the environment, under normal conditions, many organisms can protect themselves from UV radiation by skin pigments or integuments or even by their behaviour. But, organisms exposed to high UV radiation may activate protective responses to tolerate this imbalance. Plants generally adapt to changes in UV radiation by several ways, by morphological changes (Ballare et al., 1996; Liao and Lin, 1995), increased DNA repair, (Britt and Fiscus, 2003) induction of protective compounds (Middleton and Teramura, 1993) and increased levels of antioxidant compounds(Agrawal and Mishra, 2009). Soil organisms are known to be more “tolerant” to UV radiation when compared to aquatic organisms (Gies et al., 1995), but this assumption is possibly derived from a less probability of exposure. Anyway, some edaphic organisms may developed behavioural changes as S-shaped movement and jumping, when exposed to UV radiation, as it was found in the case of earthworms (Chuang et al., 2006). Others organisms developed microhabitat selection and migrations (Callaghan et al., 2004) to prevent this stress.

Considering the environmental effects of ozone depletion and global warming, the interaction of several extreme abiotic factors is prompted to occur in terrestrial ecosystems. High temperature accompanied by drought or flood, added to high ultraviolet radiation are some scenarios that we find in the field as a result of environmental global changes. These interactions are prone to impact the fauna and flora at all different biological levels.

Several studies have drawn attention to the environmental risk caused by the interaction of these factors. It is predicted that temperature and water stress will cause extinction of some amphibians and other aquatic species in Costa Rica, Spain and Australia (Pounds et al., 2006). As a result to high temperature and changes in rainfall patterns in South America, it is predicted a replacement of some Amazonian forests by ecosystems more resistant to these stressors (IPCC, 2001; Rowell and Moore, 2000).

In natural conditions, effects of UV-B radiation on plants are related to other environmental factors, as CO2, drought, temperature and nutrition (Allen, 1999; Caldwell et al., 2007; Conner and Zangorini, 1998.; Koti et al., 2005; Zhao et al., 2004). These stress factors have been shown to modify or mask the UV radiation effects (Conner and Zangorini, 1998.).

1.1.3 Effects of climate change on chemical fate and behavior in the environment.

Regarding the above mentioned, changing the abiotic factors will change also the diversity of species inhabiting several regions and the occurrence of crop plagues are more prone to happen. Therefore the application of pesticides is also predicted to suffer some increase within the near future. Another consequence of climate change is its potential to alter the fate and behaviour of chemical compounds. Temperature and precipitation have great influence on the partitioning of chemicals (Noyes et al., 2009), because high temperature may reduce the parental pesticide concentrations by increased volatilization and degradation (Komprda et al., 2013) and the increase on precipitation rates enables the wet deposition of pesticides on terrestrial ecosystems (Asman et al., 2001).

Regions with elevated soil moisture and high precipitation could also enhance the degradation of pesticides by hydrolyses, and show higher pesticide volatilization than dry soils, due the polar water molecules that are strong competitors for adsorption sites on the soil (Bailey, 2004; Dörfler et al., 1991; Van den Berg et al., 1999).

The concern about the influence of environmental factors on chemical toxicity is not recent, and studies have been reported since early 20th century (Gordon, 2003). Several studies have shown that temperature can affect the toxicity of a variety of chemical compounds (e.g.,Baetjer and Smith, 1956; Doull, 1972; Fuhrman, 1946; Weihe, 1973), but the majority of these studies are related to small mammals or human health. Recently there has been greater attention and concern about the interaction of natural and chemical stressor on different terrestrial and aquatic organisms (Bednarska et al., 2009; Bindesbol, 2005; Boone and Bridges, 1999; Boone and James, 2003; Chen et al., 2003; Coors and De Meester, 2008; Crain et al., 2008; Fischer et al., 2013; H. M. Abdel-Lateif, 1998; Holmstrup, 2010) and several outputs were obtained. For example, the bioavailability and toxicity of chemical compounds in edaphic organisms and plants increase in response to high temperatures (Bauer and Rombke, 1997; Bednarska and Laskowski, 2008; Bednarska et al., 2009; Boina et al., 2009; Boone and Bridges, 1999; H. M. Abdel-Lateif, 1998; Lima et al., 2014), possibly because temperature may alters the toxicokinetics of pollutants or may increase its uptake and elimination (Buchwalter et al., 2003; Heugens et al., 2006).

In contrast, some chemicals showed an increase on toxicity at low temperature (Cedergreen, 2013) and one of those case studies is DDT which has been reported as generally more toxic under low temperature conditions (Gordon, 2005). Increasing toxicity at low temperature is normally related to the decrease in metabolic rate and reduced chemical biotransformation in these conditions, resulting in greater persistence of toxicants into organisms body (Harwood et al., 2009).

Besides temperature, several other natural stressors (e.g. soil moisture and UV radiation) may alter physiological mechanisms in organisms, and therefore increase the deleterious effects of contaminants. Drought stress will reduce the internal water content, increasing its chemical concentration, consequently increasing the risk of intoxication

(Holmstrup, 2010). It is also known that UV-B exposure may destroy organisms' defenses, becoming more susceptible to anthropogenic stressors (Tevini, 1993).

In addition, organisms living near the boundaries to their tolerance to abiotic stress, may be more vulnerable to interaction between those natural and chemical factors (Gordon, 2003). Therefore, their ability to tolerate extreme environmental events may be impaired by the presence of the chemical compound. Holmstrup (1997) demonstrated this, after exposing the collembolan *Folsomia candida* to sublethal concentration of three soil contaminants significantly reducing their tolerance to desiccation.

Two scientific reviews from the Nomiracle FP6 project reported the interactions of natural and chemical stressors (holmstrup et al, 2009; Laskowski et al., 2010), where clear synergistic interactions, for more than 50% of the reviewed studies were highlighted, showing that the influence of natural stressors should be considered in risk assessment procedures.

1.1.4 Predictions of joint effects of natural and chemical stressors

In face of the actual global scenarios of climatic changes, a greater concern about interactions between multiple stressors, both natural and chemicals, has aroused the scientific communities to more effective and realistic environmental risk analysis by considering a larger number of factors to which organisms are exposed in the environment.

So, for the prediction of joint effects, theoretical models has been recently used for evaluate combined chemicals and natural stressor (Ferreira, 2008; Jonker, 2005; Long, 2009). This approach was based on two concepts developed for predicting mixtures toxicity in pharmacology, the concentration addition (CA) and the independent action (IA) conceptual models, based on a preliminary assumption on the similarity and dissimilarity of modes of action, respectively. The IA model, also called ”response additivity” is the most applicable in cases of predicting the combined effects of natural stressor and chemical compounds. The assumption behind independent action is that stressors do not physically, chemically or biologically interact (Bliss 1939), and the toxicity of each component is independent and the effects of two stressors can be estimated directly from the probability of responses to the each individual components (Faust et al., 2003).

The mathematical expression for the IA model is based on probability of responses and is expressed as:

$$Y = \mu_{\max} \prod_{i}^{n} = 1^{qi(ci)}$$

where Y denotes the biological response, C*i* is the concentration of chemical *i* in the mixture, q*i* (C*i*) is the probability of non-response, µmax is the control response for endpoints and ∏ the multiplication function.

Deviations from the Independent Action model indicate that the probabilities of response to the chemicals are not independent and somehow there is an interaction between them. Possible deviations from the conceptual models can be predicted using the MIXTOX tool proposed by Jonker et al., (2005) . The description behind this approach allows evaluating if some deviation patterns (from the models) occur in combined exposure. The standard deviations considered and that are then transposed from mathematical results to biologically relevant patterns are the following and are based on each stressors individual effects:

1) Synergism (S): when the combination of stressors cause a more severe effect than predicted from either reference model.

2) Antagonism (A): when the combined stressors cause a less severe effect than calculated from either reference model.

3) Dose-level dependent deviation (DL): when the deviation from either reference model is dependent of the dose of each component (e.g. the deviation at low doses levels is different from the deviation at high dose levels).

4) Dose-ratio dependent deviation: when the deviation from either reference model is dependent of the ratio of the two components (e.g., antagonism may be found when one of the stressors is the dominant in the combination, whereas synergism may be found where the toxicity is mainly caused by the other stressor.

1.2. Objective and thesis structure

This thesis is divided in six chapters, including the current General Introduction (Chapter 1), four chapters (Chapters 2 to 5) describing the main results of joint exposure of natural and chemical stressors or combined natural stressors on soil fauna and flora, in the form of five manuscripts and a General Discussion and conclusions of the main results (Chapter 6).

The main objective of this work focused on how and to what extent environmental factors (temperature, soil moisture and UV radiation) affect the toxicity of carbaryl to soil organisms and plants. For that different endpoints and species were used: survival (*Folsomia candida* and *Eisenia andrei*), reproduction (*Folsomia candida*), biomass (*Triticum aestivum*, *Brassica rapa* and *Eisenia andrei*), growth, germination and foliar changes (*Triticum aestivum* and *Brassica rapa*).

1.3 General procedures

1.3.1 Test Organisms

In this study two soil invertebrate species were used as test organisms: earthworms (*Eisenia andrei,* Bouché, 1972) and collembolans (*Folsomia candida*, Willem, 1902) and two plants species: the monocotyledonous wheat (*Triticum. aestivum*) and the dicotyledonous turnip (*Brassica rapa*). All of them are widely used as standard test species in soil ecotoxicological tests.

E. andrei (Lumbricidade, Oligochaeta) is an a ubiquitous species with resilience to wide ranges of temperature and moisture (Dominguez et al., 2005). Due to their ecological significance and ability to be cultivated in the laboratory this species is a standard test organism widely used in terrestrial ecotoxicology (OECD, 1984). In the field they are continuously exposed to contaminants through the intake of large amounts of soil or by uptaken chemicals through skin. In addition they have chemoreceptors in their body surface which allows the detection of the presence of chemicals in soil. The acute toxicity tests with earthworms allow to evaluate, short-term, mortality and loss weight, and are relevant endpoints that provide sufficient information to initiate ecotoxicological risk management.

Folsomia candida is an insect, belonging to the family Isotomidae, and it is an unpigmented, edaphic and surface-dwelling species, with a short life cycle and easy to maintain in laboratory cultures. They have been extensively used in ecotoxicology assay due to their short generation time, asexual reproduction, sensitivity to chemical exposure and easily maintenance in the laboratory. *F. candida* has optimal reproduction rates in well watered soils and at 20 ºC (Fountain and Hopkin, 2005).

T. aestivum belongs to the Poaceae family, and is one of the most important agricultural crops and most studied plants in the evaluation of responses to climate changes; therefore it has also been widely used as a standard species in ecotoxicity testing (ISO 1995). Despite being a plant of temperate environments, it is well adapted to tropical and sub-tropical areas (above 17 ºC), with high solar radiation and grows across a wide range of environments worldwide. Their optimal temperature for growth is around 25 ºC and the maximal tolerance is around 32 ºC (Briggle and Curtis, 1987), but for a good growth rate, a well moistened soil is necessary.

B. rapa belongs to the Brassicaceae family and constitutes one of the world's most economically important plant groups. It is considered a temperate species, widely adapted within a wide range of soil conditions. Air and soil temperatures influence their growth and productivity and their optimum temperature for growth and development is around 20 ºC, with the minimum being considered around 12 ºC and the maximum 30 ºC (CCME, 2009).

Rapid-cycling brassicas (within the Wisconsin Fast Plants Program) are nowadays being used in ecotoxicology because they are easily grown under laboratorial condition, where a full life cycle assessment can be carried out "from seed to seed". The early stages of plant life, as germination, root emergence and shoot development, have been considered the most sensitive endpoints within plant's life-stage (OEHHA, 2008), which justifies the large use of these endpoints in ecotoxicological tests with plants.

1.3.2 Test chemical

The insecticide and acaricide carbaryl, also known by the trade name Sevin®, is the ISO common name for 1-naphthyl N- methylcarbamate (IPCS, 1993.). It belongs to the carbamate family, known to be an inhibitor of acetilcholinesterase. The molecular formula of carbaryl (CAS registry number 63-25-2) is $C_{12}H_{11}NO_2$(FAO, 2004) and its chemical structure is presented below in figure 1.1

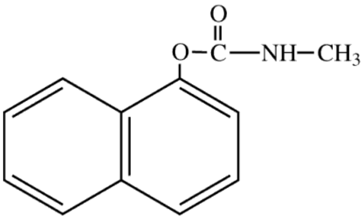


Fig. 1.1. Chemical structure of carbaryl.

Carbaryl was the first carbamate pesticide to be used successfully for the control of pests on agricultural crops, ornamental production, and residential pet and lawn uses. It was registered by Bayer CropScience and is largely used in countries worldwide, (CCME, 2009), in more than 100 species of insects that are considered plagues to crops.

Carbaryl is a lipophilic chemical, with low volatility, and it is considered a stable product under light conditions and at temperatures below 70°C (IPCS, 1993.); its half-life depends on temperature, pH and the initial concentrations. In soil the degradation half-life for carbaryl at 20± 2°C was reported to be less than 31 days, but the degradation rate was found to be 2.7 times slower with a decrease in temperature to 10°C (CCME, 2009). The degradation of carbaryl in soil is highly dependent of environmental factors, soil type and soil aeration.

The soil adsorption coefficient (Koc) is the value that determines how well the chemical binds to the soil particles. The larger the Koc, the smaller is the probability of a chemical to move along the ground. The knowledge on the sorption phenomena of pesticides in soils is very important for an accurate risk assessment procedure, because pesticide sorption affects important processes that will influence the final fate of these compounds in the soil environment (Kumar and Philip, 2006). In sandy soils, where there is a low concentration of organic matter, the mobility of carbaryl is high, and consequently a low adsorption is observed in these soils. On the other hand, adsorption of carbaryl on soils with a high organic matter content occurs more readily (IPCS, 1993.). Carbaryl has been considered to have medium mobility in silty clay loam, sandy loam, sediment and silt loam soils (CCME, 2009). Regarding soil aeration, carbaryl is degraded faster under aerobic conditions than under anaerobic conditions by microbial processes. The main metabolites from carbaryl degradation both in water and in the soil is 1-naphthol (IPCS, 1993.), which is generally more toxic than carbaryl.

Regarding the toxicity of carbaryl to soil organisms, the European Food Safety Authority (2005) considers that carbaryl represents a low acute risk for earthworms. But a potential high risk to other non-target organisms as collembola. In addition they declare that the inherent risk of carbaryl to plants is considered to be low for the representative use, once plants are exposed via spraying and therefore, exposed to lower doses of carbaryl. But these conclusions were based on experiments where abiotic factors were controlled in the laboratory, which may completely change the scenario if compared to the simultaneous presence of extreme environmental conditions.

The specific aims of each work are described below.

Chapter 2: Lima, M. P. R., Soares, A. M. V. M., Loureiro, S. (2011) Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios. *Environmental Pollution* 159**,** 1844-1851.

In order to evaluate the responses of soil organisms to the changes in soil moisture and the influence of these changes in carbaryl toxicity, the plant species *Brassica rapa* and *Triticum aestivum* and the earthworm *Eisenia andrei* were exposed to different levels of soil moisture, simulating drought and flood scenarios and a range of carbaryl concentrations. Both stress factors were tested individually, as well as combined. Acute and chronic tests were performed. When possible, data were fitted to the IA conceptual model for describing combined responses.

Chapter 3: Lima, M. P. R., Cardoso, D. N., Soares, A. M. V. M., Loureiro, S. (in press) Carbaryl toxicity prediction to soil organisms under high and low temperature regimes, *Ecotoxicology and Environmental Safety* doi: 10.1016/j.ecoenv.2014.04.004.

The toxic effects of the pesticide carbaryl were evaluated under different temperature regimes, which are indicative of temperate and tropical climates and are relevant to climate change predictions or seasonal temperature fluctuations. Four standard organisms were used (*Folsomia candida, Eisenia andrei; Triticum aestivum* and *Brassica rapa*) and the effects were assessed using synergistic ratios, calculated from EC/LC$_{50}$ values. When possible, the MIXTOX tool was used based on the reference model of independent action (IA) and possible deviations.

Chapter 4: Lima, M. P. R., Soares, A. M. V. M., Loureiro, S. Responses of wheat (*Triticum aestivum*) and turnip (*Brassica rapa*) to the combined exposure of carbaryl and ultraviolet radiation. Paper submitted to *Environmental Toxicology and Chemistry.*

The purpose of this study was to evaluate the influence of UV radiation on carbaryl toxicity in crop plants. For that two important crop plants *Triticum aestivum* (wheat) and *Brassica rapa* (turnip) were exposed to UV radiation and carbaryl, singly and in combination. For this evaluation, plant growth, weight, leaf changes and biomass accumulation were used as response parameters. The MIXTOX tool was used to predict the combined exposures and was based on the conceptual model of Independent Action (IA), where possible deviations to synergism or antagonism, dose-ratio or dose-level response pattern were also considered.

Chapter 5: Lima, M. P. R., Soares, A. M. V. M., Loureiro, S. Drought and flooded soils affect the response of wheat (*T. aestivum*) and turnip (*B .rapa*) to ultraviolet radiation.

The purpose of this work was to analyze if the water availability can change the response of *T. aestivum* and *B. rapa* to UV radiation exposure. For that seedlings immediately after emergence were submitted to different doses of UV radiation, combined with two watering regimes (drought and flood simulations). The endpoints analyzed were length, weight, foliar changes and dry matter accumulation.

Chapter 6. A general discussion and concluding remarks of this study.

References

Abdel-Lateif, H.M ,Donker, M.H., Van Straalen, N.M., 1998. Interaction between temperature and cadmium toxicity in the isopod *Porcellio scaber.* Functional Ecology. 12**,** 521-527.

Aber, J.,Neilson, R.P., McNulty, S., Lenihan, J.M., Bachelet, D., Drapek, R.J., 2001. Forest Processes and Global Environmental Change: Predicting the Effects of Individual and Multiple Stressors We review the effects of several rapidly changing environmental drivers on ecosystem function, discuss interactions among them, and summarize predicted changes in productivity, carbon storage, and water balance. BioScience. 51, 735-751.

Agrawal, S.B., Mishra, S., 2009. Effects of supplemental ultraviolet-B and cadmium on growth, antioxidants and yield of *Pisum sativum* L. Ecotoxicology and Environmental Safety 72**,** 610-618.

Ahmad, P., Prasad, N.V., 2011. Environmental Adaptations and Stress Tolerance of Plants in the Era of Climate Change. Springer New York.

Allen, D.J., Ort, D.R., 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends in Plant Science 6**,** 36-42.

Allen, D.J., Nogues, S.; Morison, J.I.L.; Greenslade, P.D.; McLeod, A.; Baker, N.R., 1999. A thirty percent increase in UV-B has no impact on photosynthesis in well-watered and droughted pea plants in the field. Global Change Biology 5**,** 235-244.

Andresen, L.C., Michelsen, A., Jonasson, S., Schmidt, I.K., Mikkelsen, TN., Ambus, P., Beier, C., 2010. Plant nutrient mobilization in temperate heath land responds to elevated CO2, temperature and drought. Plant and Soil 328**,** 381-396.

Arnell, N.W., 1999. Climate change and global water resources. Global Environmental Change 9**,** S31-S49.

Asman, W.A.H., Felding, G., Kudsk, P., Larsen, J., Mathiassen, S., Spliid, N.H., 2001. Pesticides in air and in precipitation and effects on plant communities.57. Miljøstyrelsen.

Baetjer, A.M., Smith, R., 1956. Effect of environmental temperature on reaction of mice to parathion, an anticholinesterase agent. American Journal Physiology. 186**,** 39-46.

Bailey, S.W., 2004. Climate change and decreasing herbicide persistence. Pest management science 60, 158-162.

Ballare, C.L., Scopel, A.L., Stapleton, A.E., Yanovsky, M.J., 1996. Solar Ultraviolet-B Radiation Affects Seedling Emergence, DNA Integrity, Plant Morphology, Growth Rate, and Attractiveness to Herbivore Insects in *Datura ferox*. Plant Physiology 112, 161-170

Bannayan, M., Eyshi Rezaei, E., 2014. Future production of rainfed wheat in Iran (Khorasan province): climate change scenario analysis. Mitigation and Adaptation Strategies for Global Change 19, 211-227.

Bauer, C., Rombke, J., 1997. Factors influencing the toxicity of two pesticides on three lumbricid species in laboratory tests. Soil Biology & Biochemistry 29, 705-708.

Bayley, M., Holmstrup, M., 1999. Water vapor absorption in arthropods by accumulation of myoinositol and glucose. Science 285, 1909-11.

Bednarska, A.J., Laskowski, R., 2008. Effects of nickel and temperature on the ground beetle *Pterostichus oblongopunctatus* (Coleoptera : Carabidae). Ecotoxicology 17, 189-198.

Bednarska, A.J., Portka, I., Kramarz, P.E., Laskowski, R., 2009. Combined Effect of Environmental Pollutants (Nickel, Chlorpyrifos) and Temperature on the Ground Beetle, *Pterostichus Oblongopunctatus* (Coleoptera: Carabidae). Environmental Toxicology and Chemistry 28, 864-872.

Bindesbol, A.M., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm Dendrobaena octaedra. Environmental Toxicology and Chemistry 24, 1462-1467.

Bindesbol, A.M., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. Environmental Toxicology and Chemistry 24, 1462-1467.

Boag, B., Crawford J.W., Neilson, R., 1991. The Effect of Potential Climatic Changes On the Geographical Distribution of the Plant-Parasitic Nematodes Xiphinema and Longidor Us in Europe. Nematologica 37. 1, 312-323.

Boina, D.R., Onagbola, E.O, Salyani, M., Stelinski, L.L., 2009. Influence of Posttreatment Temperature on the Toxicity of Insecticides Against *Diaphorina citri* (Hemiptera: Psyllidae). Journal of Economic Entomology 102, 685-691.

Boone, M., Bridges, C.M., 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). Environmental Toxicology and Chemistry 18**,** 1482-1484.

Boone, M.D., James, S.M., 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecological Applications 13**,** 829-841.

Briggle, L., Curtis, B., Wheat Wordwide. In: E. G. Heyne, (Ed.), Wheat and wheat improvement. American Society of Agronomy, Madison, Wisconsin, USA, 1987.

Britt, A., Fiscus, E.L., 2003. Growth responses of Arabidopsis DNA repair mutants to solar irradiation. Physiologia Plantarum 118**,** 183-192.

Buchwalter, D.B., Jenkins, J. J., Curtis, L.R., 2003. Temperature influences on water permeability and chlorpyrifos uptake in aquatic insects with differing respiratory strategies. Environmental toxicology and chemistry 22**,** 2806-2812.

Cáceres, C.E., 1997. Dormancy in invertebrates. Invertebrate Biology,116, 371-383

Caldwell, M.M., Bornman, J.F., Ballare, C.L., Flint, S.D., Kulandaivelu, G., 2007. Terrestrial ecosystems, increased solar ultraviolet radiation, and interactions with other climate change factors. Photochemistry and Photobiology Science 6, 252-66.

Callaghan, T.V., Björn, L.O., Chernov, Y., Chapin, T., Christensen, T.R., Huntley, B., Ims R.A, Johansson, M., Jolly, D., Jonasson, S., Matveyeva, N., Panikov, N., Oechel W., Shaver, G., Elster, J., Henttonen, H., Laine, K., Taulavuori, K., Taulavuori, E., Zöckler, C., 2004. Biodiversity, distributions and adaptations of Arctic species in the context of environmental change. AMBIO: A Journal of the Human Environment 33**,** 404-417.

Cardoso, D.F.N., Bastos, A.C., Soares, A.M.V.M., Loureiro, S., 2014. Short-term exposure to carbaryl and UV radiation increases the reproduction output of the collembolan *Folsomia candida*. Journal of Soils and Sediments 14**,** 1559-1567.

CCME, Canadian Water Quality Guidelines: Carbaryl. Scientific Criteria Document. Canadian Council of Ministers of the Environment, Winnipeg., 2009.

Cecchi, L., D'Amato, G., Ayres, J.G., Galan, C., Forastiere, F., Forsberg, B., Gerritsen, J., Nunes, C., Behrendt, H., Akdis, C., Dahl, R., Annesi-Maesano, I., 2010. Projections of the effects of climate change on allergic asthma: the contribution of aerobiology. Allergy 65**,** 1073-1081.

Cedergreen, N., Nørhave, N.J., Nielsen, K., Johansson, H.K.L., Marcussen, H., Svendsen, C., Spurgeon, D.J., 2013. Low temperatures enhance the toxicity of copper and cadmium to *Enchytraeus crypticus* through different mechanisms. Environmental toxicology and chemistry 32**,** 2274-2283.

Cheddadi, R., Guiot, J., Jolly, D., 2001. The Mediterranean vegetation: what if the atmospheric CO2 increased? Landscape ecology 16**,** 667-675.

Chen, T., Qiang, W., An, L., Wang, X., Effects of cadmium combined with ultraviolet-B radiation on growth, gas exchange, and stable carbon isotope value (Î´13C) in soybean (*Glycine max* L.). Vol. 4896, 2003, pp. 210-218. Proc. SPIE 4896, Ultraviolet Ground- and Space-based Measurements, Models, and Effects II, 210 (June 26, 2003); doi:10.1117/12.466165.

Chuang, S.C., Lai, W.S., Chen, J.H., 2006. Influence of ultraviolet radiation on selected physiological responses of earthworms. Journal of experimental biology 209**,** 4304-4312.

Clark, D.A., Gabel, C.V., Lee, T.M., Aravinthan, D.T.S., 2007. Short-Term Adaptation and Temporal Processing in the Cryophilic Response of *Caenorhabditis elegans*. Journal of Neurophysiology 9, 1903-1910.

Coleman, D.C., Crossley Jr, D.A., 2004. Fundamentals of soil ecology. Academic press.

Conner, J., Zangorini, L.A., 1998. Combined effects of water, nutrient, and uv-b stress on female fitness in Brassica (Brassicaceae). American Journal of Botany 85, 925–931.

Coors, A., De Meester, L., 2008. Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. Journal of Applied Ecology 45**,** 1820-1828.

Crain, C. M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecology letters 11**,** 1304-1315.

Cunha, L., Montiel, R., Novo, M., Orozco-terWengel, P., Rodrigues, A., Morgan, A. J., Kille, P., 2014. Living on a volcano's edge: genetic isolation of an extremophile terrestrial metazoan. Heredity (Edinb) 112**,** 132-42.

Dalias, P., Anderson, J. M., Bottner, P., Coûteaux, Marie-Madeleine., 2001. Temperature responses of carbon mineralization in conifer forest soils from different regional climates incubated under standard laboratory conditions. Global Change Biology 7, 181-192.

Dominguez, J., Velando, A., Ferreiro, A., 2005. Are Eisenia fetida (Savigny, 1826) and *Eisenia andrei* (Oligochaeta, Lumbricidae) different biological species? Pedobiologia 49, 81-87.

Dore, M.H.I., 2005. Climate change and changes in global precipitation patterns: what do we know? Environment international 31, 1167-1181.

Dörfler, U., Adler-Köhler, R., Schneider, P., Scheunert, I., Korte, F., 1991. A laboratory model system for determining the volatility of pesticides from soil and plant surfaces. Chemosphere 23, 485-496.

Doull, J., 1972. The effect of physical environmental factors on drug response. Essays Toxicol 3, 37-63.

Edwards, C.A., and Bohlen, P.J., 1996. Biology and ecology of earthworms. ed. Chapman & Hall, London, UK.

EFSA, (European Food Safety Authority), Draft Assessment Report. Initial risk assessment provided by rapporteur member state Spain dor the existing active substance Carbaryl. In: of the second stage of the review programmer referred to in Article 8 (2) of council Directive 91/414/EEC (Ed.), Available from: 2005.

FAO, Food and Agricultural Organization of the United States. Specifications, Evaluations for Agricultural Pesticides, Carbaryl,. 2004.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L. H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquatic Toxicology 63, 43-63.

Ferreira, A.L.G., Loureiro, S., and Soares, A.M.V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna*. Aquatic Toxicology 89, 28-39.

Fischer, B.B., Pomati, F., Eggen, R.I.L., 2013. The toxicity of chemical pollutants in dynamic natural systems: The challenge of integrating environmental factors and biological complexity. Science of the Total Environment 449, 253-259.

Flint, S.D., Caldwell, M.M., 2003. A biological spectral weighting function for ozone depletion research with higher plants. Physiologia Plantarum 117, 137-144.

Fountain, M.T., Hopkin, S.P., 2005. *Folsomia candida* (Collembola): A "standard" soil arthropod. Annual Review of Entomology 50, 201-222.

Fuhrman, F.A., 1946. The effect of body temperature on drug action. Physiol. Rev 26, 247-274.

Ghini, R., Hamada, E., Bettiol, W., 2008. Climate change and plant diseases. Scientia Agricola 65, 98-107.

Gies, H.P., Roy, C.R., Toomey, S., Maclennan, R., Watson, M., 1995. Solar UVR exposures of three groups of outdoor workers on the sunshine coast, queensland. Photochemistry and Photobiology 62, 1015-1021.

Gill, R.A., Jackson, R.B., 2000. Global patterns of root turnover for terrestrial ecosystems. New Phytologist 147, 13-31.

Gordon, C.J., 2003. Role of environmental stress in the physiological response to chemical toxicants. Environ Research 92, 1-7.

Gordon, C.J., 2005. Temperature and Toxicology: An Integrative, Comparative, and Environmental Approach. CRC Press.pp.338.

Gregory, P.J., Ingram, J.S.I., Brklacich, M., 2005. Climate change and food security. Philosophical Transactions of the Royal Society B: Biological Sciences 360, 2139-2148.

Griffith, M., Yaish, M.W., 2004. Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci 9, 399-405.

Gudleifsson, B.E., Bjarnadottir, B., 2008. Springtail (Collembola) populations in hayfields and pastures in northern Iceland. Icelandic Agricultural Sciences 21, 49-59.

Harte, J., Rawa, A., Price, V., 1996. Effects of manipulated soil microclimate on mesofaunal biomass and diversity. Soil Biology and Biochemistry 28, 313-322.

Harwood, A.D., You, J., Lydy, M.J., 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ Toxicol Chem 28, 1051-8.

Heugens, E. H. W., Tokkie, L.T.B., Kraak, M.H.S., Hendriks, A. J., Van Straalen, N.M., Admiraal, W., 2006. Population growth of *Daphnia magna* under multiple stress conditions: joint effects of temperature, food, and cadmium. Environmental toxicology and chemistry 25, 1399-1407.

Holmstrup, M., 1997. Drought tolerance in *Folsomia candida* Willem (Collemhola) after exposure to sublethal concentrations of three soil-polluting chemicals. Pedobiologia 41**,** 361-368.

Holmstrup, M., 2002. Strategies for cold and drought tolerance in permeable soil invertebrates. Unpublished doctoral dissertation, National Environmental Research Institute Ministry of Environment, Silkeborg, Denmark.

Holmstrup, M., Bindesbol, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler Heinz-R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Science of The Total Environment 408**,** 3746-3762

Holmstrup, M., Sorensen, L.I., Bindesbol, A.M., Hedlund, K., 2007. Cold acclimation and lipid composition in the earthworm *Dendrobaena octaedra*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 147**,** 911-919.

Holmstrup M., Bindesbol, A-M., Oostingh GJ., Duschl, A,. Scheil, V., Köhler, H-R., Loureiro, S., Soares, AM.V.M., Ferreira, AL.G., Kienle, C,. Gerhardt, A., Laskowski R,. Kramarz, P.E., Bayley, M,. Svendsen, C,. Spurgeon, DJ., 2009. Interactions between effects of environmental chemicals and natural stressors: A review. Science of The Total Environment 408, 3746–3762.

IPCC, Intergovernmental Panel on Climate Change., 2001: The scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change (Houghton, J.T., Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden, X. Dai, K. Maskell, and C.A. Johnson (eds.) Vol. 881. Cambridge university press Cambridge, United Kingdom and New York, NY, USA, 2001.

IPCC, Intergovernmental Panel on Climate Change., 2007 Synthesis Report: Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change Core Writing Team. IPCC, Geneva, Switzerland.

IPCS, International Programme on Chemical Safety., 1993. Carbaryl Health and Safety World Health Organization., Geneva.

ISO 1995 Soil quality—Determination of the effects of pollutants on soil flora—Part 2: Effects of chemicals on the emergence of Toxicity Assessment of Soils from Portuguese Mines Using Plants 189 higher plants. ISO—The International Organization for Standardization. Genève, ISO 11269.

Jonker, M.J., Svendensen, C., Bedaux, J.J.M., Bongers, M., and Kammenga, J., 2005. Significance Testing of Synergistic Antagonisc, Dose Level Dependent or Dose Ratio Dependent Effects In Mixture Dose Response Analysis. Environmental Toxicology and Chemistry 24**,** 2701–2713.

Kartha, K.K., 1985. Cryopreservation of Plant Cells and Organs. CRC Press. pp.288.

Kennedy, A.D., 1993. Water as a limiting factor in the Antarctic terrestrial environment: a biogeographical synthesis. Arctic and Alpine Research 308-315.

Kerr, J.B., McElroy, C.T., 1993. Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. Science 262**,** 1032-4.

Komprda, J., Sáňka, M ., Možný, M., Nizzetto, L., 2013. Influence of Climate and Land Use Change on Spatially Resolved Volatilization of Persistent Organic Pollutants (POPs) from Background Soils. Environmental Science & Technology 47**,** 7052-7059.

Koti, S., Reddy, K.R., Reddy, V.R., Kakani, V.G., Zhao, D., 2005. Interactive effects of carbon dioxide, temperature, and ultraviolet-B radiation on soybean (*Glycine max* L.) flower and pollen morphology, pollen production, germination, and tube lengths. Journal of Experimental Botanic 56**,** 725-36.

Kumar, M., Philip, L., 2006. Adsorption and desorption characteristics of hydrophobic pesticide endosulfan in four Indian soils. Chemosphere 62**,** 1064-1077.

Lal, R., 2004. Soil carbon sequestration to mitigate climate change. Geoderma. 123**,** 1-22.

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudlek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors - A meta-analysis and case studies. Science of the Total Environment 408**,** 3763-3774.

Lavelle, P., Spain, A. V., 2001. Soil ecology. Amsterdam.

Lebedinsky, A.V., Chernyh N.A, Bonch-Osmolovskaya E.A 2007. Phylogenetic systematics of microorganisms inhabiting thermal environments. Biochemistry (Mosc). 72**,** 1299-312.

Liao, C.T., Lin, C.H., 1995. Effect of flood stress on morphology and anaerobic metabolism of *Momordica charantia*. Environmental and Experimental Botany. 35**,** 105-113.

Lima, M.P., Cardoso, D.N., Soares, A.M., Loureiro, S., 2014. Carbaryl toxicity prediction to soil organisms under high and low temperature regimes. Ecotoxicol and Environmental Safety.

Lindberg, N., Engtsson, J.B., Persson, T., 2002. Effects of experimental irrigation and drought on the composition and diversity of soil fauna in a coniferous stand. Journal of Applied Ecology. 39**,** 924-936.

Long, S.M., Reichenberg, F., Lister, L.J., Hankard, P.K., Townsend, J., Mayer, P., Wright, J., Holmstrup, M, Svendsen, C., Spurgeon, D.J., 2009. Combined Chemical (Fluoranthene) and Drought Effects on *Lumbricus Rubellus* Demonstrate the Applicability of the Independent Action Model for Multiple Stressor Assessment. Environmental Toxicology and Chemistry. 28**,** 629-636.

Mahajan, S., Tuteja, N., 2005. Cold, salinity and drought stresses: An overview. Archives of Biochemistry and Biophysics. 444**,** 139-158.

McMichael, B.L., Burke, J.J., 1998. Soil Temperature and Root Growth. HortScience. 33**,** 947-951.

Middleton, E.M., Teramura, A.H., 1993. The Role of Flavonol Glycosides and Carotenoids in Protecting Soybean from Ultraviolet-B Damage. Plant Physiology 103**,** 741-752.

Morison, J.I.L., Morecroft, M.D., 2008. Plant growth and climate change. John Wiley & Sons.

Noyes, P.D., McElweea, M.K., Millera, H.D., Clarka, B.W., Van Tiema,L.A., Walcotta, K.C., Erwina,K.N., Levina,E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environment international. 35**,** 971-986.

OECD, Earthworm Acute Toxicity Tests. OECD- Organization for Economic Cooperation and Development, Paris, 1984.

Overgaard, J., Overgaard J., Slotsbo, S.,Holmstrup, M., Bayley, M., 2007. Determining factors for cryoprotectant accumulation in the freeze•tolerant earthworm, *Dendrobaena octaedra.* Journal of Experimental Zoology Part A: Ecological Genetics and Physiology. 307**,** 578-589.

Parry, M.L., Rosenzweig, C., Iglesias, A., Livermored, M., Fischer, G., 2004. Effects of climate change on global food production under SRES emissions and socio-economic scenarios. Global Environmental Change. 14**,** 53-67.

Peijnenburg, W.J.G.M., Vijver, M.G., Earthworms and their use in eco (toxico) logical modeling. Ecotoxicology Modeling. Springer, 2009, pp. 177-204.

Pounds, J.A., Bustamante, M.R., Coloma, L.A. Consuegra, J.A., Fogden, M. P.L. Foster, P.N., Marca, E.L. Masters, K.L. Merino-Viteri,A., Puschendorf, R., Ron, S.R., Sánchez-Azofeifa, G.A., Still, C.J., Young, B.E., 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature. 439**,** 161-167.

Rowell, A., Moore, P.F., Global review of forest fires. A WWF/IUCN Report, 2000.

Sanghera, G.S., Wani, S.H, Hussain, W., Singh, N.B., 2011. Engineering cold stress tolerance in crop plants. Curr Genomics. 12**,** 30-43.

Seastedt, T.R., Crossley, D.A., 1984. The influence of arthropods on ecosystems. BioScience. 34**,** 157-161.

Shao, H. B., Chu, L.Y., Jaleel, C.A., Manivannan, P., Panneerselvam, R., Shao, M.A., 2009. Understanding water deficit stress-induced changes in the basic metabolism of higher plants - biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe. Critical Review Biotechnology. 29**,** 131-51.

Silva, A.L.P., Holmstrup, M., Amorim, M.J., 2013. Worms from the Arctic are better adapted to freezing and high salinity than worms from temperate regions: Oxidative stress responses in *Enchytraeus albidus*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 166**,** 582-589.

Skopp, J., Jawson, M.D., Doran, J.W., 1990. Steady-state aerobic microbial activity as a function of soil water content. Soil Science Society of America Journal. 54**,** 1619-1625.

Swift, M.J., Andrén, O., Brussaard, L., Briones, M., Couteaux, M. -M., Ekschmitt, K., Kjoller, A., Loiseau, P., Smith, P., 1998. Global change, soil biodiversity, and nitrogen cycling in terrestrial ecosystems: three case studies. Global Change Biology. 4**,** 729-743.

Tevini, M., 1993. UV-B radiation and ozone depletion. Effects on humans, animals, microorganisms and materials.

Van den Berg, F., Kubiak, R., Benjey, W.G., Majewski, M.S., Yates, S.R., Reeves, G.L., ... & Van der Linden, A.M.A. (1999). Emission of pesticides into the air. In: Fate of Pesticides in the Atmosphere: Implications for Environmental Risk Assessment. (pp. 195-218). Springer Netherlands.

Wardle, D.A., 2002. Communities and ecosystems: linking the aboveground and belowground components. Princeton University Press.

Weihe, W.H., 1973. The Effect of Temperature on the Action of Drugs. Annual Review of Pharmacology. 13, 409-425.

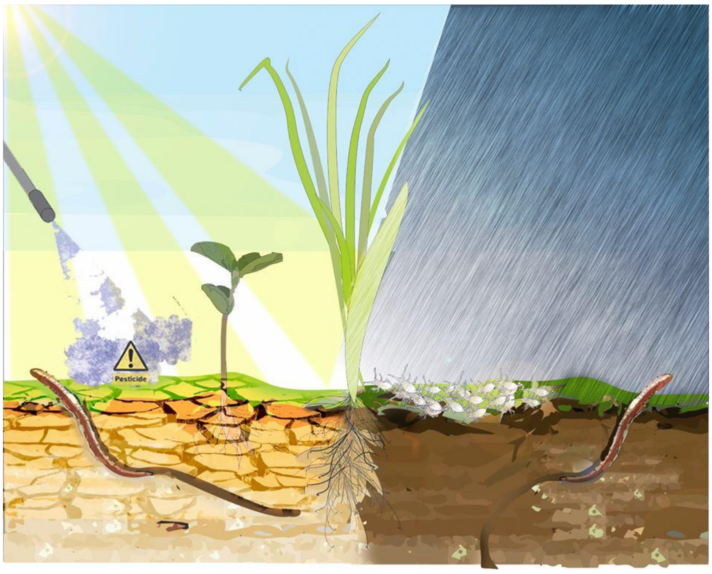
Welch, J.R., Vincent, J.R., Auffhammer, M., Moya, P.F., Dobermann,A., Dawe, D., 2010. Rice yields in tropical/subtropical Asia exhibit large but opposing sensitivities to minimum and maximum temperatures. Proceedings of the National Academy of Sciences. 107, 14562-14567.

