**Universidade de Aveiro**
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Departamento de Biologia

**Diogo Filipe Nunes Cardoso**

# Efeitos de carbaril e fatores abióticos em *Folsomia candida*.

# Combined effects of carbaryl and abiotic factors to *Folsomia candida.*

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# Combined effects of carbaryl and abiotic factors to *Folsomia candida.*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro)

**o júri**

presidente

Prof. Doutor João António de Almeida Serôdio
Professor auxiliar do Departamento de Biologia da Universidade de Aveiro

Doutora Ana Catarina Gomes Marcelo Bastos
Investigador Pós-Doutoramento, CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

Doutora Susana Patrícia Mendes Loureiro (orientador)
investigadora auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro

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**palavras-chave**

Carbaril, alterações climáticas, sobrevivência, reprodução, exposições combonadas, colembolo.

**resumo**

Os organismos terrestres podem ser expostos a uma grande variedade de stressores, como contaminantes e/ou outros stressores físicos ou biológicos que afetam a sua vida. Os organismos podem experimentar uma larga gama de flutuações ambientais como é o caso das alterações climáticas, seca e inundação de solos, ou até mesmo um aumento da radiação Ultravioleta. O objetivo deste trabalho é estudar os efeitos do carbaril no organismo terrestre *Folsomia candida* em diferentes condições abióticas, avaliando a sua sobrevivência, capacidade reprodutiva, bem como outros parâmetros. Exposições simples e combinadas foram levadas a cabo com carbaryl e dois stressores naturais: Humidade e radiação Ultravioleta. Os efeitos combinados foram comparados a exposições de carbaril sob condições standardizadas: 20ºC e 60% de humidade. Foi observado que o carbaril induz alterações na sobrevivência e produção de juvenis. Condições extremas de humidade, nomeadamente de seca e de inundação levam a uma redução da capacidade reprodutora e até mesmo da sobrevivência do organismo. De acordo com os nossos testes, descobrimos que a *Folsomia candida* foi afetada pela exposição à radiação Ultravioleta, diminuindo a sua sobrevivência a elevadas doses de UV. Além disso, as exposições combinadas de carbaril e radiação UV mostram um padrão de sinergismo. Estes resultados têm de ser analisados cuidadosamente, pois os colêmbolos possuem a capacidade de se refugiarem nas partículas do solo e consequentemente evitar a radiação. Os resultados das exposições combinadas mostraram que as flutuações das condições ambientais como na humidade e radiação UV podem induzir alterações na toxicidade dos químicos presentes nos solos.

**keywords** carbaryl, climatic changes, survival, reproduction, combined exposrures, springtail.

**abstract**

Terrestrial organisms can be exposed to a great variety of stressors, such us contaminants and/or other physical or biological stressors that affect their life traits. Organisms can experience a large range of environmental fluctuations such as temperature changes, drought and flood conditions or even UV radiation increments. The aim of this work is study the effects of carbaryl to a soil-dwelling collembolan *Folsomia candida* at different abiotic conditions, evaluating their survival, reproductive effort and other endpoints. Single and combined exposures were carried out with carbaryl and two different natural stressors: moisture and UV radiation. The combined effects were compared to carbaryl exposures under standardized condicitons: 20ºC, 60% water holding capacity. We observed that carbaryl induces changes on the survival and offspring production. Extreme conditions of drought and flood lead to a reduction of their reproduction capacity and even survival. According with our tests we found that *Folsomia candida* was affected by UV exposure decreasing their survival at highest UV doses. Moreover, the combined exposure of carbaryl and UV radiation showed a synergistic pattern. These results have to be regarded carefully as collembolans have the ability to refuge on soil particles and therefore avoid radiation. Results from the combined exposure showed that fluctuations on environmental conditions such as soil moisture or UV radiation can induce changes on chemical toxicity.