White, A., Cannell, M.G.R., Friend, A.D., 1999. Climate change impacts on ecosystems and the terrestrial carbon sink: a new assessment. Global Environmental Change. 9, Supplement 1, S21-S30.

Zhao, D., Reddy, K.R., Kakani, V.G., Mohammed, A.R., Read, J.J., Gao, W, 2004. Leaf and canopy photosynthetic characteristics of cotton (*Gossypium hirsutum*) under elevated CO2 concentration and UV-B radiation. Journal of Plant Physiology 161, 581-90.

CHAPTER 2

Combined effects of soil moisture and carbaryl to earthworms and plants: simulation of flood and drought scenarios



Chapter 2. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios

Maria P.R. Lima, Amadeu M.V.M. Soares, Susana Loureiro

published by Journal Environmental pollution. 159 (2011) 1844e1851.
doi:10.1016/j.envpol.2011.03.029

Abstract

Studying tolerance limits in organisms exposed to climatic variations is key to understanding effects on behaviour and physiology. The presence of pollutants may influence these tolerance limits, by altering the toxicity or bioavailability of the chemical. In this work, the plant species *Brassica rapa* and *Triticum aestivum* and the earthworm *Eisenia andrei* were exposed to different levels of soil moisture and carbaryl, as natural and chemical stressors, respectively. Both stress factors were tested individually, as well as in combination. Acute and chronic tests were performed and results were discussed in order to evaluate the responses of organisms to the combination of stressors. When possible, data was fitted to widely employed models for describing chemical mixture responses. Synergistic interactions were observed in earthworms exposed to carbaryl and drought conditions, while antagonistic interactions were more representative for plants, especially in relation to biomass loss under flood-simulation conditions.

Keywords: Soil moisture, Carbaryl, Synergism, Antagonism, Non-target species

2.1 Introductionn

In the last decades, several factors have had an impact on ecosystem sustainability. Among them, anthropogenic activities (e.g. agricultural practices) and environmental (including climatic) changes are important causes for such an imbalance. Global climatic changes are increasingly redirecting ecotoxicological studies, and there has been an increment on the knowledge about interactions between natural and chemical stressors, and the way they affect organisms and their performance. The response of soil fauna and flora to chemicals is dependent on the environmental conditions under which they are exposed. Environmental conditions cannot only increase the organism susceptibility to pollutants, but also become stress factors themselves (Holmstrup et al., 2007; Sjursen and Holmstrup, 2004; Spurgeon et al., 2005; Svendsen et al., 2007).

Biota play an important role in maintaining soil quality and functioning, since they intervene on the decomposition of dead organic material and nutrient cycling (Bardgett et al., 2005). The species used in this work represent different groups of organisms in terms of function, trophic level, life history strategy and route of exposure to chemicals.

Earthworms are one of the most important biocomponents of ecosystems, contributing to maintaining soil structure and fertility promote plant growth, and aid in important soil processes such as carbon and nitrogen cycling (Edwards and Bohlen, 1996). Higher plants are considered to be versatile tools for identifying and monitoring the effects of pollutants on soil (Gong et al., 2001; Loureiro et al., 2006). Plants can be used as bioindicators for toxicity assessment in both aquatic and terrestrial ecosystems (Azevedo et al., 2005; Gorsuch et al., 1991). In this context, the monocotyledonous *Triticum aestivum* and the dicotyledonous *Brassica rapa* are amongst those species more commonly used in environmental risk assessment, while being representative of economically relevant plants.

Soil available water is a key factor in determining soil fauna and plant fitness (Fragoso and Lavelle, 1992; Lavelle and Spain, 2001), while influencing the activity and habitat selection of soil organisms, as well as the behaviour and toxicity of anthropogenic contaminants towards edaphic species and plants (Højer et al., 2001;

Martikainen and Krogh, 1999). Some studies have been carried out on the influence of soil moisture on soil fauna dynamics, evaluating changes in sensitivity to soil moisture after chemical exposure or vice-versa or even the combined assessment simultaneously (Bindesbøl et al., 2005; Friis et al., 2004; Højer et al., 2001; Holmstrup, 1997; Holmstrup et al., 2007, 1998; Long et al., 2009; Maraldo et al., 2006; Sjursen and Holmstrup, 2004; Sjursen et al., 2001; Sorensen and Holmstrup, 2005). Such approaches are crucial to understand scenarios of drought and flood, which have become more frequent over the last decades.

Analysing the effects of pharmaceutical mixtures has become common practice in toxicology, with conceptual models such as concentration addition (CA) and independent action (IA) amongst those more widely used. Such models have also been transposed to environmental research, for predicting effects of chemical mixtures or combinations of natural and chemical stressors (e.g. Holmstrup, 2008; Loureiro et al., 2009; Pestana et al., 2009). The CA concept described by Loewe and Muischnek (1926) is based on the assumption that the individual components of the mixture have similar mechanisms of action. The IA principle relates to independent modes of action of the mixture components and was firstly described by Bliss (1939). Recently, both models were successfully employed as part of the European project No Miracle (2004 and 2009), for describing the combined effects of chemicals and natural stressors (Ferreira et al., 2008, 2010; Long et al., 2009).

The aim of this study was to investigate and predict the toxicity of carbaryl under different soil moisture contents, thus simulating drought and flood scenarios. Earthworms and plants were used as test-organisms. Dose-response curves for single stressors exposure was modelled using the independent action (IA) concept, and tested for possible deviations for synergism or antagonism. The carbamate insecticide carbaryl (1-naphthol N-methyl carbamate) was chosen as test-chemical, due to the fact that it is widely used in both agricultural and domestic applications, while being considered a potential neurotoxicant to non-target species (Gambi et al., 2007). The inhibition of cholinesterase (ChE) activity by carbaryl is well documented for different animal species (e.g. Caselli et al., 2006; Ferrari et al., 2004; Gambi et al., 2007; Gupta and Sundararaman, 1991). The persistence of carbaryl in plants has also been investigated, with fruit trees having shown to be able to accumulate this pesticide (Galhotra et al., 1985; Iwata et al., 1979; Rao and Ramasubbaiah, 1988). In addition, other studies have also reported the side effects of carbaryl on plant growth, e.g. apple trees (Jones et al.,1991; Murthy and Raghu,1990).

2.2 Materials and methods

2.2.1. Test substance and test species

Carbaryl (CAS No 63-25-2) was purchased from Sigma and Aldrich Ltd. (99.8% purity). Carbaryl stock solution was prepared using acetone and applied to pre-moistened soil one day before the experiment started, in order to allow the evaporation of acetone.

All experiments were carried out using the natural standard soil LUFA 2.2 from Speyer, Germany (Løkke and van Gestel, 1998). Lufa 2.2. soil is considered a standard sandy-loam soil (17% silt, 6% clay and 77% sand), with 4.4% of organic matter, a carbon/nitrogen ratio of 14, pH 5.8, water holding capacity of 55% (weight per volume) and a cation exchange capacity of 11.2 cmol/kg.

The earthworm *Eisenia andrei* Bouché was kept in laboratory cultures, in plastic boxes with a mixture of Sphagnum peat (50%) and horse dung (50%) as substrate, with pH 6.0 ± 0.05 adjusted with powdered calcium carbonate ($CaCO_3$). Organisms were fed weekly with horse dung. Cultures were maintained at 16:8 h light: dark photoperiod and at 20±2 ºC.

Seeds of *B. rapa* were purchased from Carolina Biological Supply Company (US) and *T. aestivum* from a local supplier (Aveiro, Portugal).

2.2.2. Single exposure - chemical stressor

Earthworms

Tests were performed in accordance to the OECD 207 guideline (OECD, 1984). Ten adult worms (clitellated), with individual fresh weight between 300 and 600 mg, were exposed to different carbaryl concentrations (20, 40, 60, 80, 100 mg/kg) at four replicates each. In addition to the negative control, a solvent control was prepared for comparison with 100 ml acetone/kg. The chemical exposure test was carried out in a climatic chamber at 20±2 ºC; 16h light/8h dark, and soil moisture adjusted to 60% water holding capacity (WHC). After 7 and 14 days of exposure, surviving worms were counted, and at the end of the test (14 day), earthworms were pooled weighted and their mean biomass (mg) reported. For the earthworms, loss of weight was calculated using the equation:

$$LW = \frac{(Fw_i - Fw_f)}{Fw_i}$$

where LW is the mean loss of weight; Fw_f is the mean weight after 14 days; Fw_i is the mean weight at the beginning of the test.

Plants

The plant tests were performed following the protocol ISO 11269-2 (ISO, 1995). For each species, ten seeds were placed per plastic pot with 500 g of soil at a depth of 1 cm from the soil surface. Four replicates/pot per treatment were used. Carbaryl exposure treatments ranged from 50 to 150 mg/kg. In addition to the negative control, a solvent control was prepared with 100 ml acetone/kg. Bioassays were carried out at 20±2 ºC, at 12000 lx, in a 16:8 (light: dark) photoperiod. The test duration was 14 days after 50% of seeds had emerged in the control soil. In the first 7 days, seeds' germination was reported. The soil moisture was maintained by capillary action, through a fibreglass wick (between 5 and 10 mm ø) located at the pot's bottom (Loureiro et al., 2006). At the end of the tests, individual growth (shoot length), fresh and dry weight were recorded and the hydric content (HC) calculated using the equation:

$$HC = \frac{FW - DW}{FW} \times 100$$

where DW is the plant dry weight and FW the plant fresh weight.

2.2.3. Single exposure - natural stressor

The tests with earthworms and plants were adapted from that described in the OECD 207 (OECD, 1984) and ISO 11269-2 (ISO, 1995), respectively. Earthworms and plants were exposed to different soil moisture contents, simulating drought (10, 20 and

40% of the WHC), as well as flood conditions (80, 100 and 120% of the WHC). In both approaches, a control (60% of the WHC), similarly to that described in the chemical exposure bioassays. To control moisture levels during the experiments, soil pots were weighted daily and water added when needed.

2.2.4. Combined exposure

The procedures for the tests with earthworm and plants were carried out in accordance to that described in the OECD 207 (OECD, 1984) and ISO 11269-2 (ISO, 1995), with further adaptations. Dry soil was contaminated with different carbaryl concentrations dissolved in acetone, left to evaporate for one day and then moistened with deionized water in order to obtain 10, 20, 40, 60 (control), 80, 100 and 120% WHC

All procedures were carried out as previously described for the single stressors exposures.

2.2.5. Statistical analysis

One way (ANOVA), followed by Dunnett's test, was used to analyze differences between control and treatments. Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal-Wallis ANOVA on Ranks was performed (Zar, 1996), followed by the Dunn's method when significant differences were found. Differences between control and solvent control were analyzed using a t-test or a Mann-Whitney Rank test, when normality failed ($\alpha = 0.05$). For this statistical analysis, the software package Sigma Stat was used (SPSS, 1995).

EC_{50} values were calculated using a sigmoidal (logistic, 3 parameter) equation (Systat Software Inc, 2002). LC_{50} values were calculated through the Probit method (SPSS, 2003). Data from the mixture exposures were analyzed by comparing the observed data with the expected mixture effects from the IA reference model using the MIXTOX model of Jonker et al. (2005). Under this approach, in the second step, the IA model was extended according to Jonker et al. (2005), using deviation functions to describe synergistic/antagonistic interactions, dose level, and dose-ratio dependency. For that a nested framework was built with the extra parameters needed for the deviation functions. Data are fitted to the models using the method of maximum likelihood, resulting on model fits that can be statistically compared through likelihood testing. The effect patterns were then deduced directly from the parameter values (Jonker et al., 2004, 2005).

When dose-response curves where not established for one of the stressors or parameter EC_{50}/LC_{50} values for carbaryl were compared between soil moisture treatments to detect shifts in toxicity.

2.3. Results

2.3.1. Single-exposure

There were no significant differences of the parameters measured between the control and solvent control; therefore the solvent control data will be used to compare the results obtained. The EC_{50} and LC_{50} values calculated from the single exposures to carbaryl and soil moisture, as well as the No Observed Effect Concentration (NOEC) values are presented in Table 2.1.

Earthworms' survival was significantly affected by carbaryl exposure (ANOVA, $p < 0.05$), opposed to that of biomass, which was not significantly influenced at the concentrations used (ANOVA, $p \geq 0.05$) (Figure 2.1A and B). A dose-response pattern was observed for both plant species. *B. rapa* and *T. aestivum* showed similar responses for length and biomass weight when exposed to carbaryl (Table 2.1). Plant biomass production (Figure 2.2), growth (Figure 2.3) and emergency were adversely affected and the severity of the response was directly related to increasing carbaryl concentrations. When analyzing plant water content, *T. aestivum* showed a decrease in its water content for carbaryl concentrations above 50 mg/kg, while *B. rapa* showed significant decreases from 100 mg carbaryl/kg soil (Dunn's Method, $p < 0.05$) (Figure 2.4).

The evaluation of stress induced by changes in soil moisture to organisms was carried out in two experimental setup groups: drought stress evaluation (10-40% WHC) and flood stress evaluation (80-120% WHC). Mortality and weight loss in earthworms were not significant affected by moisture, with only one earthworm having died in the 40% WHC treatment (ANOVA, $p > 0.05$).

Table 2.1 EC50, LC50 and NOEC values obtained after single exposures of Eisenia andrei, Brassica rapa and Triticum aestivum to carbaryl (mg/kg) and soil moisture (%WHC). Soil moisture is depicted as percentage of soil water holding capacity (WHC).

		Carbaryl		Drought Stress		Flood Stress	
Species	endpoint	E/LC$_{50}$ (mg/kg) (SE)	NOEC (mg/kg)	E/LC$_{50}$ (%WHC) (SE)	NOEC (mg/kg)	E/LC$_{50}$ (%WHC) (SE)	NOEC (mg/kg)
Eisenia Andrei	*survival (7d)*	53.3 (2.8)	40	n.d.	>60	n.d.	>120
	survival (14d)	45.5 (4.02)	20	n.d.	>60	n.d.	>120
	Biomass	n.d	>80	n.d.	>60	>120	>120
Brassica rapa	*Length*	66.48 (2.26)	<50	37.63 (16.50)	>40<60	120 (5.9)	100
	fresh weight	45.34 (1.81)	<50	37.2 (51.0)	40	120 (95.7)	100
	dry weight	42.31 (2.40)	<50	7.4 (22.0)	40	120 (84.8)	100
	hydric content	>150	75	n.d.	>40<60	n.d.	100
Triticum aestivum	*Length*	68.12 (2.81)	<50	8.9 (0.98)	40	120 (9.1)	80
	fresh weight	70.72 (2.55)	50	14.0 (4.0)	40	49.8 (58.3)	80
	dry weight	77.66 (2.81)	50	11.0 (1.8)	40	n.d.	80
	hydric content	>150	<50	n.d.	40	n.d.	>120

NOEC - No-observed-effect concentration; EC50 - Median effective concentration; SE - Standard Error; n.d - data not determined.

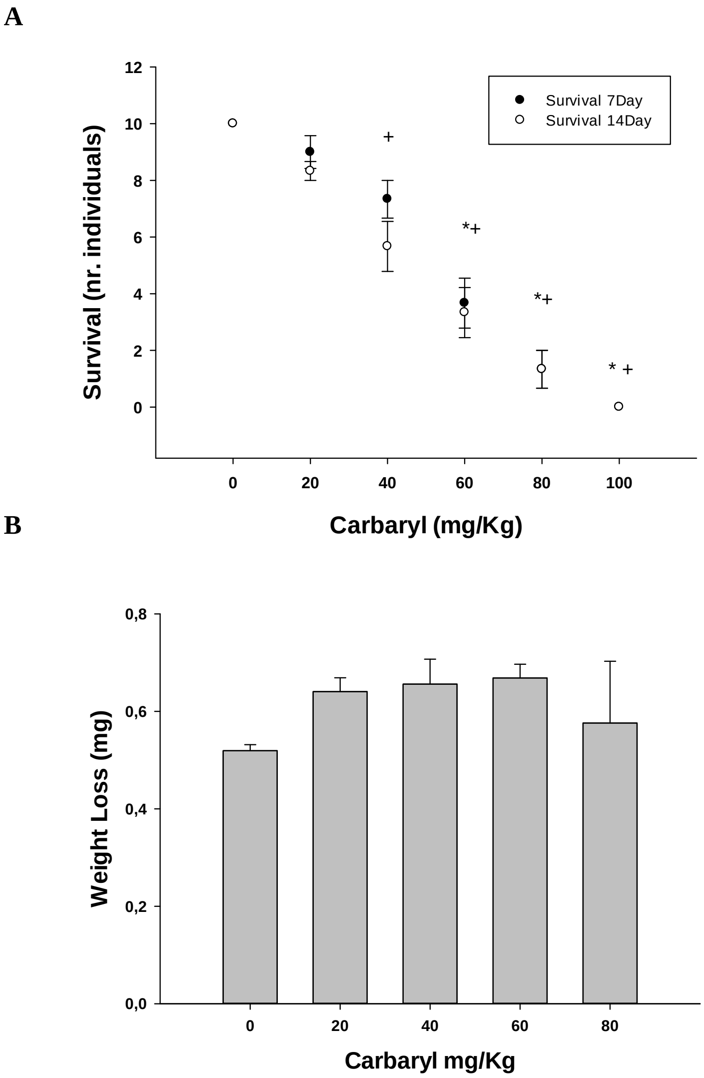
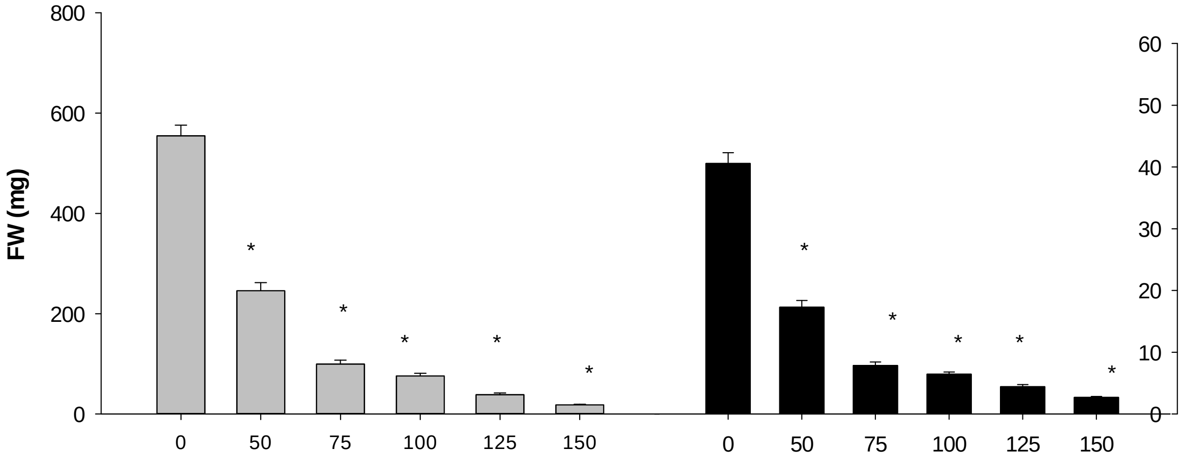


Fig. 2.1. Effect of carbaryl on Eisenia andrei survival after 7 and 14 days of exposure (A) and weight loss after 14 days of exposure (B). * and + - $p \leq 0,05$ Dunn's test, for 7 and 14 days, respectively, compared to the control.

A



B

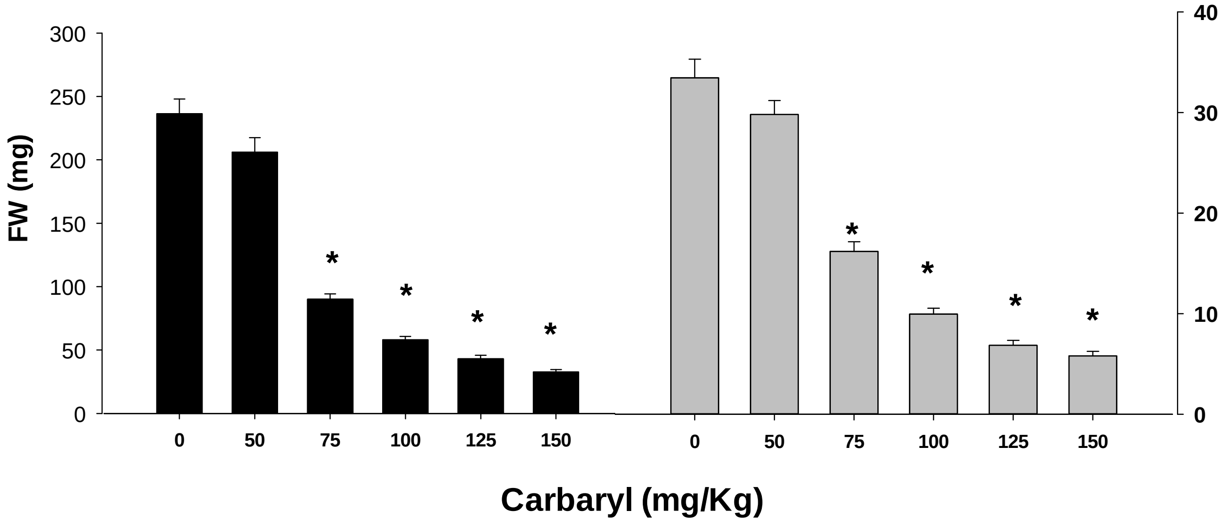


Fig. 2.2 Effect of carbaryl on the dry weight (DW) and fresh weight (FW) of *Brassica rapa* (A) and *Triticum aestivum* (B) after 14 days of exposure. * - P≤0,05 Dunn's test, comparison to the control (0 mg/Kg).

Both plant species showed similar responses to moisture, where the extreme drought and flood conditions caused low seed germination and plant development. In extreme flood stress, it was observed both a retarded seedling growth for wheat and in some cases, unsuccessful seed germination. For *B. rapa*, higher plant water content was reported in exposures to extreme stress (10, 120% WHC). In contrast, *T. aestivum* showed a different response pattern, with extreme drought stress causing a significant decrease on its hydric content, while the flood stress did not produce significant effects (ANOVA, p > 0.05).

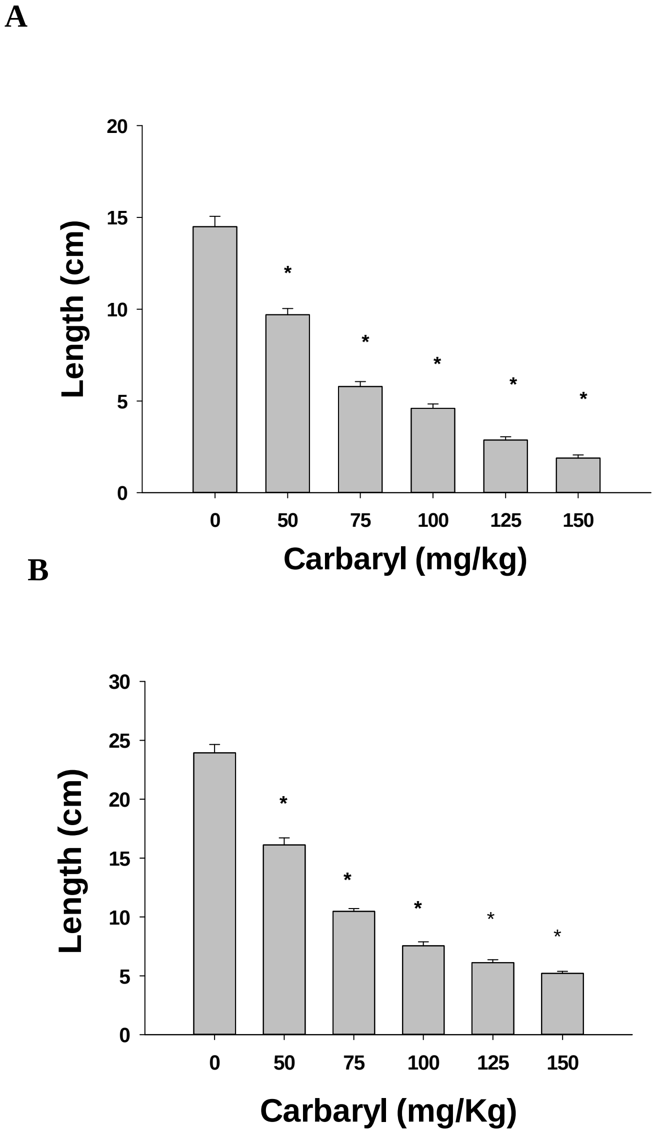


Fig. 2.3 Effect of carbaryl on *Brassica rapa* (A) and *Triticum aestivum* (B) length after 14 days of exposure. *- $p \leq 0,05$ Dunn's test, comparison to the control.

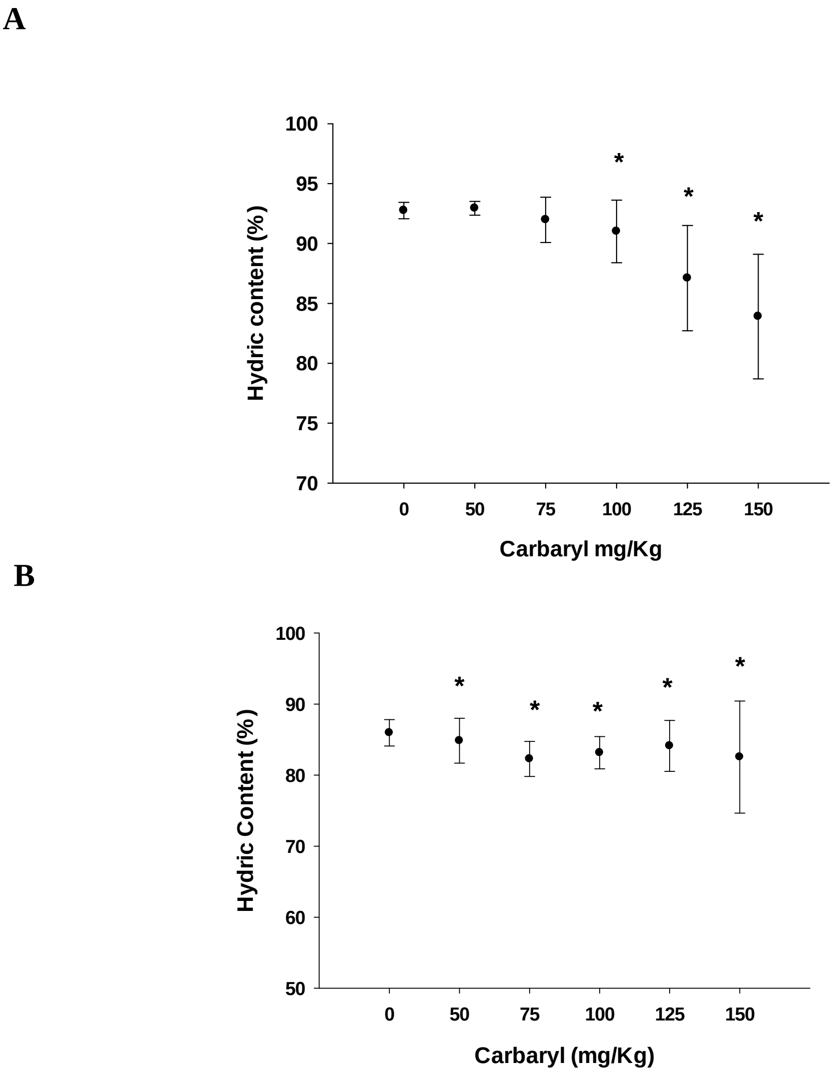


Fig. 2.4. Effect of carbaryl on the hydric content of *Brassica rapa* (A) and *Triticum aestivum* (B) after 14 days of exposure. * - $P \leq 0{,}05$ Dunn's test, comparison to the control (0 mg/Kg).

2.3.2. Combined exposure

In order to understand the response of soil organisms to the combined stressors, the IA reference model was used when dose-response curves were observed for both stressors, assuming that they do not share the same mode of action. For all case-studies, the effects of carbaryl at different soil moistures were also analyzed by calculating the corresponding LC_{50}/EC_{50} values. This output is depicted in Tables 2.2 and 2.3, respectively.

Although there were no effects induced by changes in soil moisture on the survival of earthworms, changes in soil water content induced changes in the toxicity of carbaryl. The LC_{50} of carbaryl decreased with decreasing soil moisture, suggesting a potential synergism. After 14 days of combined exposure, there was 100% mortality in the carbaryl treatments in soil at 10 and 20% WHC. In contrast, the flood-simulation scenario induced a decrease of carbaryl toxicity, showing low mortalities as response to higher concentrations after 14 days of exposure, and increasing the LC_{50} from 54.74 mg carbaryl/kg soil (60% WHC) to 89.4 mg carbaryl/kg (120% WHC). Soil pH was consistent throughout the assays (between 6 and 7).

Table 2.2 Toxicity of carbaryl to *Eisenia andrei*, *Brassica rapa* and *Triticum aestivum* in different soil moisture regimes. Data is presented as EC_{50} or LC_{50} (mg/Kg) with standard error (SE). Moisture regimes are depicted as percentage of soil water holding capacity.

		Carbaryl EC_{50}/LC_{50} (ES) for moisture conditions (mg/Kg)						
Species	endpoint	10%WHC	20% WHC	40% WHC	60% WHC	80% WHC	100% WHC	120% WHC
E. andrei	*survival (7d)*	<20 (n.d)	<20 (n.d)	49.19 (6.23)	86.5 (11.87)	>100 (n.d)	>100 (n.d)	>100 (0,39)
	survival (14d)	<20 (n.d)	<20 (n.d)	26.20 (4.52)	54.74 (5.58)	93.05 (14.27)	>100 (n.d)	89.4 (0.007)
	Biomass	n.d	n.d	n.d.	n.d	n.d	n.d.	n.d.
B. rapa	*Length*	62.4 (54.1)	n.d	59.2 (15.4)	90.5 (10.3)	99.0 (13.5)	85.4 (5.5)	72 (4.0)
	fresh weight	17.0 (25.2)	>150	45.3 (9.5)	59.7 (5.4)	63.0 (4.9)	54 (3.3)	51 (4.8)
	dry weight	n.d.	28.6 (150.1)	n.d.	49.9 (14.5)	59.4 (5.3)	56.6 (4.6)	47.5 (5.7)
	hydric content	>150	n.d	n.d.	n.d	n.d	n.d.	n.d.
T. aestivum	*Length*	>150	132.5 (41.1)	71 (8.2)	41.5 (4.1)	42.2 (7.2)	44.0 (2.8)	55.0 (4.7)
	fresh weight	n.d	145 (47.1)	56.4 (7.2)	37 (4.3)	26.7 (10.7)	31.5 (4.1)	57 (7.4)
	dry weight	105.2 (59.6)	129 (64)	65.2 (8.5)	51.5 (3.0)	55.6 (18.1)	53.3 (7.0)	89 (13.4)
	hydric content	n.d	n.d	n.d.	n.d	n.d	n.d.	n.d.

The response patterns in *B. rapa* showed that the reference model IA provided a valid estimation of the overall toxicity for combined carbaryl and drought stress ($p < 0.05$, Table 2.3); no deviation from the model was observed.

In the flood stress conditions, the mass production parameters (FW and DW) showed an antagonistic deviation from the reference model ($p < 0.05$, Table 2.3), and shoot length data revealed a dose-dependent deviation from the independent action model ($p < 0.05$; SS = 2770; $\alpha = 7.15$; $b = 1.4$; $r^2 = 0.44$; Table 2.3). In the latter case, antagonism was detected at low carbaryl concentrations and switching to a synergistic pattern for doses above the corresponding EC_{50} level, i.e. extreme water content and high carbaryl concentrations.

The combined effects of drought conditions and carbaryl in *T. aestivum* length caused a “dose ratio” deviation from the IA model ($p < 0.05$; Table 2.3). An antagonistic pattern was observed when drought stress was dominant, whereas synergism occurred when carbaryl was the dominant stressor, i.e. high doses of carbaryl and low drought stress. For the FW and DW parameters, an antagonistic effect was detected ($p < 0.05$). In flood conditions, DW data also showed an antagonistic pattern ($p < 0.05$; Table 2.3), and a “dose level” deviation from the IA model for length and FW ($p < 0.05$), with antagonism being dominant at higher dose levels and synergism at low dose levels.

Tabela 2.3. Summary of the analysis done for the effects on length, fresh weight (FW) and dry weight (DW) of *Brassica rapa* and *Triticum aestivum* exposed to the combination of carbaryl and drought or flood regimes. IA is independent action; SS is the objective function used for continuous data; r^2 is the coefficient of determination; a and b are parameters of the deviation functions; S/A is synergism/antagonism; DR and DL are “dose ratio” and “dose level” deviation from the reference model

Species and parameter	Carbaryl	IA		Deviation				
		SS	r^2	Deviation type	SS	r^2	a	b
Brassica rapa length	Drought	3.96	0.69					
	Flood	2895	0.42	DL	2770	0.44	7.15	1.4
Brassica rapa FW	Drought	149398	0.78					
	Flood	1768860	0.66	A	16×10^5	0.68	2.5	-
Brassica rapa DW	Drought	1879	0.51					
	Flood	8626	0.65	A	8307	0.66	1.7	-
Triticum aestivum length	Drought	1019	0.87	DR	8.63	0.89	3.9	-4.6
	Flood	2385	0.85	DL	1972	0.88	0.04	-
Triticum aestivum FW	Drought	87716	0.89	A	82744	0.90	1.15	- 49.5
	Flood	845314	0.71	A	691305	0.76	0.65	-
Triticum aestivum DW	Drought	2477	0.85	A	2395	0.86	1.13	-
	Flood	19390	0.61	A	16734	0.66	3.0	-

2.4. Discussion

2.4.1. Single stressor exposures

The impact of carbaryl on the inhibition of cholinesterase (ChE) activity has been well documented for various organisms, including earthworms (Caselli et al., 2006; Ribera et al., 2001; Stenersen, 1980), which are known for exhibiting high sensitivity towards chemical carbamates. Gambi et al. (2007) reported maximal cholinesterase (ChE) inhibition for carbaryl concentrations of 48.3 mg/kg, whereas Ribera et al. (2001) observed cholinesterase (ChE) inhibition at the lower dose of 12 mg carbaryl/kg soil. Although carbaryl toxicity in earthworms is caused mainly by acetylcholinesterase inhibition, LC_{50} values found in this study are above those reported in the afore mentioned studies. Edwards and Lofty (1977) have shown that carbamates are highly toxic to earthworms and can kill them rapidly.

Heimbach (1984) calculated an LC_{50} value for carbaryl exposure of 174 mg/kg, and it was cited by Jänsch et al. (2006) as the lowest LC_{50} values reported in the literature. In the present study, even lower values were calculated for 7 and 14 days of exposure using two different approaches: testing carbaryl in a single exposure experiment (53.3 and 45.5 mg/ kg, respectively) and in the combined exposure under 60% WHC (86.5 and 54.74 mg/kg, respectively). From the values calculated, lethality did not vary significantly with time of exposure (from 7 to 14 days). Carbaryl alone had no significant detrimental impact on earthworm biomass, thus concurring with the findings of Mostert et al. (2000), who reported an effect (although not always significant) on biomass relative to the control.

Regarding plant toxicity, Chakrabarti et al. (1990) reported that carbaryl inhibited the growth of germinating mustard seed, while causing accumulation of reserved triglycerides. In this study, carbaryl showed to affect plant growth and biomass production at concentrations around 65 mg/kg. It has been reported that carbaryl concentrations in cultivated soils from Udaipur in the Indian state of Rajasthan ranged from 232 to 525 mg/kg (Kavadia et al., 1978), which is above the calculated EC50 values and concentrations used in the present study.

The influence of soil moisture on soil fauna and flora has been widely described in various studies. Gunadi et al. (2003) recorded an increment in earthworms' mortality and growth rate in pig manure soils with high moisture content (90% WHC), which is not consistent with our results. On the other hand, Dominguez and Edwards (1997) found that the optimum soil moisture content for *E. andrei* growth is around 85% WHC, cultured in pig manure. Reinecke and Venter (1987) pointed out that earthworms thrive at higher soil moisture levels, showing also higher mean biomass weights. Drought is known to be an important stressor to earthworms, with small decreases in soil moisture being able to cause dramatic losses in their body water contents (Petersen et al., 2008). In this study, however, soil moisture did not affect significantly *E. andrei* survival or biomass gain/loss.

Several studies have highlighted the importance of climate change research, focussing mainly on their effects on wheat crops, which account for 21% of food and 200 million hectares of farmland worldwide (Reilly et al., 2003). In this study, *T. aestivum* and *B. rapa* emergence, growth and biomass were strongly affected by drought and flood extreme conditions. Oxidative stress during water deficit has been shown to be detrimental to plant growth and leaf photosynthesis (Dat et al., 2000; Selote and Khanna-Chopra, 2004).

Mishra et al. (1999) showed that moisture stress (10-12% WHC) reduced the relative water content in four Brassica species. This does not corroborate with that found in our study, where *B. rapa* exhibited an unexpected increase in water content under drought conditions. This might be explained as a physiological response to hydric stress, where early and progressive effects of drought are related to stomata closure for preventing water loss (Flexas and Medrano, 2002).

In contrast, this pattern was not found for *T. aestivum*, where our results corroborate those reported for *Vicia fabia* plants, in which water contents decreased with drought stress (Abdel-Basset, 1998).

A more recent study with *Brassica napus* showed that water stress (low watering) explained reductions in plant length and stem diameter, leaf area, plant dry weight, leaf weight ratio and shoot/ root weight ratio, as well as impact on parameters related to photosynthesis, such as chlorophyll-*a* and carotenoids (Sangtarash et al., 2009).

2.4.2. Combined exposures

Carbamates in general exhibit a prolonged effect on the fitness of earthworms in soil (Gilman and Vardanis, 1974), with sublethal concentrations having shown to retard their growth and reproduction (Neuhauser and Clarence, 1990). Additionally, moisture availability can strongly influence the activity of soil organisms and cause desiccation in extreme drought conditions (Hayward et al., 2004), while in plants, dehydration leads to oxidative stress (Chaves et al., 2003). Flood conditions can cause mortality due to anaerobiosis in the soil environment (Hartenstein, 1981). Exposures to extreme soil moistures and carbaryl will induce toxicity to earthworms and plants by acting in different targets (MoA). In this context, the IA reference model was chosen as a baseline, to help predicting the effects of combined stressors. This was done only when each stressor could be well described by a logistic model (Bliss, 1939).

Drought stress

Regarding the combined toxicity of carbaryl and soil moisture in earthworms, there was a significant synergism between drought stress and carbaryl on survival and weight loss of *E. andrei*. This means that the combined effects observed in our experiment were stronger than expected, considering the single exposure responses for both stressors individually. This can be explained by dehydration, whereby reducing the volume of water within the organism, leads to increasing concentration of the chemical and the risk for toxic damage to occur (Holmstrup, 2008). Various studies reported similar synergistic processes caused by interactions of desiccation and chemicals (Friis et al., 2004; Højer et al., 2001; Holmstrup, 1997; Holmstrup et al., 1998; Sjursen et al., 2001; Sorensen and Holmstrup, 2005).

Using the Independent Action model for predicting effect patterns on the combined toxicity of fluoranthene and drought stress in the reproduction (cocoon production rate) of *Lumbricus rubellus*, Long et al. (2009) showed that this model provided a good description of the combined stressors data. Although their results suggested that the IA model interprets accurately the combined effects of chemical and nonchemical stressors, authors highlighted that such an approach needs to be done cautiously, as interactions between stressors may result in deviations from the IA model predictions, as observed in the current study.

In the bioassays with plants, data from all parameters reported for *B. rapa* showed that the IA model had an accurate fit, describing the additivity of responses expressed in this model. From the EC_{50} values, we can see that as drought increases, the toxicity of carbaryl is more pronounced, as a result of combined toxicity (as additivity), demonstrating that in drought periods, carbaryl toxicity towards plants will be more marked, compared to periods of regular soil moisture.

For *T. aestivum* however, there was no additivity in the responses. Biomass parameters showed antagonism, where toxic effects were less than that expected, while for the shoot length, carbaryl was responsible for the increase in toxicity under combined exposures.

As far as we know, there are no studies published on the combined effects of chemicals and different soil moisture regimes in plant species, including those used in this study. There are several hypotheses that can be raised towards these results. One hypothesis can be that at low moisture regimes, less water is available for uptake by plants. As chemicals enter plants as water solutions, under a contaminated scenario and low water levels in soil, less water will be available for uptake by the roots, thus less chemical will enter.

Flood stress

Flood scenarios are not usually considered as stressors in environmental risk assessment. Although the water excess will not jeopardize plants over short-term periods, its combined exposure should be evaluated and considered.

Regarding the increase of water in soil and the presence of carbaryl, one can hypothesize that when the concentration of chemical in pore water is reduced (higher dilution), then we will see lower than the expected toxicity. In this study, there were no differences on the LC_{50} of the earthworms exposed to carbaryl under simulated flood scenarios. The relatively low coefficient of adsorption of carbaryl allows its dissolution and transport in water (Lin et al., 2007). This might raise also a problem for carbaryl in soil when flood scenarios occur, related to groundwater contamination.

In a study where the collembolan *Folsomia candida* was exposed to cadmium at different ranges of soil moisture (from 74 to 162% of field capacity), there were no clear effects of soil moisture on the bioavailability and toxicity of cadmium to *F. candida* (van Gestel and van Diepen, 1997) which is not in accordance with the findings reported here.

For the plants bioassay, antagonism was the pattern that showed more consistency, appearing as a predictability pattern in four out of the six evaluations. This antagonism was observed for both plant species parameters related to biomass weight (fresh and dry). For the shoot length, antagonism was the main pattern observed, although synergism became dominant when high doses of carbaryl were applied at high moisture levels. This might be related to lower concentrations of carbaryl in soil pore water due to higher moisture contents in soils, i.e. water dilution effect, with consequent decrease on carbaryl uptake by plants.

2.5. Conclusions

The present study showed that soil moisture content has a strong influence on the toxicity of carbaryl. A synergistic effect was associated to extreme drought conditions, increasing the toxicity of carbaryl towards earthworms, as confirmed by the decrease of the LC_{50} values observed under drought conditions.

The reference model, Independent Action (IA), gave a valid estimation for the combined toxicity under simultaneous exposure trials of this chemical and drought stressors in *B. rapa*. In flood stress conditions, deviations from reference models were found (antagonism, “dose ratio” and “dose level” dependence), despite that antagonistic deviations were verified for the majority of combinations studied.

The pronounced deleterious effects on earthworms from carbaryl exposure under drought soil conditions, highlighted the importance of climate change in the performance of pesticides in soil organisms. However, the lesser deleterious effects than expected under flood stress in plants, may be explained by carbaryl dilution in soil pore water. Therefore, lixiviation of carbaryl must be considered and evaluated in future studies as it can represent a potential risk to underground water.

Finally drought and flood scenarios should also be included as stress parameters in risk assessment procedures, as they can possibly induce additive responses or increase toxicity, compared to that expected (i.e. higher toxicity than the sum of responses of individual stressors). Similarly, flood scenarios should also be taken into consideration, as these can contribute for increasing leaching of chemicals that have high mobility in soils, and low adsorption to soil particles.

Acknowledgements

This study was complementary supported by a PhD grant from ALBAN (REF E07D402936BR), a PhD grant from FCT (SFRH/BD/ 65274/2009) and by the project CLIMAFUN e CLIMAFUN-CLImate Changes and Potential Impact on Soil Functional Ecology (PTDC/ AAC-CLI/104960/2008) funded by the Portuguese Science and Technology Foundation (FCT). The authors would like to thank Ana Bastos for the proof-reading that surely improved the manuscript.

2.6 Supplementary Data

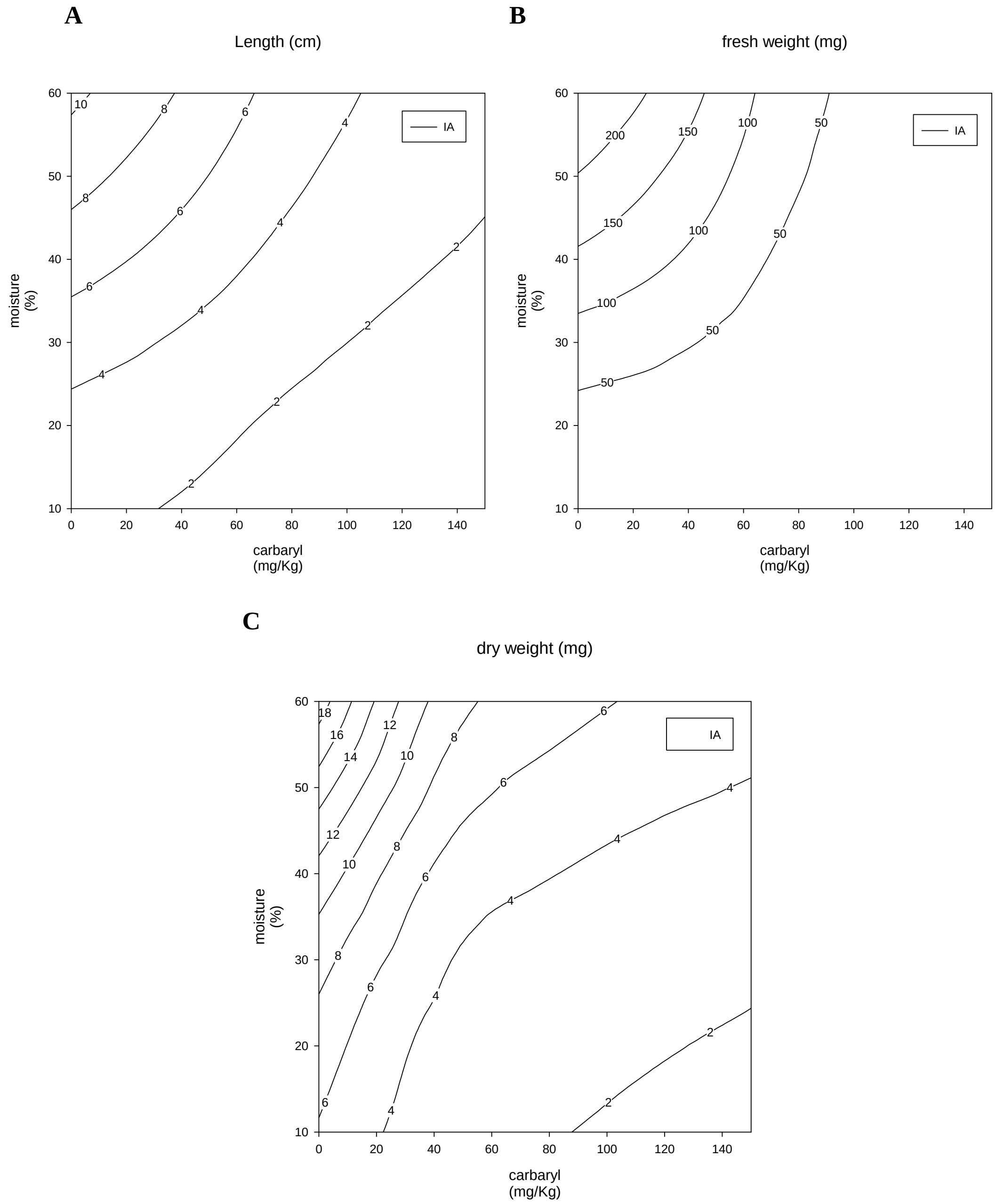


Fig. S 2.1. Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Brassica rapa* exposed to carbaryl under several drought regimes [10, 20, and 40% Water Holding Capacity (WHC)]: independent action (IA) model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3.

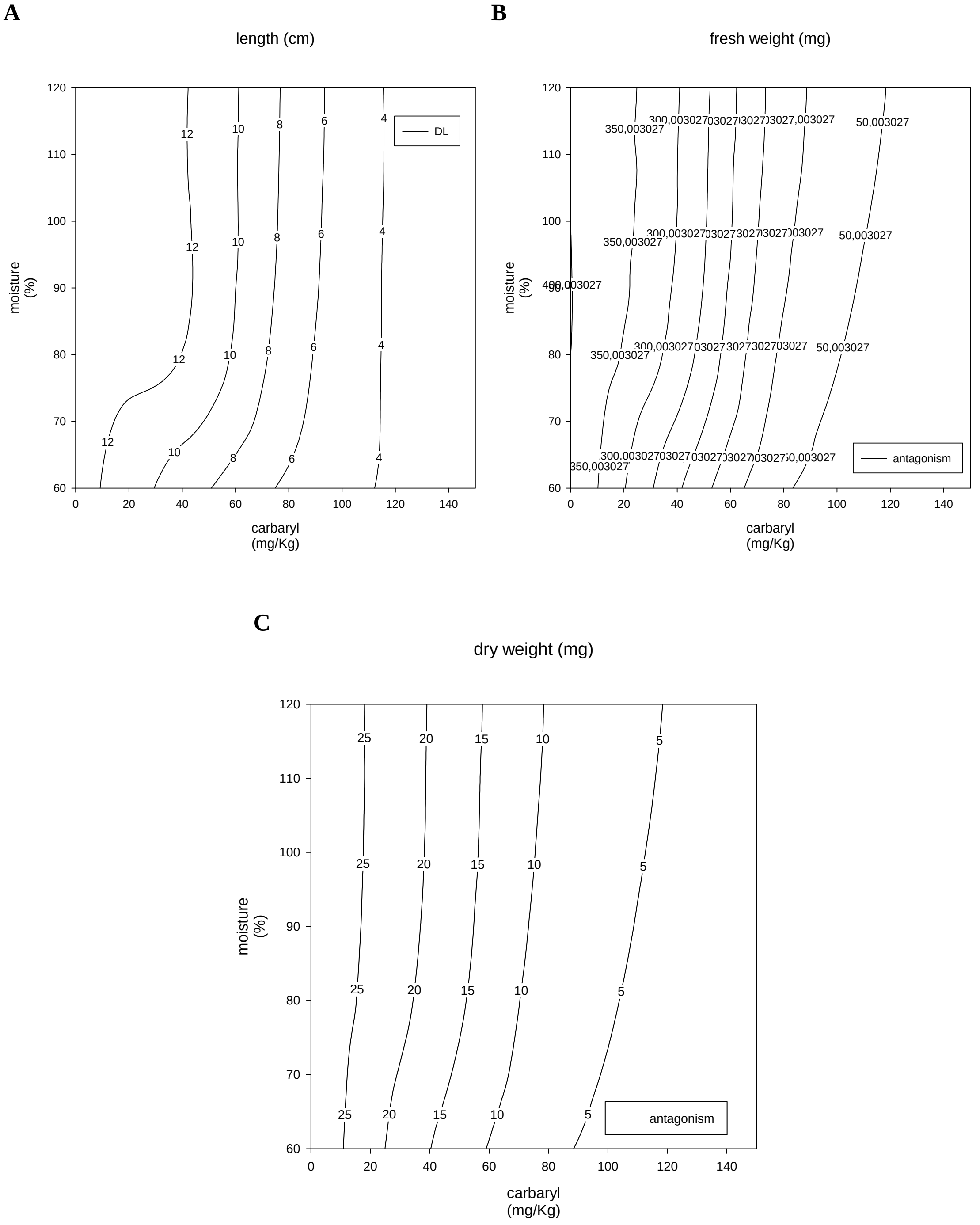


Fig. S 2.2 Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Brassica rapa* exposed to carbaryl under several flood regimes [80, 100, and 120% Water Holding Capacity (WHC)]: dose level (DL) deviation (A), antagonism deviation (B and C) and after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3.

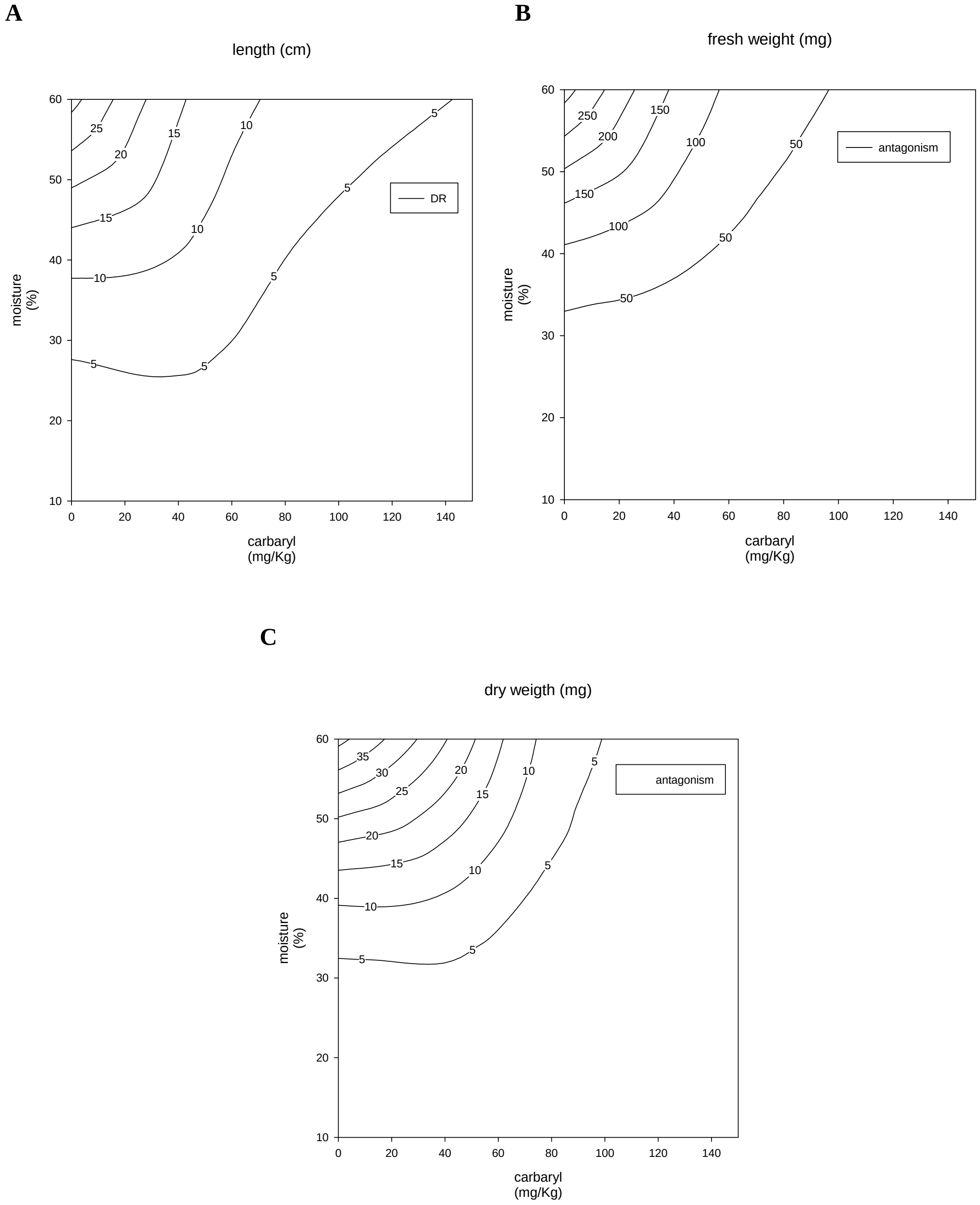


Fig. S 2.3 Concentration–response for length (A), fresh weight (B) and dry weight (mg) of *Triticum aestivum* exposed to carbaryl under several drought regimes [10, 20, and 40% Water Holding Capacity (WHC)]: dose ratio (DR) deviation (A), antagonism deviation (B and C) after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3.

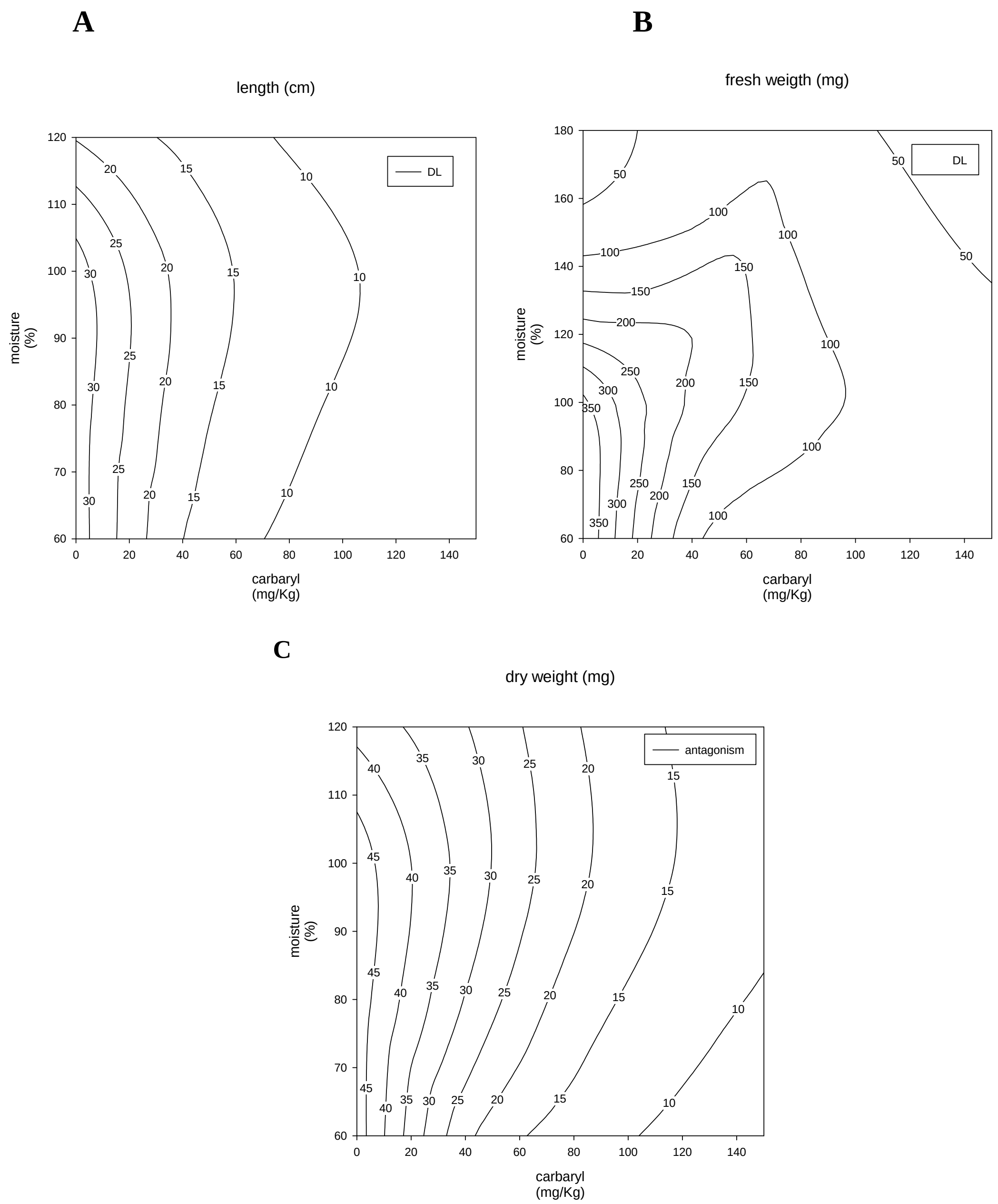


Fig. S 2.4. Concentration–response for length (A), fresh weight (B) and dry weight (C) of *Triticum aestivum* exposed to carbaryl under several flood regimes [80, 100, and 120% Water Holding Capacity (WHC)]: dose level (DL) deviation (A and B) and antagonism (DL) deviation (C) after independent action model fit to the data (2D Isobolic Surface). 60% of WHC was considered the control situation. Concentrations and moisture levels are reported as nominal values. Parameters from the MIXTOX model can be checked on Table 2.3.

References

Abdel-Basset, R., 1998. Calcium channels and membrane disorders induced by drought stress in *Vicia faba* plants supplemented with calcium. Acta Physiologiae Plantarum 20, 149-153.

Azevedo, H., Gomes, C., Pinto, G., Fernandes, J., Loureiro, S., Santos, C., 2005. Cadmium effects on sunflower growth and photosynthesis. Journal of Plant Nutrition 28, 2211-2220.

Bardgett, R.D., Bowman, W.D., Kaufmann, R., Schmidt, S.K., 2005. A temporal approach to linking aboveground and belowground ecology. Trends in Ecology and Evolution 20, 634-641.

Bindesbøl, A.M., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. Environmental Toxicology and Chemistry 24, 1462-1467.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annals of Biology 26, 585-615.

Caselli, F., Gastaldi, L., Gambi, N., Fabbri, E., 2006. In vitro characterization of cholinesterases in the earthworm *Eisenia andrei*. Comparative Biochemistry and Physiology C - Toxicology & Pharmacology 143, 416-421.

Chakrabarti, A., Sengupta, P.K., Banerjee, S.K., 1990. Influence of toxic concentrations of carbaryl on lipid breakdown in germinating seeds of mustard *Brassica nigra*. Indian Journal of Experimental Biology 28, 1088-1089.

Chaves, M.M., Maroco, P.J., Pereira, J.S., 2003. Understanding plant responses to drought e from genes to the whole plants. Functional Plant Biology 30, 239-264.

Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D., Van Breusegem, F., 2000. Dual action of the active oxygen species during plant stress responses. Cellular and Molecular Life Sciences 57, 779-795.

Dominguez, J., Edwards, C.A., 1997. Effects of stocking rate and moisture content on the growth and maturation of *Eisenia andrei* (Oligochaeta) in pig manure. Soil Biology & Biochemistry 29, 743-746.

Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms. Vol.3 Springer.

Edwards, C.A., Lofty, J.R., 1977. Biology of Earthworms (2nd. ed.).Chapman and Hall., New York., Wiley, London.

Ferrari, A., Anguiano, O.L., Solenõ, J., Venturino, A., D'Angelo, A.M.P., 2004. Different susceptibility of two aquatic vertebrates (*Oncorhynchus mykiss* and *Bufo arenarum*) to azinphos methyl and carbaryl. Comparative Biochemistry and Physiology a e Molecular & Integrative Physiology 139, 239-243.

Ferreira, A.L.G., Loureiro, S., Soares, A.M.V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna.* Aquatic Toxicology 89, 28-39.

Ferreira, A.L.G., Serra, P., Soares, A.M.V.M., Loureiro, S., 2010. The influence of natural stressors on the toxicity of nickel to *Daphnia magna.* Environmental Science & Pollution Research 17, 1217-1229.

Flexas, J., Medrano, H., 2002. Drought-inhibition of photosynthesis in C-3 plants: stomatal and non-stomatal limitations revisited. Annals of Botany 89, 183-189.

Fragoso, C., Lavelle, P., 1992. Earthworm communities of tropical rain-forests. Soil Biology & Biochemistry 24, 1397-1408.

Friis, K., Damgaard, C., Holmstrup, M., 2004. Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea caliginosa* during drought. Ecotoxicology and Environmental Safety 57, 65-73.

Galhotra, R., Garg, P.K., Halve, A.K., Pandey, S.Y., Stvasankaran, K., 1985. Carbaryl and lindane residues in potato crop. Journal of the Entomological Research 9, 57-59.

Gambi, N., Pasteris, A., Fabbri, E., 2007. Acetylcholinesterase activity in the earth-worm *Eisenia andrei* at different conditions of carbaryl exposure. Comparative Biochemistry and Physiology C - Toxicology & Pharmacology 145, 678-685.

Gilman, P., Vardanis, A., 1974. Carbofuran. Comparative toxicity and metabolism in the worms *Lumbricus terrestris* and *Eisenia fetida*. Journal of Agricultural and Food Chemistry 22, 625-628.

Gong, P., Wilke, B.-M., Strozzi, E., Fleischmann, S., 2001. Evaluation and refinement of a continuous seed germination and early seedling growth test for the use in the ecotoxicological assessment of soils. Chemosphere 44, 491-500.

Gorsuch, J.W., Lower, W.R., Lewis, M.A., Wang, W., 1991. Plants for Toxicity Assessment, vol. 2. ASTM International, Gunadi, B., Edwards, C.A., Blount, C., 2003. The influence of different moisture levels on the growth, fecundity and survival of *Eisenia fetida* (Savigny) in cattle and pig manure solids. European Journal of Soil Biology 39, 19-24.

Gupta, S.K., Sundararaman, V., 1991. Correlation between burrowing capability and ache activity in the earthworm, *pheretima posthuma,* on exposure to carbaryl. Bulletin of Environmental Contamination and Toxicology 46, 859-865.

Hartenstein, R., 1981. Sludge decomposition and Stabilization. Science, New Series 212, 743-749.

Hayward, S.A.L., Worland, M.R., Convey, P., Bale, J.S., 2004. Habitat moisture availability and the local distribution of the Antarctic Collembola *Cryptopygus antarcticus* and *Friesea grisea*. Soil Biology & Biochemistry 36, 927-934.

Heimbach, F., 1984. Correlation between three methods for determining the toxicity of chemicals by earthworms. Pesticide Science 15, 605-611.

Højer, R., Bayley, M., Damgaard, C.F., Holmstrup, M., 2001. Stress synergy between drought and a common environmental contaminant: studies with the collembolan *Folsomia candida.* Global Change Biology 7, 485-494.

Holmstrup, M., 1997. Drought tolerance in *Folsomia candida* Willem (Collembola) after exposure to sublethal concentrations of three soil-polluting chemicals. Pedobiologia. 41, 361-368.

Holmstrup, M., 2008. Strategies for cold and drought tolerance in soil invertebrates: different stressors but same mechanisms. Comparative Biochemistry and Physiology A -Molecular & Integrative Physiology 150, S155.

Holmstrup, M., Maraldo, K., Krogh, P.H., 2007. Combined effect of copper and prolonged summer drought on soil Microarthropods in the field. Environmental Pollution 146, 525-533.

Holmstrup, M., Petersen, B.F., Larsen, M.M., 1998. Combined effects of copper, desiccation, and frost on the viability of earthworm cocoons. Environmental Toxicology and Chemistry 17, 897-901.

ISO, 1995. Soil Quality e Determination of the Effects of Pollutants on Soil Flora e Part 2: Effects of Chemicals on the Emergence and Growth of Higher Plants. International Organization for Standardization, Geneva, Switzerland.

Iwata, Y., Dusch, M.E., Garman, G.E., Gunther, F.A., 1979. Worker environment research: residues for carbaryl, chlorobenzilate, dimethoate and trichlorfon applied to citrus trees. Journal of Agricultural and Food Chemistry 27, 1141-1145.

Jänsch, S., Frampton, G.K., Römbke, J., Van den Brink, P.J., Scott-Fordsmand, J.J., 2006. Effects of pesticides on soil invertebrates in model ecosystem and field studies: a review and comparison with laboratory toxicity data. Environmental Toxicology & Chemistry 25, 2490-2501.

Jones, K.M., Koen, T.B., Bound, S.A., Oakford, M.J., 1991. Efffects of paclobutrazol and carbaryl on the yield of Hi-early red ‘Delicious’ apples. Journal of Horticultural Science 66, 159-163.

Jonker, M.J., Piskiewick, A.M., Castellà, N.I., Kammenga, J.E., 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environmental Toxicology and Chemistry. 23, 152-1537.

Jonker, M.J., Svendensen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J., 2005. Significance testing of synergistic antagonistic, dose level dependent or dose ratio dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry 24, 2701-2713.

Kavadia, V.S., Gangwar, S.K., Srivastava, B.P., Kathpal, T.S., Gupta, H.C.L., 1978. Persistence of carbaryl in clay loam soil and its uptake in maize and root crops. Indian Journal of Entomology 40, 265-272.

Lavelle, P., Spain, A.V., 2001. Soil Ecology. Springer. Amsterdam.

Lin, C.C., Hui, M.N.Y., Cheng, S.H., 2007. Toxicity and cardiac effects of carbaryl in early developing zebrafish (*Danio rerio*) embryos. Toxicology and Applied Pharmacology 222, 159-168.

Loewe, S., Muischnek, H., 1926. Combined effects I announcement e implements to the problem. Nanyn-Schmiedebergs Archive der Experimentellen Pathologie und Pharmakologie 114, 313-326.

Løkke, H., Van Gestel, C.A.M., 1998. In: Handbook of Soil Invertebrate Toxicity Tests. Wiley & Sons, Chichester.

Long, S.M., Reichenberg, F., Lister, L.J., Hankard, P.K., Townsend, J., Mayer, P., Wright, J., Holmstrup, M., Svendsen, C., Spurgeon, D.J., 2009. Combined chemical (fluoranthene) and drought effects on *Lumbricus rubellus* demonstrate the applicability of the independent action model for multiple stressor assessment.

Environmental Toxicology and Chemistry 28, 629-636.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environmental Pollution 157, 625-636.

Loureiro, S., Santos, C., Pinto, G., Costa, A., Monteiro, M., Nogueira, A.J.A., Soares, A.M.V.M., 2006. Toxicity assessment of two soils from Jales mine (Portugal) using plants: growth and biochemical parameters. Archives of Environmental Contamination and Toxicology 50, 182-190.

Maraldo, K., Christensen, B., Strandberg, B., Holmstrup, M., 2006. Effects of copper on Enchytraeids in the field under differing soil moisture regimes. Environmental Toxicology and Chemistry 25, 604-612.

Martikainen, E.A.T., Krogh, P.H., 1999. Effects of soil organic matter content and temperature on toxicity of dimethoate to *Folsomia fimetaria* (Collembola: Isotomiidae). Environmental Toxicology and Chemistry 18, 865-872.

Mishra, R.S., Abdin, M.Z., Uprety, D.C., 1999. Interactive effects of elevated CO_2 and moisture stress on the photosynthesis, water relation and growth of Brassica species. Journal of Agronomy and Crop Science 182, 223-229.

Mostert, M.A., Schoeman, A.S., Van der Merwe, M., 2000. The toxicity of five insecticides to earthworms of the Pheretima group, using an artificial soil test. Pest Management Science 56, 1093-1097.

Murthy, N.B.K., Raghu, K., 1990. Effect of carbaryl and 1-naphthol on seedling growth of barley, and on growth and nodulation of groundnut in two soils. Proceedings of the Indian Academy of Sciences Plant Sciences 100, 11-16.

Neuhauser, E.F., Clarence, C.A., 1990. Soil Biology and Biochemistry. 22, 175-179.

OECD, 1984. Earthworm Acute Toxicity Tests. OECD- Organization for Economic Cooperation and Development, Paris.

Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquatic Toxicology 93, 138-149.

Petersen, C.R., Holmstrup, M., Malmendal, A., Bayley, M., Overgaard, J., 2008. Slow desiccation improves dehydration tolerance and accumulation of compatible osmolytes in earthworm cocoons (*Dendrobaena octaedra* Savigny). Journal of Experimental Biology 211, 1903-1910.

Rao, B.N., Ramasubbaiah, K., 1988. Dissipation of monocrotophos and carbaryl in tomato *Lycopersicon lycopersicum* L. Indian Journal of Entomology 50, 209-214.

Reilly, J., Tubiello, F., McCarl, B., Abler, D., Darwin, R., Fuglie, K., Hollinger, S., Izaurralde, C., Jagtap, S., Jones, J., Mearns, L., Ojima, D., Paul, E., Paustian, K., Riha, S., Rosenberg, N., Rosenzweig, C., 2003. US agriculture and climate change: new results. Climatic Change 57, 43-69.

Reinecke, A.J., Venter, J.M., 1987. Moisture preferences, growth and reproduction of the compost worm *Eisenia fetida* (Oligochaeta)*. Biology and Fertility of Soils 3, 135-141.

Ribera, D., Narbonne, J.F., Arnaud, C., Saint-Denis, M., 2001. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biology & Biochemistry 33, 1123-1130.

Sangtarash, M.H., Qaderi, M.M., Chinnappaa, C.C., Reida, D.M., 2009. Differential sensitivity of canola (*Brassica napus*) seedlings to ultraviolet-B radiation, water stress and abscisic acid. Environmental and Experimental Botany 66, 212-219.

Selote, D.S., Khanna-Chopra, R., 2004. Drought-induced spikelet sterility is associated with an inefficient antioxidant defence in rice panicles. Physiologia Plantarum 121, 462-471.

Sjursen, H., Holmstrup, M., 2004. Cold and drought stress in combination with pyrene exposure: studies with *Protaphorura armata* (Collembola: Onychiuridae). Ecotoxicology and Environmental Safety 57, 145-152.

Sjursen, H., Sverdrup, L.E., Krogh, P.H., 2001. The effects of polycyclic aromatic compounds on the drought tolerance of *Folsomia fimetaria* (Collembola: Isotomidae). Environmental Toxicology & Chemistry 20, 2899-2902.

Sorensen, T.S., Holmstrup, M., 2005. A comparative analysis of the toxicity of eight common soil contaminants and their effects on drought tolerance in the collembolan *Folsomia candida*. Ecotoxicology and Environmental Safety 60, 132-139.

SPSS, 1995. In: Science, S. (Ed.), SigmaStat for Windows (Version 2.03) IL.

SPSS, 2003. Statistical Package for the Social Sciences e SPSS 12.0 for Windows. SPSS Inc.

Spurgeon, D.J., Svendsen, C., Lister, L.J., Hankard, P.K., Kille, P., 2005. Earthworm responses to Cd and Cu under fluctuating environmental conditions: a comparison with results from laboratory exposures. Environmental Pollution 136, 443e452.

Stenersen, J., 1980. Esterases in earthworms. Part I: characterization of the cholin-esterase in *Eisenia foetida* (Savigny) by substrated and inhibitors. Comparative Biochemistry and Physiology 66, 37-44.

Svendsen, C., Hankard, P.K., Lister, L.J., Fishwick, S.K., Jonker, M.J., Spurgeon, D.J., 2007. Effect of temperature and season on reproduction, neutral red retention and metallothionein responses of earthworms exposed to metals in field soils. Environmental Pollution 147, 83-93.

Systat Software Inc, 2002. In: Richmond, P. (Ed.), SigmaPlot for Windows (Version 8.02) CA, USA.

Van Gestel, C.A.M.,Van Diepen, A.M.F., 1997. The influence of soil moisture content on the bioavailability and toxicity of cadmium for *Folsomia candida* Willem (Collembola: Isotomidae). Ecotoxicology and Environmental Safety 36, 123-132.

Zar, J.H., 1996. Biostatistical Analysis. Prentice-Hall International, Inc, New Jersey.

CHAPTER 3

Carbaryl toxicity prediction to soil organisms under high and low temperature regimes

Chapter 3 Carbaryl toxicity prediction to soil organisms under high and low temperature regimes.

Lima, M.P.R., Cardoso, D.N., Soares, A.M.V.M., Loureiro, S.

published by Journal Ecotoxicology and Environmental Safety In press;
doi: 10.1016/j.ecoenv.2014.04.004

Abstract

Many studies on risk assessment of pesticides on non-target organisms have been performed based on standardized protocols that reflect conditions in temperate climates. However, the responses of organisms to chemical compounds may differ according to latitude and thus predicting the toxicity of chemicals at different temperatures is an important factor to consider in risk assessment. The toxic effects of the pesticide carbaryl were evaluated at different temperature regimes, which are indicative of temperate and tropical climates and are relevant to climate change predictions or seasonal temperature fluctuations. Four standard organisms were used (*Folsomia candida*, *Eisenia andrei*; *Triticum aestivum* and *Brassica rapa*) and the effects were assessed using synergistic ratios, calculated from EC/LC_{50} values. When possible, the MIXTOX tool was used based on the reference model of independent action (IA) and possible deviations. A decrease on carbaryl toxicity at higher temperatures was found in *F. candida* reproduction, but when the MIXTOX tool was used no interactions between these stressors (Independent Action) was observed, so an additive response was suggested. Synergistic ratios showed a tendency to synergism at high temperatures for *E. andrei* and *B. rapa* and antagonism at low temperatures for both species. *T. aestivum* showed to be less affected than expected (antagonism), when exposed to both low and high temperatures. The results showed that temperature may increase the deleterious effects of carbaryl to non-target organisms, which is important considering both seasonal and latitude related differences, as well as the global climate change context.

Keywords: **Temperature, Carbaryl, *Folsomia candida*, *Eisenia andrei*, *Triticum aestivum*, *Brassica rapa***

3.1 Introductionn

Standardized ecotoxicological tests have been conducted under strict limits on abiotic factors such as temperature, simulating temperate regions (Martikainen and Krogh, 1999; Smit and van Gestel, 1995), while risk assessment for tropical and cold temperate climates is often carried out through extrapolations from these data (de Silva et al., 2009).

Factors such as organic matter, soil moisture, UV radiation and temperature can differ widely according to the planet region. In temperate regions, soils are seasonally cold, characterized by a low biological activity (Robertson and Grandy, 2006). Whereas, tropical regions are characterized by high moisture and temperatures that cause a fast turnover of organic compounds and organic matter in soil (Ayanaba and Jenkinson, 1990; Trumbore, 1993). Fate and transport of pesticides, toxicity and exposure routes may differ between these regions, mainly due to the temperature (Bourdeau et al., 1989; Laabs et al., 2002). So, risk assessment based on these standardized tests may not be representative of tropical and cold temperate regions.

Besides differences in latitude, seasonal fluctuations and global climate change can also lead to different temperature regimes, which may therefore influence the fate and transport of chemicals in the environment.

The high temperature favours the volatilization and degradation of some organic chemicals in soil (Martikainen and Krogh, 1999; Viswanathan and Murti, 1989) and on the other hand, degradation of pesticides occur slowly at lower temperatures (Topp et al., 1997), favouring its stability in the environment (Viswanathan and Murti, 1989).

Regarding the terrestrial environmental compartment, the increase in temperature may affect the structure and dynamics of plant communities (Aerts et al., 2006) and crops (Blum et al., 1994; Ortiz et al., 2008), accelerate earthworm growth and reproduction (Butt, 1997; Fayolle et al., 1997; Presley et al., 1996; Reinecke and Kriel, 1981), affect development and reproduction of soil organisms, such as collembolan (Choi et al., 2002). Moreover, it may lead to enhanced metabolic activity of the organisms as well as the uptake rates of toxicants (Martikainen and Krogh, 1999; Smit and van Gestel, 1997).

On the other hand, low temperatures are potentially lethal for many soil organisms and plants (Holmstrup et al., 2008), causing a decrease in burrowing activity in earthworms (Perreault and Whalen, 2006), changes in membrane physical properties of plants (Crockett et al., 2001), inducing a relatively inactive state in many invertebrates (Cáceres, 1997), decreasing rates of oxygen consumption (Tripathi et al., 2011) and metabolism (Penick et al., 1998) or desiccation.

The aim of this study was to predict the toxicity of carbaryl at low and high temperature, using four standard organisms that are representative of different taxonomic levels, ecological functions, trophic level, life history and route of exposure to chemicals.

Carbaryl (1-naphthyl-N-methylcarbamate) is a carbamate insecticide commonly used in agricultural activities worldwide, known for its action on insects by inhibiting the acetylcholinesterase (AChE), an essential enzyme in the nervous system of invertebrates. Carbaryl is not considered persistent in soil, and the adsorption coefficient values (from 100 to 600) indicate that it moderately binds to soil particles (IPCS, 1994; Jana and Das, 1997). Its half-life ranges from 4 to 27 days in aerobic soils and from 72 to 78 days in anaerobic soils (IPCS, 1994; Miller, 1993), but the half-life of carbaryl in soil can be significantly reduced by increasing the temperature (Uyanik and Özdemir, 1999).

Studies on the effects of temperature on carbaryl toxicity are limited to aquatic toxicology. Sanders et al. (1983) reported that at temperatures of 7, 12 and 22 ºC, there were no differences within carbaryl toxicity to rainbow trout (*Oncorhynchus mykiss*), but for other fish species (*Lepomis macrochirus*) an increase in temperature of 12 ºC from 22 ºC increase its toxicity twice, causing mortality. Increase of temperature also affected the survival of *Rana clamitans* tadpoles (Boone and Bridges 1999), of the midge, *Chironomus riparius* (Lohner and Fisher, 1990) and the molluscs *Melanopsis dufouri* (Almar et al., 1988) exposed to carbaryl.

However studies regarding the effect of temperature on the toxicity of carbaryl to soil organisms are scarce. The toxicity of carbofuran, another carbamate pesticide, did not differ much on its acute toxicity to earthworm when exposed to tropical (26 ºC) and temperate (20 ºC) conditions (de Silva et al., 2009).

To predict joint effects of the carbaryl and temperature on the chosen test-species, the Independent Action (IA) conceptual model for mixture toxicity prediction was adopted in this study. This model has been used to evaluate mixtures of chemicals that have different modes of action and their effects are statistically independent of each other (Bliss, 1939).

3.2 Materials and methods

3.2.1. Test species

Due to their sensitivity to chemicals, important role in the soil ecosystem, rapid life cycle and ease to maintain in the laboratory, these species are widely used in ecotoxicological tests (Crouau and Cazes, 2003; Fountain and Hopkin, 2005) and so were chosen for this study.

Folsomia candida and *Eisenia andrei* were obtained from laboratory cultures. The earthworm cultures were maintained at a constant photoperiod (16:8/Light: dark) and temperature (20±2 ºC), in plastic boxes with artificial soil prepared according to OECD (1984) and fed weekly with horse manure. The springtails were kept in laboratory cultures on a moist substrate of plaster of Paris and activated charcoal, at 18 ºC in the dark, and fed weekly with dried Baker's yeast. Seeds of *Brassica rapa* were purchased from Carolina Biological Supply Company (US) and caryopsis of T*riticum aestivum* from a local supplier (Aveiro, Portugal).

Collembolans are abundant and distributed worldwide (Hopkin, 1997) whose role is to contribute to the decomposition of leaf litter (Klironomos et al., 1999), representing also a key position in the soil food web as a prey and consumer (Fountain and Hopkin, 2004). In addition, they have shown to be vulnerable to the effects of soil contamination (Fountain and Hopkin, 2005). Earthworms are ubiquitously distributed (Diao et al., 2007) and their feeding and burrowing activity facilitates the nutrient cycling, increases soil organic matter, changes the activity of microorganisms and consequently soil fertility and nutrient availability to plants (Coleman and Ingham, 1988; Haynes et al., 2003). They are generally good indicators of the relative health of soil ecosystems (Kuhle, 1983; Spurgeon and Hopkin, 1996). Also, terrestrial plants play a very important role in ecosystems, as they are an important source of organic matter, act in nutrient cycling and soil respiration (Boutin et al., 1995; Singh and Gupta, 1977), and play an important role in food supply to animals and man.

3.2.2 Test soil and test chemical

All bioassays were carried out with the natural standard soil LUFA 2.2 from Speyer, Germany (Løkke and van Gestel, 1998). This soil is considered a standard sandy-loam soil (17% silt, 6% clay and 77% sand), with 4.4% of organic matter, a carbon/nitrogen ratio of 14, pH 5.8, maximum water holding capacity (WHC) of 55% (weight per volume) and a cation exchange capacity of 11.2 mol/kg.

Carbaryl (CAS no. 63-25-2) was purchased from Sigma Aldrich Ltd. (99.8% purity). Carbaryl stock solution was prepared using acetone and applied to pre-moistened soil a day before the experiment started, in order to allow acetone evaporation. The carbaryl concentrations used for each test species was chosen based on the LC_{50}/EC_{50} previously carried out and reported by Lima et al. (2011).

Contaminated soil (100 mg/kg) exposed under the same abiotic conditions, i.e., highest temperature (28 ºC), control (20 ºC) and the lowest temperature (8 ºC) without organisms were used for carbaryl quantification, immediately after soil spiking and after 15 days of exposure with three replicates, per time and temperature. This allowed determining the degradation of carbaryl at different temperature regimes for 15 days.

Samples were analyzed at Marchwood Scientific Services, Southampton, UK. The procedure considered an initial single-phase QuEchers extraction of 10 g of soil with 10 ml acetonitrile, followed by quantification using liquid chromatography–tandem mass spectrometry (LCMS–MS), with a limit of detection of 0.1 mg/kg.

3. 2.3. Single exposure to carbaryl

Collembolan mortality and reproduction

The test was performed according to the ISO guideline 11267 (ISO, 1999). Ten springtails, 10–12 days old, were placed in a test vessel containing the pre-moistened test soil (at 60% WHC) at 2072 1C with a 16/8 h photoperiod and the food supply. The Vials were covered with parafilm with a small hole to allow air flow, limiting moisture loss. Three nominal concentrations of carbaryl (1, 4 and 7 mg/kg) plus a negative control and a solvent control of acetone were used, at five replicates per treatment. After 14 days, approximately 2 mg of dry yeast was added and moisture re-adjusted to all test containers. After 28 days, test vessels were filled with distilled water to which dark ink was added for contrast, after which contents were transferred into larger vessels. Juveniles and adults floating the surface were photographed and counted using the image analysis software SigmaScan Pro5.

Earthworm acute toxicity test

Tests were performed in accordance with the OECD 207 guideline (OECD, 1984). Ten adult worms (clitellated), with individual fresh weight between 250 and 600 mg were selected and placed within a glass vessel, containing 500 g of the test soil. The vials were covered with parafilm with a small hole to allow air flow, limiting moisture loss. A nominal concentration range of 20–100 mg/kg was used, plus a negative control and an acetone control, with four replicates per treatment.

After 7 days of exposure at 20±2 ºC with a 16/8 h photoperiod, living worms were counted and soil moisture replenished based on the vessels weight loss; after 14 days, earthworms were recounted, pooled weighted and their mean biomass (mg) reported. Earthworm's weight loss was calculated using the ratio of the difference between the initial and final weight, and the initial weight.

Plant growth and emergence test

The plant tests were performed following the protocol ISO 11269-2 (ISO, 1995). For each species, ten seeds were introduced at 1 cm depth of the soil surface, per plastic pot, containing 500 g of soil. Bioassays were carried out at 20±2 ºC, with a 16/8 h photoperiod and a light intensity of 12000 lx. Carbaryl nominal concentrations ranged from 50 to 150 mg/kg. In addition to the negative control, a solvent control (acetone) was prepared with four replicates per treatment. Soil moisture was pre-adjusted to 60% WHC and maintained using capillary action, through a fiberglass wick (between 5 and 10 mm) located at the bottom of the pot (Loureiro et al., 2006). The test duration was 14 days counted from after 50% of the seeds had emerged in the control soil. In the first 7 days, seed germination was reported. At the end of the tests the number of plants, their length, fresh weight and dry weight were recorded. The water content (WC) was also calculated using the equation:

where DW is the plant dry weight and FW is the plant fresh weight.

$$HC = \frac{FW - DW}{FW} \times 100$$

3.2.4.Single exposure to temperature

To analyze the effects of single temperature in collembolan, earthworm and plants, the tests were adapted from that described in the ISO guideline 11267 (ISO, 1999), OECD 207 (OECD, 1984) and ISO 11269-2 (ISO, 1995), respectively. In uncontaminated Lufa 2.2 soil, collembolan and earthworms were exposed to two temperature sets: low (8 ºC, 12 ºC, 16 ºC and the control 20 ºC) and high (22 ºC, 24 ºC, 26 ºC), plus the control temperature of 20 ºC. In order to analyze only the effects of extreme temperatures, plants were exposed to uncontaminated soil at 8 ºC, 28 ºC, and the control temperature of 20 ºC, for comparison. These temperatures were chosen in order to simulate typical scenarios of cold temperate, temperate and tropical climates (Lavelle and Spain, 2001; Römbke et al., 2007; Blenkinsop et al., 2008).

All tests were carried out in climatic chambers in order to maintain a constant temperature regime. To avoid drought stress at high temperatures, soil moisture (60% WHC) was controlled daily in collembola and earthworm tests. For that vessels were weighed at the beginning of the test and daily, and water loss was replenished when necessary.

3.2.5. Combined temperature and carbaryl exposure

The procedures for this test were carried out in accordance with that described in the ISO guideline 11267 (ISO, 1999), OECD 207 (OECD, 1984) and ISO 11269-2 (ISO, 1995), with adaptations. Soil was contaminated with different carbaryl concentrations dissolved in acetone (the same concentrations described above for the single carbaryl experimental set up for each species), left to evaporate for one day and then moistened with deionized water to obtain 60% of water holding capacity (WHC). After that, organisms were included in the vials and exposed to different temperatures (the same previously described for the single temperature exposure).

Temperatures and carbaryl concentrations used for all tests performed are summarized in Table 3.1.

Table 3.1 Summary of single and combined experiments for each test-species. In the combined exposures, the carbaryl ranges were used under each temperature.

Tests				
Test – species	**Uncontaminated soil**	**Contaminated soil**		
		Standard protocol conditions (20 ºC)	**Trials with different temperatures**	
F. candida	8 ºC			8 ºC
	12 ºC	Control	Control	12 ºC
	16 ºC	1 mg/kg	1 mg/kg	16 ºC
	20 ºC	4 mg/kg	4 mg/kg	20 ºC
	22 ºC	7 mg/kg	7 mg/kg	22 ºC
	24 ºC			24 ºC
	26 ºC			26 ºC
E. andrei	8 ºC			8 ºC
	12 ºC	Control	Control	12º1C
	16 ºC	20 mg/kg	20 mg/kg	16 ºC
	20 ºC	40 mg/kg	40 mg/kg	20 ºC
	22 ºC	60 mg/kg	60 mg/kg	22 ºC
	24 ºC	80 mg/kg	80 mg/kg	24 ºC
	26 ºC	100 mg/kg	100 mg/kg	26 ºC
B. rapa and T. aestivum	8 ºC	Control	Control	8 ºC
	20ºC	50 mg/kg	50 mg/kg	20 ºC
	28 ºC	75 mg/kg	75 mg/kg	28 ºC
		100 mg/kg	100 mg/kg	
		125 mg/kg	125 mg/kg	
		150 mg/kg	150 mg/kg	

3.2.5. Statistics

Differences between control and treatments were analyzed using One Way analysis of variance (ANOVA), followed by a post-hoc Dunnett's test ($\alpha < 0.05$). When the data provided were not normally distributed and data transformation was not possible, a Kruskal–Wallis ANOVA on Ranks was performed. From the ANOVA results, the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were derived. EC_{50} and LC_{50} (or ET_{50}, for temperature only induced effects) values were calculated using a sigmoidal (logistic, 3 parameter) equation (Sigma Plot 10.0).

For the data on combined exposure to carbaryl at different temperature regimes, the approach followed was dependent on the experimental setup or on results obtained from tests employing both stressors individually. When full dose/ temperature response curves were obtained, the observed combined effect of carbaryl and temperature was compared to that expected based on their individual toxicity using the reference model of Independent Action (IA) described by Bliss (1939) and by the equation below:

$$E(Cmix) = 1 - \prod_{i=1}^{n} (1 - E(Ci))$$

where E(*Cmix*) is the combined effect at the mixture concentration *Cmix*, and E(*Ci*) is the effect of the individual mixture component *i* applied at the concentration *Ci.*

Deviation functions to describe synergistic/antagonistic interactions, dose level, and dose-ratio dependency were achieved by extending the IA equation and tested using the MIXTOX tool (e.g. Jonker et al., 2005). Parameters derived from the deviation equations are then converted into biological responses as also described in several other studies (e.g. Santos et al., 2010). When a full temperature–response curve was not achieved, the EC_{50}/LC_{50} values for carbaryl were compared considering each temperature, in order to detect shifts in toxicity. In addition, synergistic ratios (SRs) were calculated by dividing the EC_{50}/LC_{50} value for carbaryl at 20 ºC by the EC_{50}/LC_{50} values for carbaryl at each temperature. Synergistic ratios (SRs) of 1.0 indicated no effects of temperature on carbaryl toxicity, whereas values >1.0 and <1.0 indicated greater and smaller effects than expected, respectively. For the plant experimental setup, where only both temperature extremes (8 ºC and 28 ºC) and control (20 ºC) were used, EC_{50} values for carbaryl were derived under 8 ºC and 28 ºC exposures, and consequently the SRs were calculated to control the 20 ºC.

3.3. Results

There were no significant differences between the control and solvent control data for all tests carried out (One Way ANOVA, $p>0.05$). The solvent control data was used in the statistical analysis to compare the performance of organisms in all carbaryl treatments.

Carbaryl exposure was determined using chemical analysis; after spiking, 3 replicates of the nominal concentration of 100 mg/ kg retrieved a mean real concentration of 118 mg carbaryl/kg (±7.9, standard deviation). Therefore, nominal concentrations were used for the parameters calculation. In the experiment where the behaviour of carbaryl was reported after 15 days from soil spiking, it was observed that carbaryl concentration in soil decreased to 30% and 33% of the initial concentration at the temperature extremes of 8 ºC and 28 ºC, respectively, and 22.8% of the initial concentration under a 20 ºC temperature regime (Table S 3.1).

3.3.1. Effects of single exposure to carbaryl

No significant differences between the control and solvent control (ANOVA, $p>0.05$) for the all tests and for all parameters measured, therefore the solvent control data will be used to compare the results.

The EC_{50}/LC_{50} values for the species exposed to carbaryl at 20 ºC and at different temperatures, according to the ISO guidelines, based on nominal initial concentrations are presented in Table 3.2.

Collembolans survival (One-way ANOVA, $F_{3,24}=180.911$, $p\leq0.001$) and reproduction (Kruskal–Wallis, $H=18.321$, $df=3$, $p\leq0.001$) were significantly affected by carbaryl exposure at higher concentrations (4 and 7 mg/kg) compared to the control (Figure 3.1A). The NOEC for both parameter analysed was 1 mg/kg (data not show) and The EC_{50} and LC_{50} values were 4.6 (SE=0.16) and 7.0 (SE=7.6) mg/kg, respectively (Table 3.2.)

Table 3.2 EC/LC$_{50}$ values (mg/kg) and synergistic ratios (SR) calculated for carbaryl toxicity co-occurring at each temperature in tested species. Data from 20 ºC was used as the control to calculate the SR. Gray areas represent conditions that were not present in the experimental setup.

Test - species	Endpoint	Temperature (ºC)							
		8	12	16	20	22	24	26	28
F. candida	Survival LC$_{50}$ (SE)	10.1 (7.7)	8.0 (1.4)	7.5 (1.2)	7.0 (7.6)	8.1 (0.92)	7.8 (2.2)	n.d.	
	SR	**0.69**	**0.88**	**0.93**		**0.88**	**0.9**	**n.d.**	
	Reproduction EC$_{50}$ (SE)	4.1 (0.3)	4.0 (0.2)	6.0 (0.6)	4.6 (0.22)	8.0 (0.81)	6.8 (1.7)	7.3 (2.9)	
	SR	**1.12**	**1.15**	**0.85**		**0.57**	**0.68**	**0.63**	
E. andrei	Survival 7days LC$_{50}$ (SE)	>100	>100	>100	51.28 (2.6)	42.1 (5.3)	42.1 (7.0)	26.7 (4.4)	
	SR	**0.5**	**0.5**	**0.5**		**1.02**	**1.02**	**1.61**	
	Survival 14 days LC$_{50}$ (SE)	69.1 (5.1)	99.3 (1.7)	87.5 (4.8)	43.2 (3.53)	32.3 (3.8)	33.3 (6.4)	22.1 (3.5)	
	SR	**0.62**	**0.43**	**0.49**		**1.33**	**1.29**	**1.9**	
	Biomass EC$_{50}$ (SE)	23.5 (9.2)	54.6 (68.6)	52.6 (209)	>100	68.7 (7.3)	n.d.	50.8 (5.3)	
	SR	**>4.3**	**>1.8**	**>1.9**		**>1.5**	**n.d.**	**>2.0**	
B. rapa	Length EC$_{50}$ (SE)	72.2 (4.4)			66.4 (2.2)				26.5(4.1)
	SR	**0.91**							**2.5**
	Fresh weight EC$_{50}$ (SE)	43.4 (7.0)			45.42 (1.7)				17.2 (9.0)
	SR	**1.04**							**2.64**
	Dry weight EC$_{50}$ (SE)	>150			42.4 (2.3)				6.6 (9.1)
	SR	**0.28**							**6.4**
	Water content EC$_{50}$	>150			>150				>150
	SR	**n.d.**							**n.d.**
T. aestivum	Length EC$_{50}$ (SE)	82.2 (6.0)			61.04 (1.7)				73.4 (2.3)
	SR	**0.74**							**0.83**
	Fresh weight EC$_{50}$ (SE)	87.5 (7.9)			48.8 (2.0)				56.7 (2.7)
	SR	**0.56**							**0.86**
	Dry weight EC$_{50}$ (SE)	110.4 (8.5)			53.7 (1.9)				61.5 (3.4)
	SR	**0.49**							**0.87**
	Water content EC$_{50}$	>150			>150				>150
	SR	**n.d.**							**n.d.**

n.d.=data not determined.

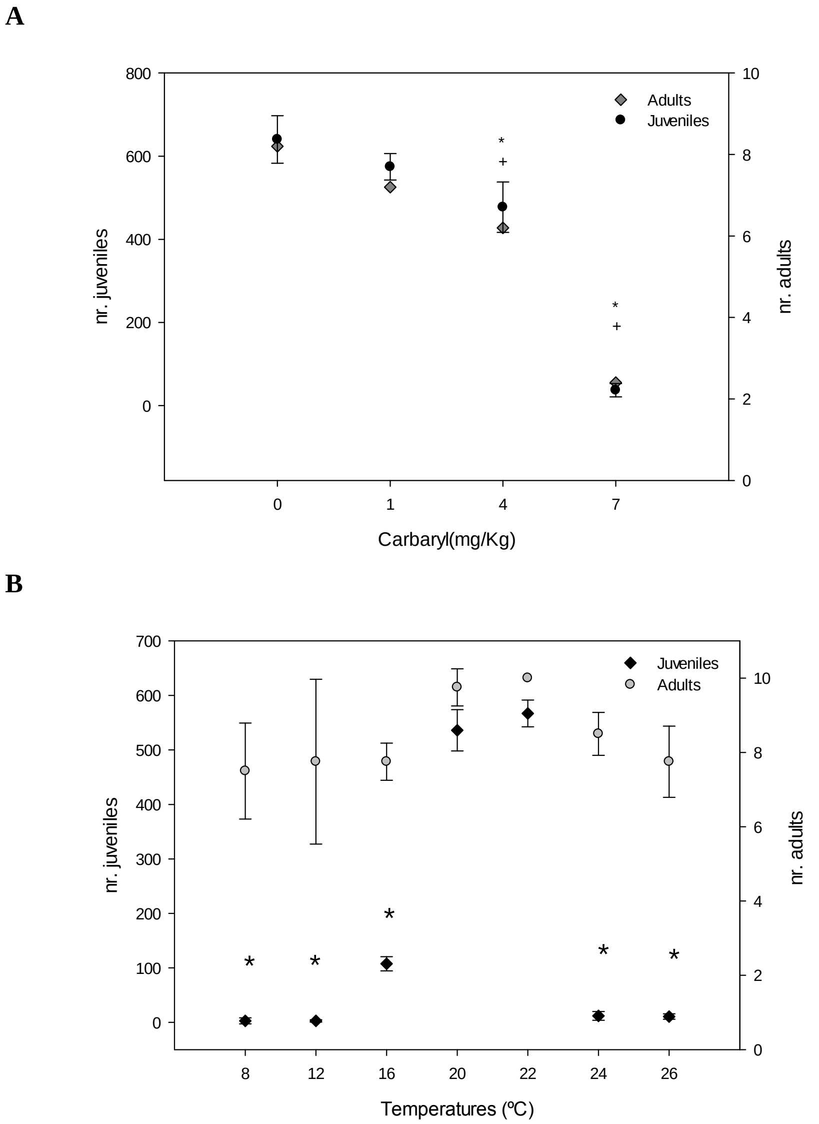


Fig. 3.1. *Folsomia candida* survival and reproduction. Effect of single carbaryl exposure (A) and single temperature exposure (B). Data is shown as mean value ± std. error. */+$p \leq 0.05$. Dunn's test: when temperatures are employed the 20 ºC was used as control and for the carbaryl treatments the solvent control was used for comparison.

Earthworm's biomass was not affected by the tested carbaryl concentrations (Kruskal–Wallis, H = 10.104, df=5, p=0.072), but a negative effect on earthworms' survival was observed with increasing concentration from 80 mg/kg at 7 days of exposure (Kruskal–Wallis, H=16.331, df=5, p =0.006), and from 40 mg/kg at 14 days of exposure, The NOEC was 60 mg/kg and 20 mg/kg respectively (one-way ANOVA, $F_{5,17}$ =45.179, $p \leq 0.001$). The LC_{50} value for 7 days was 51.28 mg/kg (SE=2.6) and for 14 days was 43.2 mg/kg (SE=3.53) (Table 3.2.)

Carbaryl affected *T. aestivum* at concentrations above 50 mg/kg in respect to all parameters analyzed (Dunnett's method, $p<0.05$). For *B. rapa* there was a decrease in plant growth (Kruskal–Wallis, H=193.001, df=5, $p \leq 0.001$), and fresh weight (Kruskal–Wallis, H=198.221, df=5, $p \leq 0.001$), for carbaryl concentrations higher than 75mg/kg, while water content significantly decreased at above 100 mg/kg of carbaryl (Kruskal–Wallis, H=121.493, df=5, $p \leq 0.001$). Both plant species showed similar EC_{50} values (Table 3.2). In like manner, seed emergence of both species did not present significant differences between the concentrations used and the control (ANOVA, $p>0.05$).

3.3.2. Effects of single temperature on test species

The ET_{50} and LT_{50} for collembolan and earthworms exposed to low and high temperatures are summarized in Table 3.3. For *F. candida*, the tested temperatures did not affect survival (Kruskal–Wallis, H=15.910, df=6, p=0.014), although both the lower (8, 12 and 16 °C) and the two highest temperatures (24 and 26 °C) inhibited their reproduction (Figure 3.1B, One Way ANOVA, $p<0.05$). The optimum temperatures for reproduction were 20 and 22°.

In addition, the temperatures used in this study did not cause significant effects on earthworms' mortality (Kruskal–Wallis, H=17.965, df=6, p=0.006) when compared to the control (20 °C), despite a significant weight loss (26%) was observed under the 26 °C exposure (One-way ANOVA, $F_{3,15}$ =4.11, $p<0.05$) (data not show).

With regard to plant length parameters, *T. aestivum* was shown to be sensitive only in cold temperatures (Kruskal–Wallis, H=46.047, df=2, $p<0.001$), lower temperatures induced also a negative effect on *B. rapa* length (Kruskal–Wallis, H=68.105, df=2, $p<0.001$), but *B. rapa* was equally sensitive to both conditions (Figure 3.2A). There was an increase in the water content of *B. rapa* at 8 °C and 28 °C (One-way ANOVA, $F_{2,94}$ =88.6 $p<0.001$), whereas such an increase was observed for *T. aestivum* only at 28 °C (Kruskal–Wallis, H=21.656, df =2, $p<0.001$)(Figure 3.2B). Bio-mass production of both species was affected as at 8 °C and 28 °C (ANOVA, $p<0.05$; Figure 3.2C and D).

Table 3.3 Median lethal temperature (LT_{50}) and median effective temperature (ET_{50}) values for the exposures of *Folsomia candida* and *Eisenia andrei* to low (8, 12,16 ºC) and high temperatures (22, 24, 26 ºC).

Species	Endpoint	**High temperatures** (ºC) (SE)	**Low temperatures** (ºC) (SE)
Folsomia candida	Survival (LT_{50})	>26	<8
	Reproduction (ET_{50})	23.68 (4 x 10^{-6})	16.9 (0.65)
Eisenia andrei	Survival (7days) (LT_{50})	n.d.	12.1 (3.7)
	Survival (14days) (LT_{50})	>26	n.d
	Biomass (ET_{50})	>26	16.3 (2.5)

LT_{50} = median lethal temperature; ET_{50} = median effective temperature; SE = standard error;

n.d.= not determined.

Seed's emergence is summarized in Table S3.2. At the control temperature (20 ºC) both species germinated within 3 days, the same time for emergence was observed in *T. aestivum* at 28 ºC, but *B. rapa* in these conditions, emerged right after 1 day of exposure. On the other hand, colder conditions delayed emergence of both species (7 days). Seeds of *B. rapa* showed 100% of emergence for all temperatures tested, while *T. aestivum* showed 100% of emergence in the control temperature (20 ºC), whereas at 8 ºC emergence significantly decreased to 70% and at 28 ºC it decreased to 86% (ANOVA, $p<0.05$).

3.3.3. Effects of combined exposure of carbaryl and temperature

To understand collembolan response to the combined stressors exposure, the reference model for Independent Action (IA) was used, because a dose–response for both stressors was obtained. A clear adjustment to the IA model was observed for the reproduction data under heat (sum of squares=14101; r^2 =0.98; $p<0.05$) and cold (sum of squares=6620; r^2=0.99; $p\leq0.05$) stress. A similar observation was made concerning data for survival under heat and cold stress (sum of squares=1668; r^2=0.66; $p<0.05$ and sum of squares=812; r^2=0.74; $p<0.05$, respectively).

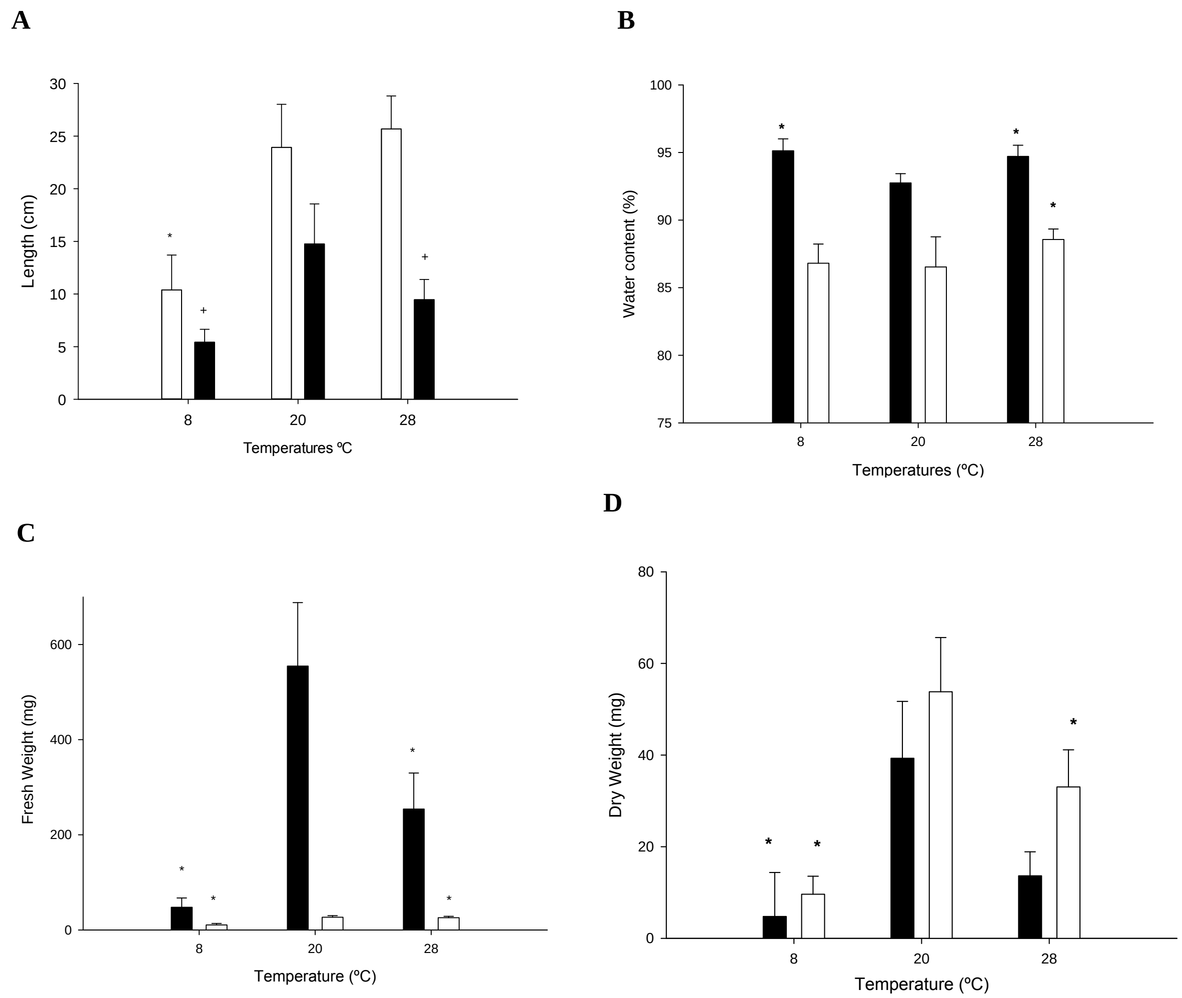


Fig. 3.2. Effects of single temperature exposure on *Brassica rapa* (■) and *Triticum aestivum* (□) length (A), water content (B), fresh weight (C) and dry weight (D). Data is shown as mean value ± std. error. */ $p \leq 0.05$. Dunn's test, compared to the control (20ºC)

The earthworm response to the combined stressors exposure was also analyzed by calculating synergistic/antagonistic ratios, because a dose–response to both stressors was not possible, so the reference model for Independent Action (IA) was not used. The synergistic/antagonistic ratios for earthworms' survival after 7 and 14 days of exposure to carbaryl and biomass were summarized in Table 3.2. For survival, the synergistic ratio (SR) increased with increasing temperature while at low temperatures a decrease in SR ratio was observed. For the earthworms' biomass, both high and low temperatures induced an increase of the SR.

The plant species exposed to carbaryl at low and high temperatures showed a different pattern of response. At low temperatures, synergism/antagonism ratios for both species, indicated low sensitivity in respect to almost parameters analyzed. At higher temperatures, however, a greater effect (trend to synergism) was observed in *B. rapa* in contrast to that observed in *T. aestivum* (trend to antagonism) for all parameters analyzed (Table 3.2).

The emergence of both plant species was influenced by the combined exposure of carbaryl and the extreme temperature.

B. rapa was strongly affected by the exposure to carbaryl at 28 ºC. At the two highest concentrations (125 and 150 mg/kg), the emergence was 96% and 70%, respectively, but before the end of the test, mortality was observed for many seedlings at concentrations above 75 mg/kg (Dunnett's method, $p<0.05$) (Table S3.2).

3.4. Discussion

3.4.1. Effects of single carbaryl exposure

The high toxicity of carbaryl to soil invertebrates and plants is well reported in the literature (Ferrari et al., 2004; Fukuto, 1990; Gambi et al., 2007; Gupta and Sundararaman, 1991; Jones et al., 1991; Lima et al., 2011; Murthy and Raghu, 1990; Ribera et al., 2001). However, to our knowledge, there are no data available on the toxicity of carbaryl to *F. candida* survival or reproduction in laboratory conditions. Few studies reported the sensitivity of collembolans to carbaryl when applied in the field, showing a significant reduction in the population, similar to other non-target species such as mites (Bishop et al., 1998; Hoy and Shea, 1981; Schulze et al., 2001; Spain, 1974; Stegeman, 1964). Spain (1974) reported a noticeable reduction in collembolan populations exposed to 0.11 g/m^2 of carbaryl.

Moreover, our results showed a high mortality rate of collembolan at higher concentrations of carbaryl. Therefore, results concerning the production of juveniles, at these concentrations, must be regarded carefully as it is not clear if carbaryl was affecting reproduction directly, if juveniles were not produced due to adult mortality or if juveniles died upon exposure. This can be highlighted using the similar effect values obtained for lethality and reproduction reduction.

The high toxicity of carbaryl to earthworms is also well reported. Gambi et al. (2007) showed that carbaryl concentrations of 48.3 mg carbaryl/kg of natural soil, comparable to the LC$_{50}$ values found in this study (43.2 mg/kg), led to a maximal cholinesterase (ChE) inhibition in *E. andrei*, while the epigeic earthworm *Perionyx excavatus* exposed in grasslands soil to sub-lethal doses of carbaryl (3.03 mg/kg) showed significant loss of weight compared to the respective control (Dasgupta et al., 2012). On the other hand, Gupta and Saxena (2003) showed that a 20 min exposure to a low concentration of carbaryl (0.125 mg/kg, which is 160 times smaller than our lowest concentration) lead to a decrease in locomotion and geotaxis of the cosmopolitan earthworm *Metaphire posthuma*. Similarly, Gupta and Sundararaman (1991) reported the reduction in the burrowing behavior of *Pheretima posthuma* exposed for 5 min to soil contaminated with 1 mg carbaryl/kg.

Furthermore, Mostert et al. (2002) reported a lower LC_{50} value (of 16 mg/kg) for the earthworm *Pheretima sp.*, after a 7 days exposure to carbaryl when compared to that calculated in the present study for *E. andrei* (LC_{50} of 51.28 mg/kg).

Many studies have been carried out in relation to carbaryl residues in plants (Argente and Heinrichs, 1983; Demirbas, 2000; Galhotra et al., 1985; Iwata et al., 1979; Pieper, 1979; Ruiz-Medina et al., 2012), but few have reported the deleterious effects caused by carbaryl on plant growth (Jones et al., 1991; Murthy and Raghu, 1990). Murthy and Raghu (1990) reported a NOEC value of 2.5 mg/kg for seedling length of barley, while higher concentrations of 25 and 100 mg/kg caused inhibitory effects in this parameter. These data are consistent with this study results, since the LOEC values for *T. aestivum* and *B. rapa* were 50 mg/kg and 75 mg/kg respectively, with regard to length and weight parameters, and 50 mg/kg and 100 mg/kg, respectively, with regard to water content.

3.4.2. Effect of single temperature exposure

Collembolans and earthworms were shown to be tolerant with regards to their survival to both high and low temperatures within the studied range. These effects may be more visible in long term exposures to these temperatures. Snider and Butcher (1973) isolated collembolan juveniles and observed their longevity at 15 and 26 ºC, reporting that survival was drastically reduced at 26 ºC, with 50% of the individuals mortality reached after 70 days compared with control (21 ºC) where 50% of the animals had died after 150 days. Hutson (1978) showed that collembolan survival was affected by temperature, where adults survived for longer than 400 days at 15 and 20 ºC, compared to the 126 days (mean value) at 25 ºC. Considering the duration of the present study (28 days) and the lack of observed effects on survival, one can conclude that time is also an important factor to consider when different temperature regimes are present, as they can also influence collembolan lifespan and responses to stress conditions.

However, with regard to the reproduction data, our results suggest that 26 ºC is a possible upper limiting temperature for the *F. candida* offspring production. Martikainen and Rantalainen (1999) using field soil, and temperatures of 13, 16 and 19 ºC found that temperature was positively correlated with reproduction. According to Fountain and Hopkin (2005) the optimal temperature for *F. candida* hatching success is 21 ºC which is in agreement with our results, showing the highest production of juveniles at 20 ºC and 22 ºC.

Temperature tolerance and preference in earthworms vary from species to species. *Dendrobaena octaedra* is known to be a freeze tolerant earthworm species (Bindesbøl et al., 2009); While *Lumbricus rubellus* is a soil dwelling species, with an optimal temperature around 15 ºC (Butt, 1991); *Eisenia fetida* lives in compost and manure heaps, usually at higher temperatures due to heat generated from decomposition processes. While their optimum temperature is considered to be between 20 and 25 ºC (Presley et al., 1996), *E. fetida* has shown to survive at 43 ºC in a work undertaken outdoors in artificial containers (Reinecke et al., 1992).

In the present study, the tolerance of *E. andrei* to extreme temperature was expected, as it is considered an ubiquitous species with resilience to wide ranges of temperature and humidity (Domínguez et al., 2005). The weight loss caused by the highest temperature (26 ºC) is also in accordance with that described by Presley et al. (1996), where the authors linked the lower growth rates and weight loss of *E. fetida* exposed to 28 ºC in peat moss under laboratory conditions.

The effects of temperature in plants have already been reported. The rate of growth and development processes is controlled by air or soil temperature (Wheeler et al., 2000). Similarly, warmer temperatures can result in the reduction of leaf area, influencing photosynthesis, transpiration and respiration (Dat et al., 2000; Mittler, 2002; Rizhsky et al., 2002; Vierling, 1991), producing smaller grain weights, due to increase in growth rate and consequent shorter grain-filling durations (Al-Khatib and Paulsen, 1984; Blum et al., 1994; Hakim et al., 2012), leading to reduction in yield (Entz and Fowler, 1991) and total plant biomass (Hossain et al., 2012).

The plant species used in this study showed different sensitivities to temperature. *B. rapa* is a temperate species, therefore, its response to high temperatures as found in this study, with reduced growth, biomass and germination, has been reported by others authors (Morrison, 1993; Xiang Yu et al., 2012). *T. aestivum* is also a species of temperate climates, but its high temperature tolerance is expected, since it grows well in tropical and subtropical areas, at temperatures above 17 ºC (Ortiz el at., 2008). Accordingly, in the present study *T. aestivum* showed no changes in length, but a decrease in fresh and dry weight at high temperatures. Its optimum growth and development temperature has been reported to be between 20 ºC and 25 ºC (Acevedo et al., 2002; Hakim et al., 2012; Hossain et al., 2012), with an upper tolerance limit reported for temperatures of 34.3 ºC (± 2.6) (Farooq et al., 2011).

Low temperature is also an abiotic stress known to induce negative effects on crops, by inducing changes in membrane lipid composition (Tasseva et al., 2004). In the present study the effects of low temperature induced growth inhibition, reduction of biomass, delay in germination for both species and increased water content in *B. rapa*.

Temperature is an important factor in germination of many plant species and may affect the absorption of water and other compounds essential for growth and development (Essemine et al., 2002; Walbot, 2011; Wanjura and Buxtor, 1972). Delay in germination at low temperatures has been described by others authors (Al-Qasem et al., 1999; Hossain et al., 2012). For instance, Al-Qasem et al. (1999) obtained similar results, with no germination observed in spring wheat cultivars at 5 °C, but starting on day 7 at 10 °C, and on day 2 and 3 at 20 and 30 ºC, respectively.

3.4.3 Combined exposure of Carbaryl and temperature.

The analysis of carbaryl in soil showed an increased degradation over time, however, differences between control and test temperatures were not significant. This was somehow unexpected, since pesticide degradation would be expected to be greater in tropical conditions (Racke et al., 1997). Nevertheless, Odeyemi (1982) reported that carbaryl disappeared in an agricultural soil after 53 days of incubation in tropical ecosystems. Uyanik and Özdemir (1999) reported that carbaryl half-life was higher than four weeks under temperatures of 40 ºC, 16 ºC and 5 ºC; but a remarkable decrease was found with increasing temperature. In the present study the exposure time of 15 days was possibly not sufficient to observe significant effects on the degradation of the chemical as reported for longer time period studies.

Considering that carbaryl is used worldwide, under a range of temperature and seasonal conditions, while accounting for temperature shifts associated to global climatic changes, studies like the present are important contributions to more representative predictions of pesticide toxicity.

To our knowledge, there are no existing reports on the effects of temperature on the carbaryl toxicity of non-target organisms, but the influence of a post-treatment of temperature on the toxicity of carbaryl to the insect *Diaphorina citri* was studied by Boina et al. (2009), reporting an increased toxicity with increasing temperature from 17 to 37 ºC.

Many studies have reported the effects on collembolan reproduction upon metal combined with temperature exposure (Sandifer and Hopkin, 1997; Smit and van Gestel, 1995, 1997), but similar studies using pesticides are scarce.

Martikainend and Krogh (1999) reported an increase in the toxicity of the organophosphate insecticide dimethoate to *Folsomia fimetaria* with low temperatures, while Sjursen and Holmstrup (2004) observed that the springtail *Protaphorura armata* exposed to pyrene at low temperatures (±1 ºC and 3 ºC) showed higher survival rates, when compared to a standardize temperature (20 ºC). These results corroborate with the present study, where the highest LC_{50} value for adult survival was found at the lowest temperature (8 ºC).

Martikainend and Krogh (1999) showed a delay in the reproduction of *F. fimetaria* when temperature decreased from 20 ºC to 15 ºC in the presence of dimethoate, and the same was found in the present study where the EC_{50} values for reproduction decreased at low temperatures. Nevertheless, the interpretation of modeled data by the MIXTOX tool suggests that there was no interaction between stressors (independent action) for all parameters analyzed and, probably, even if an additive effect occurred.

Synergistic effects were found by Jensen et al. (2009) when the freeze tolerant earthworm *Dendrobaena octaedra* was exposed to the surfactant nonylphenol (NP) combined with high (25–35 ºC) temperatures. The same species exposed to Phenanthrene at freezing temperatures showed an antagonistic effect and when exposed to Carbendazim no interactions were found (Bindesbol et al., 2009).

In the present study *E. fetida* showed a tendency to synergism (SR>1) when exposed to combined carbaryl and high temperatures, and antagonism when exposed to carbaryl and low temperatures. These results corroborate with some of the literature cited above and with the review conducted by Holmstrup et al. (2010), with reports of synergistic interactions between chemicals and heat stress. In fact, this pattern has been reported for several earthworm species exposed to chemicals at higher temperatures. For instance, Khan et al. (2007) found a synergistic effect when *Lumbricus terrestris* were exposed to lead or zinc at higher temperatures (22 ºC) compared to procedures of standardized protocols.

The synergistic interaction may be associated with increased metabolism of earthworms at high temperatures, which may promote an increase in the uptake of pesticides through the earthworm skin (Khan et al., 2007; Rombke et al., 2007). Similarly, the trend found for antagonism at low temperatures may be due to the reduced metabolism and consequently reduced absorption of carbaryl by earthworms. In addition, one can hypothesize about the possible metabolites arising from carbaryl degradation. One of the metabolites of carbaryl is 1-naphthol (IPCS, 1993) which has been found to be more toxic than carbaryl itself for some aquatic species (CCME, 2009), but to our knowledge no information on their toxicity in soil organisms is available.

There are many studies about the effects of temperature on plant development (e.g. Angadi et al., 2000; Buriro et al., 2011; Hossain et al., 2012) and on herbicide efficacy (e.g. Mervosh et al., 1995; Olson et al., 2000), but little is known about the effects of other chemicals on plants influenced by temperature.

B. rapa and *T. aestivum* showed the same response when exposed to carbaryl at low temperatures (antagonism), whereas an opposite response between the two species was observed when exposed to carbaryl at higher temperatures, with synergism for *B. rapa* and antagonism for *T. aestivum*. These results suggest that the influence of temperature on chemical toxicity depends on the plant species possibly due to the differences between the root system of monocotyledon and dicotyledon species. Sadana et al. (2003) analyzing the manganese efficiency, showed a more efficient uptake kinetics in *Brassica juncea* L. than *Triticum aestivum* due, among others, to its root system. Aroca et al. (2001) showed that two maize genotypes differed in the response of their root water uptake rate when exposed to abiotic stress.

The combined exposure to carbaryl and high temperatures for *B. rapa* may have increased its transpiration rate, which consequently increased the need for water, causing a higher chemical uptake that lead to a stronger effect than expected (Kerstien, 2006; Rizhsky et al., 2002) while for *T. aestivum* an increased in chemical metabolisation may have occurred, causing a reduction of the chemical in their tissue (Collins et al., 2006).

The antagonism found at low temperatures for both species is expected since low temperatures induce changes in the cell membrane, reducing its permeability and also causing a higher water viscosity, which difficult transport within the plant system and consequently chemical uptake (Norisada et al., 2005; Pavel and Fereres, 1998; Simon, 1974).

3.5. Conclusions

It was concluded from the present study that carbaryl is toxic to non-target species, representing an environmental risk which can also be increased when combined with temperature. Synergistic effects were observed at higher temperatures for *Eisenia andrei* and *Brassica rapa*, while antagonism occurred at higher temperatures in *Triticum aestivum* and at low temperatures for all species studied, except for *F. candida*. No interactions between stressors were observed for *F. candida* exposures and an addictive response was suggested regarding survival and reproduction.

Therefore, it is important to consider both seasonal and latitude-related differences, as well as the global climate change context. This highlights the importance of studies that allow predicting toxicological impacts under a range of temperatures that are representative of tropical and temperate regions, favoring a better assessment of environmental risk and contributing to improve agricultural production and ecosystem maintenance.

Acknowledgments

This study was supported by a Ph.D. Grant (SFRH / BD / 65274 / 2009) attributed to Maria Lima by the Portuguese Science and Technology Foundation (FCT), through the research project CLIMAFUN (FCOMP-01-0124-FEDER-008656), by funding FEDER through COMPETE - Programa Operacional Factores de Competitividade, and Amadeu M. V. M. Soares is "Bolsista CAPES/BRASIL", Project NºA058/2013. We would like to thank Ana Catarina Bastos for the suggestions and revision of the manuscript.

3.6 Supplementary Data

Table S 3.1 - Carbaryl concentrations (mg/kg) in Lufa 2.2 soil after soil spiking and after 15 days of exposure under 8ºC, 20ºC and 28ºC. Data is presented as mean values and standard values are in brackets.

Time	8ºC	20ºC	28ºC
after spiking	-	118.13	-
		(7.90)	
after 15 days	82.97	91.23	78.70
	(7.70)	(1.29)	(7.76)

Table S 3.2 - Seed germination (days to germinate and % of success) and plant survival of *Brassica rapa* and *Triticum aestivum* after exposure to carbaryl at different temperatures.

plant species	**Carbaryl (mg/kg)**	**Days to germination**			**Germination (%)**			**Survival (%)**		
		8ºC	**20ºC**	**28ºC**	**8ºC**	**20ºC**	**28ºC**	**8ºC**	**20ºC**	**28ºC**
B. rapa	0	7	3	1	100%	100%	100%	100%	100%	100%
	50	7	3	3	100%	100%	96%	100%	100%	100%
	75	8	3	3	90%	100%	100%	100%	100%	90%
	100	8	3	3	100%	97%	90%	100%	100%	89%
	125	8	3	3	90%	97%	96%	100%	100%	58%
	150	8	4	3	100%	90%	70%	100%	100%	32%
T. aestivum	0	7	3	3	95%	97%	86%	100%	100%	100%
	50	7	3	3	83%	95%	86%	100%	100%	100%
	75	9	3	3	90%	87%	86%	100%	100%	100%
	100	9	3	4	93%	85%	60%	100%	100%	100%
	125	9	3	4	70%	85%	76%	100%	100%	100%
	150	12	4	4	70%	85%	66%	100%	100%	100%

References

Acevedo, E., Silva, P., Silva, H., 2002. Wheat growth and physiology. In: Curtis, BC, Rajaram, S, Macpherson, H Gomez (Eds.), “Bread” Wheat. Improvement and Production. Food and Agriculture Organization, Rome.

Aerts, R., Cornelissen, J.H.C., Dorrepaal, E., 2006. Plant performance in a warmer world: general responses of plants from cold, northern biomes and the importance of winter and spring events. Plant Ecology 182, 65–77.

Aroca, R., Tognoni, F., Irigoyen, J.J., Sànchez-Díaz, M., Pardossi, A., 2001. Different root low temperature response of two maize genotypes differing in chilling sensitivity. Plant Physiology and Biochemical 39, 1067–1073.

Al-Khatib, K., Paulsen, G.M., 1984. Mode of high temperature injury to wheat during grain development. Physiologia Plantarum 61, 363–368

Almar, M.M., Ferrando, M.M.D., Alarcon, V., Soler, C., Andreu, E., 1988. Influence of temperature on several pesticides toxicity to *Melanopsis dufouri* under laboratory conditions. Journal of Environmental Biology 9, 183–190.

Al-Qasem, H., Kafawin, O., Duwayri, M., 1999. Effects of seed size and temperature on germination of two wheat cultivars. Dirasat Agricultural Science 26, 1–7.

Angadi, S.V., Cutforth, H.W., Miller, P.R., McConkey, B.G., Entz, M.H., Brandt, S.A., Volkmar, K.M., 2000. Response of three Brassica species to high temperature stress during reproductive growth. Canadian Journal of Plant Science 80, 693–701.

Argente, A.M., Heinrichs, E.A., 1983. Residues of carbaryl, lindane and monocrotophos on rice treated for control of rice bug, *Leptocorisa oratorius* (Fab.). Crop Protection. 2, 361–369.

Ayanaba, A., Jenkinson, D.S., 1990. Decomposition of carbon-14 ryegrass and maize under tropical conditions. Soil Science Society of America Journal. 54, 112–115.

Bindesbøl, A.M., Bayley, M., Damgaard, C., Hedlund, K., Holmstrup, M., 2009. Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sublethal copper concentrations. Environmental Science Technology 43, 5495–5500.

Bishop, A.L.M., McKenzie, H.J., Barchia, I.M., Spohr, L.J., 1998. Efficacy of insecticides against the lucerne flea, *Sminthurus viridis* (L.) (Collembola: Sminthuridae), and other arthropods in lucerne. Australian Journal of Entomology. 37, 40–48.

Blenkinsop, S.F., Fowler, H.J., Dubus, I.G., Nolan, B.T., Hollis, J.M., 2008. Developing climatic scenarios for pesticide fate modelling in Europe. Environmental Pollution 154, 219–231.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annal of Applied Biology 26, 585–615

Blum, A., Sinmena, B., Mayer, J., Golan, G., Shpiler, L., 1994. Stem reserve mobilization supports wheat-grain filling under heat stress. Australian Journal of Plant Physiology 21, 771–781.

Boina, D.R., Onagbola, E.O, Salyani, M., Stelinski, L.L., 2009. Influence of posttreatment temperature on the toxicity of insecticides against *Diaphorina citri* (Hemiptera: Psyllidae). Journal of Economic Entomology 102, 685–691.

Boone, M.D., Bridges, C.M., 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). Environmental Toxicology and Chemistry 18, 1482–1484.

Bourdeau, P., Haines, J.A., Klein, W., Krishnamurti, C.R., 1989. Ecotoxicology and climate. In: Proceedings of SCOPE 38 IPCS Joint Symposia 9, Chichester, UK, p. 392.

Boutin, C., Freemark, K.E., Keddy, C.J., 1995. Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environmental Toxicology and Chemistry 14, 1465–1475.

Butt, K.R., 1991. The effects of temperature on the intensive production of *Lumbricus terrestris* (Oligochaeta, Lumbricidae) Pedobiologia 35, 257–264.

Butt, K.R., 1997. Reproduction and growth of the earthworm *Allolobophora chlorotica* (Savingy, 1826) in controlled environments. Pedobiologia 41, 369–374.

Buriro, M., Oad, F.C., Keerio, M.I., Tunio, S., Gandahi, A.W., Hassan, S.W.U., Oad, S.M., 2011. Wheat seed germination under the influence of temperature regimes. Sarhad Journal Agriculture 27, 539–543.

Cáceres, C.E., 1997. Dormancy in invertebrates. Invertebrate Biology 116, 371–383.

CCME, 2009. Canadian Water Quality Guidelines: Carbaryl. Scientific Criteria Document. Canadian Council of Ministers of the Environment, Winnipeg.

Choi, W.I., Ryoo, M.I., Kim, J., 2002. Biology of *Paronychiurus kimi* (Collembola: Onychiuridae) under the influences of temperature. Pedobiologia 46, 548–557.

Coleman, D.C., Ingham, E.R., 1988. Carbon, nitrogen, phosphorus and sulfur cycling in terrestrial ecosystems. Biogeochemistry 5, 3–6.

Collins, C., Martin, I., Fryer, M., 2006. Evaluation of Models for Predicting Plant Uptake of Chemicals from Soil. Science Report. Science Report SC050021/SR. Environment Agency, Bristol.

Crockett, E.L., Dougherty, B.E., McNamer, A.N., 2001. Effects of acclimation temperature on enzymatic capacities and mitochondrial membranes from the body wall of the earthworm *Lumbricus terrestris*. Comparative Biochemistry and Physiology - Part.- B: Biochemistry and Molecular Biology 130, 419–426.

Crouau, Y., Cazes, L., 2003. What causes variability in the *Folsomia candida* reproduction test? Applied Soil Ecology 22 (2), 175–180.

Dasgupta, R.C., Partha, P., Kaviraj, A., 2012. Effects of carbaryl, chlorpyrifos and endosulfan on growth, reproduction and respiration of tropical epigeic earthworm, *Perionyx excavatus* (Perrier). Journal of Environmental Science- Health B 47, 99–103.

Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze´, D., Van Breusegem, F., 2000. Dual action of the active oxygen species during plant stress responses. Cellular and Molecular Life Science 57, 779–795.

De Silva, P.M.C.S., Pathiratne, P.A., van Gestel, C.A.M., 2009. Influence of temperature and soil type on the toxicity of three pesticides to *Eisenia andrei*. Chemosphere 76, 1410–1415.

Demirbas, A., 2000. Determination of carbaryl residues in agricultural lands in Trabzon province. Environmental Technology 21, 351–356.

Diao, X.P., Jensen, J., Hansen, A.D., 2007. Toxicity of the anthelmintic abamectin to four species of soil invertebrates. Environmental Pollution 148, 514–519.

Domínguez, J., Ferreiro, A., Velando, A., 2005. Are *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) different biological species? Pedobiologia 49, 81–87.

Entz, M.H., Fowler., D.B., 1991. Agronomic performance of winter vs. spring wheat. Agronomy Journal 83, 527–532.

Essemine, J., Ammar, S., Jbir, N., Bouzid, S., 2002. Sensitivity of two Wheat species seeds (*Triticum durum*, variety Karim and *Triticum aestivum*, Variety Salambo) to heat constraint during germination. Pakistan Journal of Biology Science 10, 3762–3768.

Farooq, M., Helen, B., Palta, J.A., Siddique, K.H.M., 2011. Heat stress in wheat during reproductive and grain-filling phases. Critical Review in Plant Science 30, 491–507

Fayolle, L., Michaud, H., Cluzeau, D., Stawiecki, J., 1997. Influence of temperature and food source on the life cycle of the earthworm *Dendrobaena veneta* (Oligochaeta). Soil Biology Biochemistry. 29, 747–750.

Ferrari, A., Anguiano, O.L., Solenõ, J., Venturino, A., D'Angelo, A.M.P., 2004. Different susceptibility of two aquatic vertebrates (*Oncorhynchus mykiss* and *Bufo arenarum*) to azinphos methyl and carbaryl. Comparative Biochemistry and Physiology C Toxicology & Pharmacology. 239–243.

Fountain, M.T., Hopkin, S.P., 2004. A comparative study of the effects of metal contamination on collembola in the field and in the laboratory. Ecotoxicology 13, 573–587.

Fountain, M.T., Hopkin, S.P., 2005. *Folsomia candida* (Collembola): a "standard" soil arthropod. Annual Review of Entomology. 50, 201–222.

Fukuto, R.T., 1990. Mechanism of action of organophosphoros and carbamate insecticides. Environmental Heath Perspective. 87, 245–254.

Galhotra, R., Garg, P.K., Halve, A.K., Pandey, S.Y., Stvasankaran, K., 1985. Carbaryl and lindane residues in potato crop. Journal of Entomology Researche. 9, 57–59.

Gambi, N., Pasteris, A., Fabbri, E., 2007. Acetylcholinesterase activity in the earthworm *Eisenia andrei* at different conditions of carbaryl exposure. Comparative Biochemestry Physiology- C Toxicology & Pharmacology. 145, 678–685.

Gupta, S.K., Sundararaman, V., 1991. Correlation between burrowing capability and Ache activity in the earthworm, *Pheretima-posthuma,* on exposure to carbaryl. Bulletin of Environmental Contamination and Toxicology. 46, 859–865.

Gupta, S.K., Saxena, P.N., 2003. Carbaryl-induced behavioral and reproductive abnormalities in the earthworm *Metaphire posthuma:* a sensitive model. Alternatives to laboratory animals. 31,587-593

Hakim, M.A., Hossain, A., Teixeira da Silva, J.A., Zvolinsky, V.P., Khan, M.M., 2012. Yield, protein and starch content of 20 wheat (*Triticum aestivum* L.) genotypes exposed to high temperature under late sowing conditions. Journal of Science Researche 4, 477–489.

Haynes, R.J., Fraser, P.M., Piercy, J.E., Tregurtha, R.J., 2003. Casts of *Aporrectodea caliginosa* (Savigny) and *Lumbricus rubellus* (Hoffmeister) differ in microbial activity, nutrient availability and aggregate stability. Pedobiologia 47, 882–887.

Holmstrup, M., Aubail, A., Damgaard, C., 2008. Exposed to mercury reduces cold tolerance in the springtail *Folsomia candida*. Comparative Biochemestry and Physiology - Part C - Toxicology & Pharmacology 148, 172–177.

Holmstrup, M., Bindesbol, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Kohler Heinz, R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Science of Total Environment 408, 3746–3762.

Hopkin, S.P., 1997. Biology of the Springtails (Insecta: Collembola). Oxford University Press, New York, United States p. 330.

Hossain, A., Teixeira da Silva, J.A., Lozovskaya, M.V., Zvolinsky, V.P., 2012. High temperature combined with drought affect rainfed spring wheat and barley in South-Eastern Russia: I. Phenology and growth. Saudi Journal of Biological Science 19, 473–487.

Hoy, J.B., Shea, P.J., 1981. Effects of lindane, chlorpyrifos, and carbaryl on a California pine forest soil arthropod community. Environmental Entomolology 10, 732–740.

Hutson, B., 1978. Influence of pH, temperature and salinity on the fecundity and longevity of four species of Collembola. Pedobiologia 18, 163–179.

IPCS (International Programme on Chemical Safety), 1994. Environmental Health Criteria, vol. 153: Carbaryl. World Health Organization, Geneva.

ISO (International Organization for Standardization), 1995. Soil Quality – Determination of the Effects of Pollutants on Soil Flora – Part 2: Effects of Chemicals on the Emergence and Growth of Higher Plants. ISO/DIS 11269-2. International Organization for Standardization, Geneva, Switzerland.

ISO (International Organization for Standardization), 1999. Soil Quality – Inhibition of Reproduction of Collembola (Folsomia candida) by Soil Pollutants. ISO/DIS 11267. International Organization for Standardization, Geneva, Switzerland.

Iwata, Y., Dusch, M.E., Garman, G.E., Gunther, F.A., 1979. Worker environment research: residues for carbaryl, chlorobenzilate, dimethoate and trichlorofon applied to citrus trees. Journal of Agricultural and Food Chemistry 27, 1141–1145.

Jana, T.K., Das, B., 1997. Sorption of carbaryl(1-napthyl n-methyl carbamate) by soil. Bulletin of Environmental Contaminant Toxicology 59, 65–71.

Jensen, D., Bayley, M., Holmstrup, M., 2009. Synergistic interaction between 4-nonylphenol and high but not low temperatures in *Dendrobaena octaedra*. Ecotoxicology and Environmental Safety 72, 10–16.

Jones, K.M., Koen, T.B., Bound, S.A., Oakford, M.J., 1991. Effects of paclobutrazol and carbaryl on the yield of hi-early red 'Delicious' apples. Journal of Horticultural Science 66, 159–163.

Jonker, M.J., Svendensen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J., 2005. Significance testing of synergistic antagonistic, dose level dependent or dose ratio dependent effects in mixture dose response analysis. Environmental Toxicology and Chemistry. 24, 2701–2713.

Khan, M.A.Q., Ahmed, S.A., Salazar, A., Gurumendi, J., Khan, A., Vargas, M., Von Catalin, B., 2007. Effect of temperature on heavy metal toxicity to earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). Environmental Toxicology 22, 487–494.

Kerstiens, G., 2006. Water transport in plant cuticles: an update. Journal of Experimental Botanic 57, 2493–2499.

Klironomos, J.N., Bednarczek, E.M., Neville, J., 1999. Reproductive significance of feeding on saprobic and arbuscular mychorrhyzal fungi by the collembolan, *Folsomia candida*. Functional Ecology 13, 756–761.

Kuhle, J.C., 1983. Ecotoxicological model studies to test the suitability of earthworms as bioindicators. Verhandlungen der Deutschen Zoologischen Gesellschaft. 76, 147–151.0

Laabs, V., Amelung, W., Pinto, A., Zech, W., 2002. Fate of pesticides in tropical soils of Brazil under field conditions. Journal of Environmental Quality 31, 256–268.

Lavelle, P., Spain, A.V., 2001. Soil Ecology. Kluwer Academic Publishers, Dordrecht; Boston

Lima, M.P., Soares, A.M.V.M., Loureiro, S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: simulation of flood and drought scenarios. Environmental Pollution 159, 1844–1851.

Lohner, T.W., Fisher, S.W., 1990. Effects of pH and temperature on the acute toxicity and uptake of carbaryl in the midge, *Chironomus riparius.* Aquatic Toxicology 16, 335–354.

Løkke, H., Van Gestel, C.A.M., 1998. Handbook of Soil Invertebrate Toxicity Tests. Wiley & Sons, Chichester.

Loureiro, S., Santos, C., Pinto, G., Costa, A., Monteiro, M., Nogueira, A.J.A., Soares, A.

M.V.M., 2006. Toxicity assessment of two soils from Jales mine (Portugal) using plants: growth and biochemical parameters. Archives of Environmental Contamination and Toxicology 50, 182–190.

Martikainen, E.A.T., Krogh, P.H., 1999. Effects of soil organic matter content and temperature on toxicity of dimethoate to *Folsomia fimetaria* (Collembola Isotomiidae). Environmental Toxicology and Chemistry 18, 865–872.

Martikainen, E.A.T., Rantalainen, M.L., 1999. Temperature–time relationship in Collembolan response to chemical exposure. Ecotoxicology and Environmental Safety 42, 236–244.

Mervosh, T., Sims, L., Stoller, E.W., G.,K., 1995. Clomazone fate in soil as affected by microbial activity, temperature, and soil moisture. Journal of Agricultural and Food Chemistry 43, 537–543.

Miller, N.E., 1993. Metabolism of 14C-Carbaryl Under Aerobic Soil Conditions, Submitted in Package of Rhone-Poulenc Ag Company. Department of Pesticide Regulation, Sacramento, CA, USA.

Mittler, R., 2002. Oxidative stress, antioxidants, and stress tolerance. Trends Plant Science 9, 405–410.

Morrison, M.J., 1993. Heat stress during reproduction in summer rape. Canadian Journal of Botany 71, 303–308.

Mostert, M.A., Schoeman, A.S., van der Merwe, M., 2002. The relative toxicities of insecticides to earthworms of the Pheretima group (Oligochaeta). Pest Management Science 58, 446–450.

Murthy, N.B.K., Raghu, K., 1990. Effect of carbaryl and 1-naphthol on seedling growth of barley, and on growth and nodulation of groundnut in two soils. Proceedings of the Indian Academy of Science (Plant Science) 100, 11–16.

Norisada, M., Hara, M., Yagi, H., Tange, T., 2005. Root temperature drives winter acclimation of shoot water relations in *Cryptomeria japonica* seedlings. Tree Physiology 25, 1447–1455.

Odeyemi, O., 1982. Relative stability of carbaryl in tropical ecosystems. Environmental Pollution 29 (3), 207–213.

OECD, 1984. Earthworm Acute Toxicity Tests. OECD – Organization for Economic Cooperation and Development, Paris.

Olson, B.L.S., Al-Khatib, K., Stahlman, P., Isakson, P.J., 2000. Efficacy and metabolism of MON 37500 in *Triticum aestivum* and weedy grass species as affected by temperature and soil moisture. Weed Science 48, 541–548.

Ortiz, R., Sayre, K.D., Govaerts, B., Gupta, R., Subbarao, G.V., Ban, T., Hodson, D., Dixon, J.M., Ortiz-Monasterio, J.I., Reynolds, M., 2008. Climate change: can wheat beat the heat? Agriculture, Ecosystem & Environment 126, 46–58.

Pavel, E.W., Fereres, E., 1998. Low soil temperatures induce water deficits in olive (*Olea europaea*) trees. Physiology Plantarum. 104, 525–532.

Penick, D.N., Spotila, J.R., O'Connor, M.P., Steyermark, A.C., George, R.H., Salice, C.J., Paladino, F.V., 1998. Thermal independence of muscle tissue metabolism in the leatherback turtle, *Dermochelys coriacea*. Comparative Biochemistry and Physiology-Part- A 120, 399–403.

Perreault, J.M., Whalen, J.K., 2006. Earthworm burrowing in laboratory microcosms as influenced by soil temperature and moisture. Pedobiologia 50, 397–403.

Pieper, G.R., 1979. Residue analysis of carbaryl on forest foliage and in stream water using Hplc. Bulletin of Environmental Contamination Toxicology 22, 167–171.

Presley, M.L., McElroy, T.C., Dieh, W.I., 1996. Soil moisture and temperature interact to affect growth, survivorship, fecundity, and fitness in the earthworm *Eisenia fetida*. Comparative Biochemistry Physiology-A, 114, 319–326.

Racke, K.D., Skidmore, M.W., Hamilton, D.J., Unsworth, J.B., Miyamoto, J., Cohen, S.Z., Holland, P.T., 1997. Pesticide fate in tropical soils. Pure and Applied Chemistry 69, 1349–1371.

Reinecke, A.J., Kriel, J.R., 1981. Influence of temperature on the reproduction of the earthworm *Eisenia foetida* (Oligochaeta). South African Journal of Zoology 16, 96–100.

Reinecke, A.J., Viljoen, S.A., Saayman, R.J., 1992. The suitability of *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia fetida* (Oligocaeta) for vermicomposting in Southern Africa in terms of their temperature requirements. Soil Biology and Biochemistry 24, 1295–1307.

Ribera, D., Narbonne, J.F., Arnaud, C., Saint-Denis, M., 2001. Biochemical responses of the earthworm *Eisenia fetid*a andrei exposed to contaminated artificial soil, effects of carbaryl. Soil Biology and Biochemistry 33, 1123–1130.

Rizhsky, L., Liang, H., Mittler, R., 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiology 130, 1143–1151.

Robertson, G.P., Grandy, A.S., 2006. Soil system management in temperate regions. In:

Ball, AS, Fernandes, E, Herren, H, Husson, O, Laing, M, Palm, C, Pretty, J, Sanchez, P, Sanginga, N, Thies, J (Eds.), Biological Approaches to Sustainable Soil Systems. Taylor & Francis, Boca Raton, FL, USA.

Römbke, J., Garcia, M.V., Scheffczyk, A., 2007. Effects of the fungicide benomyl on earthworms in laboratory tests under tropical and temperate conditions. Archives of Environmental Contamination Toxicology 53, 590–598.

Ruiz-Medina, A., Llorent-Martinez, E.J, Fernandez-de Cordova, M.L., Ortega-Barrales, P., 2012. Automated optosensor for the determination of carbaryl residues in vegetable edible oils and table olive extracts. Journal of Food Composition and Analysis. ScienceDirect 26, 66–71.

Sadana, U.S., Samal, D., Claassen, N., 2003. Differences in manganese efficiency of wheat (*Triticum aestivum* L.) and raya (*Brassica juncea* L.) as related to root- shoot relations and manganese influx. Journal of Plant Nutrition and Soil Science 166, 385–389.

Sanders, H.O., Finley, M.T., Hunn, J.B., 1983. Acute Toxicity of Six Forest Insecticides to Three Aquatic Invertebrates and Four Fishes. Technical Papers of the U.S. Fish and Wildlife Service. United States Department of the Interior, Fish and Wildlife Service, Washington, DC.

Sandifer, R.D., Hopkin, S.P., 1997. Effects of temperature on the relative toxicities of Cd, Cu, Pb, and Zn to *Folsomia candida* (Collembola). Ecotoxicology and Environmental Safety 37, 125–130.

Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010. Joint effects of three plant protection products to the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80, 1021–1030.

Simon, E.W., 1974. Phospholipids and plant membrane permeability. New Phytologist 73, 377–420.

Singh, J.S., Gupta, S.R., 1977. Plant decomposition and soil respiration in terrestrial ecosystems. Botanic Review 43, 449–528.

Sjursen, H., Holmstrup, M., 2004. Cold and drought stress in combination with pyrene exposure: studies with *Protaphorura armata* (Collembola: Onychiuridae). Ecotoxicological and Environmental Safety 57, 145–152.

Smit, C.E., Van Gestel, C.A.M., 1995. Influence of temperature and humidity fluctuations on the sensitivity of *Folsomia candida* for zinc. In: Van den Brink, W.J., Bosman, R., Arendt, F. (Eds.), Proceedings of the Contaminated Soil ’95. Kluwer Academic Publishers, Dordrecht.

Smit, C.E., Van Gestel, C.A.M., 1997. Influence of temperature on the regulation and toxicity of zinc in *Folsomia candida* (Collembola). Ecotoxicology and Environmental Safety 37, 213–222.

Snider, R., Butcher, J., 1973. The life history of *Folsomia candida* (Willem) (Collembola: Isotomidae) relative to temperature. Great Lakes Entomologist 6, 97–106.

Spain, A.V., 1974. The effects of carbaryl and DDT on the litter fauna of a Corsican pine forest (*Pinus nigra* var. maritima): a multivariate comparison. Journal of Applied Ecology 11, 467–481.

Spurgeon, D.J., Hopkin, S.P., 1996. Effects of metal-contaminated soils on the growth, sexual development and early cocoon production of the earthworm *Eisenia fetida*, with particular reference to zinc. Ecotoxicology and Environmental Safety 35, 86-95.

Stegeman, L.R.C., 1964. The effects of the carbamate insecticide carbaryl upon forest soil mites and Collembola. Journal of Economic Entomology 57, 803–808.

Schulze, T.L., Jordan, R.A., Robert, W., Hungb, R., Chadwick, T., Daniel, M., Martin, S. C., 2001. Efficacy of granular deltamethrin against *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) Nymphs. Journal of Medical Entomology 38, 344–346.

Tasseva, G., de Virville, J.D., Cantrel, C., Moreau, F., Zachowski, A., 2004. Changes in the endoplasmic reticulum lipid properties in response to low temperature in *Brassica Napus*. Plant Physiology and Biochemistry 42, 811–822.

Topp, E., Vallaeys, T., Soulas, G., 1997. Pesticides: microbial degradation and effects on microorganisms. In: van Elsas, JD, Trevors, JT, Wellington, EMH (Eds.), Modern Soil Microbiology. Dekker, New York.

Tripathi, G.K.N., Dabi, I., Bandooni, N., 2011. Temperature-dependent alterations in metabolic enzymes and proteins of three ecophysiologically different species of earthworms. Brazilian Archives of Biology and Technology 54, 769–776.

Trumbore, S.E., 1993. Comparison of carbon dynamics in tropical and temperate soils using radiocarbon measurements. Global Biogeochemical Cycles 7, 275–290.

Uyanik, A., Özdemir, M., 1999. Effect of the environmental temperature on the degradation period of carbaryl. Turkish Journal of Agricultural and Forestry 23, 579–584.

Vierling, E., 1991. The roles of heat shock proteins in plants. Annual Review of Plant

Physiology and Plant Molecular Biology 42, 579–620.

Viswanathan, P.N., Krishna Murti, C.R., 1989. Effects of temperature and humidity on ecotoxicology of chemicals. In: Bourdeau, P., Haines, J.A., Klein, W., Krishanamurti, C.R. (Eds.), In: Ecotoxicology and Climate with Special Reference to Hot and Cold Climates, 1989. John Wiley & Sons, New York, NY, USA.

Yu, Xiang, Wang, Han, Lu, Yizhen, Ruiter, M., Cariaso, M., Prins, M., van Tunen, A., He, Yuke, 2012. Identification of conserved and novel microRNAs that are responsive to heat stress in *Brassica rapa*. Journal of Experimental Botanic 63, 1025–1038.

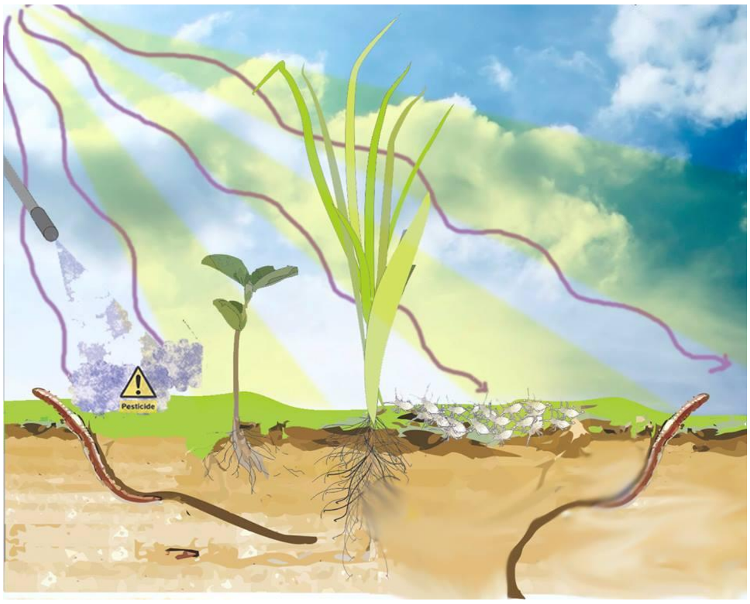
Walbot, V., 2011. How plants cope with temperature stress. BMC Biology C7 – 79 9, 1–4.

Wanjura, D.F., Buxtor, D.R., 1972. Hypocotyl and radicle elongation of cotton as affected by soil environment. Agronomy Journal 64, 431–435.

Wheeler, T.R.C., Peter, Q., Ellis, R.H., Porter, J.R., Vara Prasad, P.V., 2000. Temperature variability and the yield of annual crops. Agriculture, Ecosystem & Environment 82, 159–167

CHAPTER 4

Responses of wheat (*Triticum aestivum*) and turnip (*Brassica rapa*) to the combined exposure of carbaryl and ultraviolet radiation.



Chapter 4. Responses of wheat (*Triticum aestivum*) and turnip (*Brassica rapa*) to the combined exposure of carbaryl and ultraviolet radiation.

Under review in Environmental Toxicology and Chemistry

Abstract

The increase of ultraviolet radiation reaching the Earth's surface due to the increase of ozone layer depletion have affected crop production systems, and in combination with pesticides, used in agricultural activities, can lead to greater risks to the environment. The impact of ultraviolet radiation and carbaryl singly and in combination on *Triticum aestivum* (wheat) and *Brassica rapa* (turnip) was studied. The combined exposure was analyzed using the MIXTOX tool and was based on the conceptual model of Independent Action (IA) where possible deviations to synergism or antagonism, dose-ratio or dose-level response pattern were also considered. Compared to the control, carbaryl and ultraviolet radiation (UV radiation) individually led to reductions in growth, fresh and dry weight and water content for both species. Combined treatment of ultraviolet and carbaryl were more deleterious when compared to single exposure. For *T. aestivum* length an additive effect (Independent Action) between the two stressors was found and analyzing weight parameters a synergism occurred at low concentrations of carbaryl combined with low UV intensities. Synergistic patterns were observed for *B. rapa* length and dry weight when UV radiation was the dominant stressor and carbaryl was present at low concentrations.

Key words: *Brassica rapa, Triticum aestivum,* Ultraviolet radiation, carbaryl, Independent Action, Synergism.

4.1 Introduction

The increase of ultraviolet radiation on the Earth's surface due to the depletion of the ozone layer has caused a great impact on terrestrial and aquatic ecosystems (Ballaré et al., 2001; Caldwell et al., 2007 Zaller et al., 2002) and in crop production (Yao et al. 2013). In the environment, ultraviolet radiation is an additional stress besides the stress caused by the presence of pollutants to which the organisms are commonly exposed (Dubé and Bornman, 1992; Misra et al., 2005) and a synergistic or additive effect between these stressors may be found, causing higher environmental hazards than those predicted for chemical exposure and also may lead to severe economic consequences.

To accurately relate photobiological effects with UV radiation, ultraviolet spectral functions need to be implemented and integrated, describing the relative effectiveness of a spectrum band in relation to a specific biological response (Diffey, 1991). In the case of humans, this response is standardized by the erythemic action spectrum, which is the skin's sensitivity to UV radiation absorbed (McKinlay and Diffey, 1987). The spectral irradiance UV should be weighted by the action spectrum in order to obtain the biologically active irradiance.

The biological spectral weighting function most commonly used for plants is the generalized plant response developed by Caldwell (1971), which is composed by nine different UV-B responses. But this methodology involves several constraints being one of them the exclusion of the responses regarding plant growth and also take into account only the effects of wavelengths up to 313 nm (Flint and Caldwell, 2003), when biological responses in plants have been reported to occur at the UV-A region (320-400nm) (Fagerberg, 2007; Flint and Caldwell, 1996; 2003; Kataria and Guruprasad, 2012).

Studies have reported effects of high UV-B radiation on the physiology, growth and development of plants which have also been modified by the presence of heavy metals (Agrawal and Mishra, 2009; Dubé and Bornman, 1992; Shahbaz et al., 2012; Shweta and Agrawal, 2006; Srivastava et al., 2012). However, from our knowledge, no work has been done on the combined exposure of ultraviolet radiation and pesticides in plants.

Given this, an evaluation of the effects caused by pollutants on crop plants under different doses of ultraviolet radiation, using the more recent plant action spectrum described by Flint and Caldwell (2003), which include the effects of wavelengths until 390 nm, is very important to acquire a more realistic view of the current scenario of environmental changes induced by the ozone depletion. So, the purpose of this study was to analyze the individual and combined effects of different and realistic doses of ultraviolet radiation (UVA and UVB) and carbaryl on two species of plants, very important for global food crop, the monocotyledonous *Triticum aestivum* and the dicotyledonous *Brassica rapa*. Carbaryl is a widely used pesticide in agriculture worldwide, considered for many insect pests of fruits, vegetables, cereals, and more than 100 crop species (Branch and Jacqz, 1986; Jana and Das, 1997).

In order to predict the joint effects of UV radiation and carbaryl on these plant species, the conceptual model of Independent Action (IA) was adopted. The IA principle is that the stressors do not interact physically, chemically or biologically (Bliss, 1939), and assumes that stressors affect organisms through different modes of action (Loureiro et al., 2010).

4.2 Material And Methods

4.2.1 Plants growth bioassay

The plants' bioassay methodology was adapted from that described in ISO 11269-2 (ISO 1995). The experimental designs of single and combined exposure to carbaryl and UV radiation were performed in a laboratory room at 20± 2°C, at 12000 lx, in a 16:8 (light: dark) photoperiod.

The caryopses of *Triticum aestivum* (wheat) were obtained from an agricultural store (Aveiro, Portugal), whereas the seeds of *Brassica rapa* (turnip) were purchased from Carolina Biological Supply Company (US). The tests were performed with LUFA 2.2 soil, commercialized by the German Institution LUFA Speyer, with the properties: pH = 5.5 ± 0.2 (0.01 M CaCl2), water holding capacity = 41.8 ± 3.0 (g/100 g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. Each test pot contained 500g of natural Lufa 2.2 soil and ten caryopses/seeds were placed per pot at a maximum depth of 1 cm from the soil surface; the bottom of each pot was perforated and a fiberglass wick was placed in direct contact with the soil (5-10 mm in diameter). These pots were then placed on the top of a container filled with water, and the wick maintained the soil moisture (60%WHC) by capillarity. Seeds and caryopses germination time was reported. After 14 days, plants were harvested and growth parameters (individual shoot length, dry and fresh weight) were recorded. The water content (WC) was calculated using the following equation:

$$WC = \frac{FW - DW}{FW} \times 100$$

DW = plant's dry weight

FW = plant's fresh weight.

Visible changes in plant color or other symptoms of foliar morphology or death were also recorded for all plants.

4.2.2 Stressors: test chemical and UV radiation

Carbaryl (CAS No 63-25-2) was purchased from Sigma-Aldrich Ltd. (99.8% purity). The pesticide was applied in pre-moistened soil one day before the experiment started, in order to allow the evaporation of acetone. In addition to the negative control, a solvent control was prepared with 100 ml acetone/kg. Nominal concentrations used ranged from 50 to 150 mg/kg and four replicates for treatments were used. These concentrations were the same used in previous chapter (2 and 3).

The UV radiation was produced by a UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 313 NM and 365nm corresponding UV-B and UV-A), applied on metal frames and suspended 30 cm above the plant apex and kept constant throughout the experiment. A clear cellulose acetate sheet (0.003 mm, Grafix plastics, USA) was used for cut-off UV-C range wavelengths and was previously UV irradiated for 12 h before being used in the experiments to minimize differences in UV radiation intensity. The sheets were changed frequently to avoid aging effects on the spectral transmission of UV-B. The intensities of transmitting UV and visible light at the top of the plant was measured with a Spectroradiometer connected to a monochromator and spectral radiance was obtained by the BenWin+ Software (Bentham Instruments, Reading, UK). Intensity values were converted into biologically effective UV radiance by using the plant growth BSWF (Flint and Caldwell, 2003). Since research on the effects of UV radiation on terrestrial plants different action spectra are used, as biological spectral weighting functions (BSWFs), a summary of the effective doses used in this work, calculated with different BSWFs is shown in Supporting Information Table S 4.1, which will also allow a better comparison with other works.

For UV experiments, seedlings, immediately after emergence, were irradiated daily for 4h, 6h and 8h, corresponding to three levels of ultraviolet radiation. The total biologically effective doses were 14.2 $kJm^{-2}\ d^{-1}$, 21.3 $kJm^{-2}\ d^{-1}$ and 28.4 $kJm\text{-}^{2}\ d^{-1}$, and were calculated as follows:

$$UV_{BE}\,dose\ (Jm^{-2}) = \frac{I_{BE}\,(mWcm^{-2})\ X\ Time\ of\ \exp osure\,(s)}{1.000} \times 100$$

Where I_{BE} is the biologically effective UV irradiance. Plants irradiated with PAR light were kept as control.

These doses used in this work are relevant, considering as an example the mean effective daily ambient UV-B dose for Portugal, weighted with generalized plant spectrum, reported for a day summer solstice, with clear sky conditions, as 6.84 kJ m^{-2} (Correia et al., 2012).

4.2.3 Joint toxicity of carbaryl and UV radiation

Immediately after emerging in clean or carbaryl contaminated soil, seedlings were exposed to different doses of UV radiation to evaluate the joint effects of carbaryl and UV radiation. Carbaryl analysis in soil was performed after 15 days of exposure. Soil samples (three replicates) of spiked soil (time zero), soil without UV exposure (at day 15) and soil exposed to the highest UV radiation dose (28.5 $kJm^{-2}d^{-1}$) (at day 15) were sent for chemical analysis at Marchwood Scientific Services, Southampton, UK. The procedure involved initial single-phase QuEchers extraction of 10 g of soil with 10 mL acetonitrile, followed by analysis using liquid chromatography-tandem mass spectrometry (LCMS-MS), with a limit of detection of 0.1 mg/kg

4.2.4 Statistical analysis

Data for both single and combined exposures were analyzed by one way ANOVA (Systat, 2006). When data were normally distributed, the Dunnett's test was used to analyze differences between control and treatments. For data that failed the normality test and data transformation did not correct for normality, a non-parametric Kruskal–Wallis test was performed, followed by the Dunn's method when significant differences were found (Systat, 2006).

EC_{50} values were calculated using a non-linear regression, a sigmoidal logistic 3-parameter equation (Systat, 2006). Lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) were also derived from the multiple comparison test (Dunn's or Dunnett's test, $\alpha=0.05$).

The sensitivities of *B. rapa* and *T. aestivum* to UV radiation were assessed using two methods:

1) The changes in dry weight and plants length were calculated for all UV-radiation doses used and an UV sensitivity index (UV-SI) was determined by the equation (Adapted from Saile- Mark and Tevini, 1997):

$$UV-SI=\frac{Shoot\ length\ treatment}{Shoot\ length\ control}+\frac{Dry\ weight\ \ treatment}{Dry\ weight\ \ control}$$

When UV-SI<1, then plants are classified as sensitive to the exposure conditions; when UV-SI≥1, plants are considered tolerant. This limit value of 1 was chosen based on the fact that when treatment plants have half the parameter values when compared to the control (equivalent to an EC_{50}), their sum will be 1.

2) The UV sensitivity was determined by the biomass accumulation from shoot dry weight (Smith et al., 2000)

It has been shown that biomass accumulation is a good parameter to evaluate UV sensitivity in plants (Smith et al., 2000), because a decrease in biomass accumulation indicates some damage to physiological functions caused by ultraviolet radiation. (Smith et al., 2000; Visser, 1997).

Conceptual models have been used to predict the mixture toxicity and also the combined effects of natural and chemical stressor to several organisms (Ferreira et al., 2008; Lima et al., 2011; 2014; Ribeiro et al., 2011;). Deviations from the model as synergism, antagonism, dose-level and dose-ratio dependency can be characterized by a nested approached using the MIXTOX tool that can be further explored in Jonker et al., (2005).

4.3 Results

The data from chemical analysis to carbaryl spiked soil showed that although there was a decrease on carbaryl concentration after 15 days of exposure (in both UV irradiated and non-irradiated trials), no significant changes were attained (One Way ANOVA, $F_{2,6}=2.26$; $p>0.05$, Table 4.1). Due to the high variability of results obtained on the soils after 15 days and no UV irradiation, a t-test was performed between data in time zero and UV irradiated soil after 15 days, and a significant decrease of 25% was achieved (t-test, p=0. 027).

Table 4.1. Concentrations of carbaryl in soil after spiking (time zero-T0), after 15 days of exposure without UV-radiation (15d without UV) and exposed to UV-radiation (15d with UV), and carbaryl degradation (%) compared to T0. Data is presented as mean values and standard error in brackets.* p<0.05, t-test with T0.

Treatments	[Carbaryl] (mg/kg)	% degradation (related to T0)
T0	152.3 (11.18)	
15d without UV	144.8 (20.36)	4.95% (13.36)
15d with UV	114.3 (0.15)	24.96%* (0.10)

4.3.1 Single exposure to carbaryl and UV radiation.

Growth parameters

Growth was adversely affected by carbaryl applied alone for both plant species. Shoot length decreased in both *T. aestivum* and *B. rapa* under carbaryl exposure of 50 mg/kg and from 75 mg/kg onwards (Dunnett's, $p<0.05$) respectively, compared to the control (Table 4.2). *T. aestivum* was considered more sensitive than *B. rapa* for the length parameter with EC_{50}= 62.0± 2.0 mg/kg and 76.9±2.5 mg/kg (mean ± St. Err.), respectively. The fresh and dry weight of *T. aestivum* was significantly reduced from 75 mg/kg onwards and the water content from 50 mg/kg onwards (Dunnett's, $p<0.05$), and *B. rapa* all these parameters were affected from 75 mg/kg onwards (Table 4.2). The fresh weight parameter, showed that *B. rapa* was more sensitive than *T. aestivum* with an EC_{50} values of 48 ±2.3 mg/kg and 65.1 ±3 mg/kg (mean ± St. Err.), for the dry weight, the EC_{50} value for *T. aestivum* was also lower (78.0 ±3 mg/kg) than *B. rapa*, which could not be calculated, being higher than the highest concentrations (>150 mg/kg) (Table 4.2).

UV irradiation showed significant reductions in shoot length for both species, from the lowest dose used (14.2 kJm^{-2} d^{-1}) onwards (Dunnett's, $p<0.05$) (Figure 4.1A and D). *T. aestivum* showed high tolerance to UV radiation, where the ED_{50} could not be calculated within the intensities used, being higher than the highest dose of UV radiation; for *B. rapa* the ED_{50} for shoot length was 21.8±0.7 $kJm^{-2}d^{-1}$ (Table 4.2). UV irradiation also caused a reduction in fresh weight (Figure 1B and E) for both species. Again, *B. rapa* was more affected, and a significant reduction of the fresh weight was found from 14.2 $kJm^{2}d^{-1}$ onwards, with a decrease of 90.9% on at the highest UV dose, when compared to control (Figure 1E). *T. aestivum* was affected only at the highest dose (28.4 $kJm^{2}d^{-1}$), with a lower decrease in fresh weight (30.6%) compared to controls. (Figure 1 B). Analyzing the ED_{50} values for these parameters, *T. aestivum* was more tolerant to UV radiation than *B. rapa* with an ED_{50} higher than the highest dose used (28.4 $kJm^{2}d^{-1}$) for fresh and dry weight, and water content (Table 4.2).

UV sensitivity index.

Table 4.3 shows the sensitivity indexes of *B. rapa* and *T. aestivum* to UV radiation. The calculated UV sensitivity index (UV-SI) showed that *T. aestivum* is more tolerant to UV radiation than *B. rapa* for all doses used. The lowest UV-SI was 0.35 for *B. rapa* exposed to 28.4 $kJm^{-2}d^{-1}$,whereas for *T. aestivum* exposed to same UV radiation the UV index was 1.48 (the lowest UV-IS for this species).

The highly significant reductions in biomass and highest sensitivity to UV-radiation were observed in *B. rapa* from 14.2 $kJm^{-2}d^{-1}$ onwards, accumulating only 58.9% of the biomass of controls, while *T. aestivum* showed a decrease in biomass only at the dose of 28.4 $kJm^{-2}d^{-1}$ (Table 4.3), accumulating in this UV-dose 70.3% of the biomass of control.

Foliar changes

T. aestivum and *B. rapa* exposed to single carbaryl did not show any visible changes in plant morphology or death. But in the exposure to UV radiation, both species showed changes in their leaf morphology. *T. aestivum* showed foliar changes even at the lower radiation used (14.2 $kJm^{-2}d^{-1}$): shorter stems, smaller leaves, serrated tips and rolled down leaves. Necrosis of tip leaves was found at the highest dose (28.4 $kJm^{-2}d^{-1}$). Foliar changes in *B. rapa* appeared only after the UV dose of 21.3 $kJm^{-2}d^{-1}$, with shorter stems, smaller leaves, and rolled down leaves. Despite of these morphological changes, none of emerging seedlings died by the end of the test for both species.

Table 4.2. EC_{50}/ED_{50} values with standard error values (under brackets) and LOECs for the exposures of *Triticum aestivum* and *Brassica rapa* to carbaryl and UV radiation alone and upon a combined exposure.

Species	**Endpoint**	**UV Single** ED_{50} (kJ/m^2d^{-1})	**Carbaryl Single** EC_{50} (mg/kg)	**EC_{50} Carbaryl + 14.2 kJ/m^2d^{-1}**	**EC_{50} Carbaryl + 21.3 kJ/m^2d^{-1}**	**EC_{50} Carbaryl + 28.4 kJ/m^2d^{-1}**
Triticum aestivum	*Length*	>28.4 LOEC=14.2	62.0 (2.0) LOEC=50	62.1 (2.9) LOEC=50	59.2 (3.3) LOEC=50	72.1 (4.3) LOEC=50
	Fresh weight	>28.4 LOEC=28.4	65.1 (3.0) LOEC=75	40.5 (3.8) LOEC=50	39.9 (4.9) LOEC=50	23.5 (8.6) LOEC=50
	Dry weight	>28.4 LOEC=28.4	78.0 (3.7) LOEC=75	50.9 (4.9) LOEC=50	47.8 (6.0) LOEC=50	43.6 (8.9) LOEC=50
	Water Content	>28.4 LOEC=28.4	>150 LOEC=50	>150 LOEC=50	>150 LOEC=75	>150 LOEC=50
Brassica rapa	*Length*	21.8(0.7) LOEC=14.2	76.9 (2.5) LOEC=75	75.2 (6.6) LOEC=50	67.0 (21.8) LOEC=50	>150 LOEC=125
	Fresh weight	15.3 (0.7) LOEC=14.2	48.0 (1.8) LOEC=75	34.6 (6.2) LOEC=50	24.9 (13.4) LOEC=50	34.4 (15.3) LOEC=50
	Dry weight	15.4 (0.76) LOEC=14.2	46 (2.3) LOEC=75	80 (10.4) LOEC=50	n.d LOEC>150	>150 LOEC=>150
	Water content	n.d LOEC=21.3	>150 LOEC=75	n.d LOEC=50	>150 LOEC=50	>150 LOEC=50

EC_{50}= Median effective concentration; ED_{50}= Median effective Dose UV-B; LOEC- Lowest Observed Effect Concentration; n.d = not determin

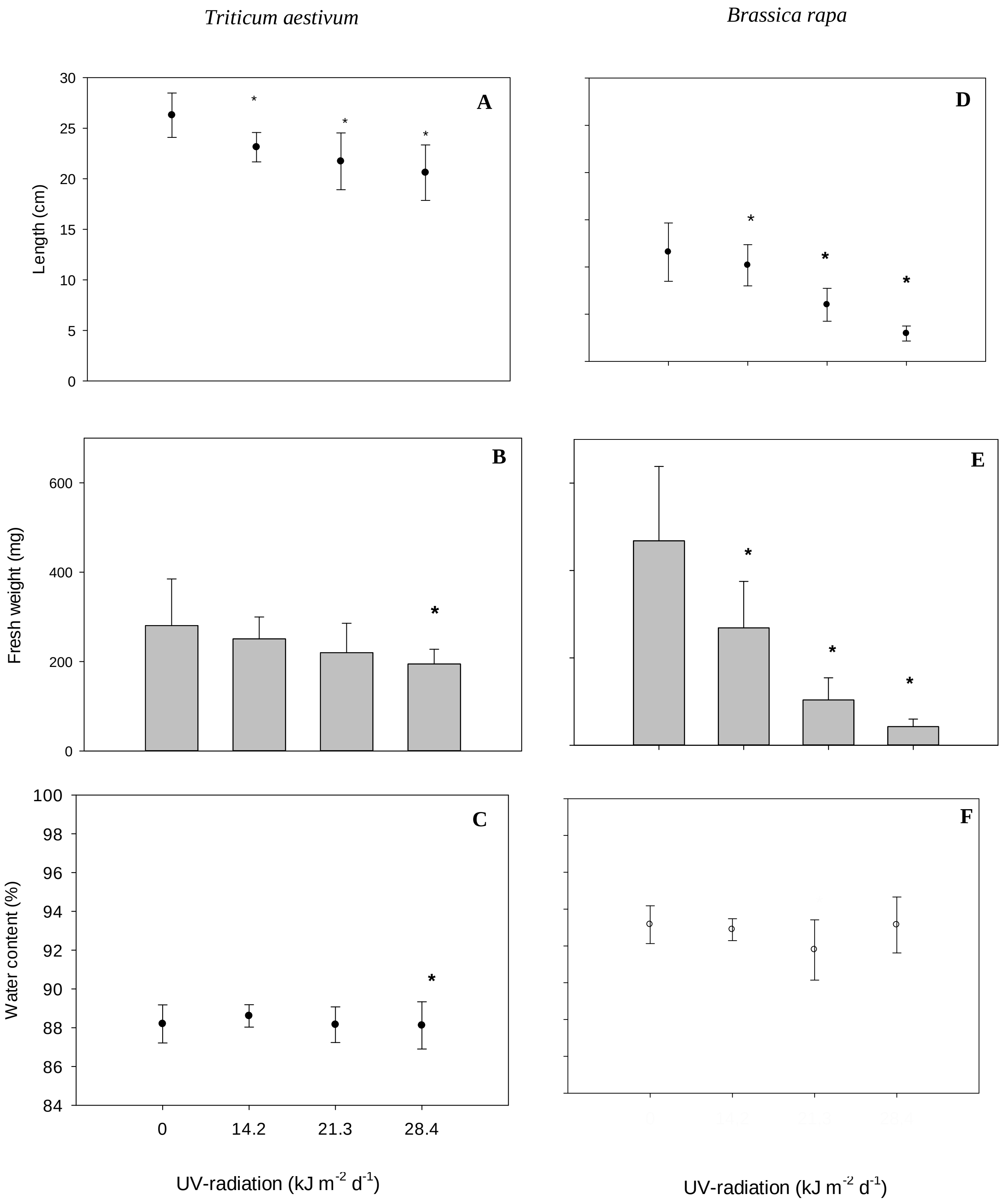


Fig. 4.1. The effects of UV radiation after 14 days of exposure on *Triticum aestivum* shoot length (A), fresh weight (B) and water content (C) and *Brassica rapa* shoot length (D), fresh weight (E) and water content (F) respectively. Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test).

Table 4 3 Sensitivity of *Brassica rapa* and *Triticum aestivum* to UV radiation, using the percentage of changes in DW and plants length and the biomass accumulation (dry weight) for all UV radiation doses used.

		UV Sensitivity index		Biomass accumulation	
	UV- radiation		Sensitivity	%	Sensitivity
Brassica rapa	14.2 kJ $m^{-2}d^{-1}$	1.48	T	58.9*	S
	21.3 kJ $m^{-2}d^{-1}$	0.78	S	25.4*	S
	28.4 kJ $m^{-2}d^{-1}$	0.35	S	8.7*	S
Triticum aestivum	14.2kJ $m^{-2}d^{-1}$	1.75	T	87.3	T
	21.3kJ $m^{-2}d^{-1}$	1.6	T	79.2	T
	28.4kJ $m^{-2}d^{-1}$	1.48	T	70.3*	S

*$P<0.05$ (Dunn's Test); T= Tolerant; S= sensitive; Tolerant : UV-SI > 1; Sensitivy: UV-SI < 1

4.3.2 Combined exposure to carbaryl and UV radiation

The joint toxicity was predicted by using the MIXTOX tool and the data set was fit to generate the best description of the biological response of *B. rapa* and *T. aestivum* to this combination. The Independent Action (IA) was chosen considering that UV radiation and carbaryl have different modes of action on plants. All parameters generated from the MIXTOX tool are presented in Table 4.4.

Table 4.4 Summary of carbaryl and ultraviolet radiation effect on *Triticum aestivum and Brassica rapa*. IA is independent action; SS is the objective function used for continuous data; r^2 is the coefficient of determination; *a* and *b* are parameters of the deviation functions; S is synergism; DR and DL are "dose ratio" and "dose level" deviation from the reference model.

Species	Endpoints	Reference Model (IA)		Deviation				
		SS	r^2	Type	SS	r^2	*a*	*B*
Triticum aestivum	Length	9921	0.64					
	Fresh weight	910134	0.83	DL	8055370	0.85	-8.2	1.2
	Dry weight	14774.2	0.82	DL	13146	0.85	-7.6	1.3
Brassica rapa	Length	1804	0.70	DR	1800	0.72	- 4.7	8.7
	Fresh weight	2119371	0.77					
	Dry weight	19707	0.55	DR	16462	0.63	- 2,8	8,6

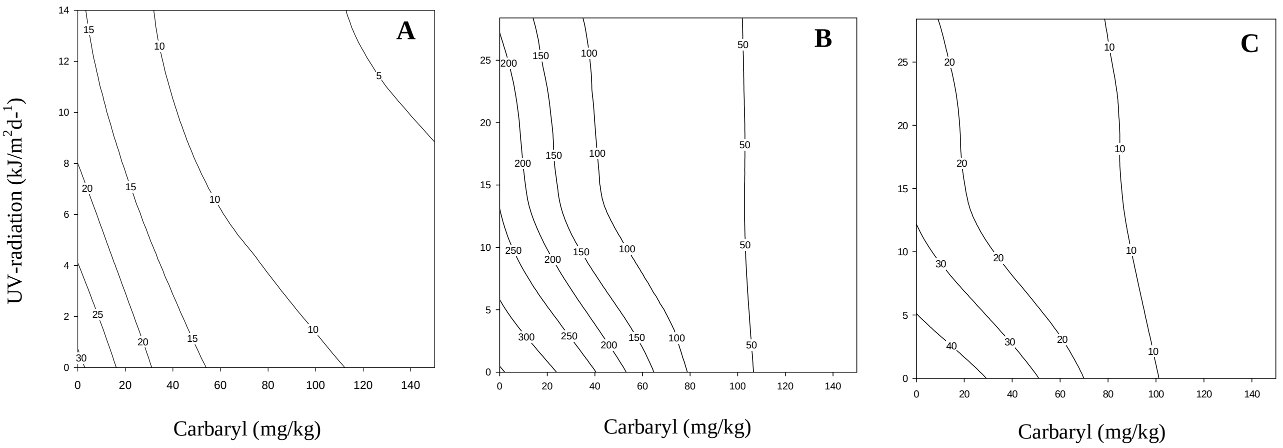


Fig. 4.2 Dose-response relationship for the combination of carbaryl and UV- radiation *on T. aestivum*, showing an additive effect (Independent Action) for the parameter length (A) and dose- level dependent deviations for the parameters fresh weight (B) and dry weight (C).

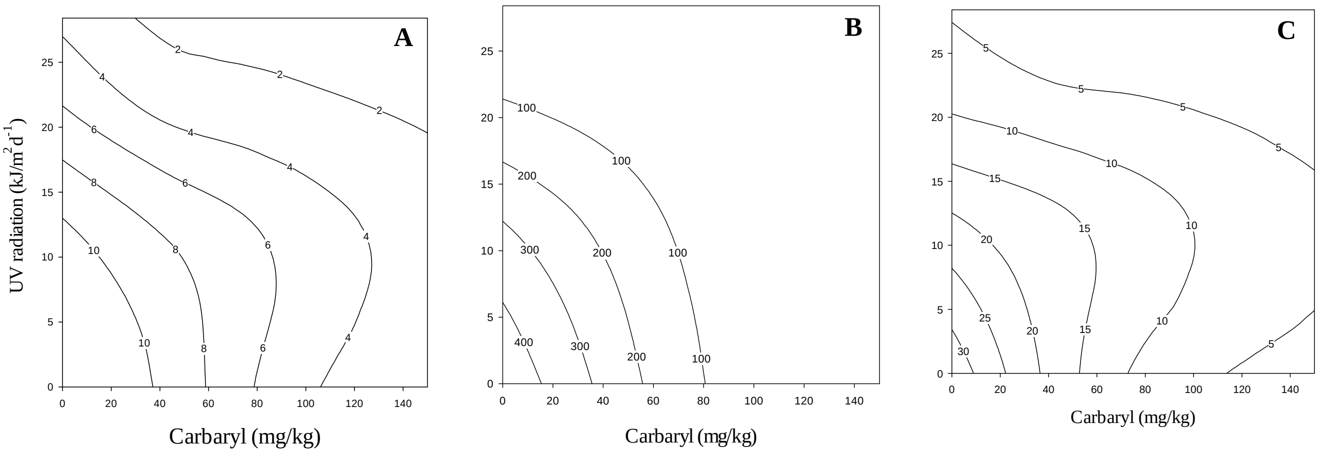


Fig. 4.3 Dose-response relationship for the combination of carbaryl and UV- radiation on *B.rapa*, showing a dose-ratio dependent deviation from the IA model for the parameter length (A) and dry weight (C) and an additive effect (Independent Action) for the parameter fresh weight (B)

Growth parameters

The combined exposure to both stressors induced an additive effect regarding the *T. aestivum* shoot length, with a good estimation by the IA model ($p=1.\ 11\times10^{-128}$; Table 4.4 and Figure 4.2A). For *B. rapa* shoot length the effects of combined exposure fitted by Independent Action showed a SS value of 1804 and by adding the parameters α and b_{DR} to the nested framework a dose-ratio dependent deviation was achieved, with a decrease in the SS value and increase on the r^2 of the fit (SS=1800; r^2=0. 72; P=2.1

$\times 10^{-10}$; Table 4.4). In this case, the values of ɑ (-4.7) and b (8.7) parameters indicate an increase on the toxicity (synergism) when UV radiation was the dominant stressor, i.e. low carbaryl concentrations combined with high UV radiation doses (Figure 4.3A)

T. aestivum fresh and dry weight data were fitted by the IA model (SS= 910134; r^2=0.82 and SS=14774.2; r^2=0.82, respectively) but a significantly better fit was obtained after adding the parameter ɑ and b_{DL} (SS=805537; r^2=0.85 and SS=13146; r^2 =0.85, respectively, Table 4.4), showing a deviation dependency on the dose level present. The negative parameter ɑ was indicative of a synergism occurring at low dose levels of both stressors and antagonism at higher doses of both stressors (Figure 4.2B and C).

The combined exposure to both stressors induced an additive effect regarding *B. rapa* fresh weight, with a good estimation by the IA model (p=1. 1×10^{-188}; Table 4.4; Figure 4.3B). Looking at *B. rapa* dry weight and after the data fit to the IA model (SS=19707; r^2= 0.55), a dose-ratio dependent deviation was observed with parameters ɑ and b_{DR} provides a better estimation of the joint toxicity (SS=16462; r^2=0. 63; P=3. 3×10^{-24} Table 4.4 and Figure 4.3C). The values of ɑ (-2.8) and b (8.6) parameters indicate an increase on the toxicity (synergism) when UV radiation was the dominant stressor.

Foliar changes and mortality

The combined action of UV radiation and carbaryl on leaf morphology of *T. aestivum* was greater than the individual effects of these stressors, and were visible earlier, at lower doses of UV radiation (14.2 $kJm^{-2}d^{-1}$) and intensified at higher doses of UV radiation (28.4 $kJm^{-2}d^{-1}$). In this scenario all leaves showed yellowing and in some plants yellow patch turned into necrotic regions. Foliar changes in *B. rapa* only appeared from the UV radiation of 21.3 $kJm^{-2}d^{-1}$ onwards, similar to what occurred in the single UV exposure, but were intensified at carbaryl concentration of 75 mg/kg and onwards. Foliar changes included yellowing leaves, loss of leaf rigidity, some wilting and dried leaves, and the leaf edges curled down. Before the end of the test, under a UV radiation dose of 28.4 $kJm^{-2}d^{-1}$ combined with 125 mg carbaryl/kg, 5.5% of the *B. rapa* seedlings died and 24.5% of the plants died at this UV dose combined with 150 mg/kg. No dead plants were registered on the *T. aestivum* trial.

4.4 Discussion

4.4.1 Single exposure

The exposures to carbaryl and UV radiation as single stressors showed a negative influence on growth, water content and biomass. Some studies have already reported that carbaryl severely affects plant growth, water content, emergency and biomass production (Jones et al., 1991; Lima et al., 2011; Murthy, 1990;). Murthy and Raghu (1990) reported a high LOEC (100 mg/kg of carbaryl) and significant decrease in barley dry weight of 32.6%, compared to the control, in sandy loam soils. Comparing with our results, a low LOEC was found (75 mg/kg) for both *T. aestivum* and *B. rapa*, and a high and significant reduction in their dry weight in this concentration, compared to the control, of 52% and 78.5%, respectively (data not show).

Inconsistent UV-B doses and different BSWFs used in studies on the effects of UV-radiation in plants has generated many different outputs which lead to a difficult comparison and conclusions regarding the response of plants to this stressor. Furthermore, many these studies. e.g., (Correia, 2012; Fagerberg, 2007; Li et al., 2000; Qaderi and Reid, 2005; Qaderi et al., 2007; Singh et al., 2012) have used only UV-B wavelengths and exposure to just one level, comparing with and without UV irradiation, but different biological response can be found at different levels of UV radiation (Frohnmeyer and Staiger, 2003) and under UV-A wavelengths (Flint and Caldwell, 2003).

Studies indicate that ultraviolet radiation affects mainly the photosystem II (Booij-James at al, 2000), and the main plant protection mechanisms to that stress is the biosynthesis of UVabsorbing compounds, as phenolic compounds and flavonoids, that attenuate the penetration of the UV-B range of the solar spectrum through the leaf (Frohnmeyer, and Staiger, 2003).

Among the negative effects of UV radiation on the plants are: reduced plant growth, reproduction and development, reducing photosynthesis and decreasing plant productivity (Caldwell et al., 2007; Rousseaux et al., 1999; Srivastava et al., 2012; Stapleton, 1992; Zuk-Golaszewska et al., 2003) but the effect varies among species (Li et al., 2000) In present study UV radiation had a significant inhibitory effect on shoot length, fresh and dry weight of both plant species and this agrees with results from other works (Alexieva et al., 2001; Li et al., 2000; Qaderi et al., 2007; Yuan, 1998) where *T. aestivum* and *B. rapa* also were negatively affected in their growth by UV radiation. At the highest UV radiation, the decrease in *B. rapa* shoot length was more accentuated (75%), while *T. aestivum* at the same conditions decreased their shoot length in 16.3%, when compared to the control. This reduction in shoot length has been commonly found in other plant species exposed to UV radiation (Dai et al., 1994; Lydon, 1986) and may be related to several factors such as photooxidative stress, DNA damage (Rozema et al., 1997; Rousseaux et al., 1999, Strid, 1993), decrease in cell division (Hopkins et al., 2002) and energy allocation to others routes, as production of ultraviolet-absorbing sunscreens (Jaakola and Hohtola, 2010; Mazza et al., 2000).

Tian and Lei (2007) reported a decrease of 29.1% on *T. aestivum* fresh weight exposed to 3.5 kJm^2 of UV-B (weighted with the generalized plant response action spectrum) for 7 days of exposure, which is above that was reported in the present study, for the same species in more days of exposure (14 days). The dose used by the author mentioned above corresponds approximately to our dose of 14.2 kJm^2d^{-1} where a decrease in fresh weight of 12.2% and 10.7% was observed in *T. aestivum* and *B. rapa*, respectively. In table S 4.1 we can compare the doses used by the authors with the doses used in this study, since different BSWFs were used.

Our results also showed that *T. aestivum* was less affected regarding the water loss, with a LOEC of 28.4 kJm^2d^{-1}, which corroborates with other studies where no changes were observed on leaf water content of wheat after UV-B treatments (Alexieva et al., 2001), and only one cultivar, among three spring wheat (*T. aestivum* L.) cultivars, was significantly affected in their water content by UV radiation (Feng et al., 2007).

The reduction in plant height and dry weight are two parameters that have often used to assess the plant's sensitivity to UV radiation (Biggs et al., 1981; Mishra and Agrawal, 2006; Teramura, 1983). The dry weight represents the adverse effects of cumulative radiation on some functions of plants (Smith et al., 2000) and reduction in plant length are a characteristic of UV radiation sensitive plants (Tevini and Teramura, 1989). In the present work, these two endpoints were used to evaluate the differences in UV sensitivity of both plants species. In table 4.3 is presented a index of sensibility to UV and the biomass accumulation of both species, and have proved to be good indicators of the UV sensitivity. Both indicators reported the low tolerance of *B. rapa* to ultraviolet radiation.

The tolerance of plants to UV radiation vary inter and intraspecifically (Li et al., 2000; Lv et al., 2013; Yuan et al., 2000) and plants grown at higher altitudes, continuously exposed to higher levels of UV-B, are commonly more tolerant to this radiation than plants grown at lower altitudes, with low UV-B exposure (Jordan, 1996). Several studies have reported high UV sensitivity of the family Brassicaceae (Tevini et al., 1981; Van et al., 1976), and also regarding wheat species (Yuan, 1998), but to our knowledge, there is no study comparing the tolerance between these two species to UV radiation. Our results showed that *T. aestivum* can be considered more tolerant to UV radiation for the doses used when compared to *B. rapa*.

In contrast, monocotyledonous as *T. aestivum* are, generally, more sensitive to UV radiation, when regards to morphology endpoints, than dicotyledonous as *B. rapa* (Barnes et al., 1990). This was observed in our study, where visible foliar changes occurred in *T. aestivum* at lower UV radiation (14.2 kJm^2d^{-1}) and the more severe visible effects, such as leaf necrosis, were found at the highest dose of UV radiation (28.4 kJm^2d^{-1}). In the case of *B. rapa* the lowest UV radiation used to have no effect on leaf morphology, and no necrosis was found in a single UV radiation exposure. The foliar changes for this species occurred from 21.3 kJm^2d^{-1} onwards.

The changes in leaf color that occurred in both species have been reported also for other species exposed to UV radiation, like a visible foliar bronzing of irradiated pea leaves (Strid and Porra, 1992) or the appearance of black leaf symptoms in grape *Vitis vinifera* L. (Lang et al., 2000). The leaf curling, found in the present study for *B. rapa* exposed to high UV radiation, was also reported in other Brassicacea species treated with UV-B as *Arabidopsis thaliana* (Boeger and Poulson, 2006) and *Brassica napus* L. (Wilson and Greenberg, 1993), where it was suggested an association with the degradation of auxin in the adaxial epidermis. On the other hand, Zlatev et al. (2012) suggested that this symptom can be a result of a photomorphogenic response that helps diminish the leaf area exposed to UV radiation. The shorter stem and smaller leaves that occurred in both species exposed to UV radiation were also reported as defense mechanisms developed in plants to protect them against UV radiation (Bornman and Vogelmann, 1991; Laakso and Huttunen, 1998; Stratmann, 2003). These morphological changes can result in a decrease in photosynthesis (Barnes et al., 1990; 1996), which also affects plant growth.

4.4.2 Combined exposure

UV radiation is responsible for most of the pesticide photolysis (Crosby, 1969), and carbaryl has a high absorbance within the UV- B range (300 nm) (Addison et al., 1974; Zaga et al., 1998), which facilitates its degradation. Our data showed a higher degradation of carbaryl (25%) when soil was irradiated with UV light, while in the non-exposed soil only almost 5% was lost. Therefore, the increase on toxicity found in the UV irradiated trial can be seen in light of the effects of UV combined with carbaryl or with its degradation products. Although carbaryl metabolites are considered generally less toxic than the original compound (WHO, 1993) the main decomposition product 1-naphthol is the exception, being considered more toxic than carbaryl (Blaustein et al., 2003; CCME, 2009). This was highlighted by Crosby et al. (1965) where it was reported modifications on carbaryl molecule by UV radiation, increasing the formation of 1-naphtol, and therefore inducing higher toxicity than the original compound.

There have been some reports on the increase of carbaryl toxicity when combined with temperature to the earthworm *Eisenia andrei*, the plant *Brassica rapa* (Lima et al., 2014) and to the Hemiptera *Diaphorina citri* (Boina, 2009). In addition, when combined with UV radiation Zaga et al. (1998) suggested that carbaryl was photoactivated by UV-B, causing synergistic effects in anuran embryos of *Xenopus laevis* when carbaryl was irradiated, by inducing higher mortality. The only study reporting the combined effect of carbaryl and UV radiation for soil organisms showed that a short exposure of the Collembola *Folsomia candida* to these stressors, caused an increase in reproduction and decrease in hatch success (Cardoso et al., 2014).

In the present study, the combination of carbaryl and UV radiation showed that the response depends on the species studied and the endpoint analyzed, and this has already been reported by other studies using the combined effects of chemicals and abiotic stressors (Holmstrup, 2010; Lima et al., 2011; 2014). Synergistic patterns were observed for *B. rapa* length and dry weight when UV radiation was the dominant stressor and carbaryl was present at low concentrations. Decreasing in the shoot height and synergism was also reported by others authors when UV radiation was combined with metals exposure in spruce seedlings of *Picea abies* (Dubé and Bornman, 1992) and in the soybean *Glycine max* L. (Chen et al., 2003).

Analyzing weight parameters, *T. aestivum* showed synergism at more relevant scenarios, where low concentrations of carbaryl are combined with low UV intensities.

Other studies have reported the synergistic effect of UV radiation and metals on biomass production and accumulation in soybean (*Glycine max* L) (Chen et al., 2003), *Triticum aestivum* seedlings (Shukla et al., 2002) and in the spinach *Spinacia oleracea* L (Mishra and Agrawal, 2006).

In foliar morphology an effect higher than the predicted based on each single stressor was observed. Yellow patches on leaves and necrotic regions found only at the highest dose of UV radiation for *T. aestivum* (single exposure), were also found at lower UV intensities when combined with carbaryl, being then intensified at higher UV levels reaching necrosis. Loss of stiffness of *B. rapa* leaves were found in combined exposure and it can be related to decrease on fresh weight and water content in this species. The mortality of *B. rapa* seedlings found at high doses of UV radiation and high concentration of carbaryl (from 125 mg/kg onwards) showed the high sensitivity of these species to combined exposure and it can be related to the synergism output for plant fresh weight. The increase of carbaryl toxicity to these plants can be due to changes in membrane permeability of plants exposed to UV radiation, facilitating the uptake of carbaryl. This has been reported previously by Agrawal and Mishra (2009) when *Pisum sativum* was exposed to Cd after UV-B irradiation.

As a conclusion, this study highlights the importance of studying the combined effects of UV radiation and pesticides to crop species, as most of the studies are carried under controlled conditions disregarding UV irradiation. Important results were achieved, considering that interactions between both stressors inside plants occurred, with synergism showing up as the main pattern related to UV irradiation.

Acknowledgments

This study was supported by a Ph.D. grant (SFRH / BD / 65274 / 2009) attributed to Maria Lima by the Portuguese Science and Technology Foundation (FCT) and through the research project CLIMAFUN (FCOMP-01-0124-FEDER-008656), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade. AMVMS is "Bolsista CAPES/BRASIL", Project NºA058/2013.

4.5 Supplementary Data

Table. S 4.1 - Comparative summary of the biologically effective UV radiation doses using different biological spectral weighting functions (BSWFs) commonly used for terrestrial plants in each time of exposure used in the present work.

	Authors	**Wavelength range (nm)**	**4h exposure**	**6h exposure**	**8h exposure**
Unweighted	—	280-400	65 kJm^2 d^{-1}	97.5 kJm^2 d^{-1}	130 kJm^{2}d^{-1}
Spectrum induced erythema in human skin	(McKinlay and Diffey, 1987)	280-400	8.5 kJm^2 d^{-1}	12.8 kJm^2 d^{-1}	97.5 kJm^2 d^{-1}
Generalized plant action spectrum	(Caldwell, 1971)	286-313	3.1 kJm2 d-1	4.7 kJm^2 d^{-1}	6.2 kJm^2 d^{-1}
The plant growth Action spectrum *	(Flint and Caldwell, 2003)	286-390	14.2 kJm^2 d^{-1}	21.3 kJm^2 d^{-1}	28.4 kJm^2 d^{-1}

*BSWF used in the present work

References

Addison, J.B., Silk, P.J., Unger, I., 1974. The photochemical reactions of carbamates II. The solution photochemistry of matacil (4-dimethyl-amino-m-tolyl-N-methyl carbamate) and Landrin (3, 4, 5-trimethylphenyl-N-methyl carbamate). Bulletin of environmental contamination and toxicology, 11, 250-255.

Agrawal, S.B., Mishra, S., 2009 Effects of supplemental ultraviolet-B and cadmium on growth, antioxidants and yield of *Pisum sativum* L. Ecotoxicology and Environmental Safety, 72, 610-618.

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell & Environment, 24, 1337-1344.

Ballaré, C.L., Rousseaux, C.M. Searles, P.S. Zaller, J.G. Giordano, C.V. Robson, M.T. Caldwell, M.M. Sala, O.E., Scopel, A.L., 2001. Impacts of solar ultraviolet-B radiation on terrestrial ecosystems of Tierra del Fuego (Southern Argentina): An overview of recent progress. Journal of Photochemistry and Photobiology B: Biology 62, 67-77.

Barnes, P.W., Flint, S.D., Caldwell, M.M., 1990. Morphological responses of crop and weed species of different growth forms to ultraviolet-B radiation. American Journal of Botany 77, 1354-1360.

Barnes, P.W., Ballaré, C.L., Caldwell, M.M., 1996. Photomorphogenic effects of UV-B radiation on plants: consequences for light competition. Journal of Plant Physiology 148, 15-20.

Biggs, R.H., Kossuth, S.V., Teramura, A.H., 1981. Response of 19 cultivars of soybeans to ultraviolet-B irradiance. Physiologia Plantarum 53,19-26.

Blaustein, A.R., Romansic, J.M. Kiesecker, J.M., Hatch, A.C., 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity and distributions 9,123-140.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Annals of Applied Biology 26, 585-615.

Boeger, M.R.T., Poulson, M.E., 2006. Efeitos da radiação ultravioleta-B sobre a morfologia foliar de *Arabidopsis thaliana* (L.) Heynh.(Brassicaceae). Acta Botanica Brasilica 20, 329-338.

Boina, D.R., Onagbola, E.O; Salyani, M. Stelinski, L. L., 2009. Influence of Posttreatment Temperature on the Toxicity of Insecticides Against *Diaphorina citri* (Hemiptera: Psyllidae). Journal of Economic Entomology 102,:685-691.

Booij-James, I.S., Dube, S.K., Jansen, M.A.K., Edelman, M., & Mattoo, A.K., 2000. Ultraviolet-B Radiation Impacts Light-Mediated Turnover of the Photosystem II Reaction Center Heterodimer in *Arabidopsis Mutants* Altered in Phenolic Metabolism.Plant Physiology,124: 1275–1284.

Bornman, J.F., Vogelmann, T.C., 1991. Effect of UV-B radiation on leaf optical properties measured with fibre optics. Journal of Experimental Botany 42, 547-554.

Branch, R.A., Jacqz, E., 1986. Is carbaryl as safe as its reputation? Does it have a potential for causing chronic neurotoxicity in humans? American Journal of Medicine 80, 659-64.

Caldwell, M.M., 1971. Solar UV irradiation and the growth and development of higher plants. p. 131-177, In A. Giese, ed. Photophysiology, Vol. 6. Academic Press, NEw York.

Caldwell, M.M., Bornman, J.F., Ballare, C.L., Flint, S.D., Kulandaivelu, G., 2007. Terrestrial ecosystems, increased solar ultraviolet radiation, and interactions with other climate change factors. Photocheistry and Photobiology Science 6, 252-66.

Cardoso, D.N., Bastos, A., Soares, A.V.M., Loureiro, S., 2014. Short-term exposure to carbaryl and UV radiation increases the reproduction output of the collembolan *Folsomia candida.* Journal of Soils and Sediments 14, 1-9.

CCME., 2009. Canadian Water Quality Guidelines: Carbaryl. Scientific Criteria Document. Canadian Council of Ministers of the Environment, Winnipeg (ed.) 2003.

Chen, T., Qiang, W., An, L., Wang, X., 2003. Effects of cadmium combined with ultraviolet-B radiation on growth, gas exchange, and stable carbon isotope value (δ13C) in soybean (*Glycine max* L.). Proc. SPIE 4896, Ultraviolet Ground- and Space-based Measurements, Models, and Effects II, 210 (June 26, 2003); doi: 10.1117/12.466165.

Correia C.M.,Coutinho, J. F; Bacelar, E. A; Gonçalves, B. M; Björn, L. O; Pereira, J. M., 2012. Ultraviolet-B Radiation and Nitrogen Affect Nutrient Concentrations and the Amount of Nutrients Acquired by Above-Ground Organs of Maize. The Scientific World Journal 2012, 1-11.

Crosby, D., 1969. Experimental approaches to pesticide photodecomposition. In: Residues of Pesticides and Other Foreign Chemicals in Foods and Feeds /Rückstände von Pesticiden und anderen Fremdstoffen in Nahrungs- und Futtermitteln (Ed F. Gunther), Springer, New York.

Crosby, D.G., Leitis, E., Winterlin, W.L., 1965. Carbamate Insecticides, Photodecomposition of Carbamate Insecticides. Journal of Agricultural and Food Chemistry 13, 204-207.

Dai, Q., Shaobing, P., Chavez, A.Q., Vergara, B.S., 1994. Intraspecific responses of 188 rice cultivars to enhanced UVB radiation. Environmental and Experimental Botany 34, 433-442.

Diffey, B.L., 1991. Solar ultraviolet radiation effects on biological systems. Physics in Medicine and Biology 36, 299-328.

Dubé, S., Bornman, J., 1992. Response of spruce seedlings to simultaneous exposure to ultraviolet-B radiation and cadmium. Plant Physiology and. Biochemical 30, 761-767.

Fagerberg, W.R., 2007. Below-ambient levels of UV induce chloroplast structural change and alter starch metabolism. Protoplasma 230, 51-59.

Feng, H., Li, S., Xue, L., An, L., Wang X., 2007, The interactive effects of enhanced UV-B radiation and soil drought on spring wheat. South African Journal of Botany, 73, 429-434.

Ferreira, A.G., Serra, P., Soares ,A.V.M., Loureiro, S. 2008. The influence of natural stressors on the toxicity of nickel to *Daphnia magna*. Environmental Science and Pollution Research 17, 1217-1229.

Flint, S.D., Caldwell, M.M., 1996. Scaling Plant Ultraviolet Spectral Responses from Laboratory Action Spectra to Field Spectral Weighting Factors. Journal of Plant Physiology, 148, 107-114.

Flint S.D., Caldwell M.M., 2003. A biological spectral weighting function for ozone depletion research with higher plants. Physiologia Plantarum 117, 137-144.

Frohnmeyer, H., Staiger D., 2003. Ultraviolet-B Radiation-Mediated Responses in Plants. Balancing Damage and Protection. Plant Physiology 133, 1420-1428.

Holmstrup, M., Bindesbol, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler H.R., Loureiro S., Soares A.M., Ferreira, A.L., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Science of The Total Environment 408, 3746-3762

Hopkins, L., Bond, M.A., Tobin, A.K., 2002 Ultraviolet-B radiation reduces the rates of cell division and elongation in the primary leaf of wheat (*Triticum aestivum* L. cv Maris Huntsman). Plant, Cell & Environment 25, 617-624.

ISO, 1995 Soil quality—Determination of the effects of pollutants on soil flora—Part 2: Effects of chemicals on the emergence of Toxicity Assessment of Soils from Portuguese Mines Using Plants 189 higher plants. ISO—The International Organization for Standardization. Genève, *ISO* 11269.

Jaakola, L., Hohtola, A., 2010. Effect of latitude on flavonoid biosynthesis in plants. Plant, Cell & Environment 33, 1239-1247.

Jana, T.K., Das, B., 1997. Sorption of carbaryl (1-napthyl N-methyl carbamate) by soil. Bulletin of Environmental Contamination and Toxicology 59, 65-71.

Jones, K.M., Koen, T.B., Bound, S.A., Oakford, M.J., Pettenon, S., Rudge, T., 1991. Effects of paclobutrazol and carbaryl on the yield of Hi-early red ‘Delicious’ apples. Journal of Horticultural Science 66, 159-163.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environmental Toxicology and Chemistry 24, 2701-2713.

Jordan, B.R., 1996. The effects of ultraviolet-B radiation on plants: a molecular perspective. Advances in botanical research 22, 97-162.

Kataria, S., Guruprasad, K.N., 2012. Solar UV-B and UV-A/B exclusion effects on intraspecific variations in crop growth and yield of wheat varieties. Field Crops Research 125, 8-13.

Laakso, K., Huttunen, S. 1998. Effects of the ultraviolet-B radiation (UV-B) on conifers: a review. Environmental Pollution 99, 319-328.

Lang, N.S., Mills, L., Wample, R.L., Silbernagel, J., Perry, E.M., Smithyman, R., 2000. Remote image and leaf reflectance analysis to evaluate the impact of environmental stress on grape canopy metabolism. Hort Technology 10, 468-474.

Li, Y., Yanqun, Z., Haiyan, C., Jianjun, C., Jilong, Y., Zhide, H., 2000. Intraspecific responses in crop growth and yield of 20 wheat cultivars to enhanced ultraviolet-B radiation under field conditions. Field Crops Research 67, 25-33.

Lima M.P.R., Soares A.M.V.M., Loureiro S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios. Environmental Pollution 159, 1844-1851.

Lima, M.P., Cardoso, D.N., Soares, A.M.V.M., Loureiro, S., 2014. Carbaryl toxicity prediction to soil organisms under high and low temperature regimes. Ecotoxicology and Environmental Safety."*in press*" DOI:10.1016/j.ecoenv.2014.04.004.

Loureiro, S., Svendsen, C., Ferreira, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to *Daphnia magna*: Comparing chemical modes of action and deviations from conceptual models. Environmental Toxicology and Chemistry 29, 1716-1726.

Lv, Z., Zhang, X., Liu, L., Guo, Y., Fan, Y., Yang, X., Li, Y., Zhang, W., 2013. Comparing intraspecific responses of 12 winter wheat cultivars to different doses of ultraviolet-B radiation. J Photochemistry and Photobiology B, 119, 1-8.

Mazza, C.A., Boccalandro, H.N.E., Giordano, C.V., Battista, D., Scopel, A.L., Ballaré, C.L., 2000. Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field-grown soybean crops. Plant Physiology 122, 117-126.

McKinlay, A.F., Diffey, B.L., 1987. A reference action spectrum for ultra-violet induced erythema in human skin. In: Human Exposure to Ultraviolet Radiation: Risks and Regulations. (Ed B.F.M.B. W. F. Passchier), Elsevier, Amsterdan.

Misra, R.B., Lal, K., Farooq M., Hans R.K., 2005. Effect of solar UV radiation on earthworm (*Metaphire posthuma*). Ecotoxicology and Environmental Safety 62, 391-396.

Mishra, S., Agrawal, S.B., 2006. Interactive effects between supplemental ultraviolet-B radiation and heavy metals on the growth and biochemical characteristics of *Spinacia oleracea* L. Brazilian Journal of Plant Physiology 18, 307-314.

Murthy, N.B.K., Raghu, K., 1990. Effect of carbaryl and 1-naphthol on seedling growth of barley, and on growth and nodulation of groundnut in two soils. Proceedings of the Indian Academy of Sciences, Plant Sciences 100, 11-16.

Qaderi, M.M., Reid, D.M., 2005. Growth and physiological responses of canola (*Brassica napus*) to UV-B and CO2 under controlled environment conditions. Physiologia Plantarum 125, 247-259.

Qaderi, M.M., Reid, D.M., Yeung, E.C., 2007. Morphological and physiological responses of canola (*Brassica napus*) siliquas and seeds to UVB and CO2 under controlled environment conditions. Environmental and Experimental Botany 60, 428-437.

Ribeiro, F., Ferreira, N.C., Ferreira, A., Soares, A.M.V.M., Loureiro, S., 2011. Is ultraviolet radiation a synergistic stressor in combined exposures? The case study of *Daphnia magna* exposure to UV and carbendazim. Aquatic Toxicology 102, 114-122.

Rousseaux, M.C., Ballaré, C.L., Giordano, C.V., Scopel, A.L., Zima, A.M., Szwarcberg-Bracchitta M., Searles, P.S., Caldwell, M.M., Diaz S.B., 1999. Ozone depletion and UVB radiation: Impact on plant DNA damage in southern South America. Proceedings of the National Academy of Sciences 96, 15310-15315.

Rozema, J., van de Staaij, J., Björn, L.O., Caldwell, M., 1997. UV-B as an environmental factor in plant life: stress and regulation. Trends in Ecology & Evolution 12, 22-28.

Saile-Mark M, Tevini M (1997) Effects of solar UV-B radiation on growth, flowering and yield of central and southern European bush bean cultivars (*Phaseolus vulgaris* L.). Plant Ecol. 128:115-125.

Shahbaz, M., Parmar, S., Stuiver, C.E.E., Hawkesford, M.J., De Kok, L.J., 2012. Copper toxicity and sulfur metabolism in Chinese cabbage are affected by UV radiation. Environmental and Experimental Botany 88, 60-70.

Shukla, U.C., Joshi, P.C., Kakkar, P., 2002. Synergistic Action of Ultraviolet-B Radiation and Cadmium on the Growth of Wheat Seedlings. Ecotoxicology and Environmental Safety 51, 90-96.

Shweta, M., Agrawal, S.B., 2006. Interactive effects between supplemental ultraviolet-B radiation and heavy metals on the growth and biochemical characteristics of Spinacia oleracea L. Brazilian Journal of Plant Physiology 18, 307-314.

Singh,M., Singh, S., Agrawal, S.B., 2012. Intraspecific responses of six cultivars of wheat (*Triticum aestivum* L.) to supplemental ultraviolet-B radiation under field conditions. Acta Physiologiae Plantarum 34, 65-74.

Smith, J.L., Burritt, D.J., Bannister, P., 2000. Shoot Dry Weight, Chlorophyll and UV-B-absorbing Compounds as Indicators of a Plants Sensitivity to UV-B Radiation. Annals of Botany 86, 1057-1063.

Srivastava, G., Kumar, S., Dubey, G., Mishra, V., Prasad, S.M., 2012. Nickel and ultraviolet-B stresses induce differential growth and photosynthetic responses in *Pisum sativum* L. seedlings. Biological Trace Element Research 149, 86-96.

Stapleton, A.E., 1992. Ultraviolet radiation and plants: burning questions. The Plant Cell 4, 1353.

Stratmann, J., 2003. Ultraviolet-B radiation co-opts defense signaling pathways. Trends in plant science 8, 526-533.

Strid, Â., 1993. Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet-B radiation. Plant and Cell Physiology 34, 949-953.

Strid, Â., Porra, R.J., 1992. Alterations in Pigment Content in Leaves of *Pisum sativum* After Exposure to Supplementary UV-B. Plant and Cell Physiology 33, 1015-1023.

Systat., 2006. SystatSoftware, Incorporation. In: Sigma Plot for Windows

Teramura, A.H., 1983. Effects of ultraviolet-B radiation on the growth and yield of crop plants. Physiologia Plantarum 58, 415-427.

Tevini, M., Iwanzik, W., Thoma, U., 1981. Some effects of enhanced UV-B irradiation on the growth and composition of plants. Planta 153, 388-394.

Tevini, M., Teramura, A.H., 1989. UV-B effects on terrestrial plants. Photochemistry and Photobiology 50, 479-487.

Tian, X.R., Lei, Y.B., 2007. Physiological responses of wheat seedlings to drought and UV-B radiation. Effect of exogenous sodium nitroprusside application. Russian journal of plant physiology 54, 676-682.

Van, T.K., Garrard, L.A., West, S.H., 1976. Effects of UV-B radiation on net photosynthesis of some crop plants. Crop Science 16, 715-718.

Visser, A.J., Tosserams, M., Groen, M.W., Kalis, G., Kwant, R., Magendans, G.W.H., Rozema, J., 1997. The combined effects of CO2 concentration and enhanced UV-B radiation on faba bean. 3. Leaf optical properties, pigments, stomatal index and epidermal cell density. In: UV-B and Biosphere, Springer Netherlands.

WHO-World Health Organization., 1993. In: Environmental Health Criteria., pp. 40. International Program on Chemical Safety, Geneva.

Wilson, M.I., Greenberg, B.M., 1993. Protection of the D1 photosystem II reaction center protein from degradation in ultraviolet radiation following adaptation of *Brassica napus* L. to growth in ultraviolet-B. Photochemistry and Photobiology 57, 556-563.

Yao, X., Jianzhou, C., Xueli, H., Binbin, L., Jingmin, L., Zhaowei, Y., 2013. Effects of selenium on agronomical characters of winter wheat exposed to enhanced ultraviolet-B. Ecotoxicology and Environmental Safety 92, 320-326.

Yuan, L., Yanqun, Z., Haiyan C., Jianjun, C., Jilong, Y., Zhide, H., 2000. Intraspecific responses in crop growth and yield of 20 wheat cultivars to enhanced ultraviolet-B radiation under field conditions. Field Crops Research 67, 25-33.

Yuan, L.Y., M.; Wang, X., 1998. Effects of enhanced ultraviolet-B radiation on crop structure, growth and yield components of spring wheat under field conditions. Field Crops Research 57, 253-263.

Zaga, A., Little, E.E., Rabeni, C.F., Ellersieck, M.R., 1998. Photo enhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. Environmental Toxicology and Chemistry 17, 2543-2553.

Zaller, J.G., Caldwell, M.M., D. Flint, S., Scopel, A.L., Salo, O.E., Ballaré, C.L., 2002. Solar UV-B radiation affects below-ground parameters in a fen ecosystem in Tierra del Fuego, Argentina: implications of stratospheric ozone depletion. Global Change Biology 8, 867-871.

Zlatev, Z.S., Lidon F.J.C., Kaimakanova, M., 2012. Plant physiological responses to UV-B radiation. Emirates Journal of Food and Agriculture, 24.

Zuk-Golaszewska, K., Upadhyaya, M.K., Golaszewski, J., 2003. The effect of UV-B radiation on plant growth and development. Plant Soil And Environment 49, 135-140.

CHAPTER 5

Drought and flood conditions in soils affect the responses of crop plants to ultraviolet radiation.

Chapter 5. Drought and flood conditions in soils affect the responses of crop plants to ultraviolet radiation

Abstract

The effect of different doses of ultraviolet radiation (UVR), combined with two watering regimes (drought and flood) on two crop species (*T. aestivum* and *B. rapa*) was investigated. Seedlings immediately after emergence were exposed to single and combined stressors. In single exposure UVR caused significant decrease on the growth of both plant species, although *T. aestivum* was more tolerant than *B. rapa* to UVR effects. Drought and flood significantly affected growth and biomass production for both species. A significant interaction between UVR and soil moisture was found and the two plant species responded differently to combined exposure. The drought stress alleviated the deleterious effect of UVR on *T. aestivum* length and biomass production and the flood stress increased its effects. For *B. rapa,* both water stresses caused an increase of the deleterious effects of UVR for all endpoints evaluated, showing potential synergistic interaction in all combined exposure.

Key words: *Brassica rapa*, *Triticum aestivum,* Ultraviolet radiation, Drought, Flood, Soil moisture.

5.1 Introduction

Climate change is a result of human activities over the decades, such as forest destruction, burning of fossil and the release of chlorofluorocarbon, that have led to atmospheric increases in CO_2, and accumulation of greenhouse gases (Molina and Rowland, 1974; Worrest, 1989). The amounts of these gases induce or increment the depletion of the ozone layer and consequent incoming of solar radiation to reach the Earth's surface, producing global warming.

In addition to warming effect and also as a consequence of climate change significant changes in precipitation patterns are expected (Caldwell et al., 2007), causing annual variations and local changes in precipitation, exposing plants to flood scenarios (Walther, 2002). Beyond that, extreme drought events are projected to increase in future also due the climatic change (Tao et al., 2003).

So, in field, several environmental stressors, sequentially or simultaneously, may affect plant development, which will lead to socio-economic crucial issues. Factors as temperature, water deficit and increased of UV radiation reaching the Earth surface are important factors limiting productivity of crops in many regions (Caldwell et al., 2007; Farooq et al., 2009).

Some studies have shown that natural stressors as soil moisture (Balakumar, 1993; Mark and Tevini, 1996), temperature (Takeuchi et al., 1993) and nutrients (Murali and Teramura, 1985) can also decrease or increase the negative effect of UV radiation on plants and alter the effect of the individual stress response.

It has been predicted that UV radiation affects plants by many pathways, reducing plant growth , reproduction (Caldwell, 1989; Tevini and Teramura, 1989), primary productivity (Bornman and Teramura, 1993; de Almeida, 2012) photosynthesis (Tevini and Teramura, 1989), by causing foliar changes as reduced leaf area (Caldwell et al., 2007) and increased epidermal thickness (Ren et al., 2007). Also, reduction in plant's dry weight is often a reliable indication of a plant's sensitivity to UVR (Smith et al., 2000).

Drought has affected agricultural production in some regions (Balouchi, 2009), impairing plant development, growth, emergence and weight (Lima et al., 2011). It causes stomatal closure, which will lead to a reduction on the CO2 diffusion into leaves

(Li et al., 2004), limiting the photosynthesis and consequently reducing cell division, leaf area and biomass (Nogués and Baker, 2000; Tsukaya, 2003). It is also known that roots exposed to flood stress may suffer hypoxia, causing morphological changes and mechanisms of anaerobic metabolism (Liao and Lin, 1995), reduction in plant growth and development (Lima et al., 2011).

Some studies have reported that UV radiation combined with drought stress have synergistic effects on plants (Feng et al., 2007), causing oxidative burst (Monk et al., 1989), reducing plant growth, biomass and water content (Bandurska et al., 2012; Feng et al., 2007; Sullivan and Teramura, 1990). Moreover, it has also been reported that natural plant adaptation to drought can provide tolerance to UVR due to induction of protective mechanisms (Alexieva, 2001; Balakumar, 1993), but the effects of flood stress combined with UV radiation on plants are still unknown.

Since crop plants are very responsive to these two important climate factors, the present study pretends to infer whether drought or flood stress can influence the sensitivity of plants to ultraviolet radiation, in order to provide a better analysis of environmental risk in the face of climate changes but also regarding seasonality and climate conditions in different countries worldwide.

5.2. Material and Methods

5.2.1 Plants and growth conditions

For both single and combined exposure, the seeds of *Brassica rapa* (turnip) were purchased from Carolina Biological Supply Company (US) and the caryopses of *Triticum aestivum* (wheat) were obtained from an agricultural store in Aveiro, Portugal. The methodology for all bioassays was adapted from the ISO 11269-2 guideline (ISO 1995).

The seeds/caryopses were sown in natural soil Lufa 2.2, obtained by the German Institution LUFA Speyer, with a pH=5.5 ± 0.2 (0.01 M CaCl2), water holding capacity=41.8 ± 3.0 (g/100 g), organic C = 1.77 ± 0.2 (%), nitrogen=0.17 ± 0.02, texture=7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. The experimental design was constituted of four replicates per treatment, being each replicate a pot filled with 500g of soil and ten caryopses/seeds. The initial soil moisture was adjusted to 60% of Water Holding Capacity (WHC) and plants were grown in a laboratory room (20± 2^{o}C; 12000 lx; photoperiod of 16:8 light: dark).

5.2.2.Single exposure

UV radiation treatments

Seedlings, immediately after emergence, were irradiated using three different doses of UV radiation, converted to kilojoules per day of exposure : 14.2 kJ m^{-2} d^{-1} (corresponding to 4h of exposure); 21.3 kJ m^{-2} d^{-1} (6h) and 28.4 kJ m^{-2} d^{-1} (8h). These UVR doses were chosen based on previous results described in chapter 4. During the single UVR exposure the soil moisture was maintained at 60%WHC.

The UV radiation was provided by a UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 313 nm and 365nm corresponding UV-B and UV-A), that was placed in mobile adjustable metal frames suspended 30 cm above the top of the plants.

To eliminate the UV-C range wavelengths, that do not reach the earth's surface, a clear cellulose acetate sheet (0.003mm) was used; and was previously UV irradiated for 12 h before use in the experiments to minimize differences in intensity of UVR that crosses the sheets. The cellulose acetate films were regularly replaced to avoid aging effects on the spectral transmission of UVR. PAR light was also used simultaneously to UVR exposure and intensities were measured at the top of the plant with a spectroradiometer connected to a monochromator; the spectral radiance was obtained by the BenWin+ Software (Bentham Instruments, Reading, UK). To yield the biologically effective dose of UV radiation, the intensity values were converted into biologically effective UV radiance by using the plant growth action spectrum BSWF (Flint and Caldwell, 2003).

For all intensities, UV doses were calculated, according to the time of exposure at that intensity and the total biologically effective dose was calculated as follows:

$$UV_{BE}\,dose\ (Jm^{-2}) = \frac{I_{BE}\,(mWcm^{-2})\ X\ Time\ of\ \exp osure\,(s)}{1.000} \times 100$$

Where I_{BE} is the biologically effective UV irradiance.

For a better comparison between the effective doses used in this study (using the BSWC as weight function) and the doses used in others studies, Table S 4.1 (chapter 4) presents comparative values, calculated using different weight functions.

Drought and flood treatments

The seeds/caryopses were sown in a pot filled with 500g of natural soil Lufa 2.2 moistened in three irrigation regimes, simulating drought stressed (10%WHC), flood stressed (120%WHC) and regular optimum conditions (control, 60%WHC). To control soil moisture levels during the experiments, test vessels were weighted daily and water added whenever needed.

5.2.3 Exposure to combined treatments

This exposure followed the same pattern described above for the single exposure, with some adjustments: All seeds and caryopsis were introduced in test pots with a soil moisture of 60%WHC; immediately after emergence, soil moisture was adjusted to the pretended soil moisture regimes and seedlings were exposed simultaneously to different doses of UV radiation and water stress (10% WHC and 120% WHC). The joint effect of UV and soil moisture exposures was compared to responses from control plants (without UV and at 60%WHC). Soil pots were weighted daily and water added whenever needed.

5.2.4 Endpoints

Plants were harvested and the shoot length, fresh and dry weight recorded after 14 days of 50% of seeds had emerged in the control soil. The water content in plants (WC) was calculated using the following equation, where DW is the plant dry weight and FW the plant fresh weight.

$$WC = \frac{FW - DW}{FW} \times 100$$

Visible foliar changes were also recorded for all plants exposed as changes in leaf edges, color of leaves and stem stiffness. The results were initially expressed in percentage of seedlings affected by symptoms.

5.2.5 Statistical analysis

Data from single exposures were analyzed by a One-way ANOVA. The Dunnett's test was used to analyze differences between control and treatments. Whenever the normality test failed and data transformation did not correct for normality, non-parametric Kruskal–Wallis test was performed, followed by the Dunn's method when significant differences were found (Systat, 2006).

A two-way ANOVA was applied to assess the interaction between UV radiation and soil moisture. The median effective dose (ED50) was calculated using a non-linear regression, a sigmoidal logistic 3-parameter equation. All analyses were performed using the software package SigmaPlot 12.5 (Systat Software, Inc.).

Synergistic ratios (SRs) were calculated, when possible, by dividing the ED_{50} value for UV radiation at 60%WHC by the ED_{50} values for UV radiation at drought and flood stress. Synergistic ratios (SRs) of 1.0 indicated no effects of water stress on UV radiation sensibility, whereas values >1.0 and <1.0 indicated greater and smaller effects than expected, respectively.

5.3. Results

5.3.1 Single exposure

UV radiation

UVR caused a significant decrease on *T. aestivum* and *B. rapa* length (Figure 5.1E and G, respectively) from 14.2 kJ m^2 d^{-1} onwards (ANOVA, $p \leq 0.05$), the FW and DW of *T.aestivum* was affected just at 28.4 kJ m^2 d^{-1} (Figure 5.2B), while *B. rapa* FW and DW decreased from 21.3 and 14.2 kJ m^2 d^{-1} onwards, respectively (Figure 5.3B). But, the plants water content did not change in any doses used for *T. aestivum* (Figure 5.1F), whereas for *B. rapa* a significant increase was found at 14.2 kJ m^2 d^{-1} and at 28.4 kJ m^2 d^{-1}(Figure 5.1H).

Foliar changes were also reported by visual observation in both species: for *T. aestivum* from 14.2 kJ m^2 d^{-1} onwards and for *B. rapa* this changes were observed at 21.3 kJ m^2 d^{-1} onwards. These changes included: shorter stems, smaller leaves, serrated tips and curled down leaves. Chlorosis and necrosis of tip leaves were found at the highest dose (28.4 kJ m^2 d^{-1}) for both species.

Soil moisture

T. aestivum length decreased significantly (ANOVA, $p \leq 0.05$) in both water extreme regimes, but the decrease caused by drought was higher (62%) than the decrease caused by flood (18%). *B. rapa* length showed similar responses to both water stress, with a significant decrease of 21.3% at drought stress and 22.5% at flood stress when compared to control (ANOVA, $p \leq 0.05$).

The fresh weight of the two plant species decreased at both water regimes. Under drought stress the decreased was of 76.2% for *T. aestivum* and 51% for *B. rapa*; in flood stress simulation, the decreased was of 28.5% and 47.1%, respectively. Regarding the dry weight, different responses were found between species. *B. rapa* was not affected by water stress (ANOVA, $p > 0.05$), while *T. aestivum* was only affected by drought, with a significant decrease of 70.7% under these conditions (ANOVA, $p \leq 0.05$). The water content of both species decreased significantly (ANOVA, $p < 0.05$) in both water stress, however, the effect was higher in *B. rapa* (5.3% under drought and 9% under flood) than in *T. aestivum* (2.2% under drought and 2.93% under flood).

T. aestivum exposed to single drought or flood stress did not show any change in leaf morphology, whereas *B. rapa* showed a few brownish leaves (10% of exposed leaves) in drought stress and wilting leaves (8%) in flooding stress (data not shown).

5.3.2 Combined exposure

The effects of different levels of UVR to both plant species depended on the level of soil moisture for all endpoints. There was a statistically significant interaction between these stressors for *T. aestivum* length (Two Way Anova, $F_{6,316}=8.94$, $p<0.001$). When under optimal water conditions (60%WHC) a decrease on its length was observed (ANOVA, $p< 0.05$) with the increase of UVR (Figure 5.1E), with the highest dose of UVR causing a reduction on their length of 25%, compared to the control. When the flood stress was added, the sensitivity to UVR was maintained (ANOVA, $p < 0.05$) and a similar response to those observed under optimal water conditions at the highest UVR dose was found (reduction of 25.8% on its length) (Figure 5.1I). But the addition of drought stress caused greater tolerance of plants to UVR, because the increase of UVR did not cause significant effects in *T. aestivum* length (ANOVA, $p > 0.05$).

The length of *B. rapa* was also affected by the interaction of stressors (Two Way Anova, $F_{6,326}=47.21$, $p<0.001$), and in both water conditions the increase of UVR caused a decrease on Brassica's length (ANOVA, $p < 0.05$). Under optimal water conditions (60%WHC) the decreased caused by the highest UVR dose was 77.3% (Figure5.1G), similar to effects under flood conditions (74.2%), compared to controls (Figure 5.1K), whereas under drought conditions the reduction was of 27.1% (Figure 5.1C). But in flood conditions, the first dose UVR caused a greater reduction in length than that recorded under ideal conditions.

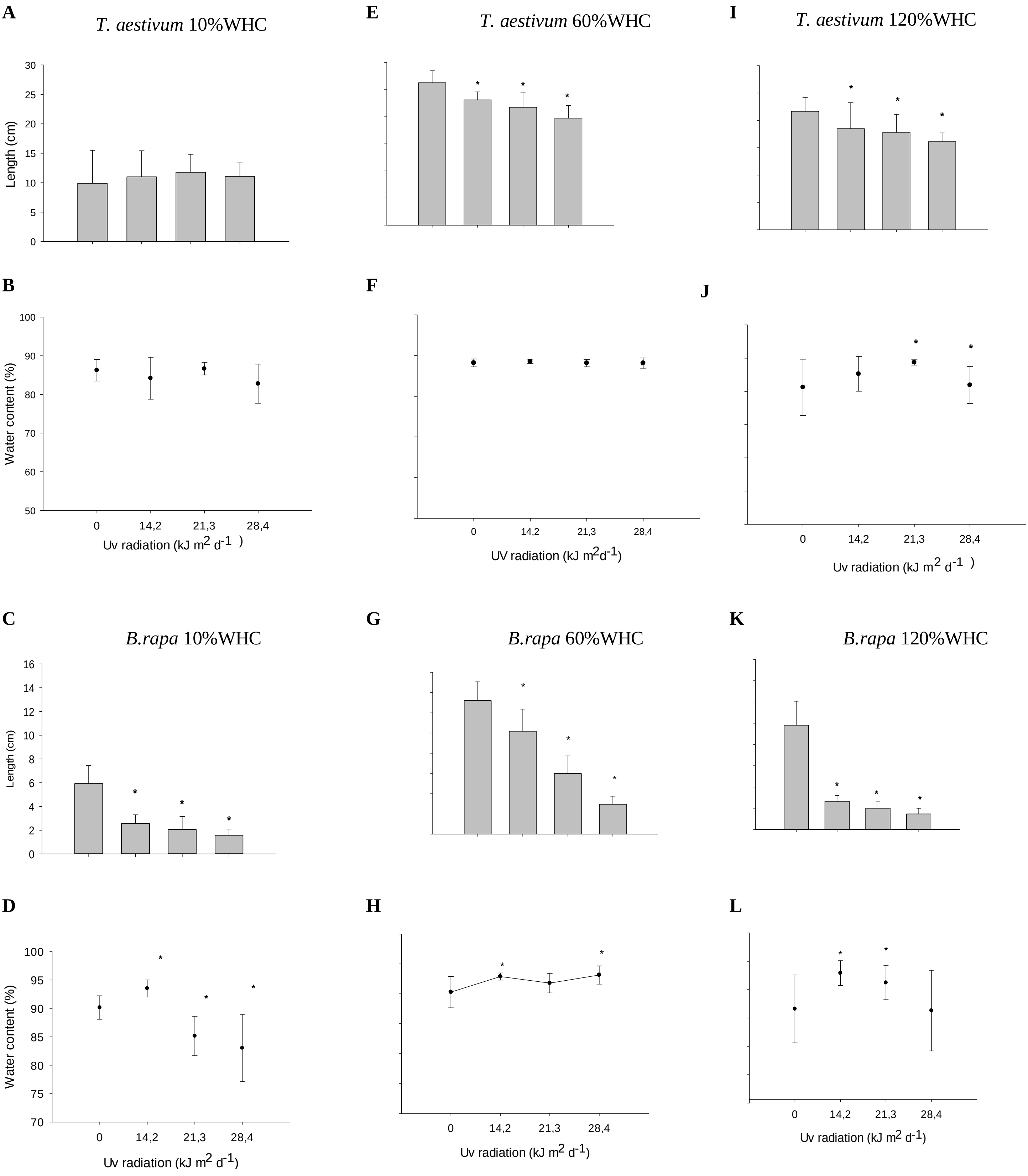


Fig. 5.1. Effects of UV radiation on *Triticum aestivum* shoot length (A, E, I) and water content (B, F, J) and *B.rapa* shoot length (C, G, K) and water content (D, H, I) subjet to different soil moisture regimes. Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test).

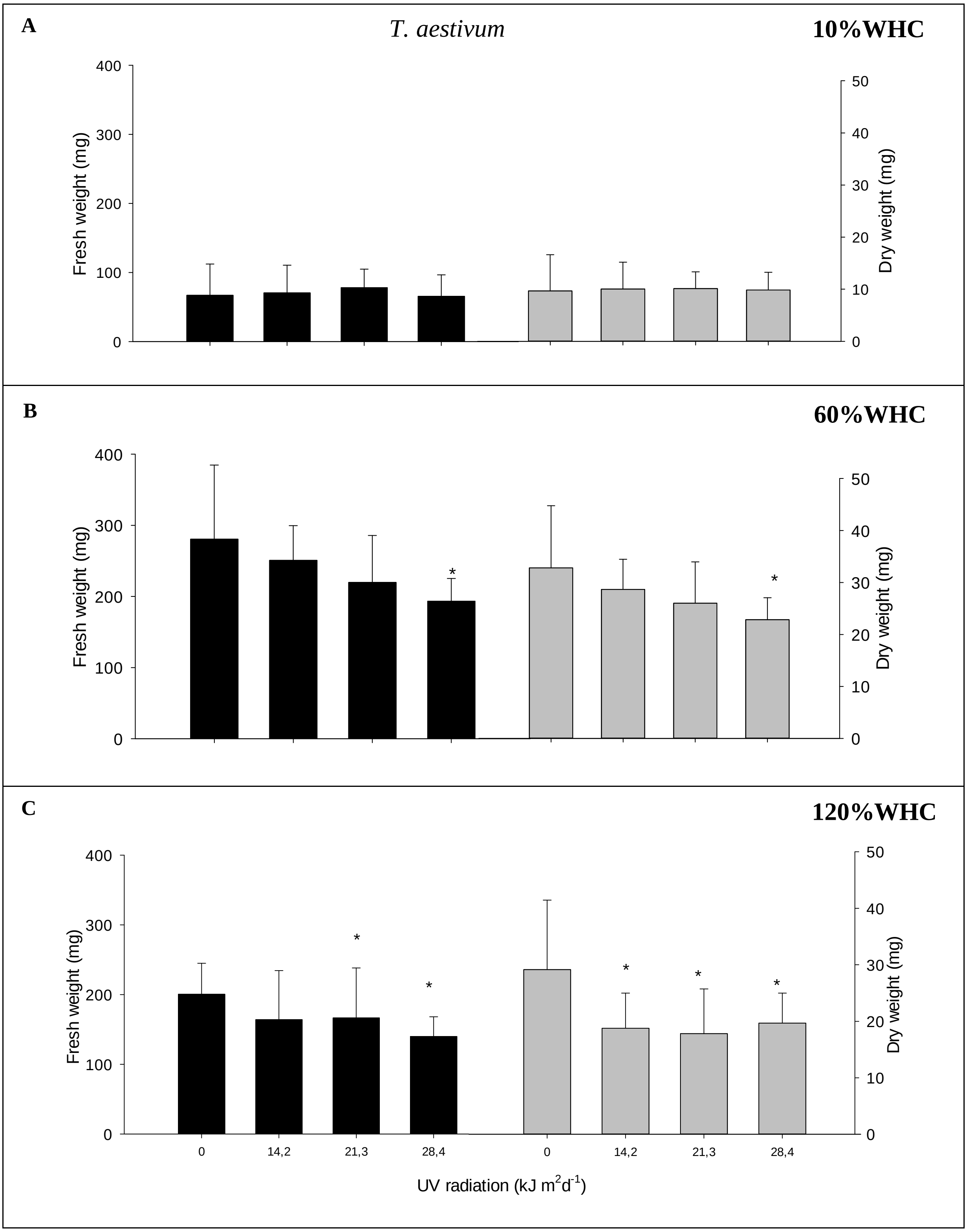


Fig. 5.2. Effects of UV radiation on *Triticum aestivum* fresh weight and dry weight in drought stress (A); control conditions (B) and flood stress (C). Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test).

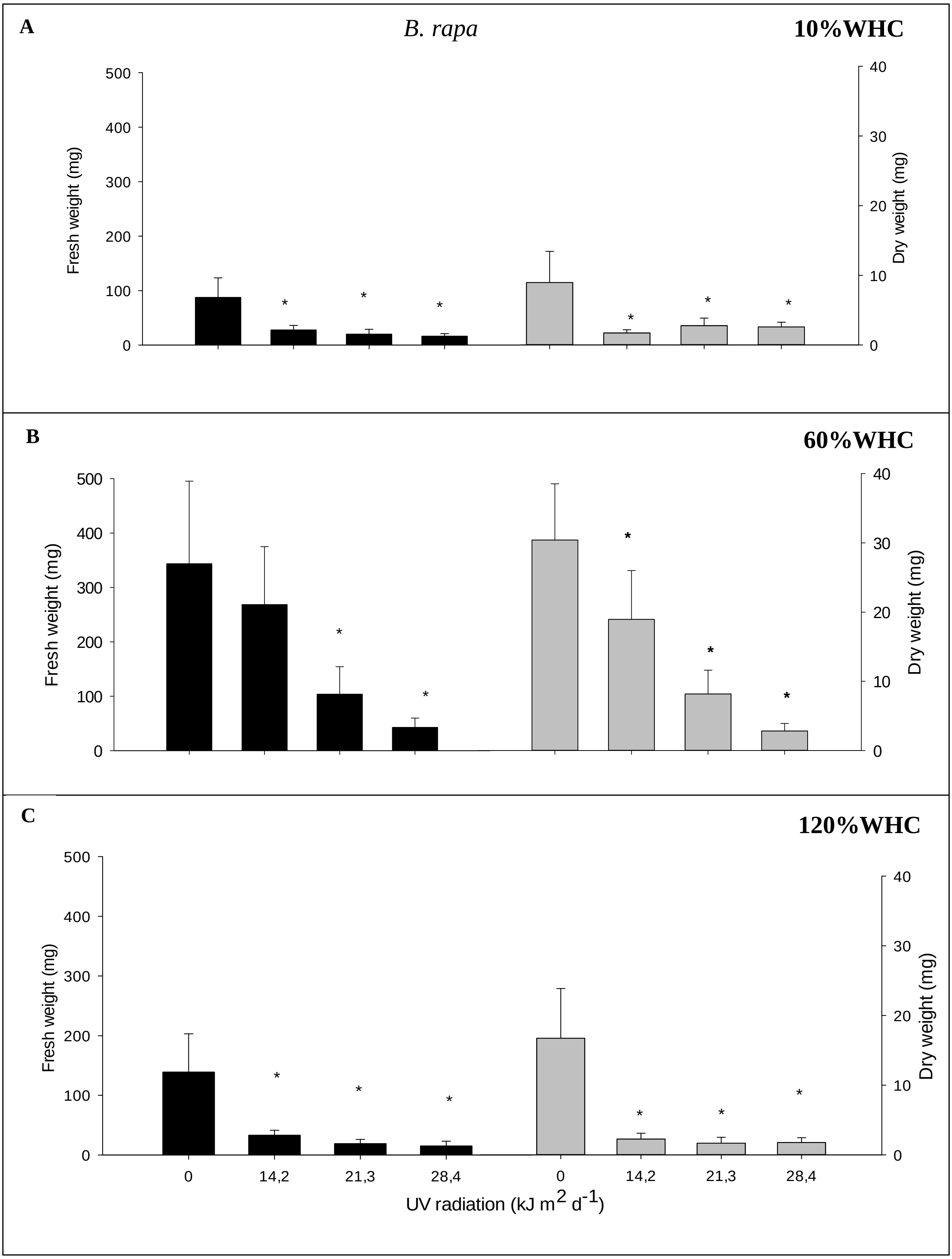


Fig. 5.3. Effects of UV radiation on *B. rapa* fresh weight and dry weight in drought stress (A); control conditions (B) and flood stress (C). Asterisks indicate significant difference from the control ($p<0.05$ – Dunnett's test).

The water content of *T. aestivum* and *B. rapa* was significantly affected by different levels of UVR and soil moisture (Two Way Anova, $F_{6,316}$=5.73, p<0.001 and $F_{6,326}$=16.7, p<0.001, respectively). Under optimal water conditions, UVR did not induce significant effects on the water content of *T.aestivum* (Figure 5.1F; ANOVA, p>0.05), under drought stress (Figure 5.1B), no significant changes were observed regarding the water content with a decrease of 2.4% at the lowest UVR dose and 3.8% at the highest dose of UVR, when compared to controls (ANOVA> 0.05), but when flood stress were added as a second stressor there was a change on its UVR sensitivity (Figure 5.1J), plants' water content slightly increased when exposed to the first two doses of ultraviolet radiation (2.3%; *P*>0.05 and 4.3%; *P*<0.05 respectively), compared to controls.

For *B. rapa*, under all water treatments, an increase of plant's water content, compared to the control, was found at the first UVR dose. Under optimal water conditions (Figure 5.1H) its increase (3%) remained in the highest UVR dose, while in the drought conditions (Figure 5.1D) the first UVR dose caused an increase of 3.7%, on its WC, followed by a significant decrease (5.5% and 7.9%) at 23.1 kJ m^2 d^{-1} and 28.4 kJ m^2 d^{-1}, respectively (ANOVA, p<0.05) and under flood conditions (Figure 5.1L), a similar increase of 3% in its WC was found in the first two UVR dose, compared to the control, but the following dose did not affect plants' water content (ANOVA, p>0.05),

The ED50 and SRs were summarized on table 5.1. Under optimal conditions of soil moisture and under flood stress the ED_{50} values for *T. aestivum* were higher than the highest dose of UVR for all endpoints, for drought stress the ED50 could not be calculated, as well as the SRs for all treatments as low levels of toxicity were observed. For *B. rapa*, under both water stress the ED_{50} values decreased for most endpoints, and a trend to synergism (SRs >1) was found in both water stress.

The interaction between UVR and drought caused foliar changes to both species from 14.2 kJ $m^{-2}d^{-1}$onwards. *B. rapa* showed curled down and wilted leaves and necrosis of tip leaves at 14.2 kJ $m^{-2}d^{-1}$ in 60% of exposed plants and at 21.3 kJ $m^{-2}d^{-1}$ onwards, dry and poorly developed leaves were observed in approximately 20% of exposed plants for both UV doses. *T. aestivum* showed foliar changes in all doses of UVR in approximately 30% of plants for each UVR treatment. These changes included withered and curled down leaves, serrated and burn tips and in some cases, the development only of the first leaf (data not shown).

The interaction between UVR and water excess also caused changes on the leaves of both species from the first UVR dose (14.2 kJ m^{-2}d^{-1}) onwards, but the frequency of occurrence was less than under drought stress. *B. rapa* showed only curled down leaves in 10% of plants in each UVR treatment. *T. aestivum* showed weak leaves, brownish and curled down in 10% of plants in each UVR treatment. Chlorosis or necrosis was not found in the combined exposure (data not shown).

Table 5.1. ED50 (SE) and Sinergistic Ratios (SRs) for the exposures of *Triticum aestivum* and *Brassica rapa* to UV radiation under different soil moisture.

Species	**Endpoint**	**Control (60% WHC)**	**Drought 10%WHC**		**Flood 120%WHC**	
		ED$_{50\ (SE)}$ (kJ/m^{2}d^{-1})	**ED$_{50\ (SE)}$ (kJ/m^{2}d^{-1})**	**SRs**	**ED$_{50\ (SE)}$ (kJ/m^{2}d^{1})**	**SRs**
Triticum aestivum	*Length*	>28.4	n.d	n.d	>28.4	n.d
	Fresh weight	>28.4	n.d	n.d	>28.4	n.d
	Dry weight	>28.4	n.d	n.d	>28.4	n.d
	Water Content	n.d	n.d	n.d	>28.4	n.d
Brassica rapa	*Length*	20.1 (0.54)	11.2 (2.06)	1.8	12.5 (1.6)	1.6
	Fresh weight	18.2 (0.86)	6.8 (3.3)	2.7	9.52 (1.3)	1.9
	Dry weight	16.2 (0.60)	n.d	n.d	1.47 (4.0)	11
	Water content	n.d	>28.4	n.d	>28.4	n.d

n.d= data not determined; SE= standard error

5.4 Discussion

5.4.1 Single exposure

The negative effects induced by UVR on growth and biomass production founded in present work for both plant species are consistent with our previous data (chapter 4) and with others for the same plant species (Ballare et al., 1996; Conner and Zangori, 1997; Lv et al., 2013), as well as for other crop species, like *Pisum sativum* (pea) (Nogues et al., 1998), Oryza sativa L. (rice) (Teramura et al., 1991), *Glycine max* L.(soybean) (Caldwell et al., 1994) and *Gossypium hirsutum* (cotton) (Coleman and Day, 2004).

The control of water content by *T. aestivum* under UVR treatments, which corroborate with our previous study (chapter 4), was also reported by Alexieva et al. (2001) for *T. aestivum* and *Pisum sativum*, and according to the authors, this water control may be related to induction by UVR of osmolytes or stress proteins. Another hypothesis is that the water regulation by *T. aestivum* may be related with stomata, since it is known that UVR causes changes in plant-water relations through the stomata (Nogues et al., 1998). The higher tolerance of *T. aestivum*, compared to *B. rapa*, for the UVR doses used in this work was also found in our previous study (chapter 4), where the UV sensitivity index (UV-SI) and biomass accumulation showed that *T. aestivum* was more tolerant to UV radiation than *B. rapa* for the same doses used in this work. This proves the reproducibility of results and reinforces the deleterious effects of UVR on crop species under ecological relevant doses.

The water stress is also harmful to plants, and the significant reduction on growth and biomass production reported here, mainly in drought conditions, corroborates with those reported in others studies (Chaves et al., 2002; Farooq et al., 2009; Lima et al., 2011; Mahajan and Tuteja, 2005). The most prominent response of plants to drought is stomatal closure and reduction of cell division and expansion (McCree and Fernandez, 1989), which result in reduced leaf area, root proliferation and biomass (Farooq et al., 2009; Liu et al., 2009). The response of plants to flood stress is related to the reduction of water absorptions due a high CO_2 concentration in the soil water, causing leaf dehydratation and stomatal closure. But these response vary with the type of plant and with duration of flooding (Kozlowski, 1984).

Our results showed that despite the two species respond similarly to water stress, and that *T. aestivum* is known as sensitive species to flood condition (Liao and Lin, 1995), the negative effects caused by flood stress were higher in *B. rapa*. These differences reinforce the discussed by Chaves et al. (2002) on plants' responses to water stress which the authors considered to be complex, and involving many factors, with tolerance varying among species and genotypes.

5.4.2 Combined exposure

The interactive effects between UVR and drought stress to plants was also reported by others authors for *T. aestivum* (Alexieva, 2001; Feng et al., 2007), *Pisum sativum* (pea) (Alexieva, 2001), *Glycine max* L. (soybean) and for *Hordeum vulgare* (barley) (Bandurska et al., 2012). But no study, so far, has shown potential effects of flood stress on UVR sensitivity.

Drought stress alleviated the detrimental effect of UVR to *T. aestivum* for most endpoints, but the same did not occur for *B. rapa*. These different growth responses between these species to combined exposure to UVR and drought was expected, since different results were found for this combined exposure in several crop species. Teramura et al. (1990) reported that the soybean cultivar (*Glycine max* L.) sensitive to UVR became more tolerant when subjected to drought stress, while a UVR tolerant cultivar became more sensitive to UVR, when subjected to drought conditions. Contrary to our findings, Feng et al. (2007) reported a decrease in *T. aestivum* growth and biomass by the combination of UVR and drought. On the other hand, no significant effect were found on the growth of Mediterranean olive (*Olea europea* L), rosemary (*Rosmarinus officinalis* L.) and lavender (*Lavandula stoechas* L.) under drought and UVR stress (Nogués and Baker, 2000). Similarly to what was found in the present study, UVR and drought stress showed a beneficial effect on the growth of wheat (*Triticum aestivum* L., cv. Centauro) (Alexieva, 2001) and cowpea (*Vigna unguiculata* L. Walp.) (Balakumar, 1993).

Although the addition of drought stress decreased the negative effects of UVR on *T. aestivum* length and weight, for its water content the addition of soil moisture stress (drought and flood) increased UVR damage, showing that the control of water content is more associated with soil moisture than UVR effects. These results are supported by those found by Bandurska et al. (2012), where it was reported for barley seedlings (*Hordeum vulgare* L. cv. Granal) an increase on effects induced by combined exposure of drought and UVR, when compared to a single exposure to UVR, that did not cause any changes on the water content.

It is known that adaptation to drought stress contributes to tolerance to UVB radiation by plants (Hofmann et al., 2003; Monk et al., 1989), and this event may be related to anatomical adjustments on leaves (Murali and Teramura, 1986), induction of stress proteins and other UV-absorbing compounds, as flavonoides (Alexieva, 2001; Balakumar, 1993; Middleton and Teramura, 1993). The latter may explain the maintenance of the dry weight with increase of UVR in *T. aestivum* and related to increased of production and accumulation of UV-screening compounds in these conditions. The accumulation of UV-absorbing compounds may have not occurred in single exposure to both stressors, once our results showed that the dry weight was clearly reduced by each stressor individually, but in combined exposure its production may be increased, and due to accumulation of flavonols by plants, which alleviates the negative effects of UVR (Klem, 2013). In addition, production of flavonoid differs according to the species (Beggs et al., 1986) and may be the cause of the great variability of UVR response between *T. aestivum* and *B. rapa*.

Although there are no studies on effects of plants exposed to UVR in flood scenarios, we hypothesize that plants could be more susceptible to UVR damage in flood conditions, because the accumulation of flavonols probably would not occur under these conditions. A study with the perennial herbaceous plant *Hypericum maculatum* and the grass *Agrostis tenuis* showed that drought treatments caused an increase on the accumulation of UV-screening compound (flavonols) compared which was not observed in the wet treatment (Klem, 2013).

The synergistic ratios calculated for *B. rapa* reinforced the discussed above, where a high sensitivity of this species to single UVR and combined UVR and water stress was observed. Here the SRs were always greater than 1 for all UVR treatments under drought and flood stress, showing synergistic interaction between these stressors. A possible explanation for the difference in sensitivity to UVR (as single as well in combined exposure) between these crop species may be the fact that dicots as *B. rapa* have a horizontal growth of leaves, thus receiving more sunlight and hence more UVR than the monocot *T. aestivum* whose vertical pattern of leaf growth are more prone to receive less direct sunlight (He et al., 1993).

The foliar changes observed in both species exposed to single UVR were reported and discussed in our previous study (chapter 4). But adding drought as a second stress, similarly to what happened with the other endpoints, showed to be more harmful to *B. rapa* than *T. aestivum*, with an increase in leaves symptoms. Some foliar changes found in both plant species, as reduced leaf area, smaller leaves and leaf curling, are considered protective responses that will reduce radiation absorption and were already reported in the literature (Teramura and Ziska, 1996), likewise reduction of the leaf size and rolling leaf which are also strategies for adaptation to drought stress (Bosabalidis and Kofidis, 2002).

5.5. Conclusions

The effectiveness of UVR on plants was dependent of soil moisture conditions, and the combination of these stressors was harmful for most simulated scenarios. But the two plants species showed different responses. Flood stress is an environmental factor that is often overlooked in environmental risk analysis, but our findings showed that flood is a weather event of high environmental risk, especially when interacting with other environmental stressor as UV radiation or chemical stressors. Therefore, there is a need for more research to understand how the combinations of several factors related with future global environmental changes may affect physiological responses in plants.

Acknowledgments

This study was supported by a Ph.D. Grant (SFRH / BD / 65274 / 2009) attributed to Maria Lima by the Portuguese Science and Technology Foundation (FCT) and through the research project CLIMAFUN (FCOMP-01-0124-FEDER-008656), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade.

Reference

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell & Environment, 24, 1337-1344.

Balakumar, T.V., Babu, H., Paliwal, K., 1993, On the interaction of UV-B radiation (280–315 nm) with water stress in crop plants. Physiologia Plantarum 87, 217-222.

Ballare, C.L., Scopel, A.L., Stapleton, A.E., Yanovsky, M. J. 1996, Solar Ultraviolet-B Radiation Affects Seedling Emergence, DNA Integrity, Plant Morphology, Growth Rate, and Attractiveness to Herbivore Insects in *Datura ferox*. Plant Physiol, v. 112, p. 161-170.

Balouchi, H.R.S., Modarres, S.A.M., Emam, Y., Dolatabadian, A., 2009, UV radiation, elevated CO_2 and water stress effect on growth and photosynthetic characteristics in durum wheat. Plant, Soil and Environment 55,. 443-453.

Bandurska, H. P.-B., M.; Cieałak, M., 2012, Response of barley seedlings to water deficit and enhanced UV-B irradiation acting alone and in combination: Acta Physiologiae Plantarum 34, 161-171.

Beggs, C. J., Schneider-Ziebert, U., Wellmann, E., 1986, UV-B Radiation and Adaptive Mechanisms in Plants, Stratospheric Ozone Reduction, Solar Ultraviolet Radiation and Plant Life: NATO ASI Series, 8, Springer Berlin Heidelberg, p. 235-250.

Bornman, J.F., Teramura, A. H., 1993, Effects of Ultraviolet-B Radiation on Terrestrial Plants, Environmental UV Photobiology, Springer US, p. 427-471.

Bosabalidis, A.M., Kofidis, G., 2002, Comparative effects of drought stress on leaf anatomy of two olive cultivars. Plant Science 163, 375-379.

Caldwell, M.M., Flint, S.D., Searles, P. S.,1994, Spectral balance and UV-B sensitivity of soybean: a field experiment. Plant, Cell & Environment 17, 267-276.

Caldwell, M.M., Teramura, A.H., Tevini, M., 1989, The changing solar ultraviolet climate and the ecological consequences for higher plants: Trends Ecol Evol. 14, 363-367.

Caldwell, M.M., Bornman, J.F., Ballare, C.L., Flint, S.D., Kulandaivelu, G., 2007. Terrestrial ecosystems, increased solar ultraviolet radiation, and interactions with other climate change factors. Photocheistry and Photobiology Science 6, 252-66.

Chaves, M.M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osório, M.L., Carvalho, I., Faria, T., Pinheiro, C., 2002, How Plants Cope with Water Stress in the Field? Photosynthesis and Growth. Annals of Botany 89, 907-916.

Coleman, R.S., Day, T. A. 2004, Response of cotton and sorghum to several levels of subambient solar UV-B radiation: a test of the saturation hypothesis. Physiologia Plantarum122, 362-372.

Conner, J.K., and L.A. Zangori, 1997, A garden study of the effects of ultraviolet-B radiation on pollination success and lifetime female fitness in *Brassica: Oecologia*, v. 111, p. 388-395.

de Almeida, S.L., Schmidt, É. C., Rodrigues, A. C., Bouzon, Z. L., 2012, Effects of Natural Radiation, PAR and Artificial Ultraviolet Radiation-B on the Ultrastructure and Histochemistry of Leaf of *Oryza sativa* L: American Journal of Plant Sciences, v. 3.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A., 2009, Plant drought stress: effects, mechanisms and management: Agronomy for Sustainable Development, 29, 185-212.

Feng, H., Li, S., Xue, L., An, L., Wang X., 2007, The interactive effects of enhanced UV-B radiation and soil drought on spring wheat. South African Journal of Botany, 73, 429-434.

He, J., Huang, L.K., Chow, W.S., Whitecross, M. I., Anderson, J. M. 1993, Effects of Supplementary Ultraviolet-B Radiation on Rice and Pea Plants. Functional Plant Biology 20, 129-142.

Hofmann, R.W., Campbell, B.D., Bloor, S.J., Swinny, E.E., Markham, K.R., Ryan, K.G., Fountain, D.W., 2003, Responses to UV-B radiation in *Trifolium repens* L. – physiological links to plant productivity and water availability: Plant, Cell & Environment 26, 603-612.

Klem, K., Effects of UV radiation and drouhgt on the accumulation of UV-screening compounds and photosynthetic parameters in selected herbs and grasses of the mountain grassland ecosystem, *in* P. RajsnerovÃ¡, J. Ã. KubÃ¡sek, P. Holub, and O. Urban, eds.: Global Change and Resilience, Centrum vÃ½zkumu globÃ¡lnÃ- zmÄ›ny, BÄ›lidla 4a, 603 00 Brno, http://www.czechglobe.cz/.

Kozlowski, T.T., 1984, Plant responses to flooding of soil. BioScience 34, 162-167.

Li, C., Liu, S., Berninger, F., 2004, Picea seedlings show apparent acclimation to drought with increasing altitude in the eastern Himalaya. Trees, 18,. 277-283.

Li, Y., Sperry, J.S., Shao, M. 2009, Hydraulic conductance and vulnerability to cavitation in corn (*Zea mays* L.) hybrids of differing drought resistance. Environmental and Experimental Botany. 66, 341-346.

Liao, C.T., Lin, C.H. 1995, Effect of flood stress on morphology and anaerobic metabolism of *Momordica charantia*. Environmental and Experimental Botany, 35, 105-113.

Lima M.P.R., Soares A.M.V.M., Loureiro S., 2011. Combined effects of soil moisture and carbaryl to earthworms and plants: Simulation of flood and drought scenarios. Environmental Pollution 159, 1844-1851.

Lv, Z., Zhang, X., Liu, L., Guo, Y., Fan, Y., Yang, X., Li, Y., Zhang, W., 2013. Comparing intraspecific responses of 12 winter wheat cultivars to different doses of ultraviolet-B radiation. J Photochemistry and Photobiology B, 119, 1-8.

Mahajan, S., Tuteja, N., 2005, Cold, salinity and drought stresses: An overview. Archives of Biochemistry and Biophysics 444, 139-158.

Mark, U., Tevini, M., 1996, Combination Effects of UV-B Radiation and Temperature on Sunflower (*Helianthus annuus* L., cv. Polstar) and Maize (*Zea mays* L, cv. Zenit 2000) Seedlings: Journal of Plant Physiology 148, 49-56.

McCree, K.J., Fernandez, C.J. 1989, Simulation Model for Studying Physiological Water Stress Responses of Whole Plants. Crop Science 29, 353-360.

Middleton, E.M., Teramura, A.H., 1993, The Role of Flavonol Glycosides and Carotenoids in Protecting Soybean from Ultraviolet-B Damage. Plant Physiology 103, 741-752.

Molina, M.J., Rowland, F.S., 1974, Stratospheric sink for chlorofluoromethanes: chlorine atomc-atalysed destruction of ozone. Nature 249, 810-812.

Monk, L.S., Fagerstedt, K.V., Crawford, R.M.M., 1989. Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. Physiologia Plantarum, 76, 456-459.

Murali, N.S,. Teramura, A.H. 1985, Effects of ultraviolet-B irradiance on soybean. VI. Influence of phosphorus nutrition on growth and flavonoid content. Physiologia Plantarum 63, 413-416.

Murali, N.S., Teramura, A.H., 1986, Effectiveness of UV-B radiation on the growth and physiology of field-grown soybean modified by water stress: Photochemistry and Photobiology 44, 215-219.

Nogues, S., Allen, D.J., Morison, J.I., Baker, N. R., 1998, Ultraviolet-B radiation effects on water relations, leaf development, and photosynthesis in droughted pea plants. Plant Physiology 117, 173-81.

Nogués, S., Baker, N.R., 2000, Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. Journal of Experimental Botany 51, 1309-1317.

Ren, J., Dai, W., Xuan, Z., Yao, Y., Korpelainen, H., Li, C., 2007, The effect of drought and enhanced UV-B radiation on the growth and physiological traits of two contrasting poplar species. Forest Ecology and Management 239, 112-119.

Smith, J.L., Burritt, D.J., Bannister, P., 2000. Shoot Dry Weight, Chlorophyll and UV-B-absorbing Compounds as Indicators of a Plants Sensitivity to UV-B Radiation. Annals of Botany 86, 1057-1063.

Sullivan, J.H., Teramura, A.H., 1990, Field Study of the Interaction between Solar Ultraviolet-B Radiation and Drought on Photosynthesis and Growth in Soybean: Plant Physiology 92, 141-146.

Takeuchi, Y., Ikeda, S., Kasahara,H., 1993, Dependence on Wavelength and Temperature of Growth Inhibition Induced by UV-B Irradiation. Plant and Cell Physiology 34, 913-917.

Tao, F., Yokozawa, M., Hayashi, Y., Lin, E., 2003, Terrestrial water cycle and the impact of climate change: AMBIO. A Journal of the Human Environment 32, 295-301.

Teramura, A.H., Sullivan, J.H. Lydon, J., 1990, Effects of UV-B radiation on soybean yield and seed quality: a 6-year field study. Physiologia Plantarum 80, 5-11.

Teramura, A.H., Ziska, L.H., Sztein, A.E., 1991, Changes in growth and photosynthetic capacity of rice with increased UV-B radiation. Physiologia Plantarum 83, 373-380.

Teramura, A. H., Ziska, L.H., 1996, Ultraviolet-B radiation and photosynthesis., *in* N. Baker, ed., Photosynthesis and the Environment: Advances in photosynthesis and respiration. Dordrecht, The Netherlands, Kluwer Academic Publishers.

Tevini, M., Teramura, A.H. 1989, Uv-B Effects On Terrestrial Plants. Photochemistry and Photobiology 50, 479-487.

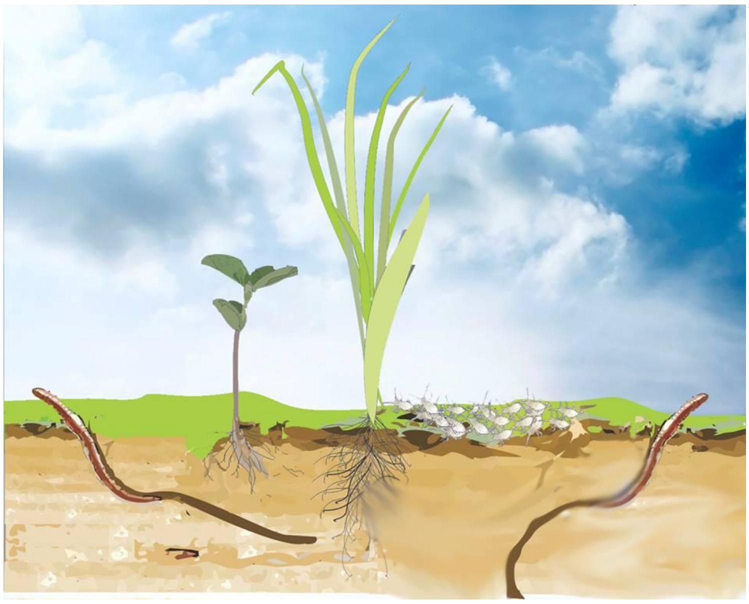
Tsukaya, H., 2003, Organ shape and size: a lesson from studies of leaf morphogenesis: Current Opinion in Plant Biology 6, 57-62.

Walther, G.-R., Eric; P., Convey, P., Menzel, A., Parmesan, C., Beebee, T.J.C., Fromentin, J-M., Hoegh-Guldberg, O.; Bairlein, F., 2002, Ecological responses to recent climate change: Nature 416, 389-395.

Worrest, R.C., Smythe, K.D., Tait, A.M., 1989, Linkages between climate change and stratospheric ozone depletion., *in* J.C.W. White, W.R.; Beal, C.N.; Center for Environmental, Information; Air Resources Information, Clearinghouse, ed., Global Climate Change Linkages: Acid Rain, Air Quality, and Stratospheric Ozone: USA, Springer.

CHAPTER 6

General discussion and final considerations



Chapter 6. General discussion and final considerations

The main aim of this dissertation was to evaluate how abiotic factors can affect the toxicity of stressors or impair soil organisms by its own, using ecotoxicological assays, and simulating more realistic environmental scenarios; this will provide a better understanding and prediction of adverse effects of chemical stressors on soil ecosystem. In the environment, soil invertebrates and plants experience the exposure to multiple stressors, as chemical and abiotic, that act individually or interact with each other causing significant effects to exposed organisms.

In the previous chapters, it has been noticed that the U.N. Intergovernmental Panel on Climate Change (IPCC) has published four assessment reports on the possible future impacts that climate change will have on the environment, even in this century, as well as mitigation measures to reduce them (IPCC, 2007). The extreme predicted climate events will consist on periods of extreme heat, cold, drought, and flooding with greater severity than historical standards (IPCC, 2013). In the present work scenarios simulating different climatic areas and also extreme events predicted for the next decades in some regions of the world were explored (Figure 6.1).

Although these scenarios for 100 years may raise several criticism (Gleditsch and Nordåsa, 2014), it is known that these changes will probably not occur exactly as predicted, since climate change depend on several factors, mainly related to human activities, but most of these scenarios may already be happening globally. Some studies of climatic change have reported a decrease in soil moisture globally since the 1970s (Sheffield et al., 2012). A study developed by Dai et al. (2004), using an index of meteorological drought, indicated that climate change is already leaving several parts of the world drier, and concluded that the percentage of land area affected by serious drought rose by more than half, between 1970 and 2000.

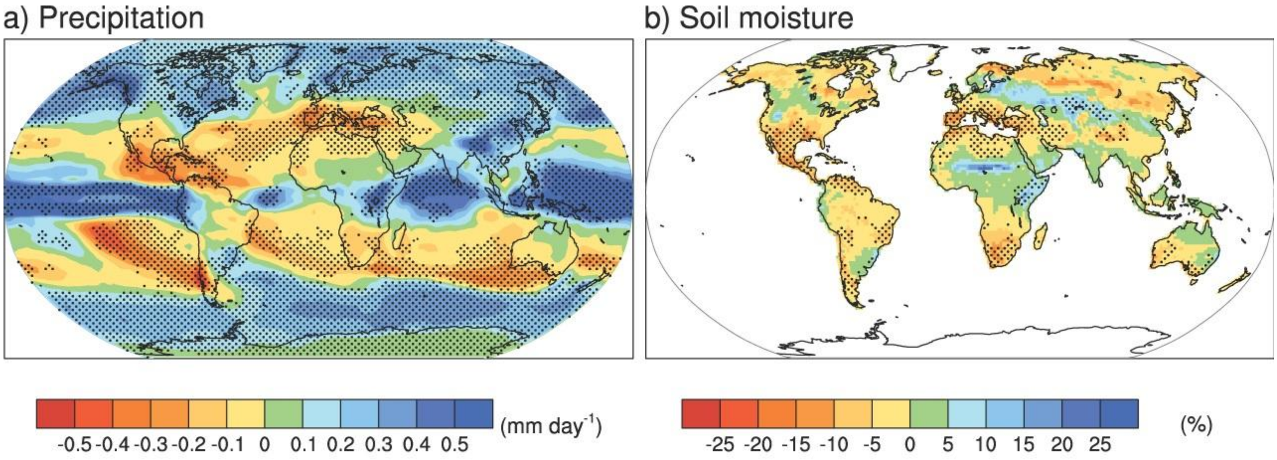


Fig. 6.1. Climate model projections of rainfall changes and soil moisture by end of 21st Century in A1B scenario (based on average over all IPCC models). Dots represent areas where at least five of the nine models agree in determining that the change is statistically significant. Source: IPCC (IPCC, 2007).

In traditional ecotoxicological studies, the use of standard guideline tests are described to be run under controlled laboratory conditions (often as close to optimum as possible) and they have proven to be an efficient toll to assess the toxicity of chemicals towards model species. But they do not address all climate scenarios or latitudinal differences and seasonal fluctuations. Therefore, the scenarios of drought and flood applied in chapter 2 and 5, will help to understand the environmental risk that may occur in organisms that lives in mediterranean areas, such as Portugal, which is expected to increase in terms of consecutive dry days in the next future, as well as in areas of high latitude, where it is predicted to experience high rainfall and consequent flood (IPCC, 2007). The scenarios of heat and cold performed in chapter 3**,** allows also the prediction of what might happen with soil organisms and plants exposed to pesticides in areas such as tropical countries, experiencing high temperatures throughout the year, as well as in countries with low temperatures or temperate regions in winter. The scenarios of UV radiation will allow inferring what can happen with plants exposed simultaneously to relevant levels of UVR and pesticides (chapter 4) or with extreme events considering drought and flood scenarios (chapter 5**).**

Our results support that single environmental abiotic factors, as soil moisture, temperature and UV radiation, may became natural stressors for soil organisms and plants, inducing significant effects on their survival, biomass acquisition, growth and development. This work showed that when soil organisms are exposed to combined natural and chemical stressors as carbaryl, significant interaction may occur, altering their response to chemical exposures. Our findings also allowed understanding the way two environmental factors (UV and water stress) can act simultaneously on plants, by increasing or decreasing the effect of each other, and causing significant changes on plant's development. Our results also showed that the intensity of response to natural and chemical stressors varies according to the species and endpoint analyzed.

The earthworm *Eisenia andrei,* which is known to have a wide temperature and soil moisture tolerance range (Dominguez et al., 2010), was not affected in terms of survival when exposed to extreme dried or flooded soils (chapter 2), nor in heat or cold scenarios (chapter 3). Only the temperature of 26ºC caused loss of weight, showing that biomass is a more sensitive endpoint than survival to temperature exposures. Despite these stressors, in general, have not caused significant effect on earthworm's survival or biomass, for the range of temperature and soil moisture examined here, long-term drought or extreme events on a large spatial scale, are not excluded of potentially affect earthworm's survival and biomass negatively. In accordance to the findings of this study, Reinecke and Venter (1985) showed that soil moisture did not cause significant effects in *E. fetida* weight upon 10 days of exposure, but after 20 days significant changes were observed, which can reinforce the idea that long term exposures can induce higher effects.

Certainly a chronic reproduction test or a biochemical approach (enzymatic activities or lipid composition) could reveal significant effects of temperature and water stress on earthworms, since Reinecke and Venter (1985) showed that soil moisture have a profound influence on the rate of clitellum development of *Eisenia fetida* and in another study (Reinecke, 1987), the optimal moisture level for growth and development of clitella were considered not necessarily optimal for the production of cocoons for this species.

Carbaryl is an insecticide registered in several countries (e.g., Canada, United States, South Africa and Portugal) and the agricultural dose recommended is 2.000 g ha^{-1} (Gupta et al., 2011). The doses used in present work were chosen based on LC_{50} and EC_{50} values for carbaryl, or others carbamates reported in literature for each tested species.

Earthworms are known to be very sensitive to sublethal concentrations of carbaryl, which acts, primarily, as an inhibitor of acetylcholinesterase (AChE) (Ribera, 2001). In chapters 1 and 2 it was discussed that very low concentrations of carbaryl, far below those used in this study, caused significant effects on locomotion, burrowers activities, inhibition of AChE, as well as lethality for some species, but information on lethality of *Eisenia andrei* exposed to carbaryl in literature are not very consistent. Studies reported that *E. andrei* and *E. fetida* have a high lethal threshold to this pesticide (Stenersen et al., 1992), Heimbach (1984) found a LC_{50} value of 174mg/kg, but the author has submitted the earthworms to different conditions from those used in this work, such as, 28 days of exposure in artificial soil (OECD) at 22ºC. However, Saxena et al. (2014) reported, recently, for *E. fetida* a LC_{50} of 26.86 mg /kg and Ribera et al. (2001) reported a LC_{50} of 50 mg/kg after 14 days of carbaryl exposure in OECD artificial soil, which are indeed more similar to our findings in the control situations of the three different approaches used: testing carbaryl in a single exposure experiment (LC_{50} = 54.74 mg/kg), in combined exposure with soil moisture (LC_{50} = 45.5 mg/kg at control conditions) and temperature (LC_{50} = 51.28 mg/kg at control conditions) for 14 days of exposure.

Ours results showed that the single exposure of earthworms to different temperatures or different soil moisture regimes, in general, have not caused significant effect on earthworm's survival and biomass, but, when these natural stressors were combined with carbaryl, the toxicity of its pesticide was strongly influenced by these environmental factors. The combined exposure of carbaryl and soil moisture was performed using the reference models of independent action (IA), based on the assumption of independently probabilities of response to both stressors. But the application of the IA model, for assess joint effects requires that there is a definable dose-response between the measured parameters and the stressor, and that these responses are reasonably modeled using a nonlinear model (Long et al., 2009), thence, since the dose response for earthworm's exposure to combined carbaryl and temperature was not possible, the interaction between stressors was assessed using synergistic ratios.

The results showed that, in general, dry soils and high temperature increased the deleterious effects of carbaryl to earthworms. These results highlighted the ecotoxicologial risks of natural stressors on chemical toxicity, because the LC_{50} for earthworms exposed to carbaryl under standard conditions (approximately 50 mg/kg), decreased considerably in dry soils (<20 mg/kg) and in hot environments (22.1 mg/kg). These results support the hypothesis that the traditional ecotoxicological studies, with optimal conditions of temperature and soil moisture, may lead to the underestimation of the general toxicity of a chemical.

Folsomia candida, as well as *Eisenia andrei,* is tolerant of a wide range of environmental conditions and when exposed to different temperatures in the work presented in chapter 2, it showed high tolerance regarding survival, but looking at the reproduction output, at temperatures below or above the optimum (20-22 ºC) it was observed a decrease on the juveniles production. Our results suggest that 8 and 26 ºC were the lower and upper limit for *F. candida* ability to reproduce. Our findings are in agreement with those reported by Martikainen and Rantalainen, (1999), where the temperature of 13 ºC to 19 ºC did not cause changes on *F. candida* survival after 28 days of exposure, but the numbers of juveniles decreased at lower temperatures (13º and 16 °C). But, regarding high temperature, Bandow et al. (2014) reported that 26 ºC did not affect *F. candida* reproduction, which does not corroborate with our results, and also with those reported by Sandifer and Hopkin (Sandifer and Hopkin, 1997), where, similarly to our results, the juvenile production of *F. candida* was very low at 25 ° C.

The single exposure of *F. candida* to carbaryl showed that these organisms are very sensitive to this pesticide, both for survival (LC_{50}=7.0 mg/kg) and for the reproduction (EC_{50}=4.6 mg/kg). Cardoso et al. (2014) showed that the short exposure (4h) of *F. candida* at the same range of carbaryl concentrations used here, cause no mortality, but affect the reproduction output (EC_{50} of 5.93 mg/kg) but in an opposite way, with an increase in the reproduction output (increase of number of eggs laid with increasing doses of carbaryl, but no influence on the hatching of the eggs). Therefore differences on mortality between both studies are probably due to time of exposure (4h vs 28 days), while differences on reproduction output can only be explained by a potential increase on the mortality of juveniles that may occur, which was not confirmed by the authors.

However, when collembolan were exposed to combined carbaryl and temperature (Chapter 3), no deviations occurred from the IA model, meaning that the temperature and carbaryl had no interactions among effects induced to organisms;

however, the negative effect of each stressor separately and independently from each other, are summed, resulting in an additive toxicity to organisms.

Regarding plants bioassay, the high and low temperatures (chapter 3), as well as extreme drought and flood conditions (chapter 2 and 5), also affected significantly their growth, biomass production and emergence, and although the two species exhibited different responses to temperature, they respond similarly to water stress. Our findings showed that high temperature induced positively the emergence of *B. rapa* but not of *T. aestivum*, while low temperature and flood conditions, in general, delayed the emergence and decreased the fitness of both species. *T. aestivum* length showed tolerance to high temperature, but *B. rapa* showed a decrease on its length at 28ºC.

The plants are divided into two categories (C3 and C4), according to the biochemical pathways of carbon dioxide assimilation by their photosynthetic systems (Rowley and Mockler, 2011), and both species used in this study are C3 plants, which differ from the C4 plants, because they have a low rate of CO_2 uptake, reduced photorespiration and photosynthesis rate. Consequently, C3 plants are sensitive to dry conditions, high temperatures (> 25ºC) and luminosity, their stomata remains partially closed during the hottest hours of the day to avoid water loss by perspiration and stay open at night when the temperature drops (Goudriaan and Zadoks, 1995). But it is also important to highlight that the plant's tolerance to abiotic factors is related to several other physiological and cellular responses, and monocots as *T. aestivum* and dicots as *B. rapa* differ on their ability to survive to environmental stressors (Rowley and Mockler, 2011). This may be one explanation for the different responses to natural stressors between these crop species in the different studies used. It is also important to note that, although the protocol for plants suggests optimum growth in soil moisture of 40-60% WHC, our results showed that both species had optimal growth and faster development in wetter soils around 80% WHC.

In chapter 4 and 5, a new biological spectral weighting function (called here $BSWF_{03}$) proposed by Flint and Caldwell (2003) for plant growth was applied, which differs from most studies on the effect of ultraviolet radiation on plants that use the generalized plant response (called here $BSWF_{71}$) of Caldwell (1971). As explained in chapter 4, this $BSWF_{71}$ is a composite of nine different UV-B responses that does not involve plant growth measurements, and furthermore, it does not take into account wavelengths greater than 313 nm (UV-A region). On the other hand, Flint and Caldwell (1996) showed that wavelengths higher than 310 nm are more effective than would be predicted by the $BSWF_{71.}$ The validity of the $BSWF_{03}$ (that was used in our study) was verified in field experiments and proved to be a realistic representation of UV-induced (UV-A and UV-B) growth and morphological responses in plants (Flint and Caldwell, 2003).

Nevertheless, many studies have been conducted with the $BSWF_{71}$, making it difficult to compare results, or used inconsistent UV-B doses and different BSWFs that generated many outputs which lead to a difficult conclusions regarding the response of plants to UV radiation.

Our results showed that the single exposure of UV radiation to these plant species (chapter 4 and 5) also showed greater influence on plants growth and biomass production. B. rapa was more sensitive to UV radiation as well as high temperature and flood conditions than T. aestivum. The UV sensitive index used in chapter 4 was an important tool to evaluate the difference in sensitivity of both species to ultraviolet radiation, considering also that the UVR doses used in present work are not as high as those found in countries like China, where the ambient UVBBE is around 11.02 kJ $m^{-2}d^{-1}$, weighted with the generalized plant action spectrum (Caldwell, 1971), on a summer solstice (An et al., 2000), but are relevant for Portugal and other European countries. These doses were sufficient to cause damage to the crop species, and we can highlight an emerging risk when ultraviolet radiation is acting simultaneously with carbaryl (chapter 4) or with natural stressors like water stress (chapter 5), which may be crucial to enhance or potentiate the effect of each other on plants.

The carbaryl toxicity to plants was explored on chapter 2, 3 and 4, and consistent responses were found in all approaches, with significant effect from the range of 50 mg/kg - 75 mg/kg onwards, showing that both plants are equally sensitive to carbaryl. But the combined exposure of this chemical with different natural stressors showed different responses regarding the endpoints and the plant species. All combined exposure affected plants emergence, except the UV radiation, where plant emergence was not assessed.

Table 6.1. Summary of the main responses of tested species to carbaryl under different natural stressors.

Bioassay	**Natural Stressor**	**Mainly response in carbaryl toxicity**	**Assessment tool**
Eisenia andrei Acute test	Drought	Increased the toxicity	EC$_{50}$ values
	Flood	Decreased the toxicity	EC$_{50}$ values
	High temperature	Synergism $_{\text{Survival and biomass}}$	SRs
	Low temperature	Antagonism $_{\text{Survival}}$ Synergism $_{\text{Biomass}}$	SRs
Folsomia candida Reproduction test	High temperature	Independent Action $_{\text{Survival and Reproduction}}$	Mixtox
	Low temperature	Independent Action $_{\text{survival and reproduction}}$	Mixtox
Brassica rapa Growth and emergence	Drought	Independent Action	Mixtox
	Flood	Antagonism $_{\text{FW and DW}}$ Synergism at high carbaryl and extreme flood $_{\text{Length}}$	Mixtox
	High temperature	Synergism	SRs
	Low temperature	Antagonism	SRs
	UV radiation	Synergism when UV is dominant	Mixtox
Triticum aestivum Growth and emergence	Drought	Antagonism $_{\text{FW and DW}}$ Synergism when carbaryl was dominant $_{\text{Length}}$	Mixtox
	Flood	Antagonism $_{\text{FW and DW}}$ Synergism at low carbaryl and low flood stress $_{\text{Length}}$	Mixtox
	High temperature	Antagonism	SRs
	Low temperature	Antagonism	SRs
	UV radiation	Synergism at low carbaryl and low UVR intensities	Mixtox

In general terms, the interactions between carbaryl and natural stressors in plants occurred with synergism showing up as the main pattern related to UV irradiation and high temperature, while antagonism showing up as the main pattern related with low temperature and flood stress. A general summary of the response for each of the tested species to combined exposure (carbaryl and natural stressors) is shown on table 6.1. In a review paper about the interactions between chemicals and natural stressors (Holmstrup et al., 2010) synergistic interactions were also found in most exposure of soil organisms to pesticides and high temperatures or drought stress, corroborating the results found in this dissertation, while no interaction was reported in pesticides exposures under cold stress.

In an overall assessment of combined exposure, the reference model of independent action (IA) gave a valid estimation for the toxicity of 23.7% of the assays performed, while synergism occurred in 16% and antagonism was the interaction described in the majority of the assays/endpoints (42.1%); although antagonism occurred most frequently, its occurrence was found mainly in simulated scenarios with low temperatures, flooded soils and low ultraviolet radiation. But our results showed that a high risk is predicted for tropical environments, where temperatures are often very high, for arid and semiarid regions, where drought is the main scenarios, as well as temperate environments, where summers will be hotter and drier each year.

Finally, in Chapter 5, another approach to natural stressors was made, where *Triticum aestivum* and *B. rapa* were exposed to the same doses of UV radiation used in the chapter 4, but under extreme drought (10% WHC), optimal (60%WHC) and flood (WHC 120%) conditions. The main objective of this approach was to evaluate whether soil moisture alters the tolerance of both plants to UV radiations, and this goal was successfully achieved. Our results revealed that a significant interaction between UVR and soil moisture was found for both plant species, and as occurred with most of the single exposure to natural stressors, plants responded differently to this combined exposure. Drought stress alleviated the deleterious effect of UVR on *T. aestivum* (antagonism) while flood stress increased its effects (synergism). But for *B. rapa*, a synergism was found when both water stress was added to UVR exposure. Within our findings it was shown that the response of one organism to one natural stressor may be changed not only by the presence of a chemical stressor, but also when other natural stressor act simultaneously.

The different sensitivity between *B. rapa* and *T. aestivum* to environmental stressors and combined exposures reported here shows the importance of including, in ecotoxicological assays, different species of plants with different photosynthetic (C3 and C4 plants) and root systems, number of cotyledons, among others.

One of the major outputs of this study was the demonstration that altered climatic conditions can dramatically alter the toxicity of pesticide (by using carbaryl as model) and impair organisms' development and survival. One novelty of this study was to evaluate the effects of flooding stress since this environmental factor is commonly disregarded, and our results showed flood as a factor increasing carbaryl toxicity, e.g., high carbaryl concentration and high soil moisture, or increase the deleterious effect of ultraviolet radiation on plants.

Our results showed also that the assessment of the toxicological risk in agricultural soils should be suitable for different climatic zones, which will ensure a better assessment of the effects of climate change on the soil ecosystem.

References

An, L., Feng, H., Tang, X., Wang, X., 2000. Changes of microsomal membrane properties in spring wheat leaves (Triticum aestivum L.) exposed to enhanced ultraviolet-B radiation. Journal of Photochemistry and Photobiology B 57, 60-5.

Bandow, C., Karau, N., Römbke, J., 2014. Interactive effects of lambda-cyhalothrin, soil moisture, and temperature on *Folsomia candida* and *Sinella curviseta* (Collembola). Environmental Toxicology and Chemistry 33, 654-61.

Caldwell, M.M., 1971. Solar UV irradiation and the growth and development of higher plants . In: A. Giese, (Ed.), Photophysiology. Academic Press, NEw York,

Cardoso, D.F.N., Bastos, A.C.; Soares, A.MV.M.; Loureiro, S., 2014. Short-term exposure to carbaryl and UV radiation increases the reproduction output of the collembolan *Folsomia candida.* Journal of Soils and Sediments. 14, 1559-1567.

Dai, A., Trenberth, K. E., Qian, T., 2004. A Global Dataset of Palmer Drought Severity Index for 1870-2002: Relationship with Soil Moisture and Effects of Surface Warming. Journal of Hydrometeorology 5, 1117-1130.

Dominguez, J., Edwards, C. A., Arancon, N. Q., Sherman, R. L., 2010. Biology and ecology of earthworm species used for vermicomposting. Vermiculture Technology: Earthworms, Organic Waste and Environmental Management. CRC press.

Flint, S.D., Caldwell, M. M., 1996. Scaling Plant Ultraviolet Spectral Responses from Laboratory Action Spectra to Field Spectral Weighting Factors. Journal of Plant Physiology 148, 107-114.

Flint, S. D., Caldwell, M. M., 2003. A biological spectral weighting function for ozone depletion research with higher plants. Physiologia Plantarum 117, 137-144.

Gleditsch, N. P., Nordas, R., 2014. Conflicting messages? The IPCC on conflict and human security. Political Geography. "in press" DOI: 10.1016/j.polgeo.2014.08.007

Goudriaan, J., Zadoks, J. C., 1995. Global climate change: modelling the potential responses of agro-ecosystems with special reference to crop protection. Environmental Pollution 87, 215-24.

Gupta, R. D., Chakravorty, P. P., Kaviraj, A., 2011. Susceptibility of epigeic earthworm *Eisenia fetida* to agricultural application of six insecticides. Chemosphere 84, 724-6.

Ribera, D., Narbonne, J.F., Arnaud, C., Saint-Denis, M., 2001. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biology & Biochemistry 33, 1123-1130.

Rowley, E.R., Mockler, T.C., Plant Abiotic Stress: Insights from the Genomics Era, . In: Prof. Arun Shanker (Ed.), Abiotic Stress Response in Plants - Physiological, Biochemical and Genetic Perspectives, 2011. Oregon, United State.

Sandifer, R.D., Hopkin, S.P., 1997. Effects of temperature on the relative toxicities of Cd, Cu, Pb, and Zn to *Folsomia candida* (Collembola). Ecotoxicology and Environmental Safety 37, 125-130.

Saxena, P.N., Gupta, S.K., Murthy, R.C., 2014. Comparative toxicity of carbaryl, carbofuran, cypermethrin and fenvalerate in *Metaphire posthuma* and *Eisenia fetida* - A possible mechanism. Ecotoxicology and Environmental Safety 100, 218-225.

Sheffield, J., Wood, E.F., Roderick, M.L., 2012. Little change in global drought over the past 60 years. Nature 491, 435-438.

Stenersen, J., Brekke, E. Engelstad, F., 1992. Earthworms for Toxicity Testing - Species-Differences in Response Towards Cholinesterase Inhibiting Insecticides. Soil Biology & Biochemistry 24, 1761-1764.