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# Chapter 1

# 1. General Introduction

## 1.1 - Soil contamination

Over the past century several activities like mining, manufacturing and urban activities contributed to extensive soil contamination (Cunningham et al. 1995). Soil is a three – dimensional body with great relevance in ecological functions. It comprises a porous matrix where water, biota and air occur at the same time, interacting (Agency EE 2000). Soil is a dynamic and complex system, functioning as habitat for microorganisms, flora, animals and humans and its contamination probably leads to groundwater contamination and biomagnification of chemical compounds through the food chain and possibly affect our health (Loureiro et al. 2005).

Environmental disturbances are capable of threatening the global environment, with climatic changes, atmospheric pollution, degradation of water and soils and the impoverishment of biodiversity having an important role. In soil biota intervenes on the decomposition of dead organic material and nutrient cycling and play an important role on the maintenance of its quality and function (Lima et al. 2011).

Disturbance caused by pollutants in soils results in quantitative and qualitative changes in soil fauna and consequently affects the soil function (Cortet et al. 1999). The increased use of pesticides has received special attention and numerous studies have been carried out in standard organisms trying to predict how the organisms are affected on real scenario (Wang et al. 2012)

## 1.2 - Interaction between chemical and natural stressors

Predominantly, standard ecotoxicological tests study the exposure of test organisms under optimal environmental conditions. However, organisms in their habitat are not subjected to optimal and linear conditions, confronting mixtures of pollutants and fluctuations of abiotic exposure conditions (Ferreira et al. 2010; Holmstrup et al. 2010). When several types of stressors are combined, their effects can sometimes result in greater effects than expected from either of the stress types alone (Holmstrup et al. 2010). Single contamination and combined exposures of chemical and natural stressors may pose a threat to human health, the environment and lead to a reduction of biodiversity (Groten 2000).

Although the previously knowledge that chemicals can interact themselves, some studies has been carried out to prove that abiotic factors can influence the toxicity of chemical contamination, with studies on terrestrial (e.g. Lima et al. 2011; Smit and Van Gestel 1997; Khan et al. 2007) and aquatic organisms (e.g. Ferreira et al. 2010; Ferreira et al. 2008; Heugens et al. 2003). When abiotic stressors are also present, chemical toxicity can be enhanced for the species than the toxicity predicted on tests with "standard conditions" where these conditions (e.g. UV, salinity, pH, moisture and temperature) are controlled (Laskowski et al. 2010).

Some toxicity tests with aquatic and terrestrial organisms are standardized, however, these tests do not represent the real scenario on real ecosystems due to the existence of different physico - chemical properties of soils or different climate conditions throughout the world, which will have influence on the bioavailability of toxicants and also on the physiological performance of organisms. So, the same total environmental concentration of a toxicant causes different effects in different exposure scenarios (Holmstrup et al. 2010). To increase the ecological relevance in toxicity studies, we must consider also several abiotic factors such as temperature, pH, moisture, salinity and UV radiation because these conditions may have influence on degradation, toxicity and bioavailability of pesticides but also that they can induce changes on organisms physiology. In addition, we must also consider the role of biotic stress, such as competition or predation under this real scenarios approaches (Pestana et al. 2009).

## 1.3 - Invertebrate bioassays to assess soil quality

With the recent development of human activities, several factors have a significant impact on ecosystem sustainability. Anthropogenic factors and changes on the environment may affect organisms and their performance (Lima et al. 2011).

Ecotoxicologial tests are used to assess the effects of chemicals on organisms, with the final aim of protecting the structure and functioning of ecosystems. These tests are necessary to complement chemical analyses because these analyses are essential for the evaluation of soil pollution but do have some drawbacks: 1) requires an extensive knowledge of the classes of pollutants to be analyzed; 2) are expensive for all the classes of pollutants potentially present in soils; 3) gives little information about the bioavailability of pollutants or their degradation products; 4) do not reveal possible synergism and antagonism interactions between pollutants and between the pollutants and the soil matrix they are mixed in with (Crouau et al. 2002; Crouau and Moịa 2006).

Generally, reproduction tests are more sensitive than mortality tests and supplies more information (Crouau et al. 1999). The reproduction tests are the preferred endpoint for chronic tests with invertebrates, being more sensitive than mortality tests because even slight impacts can influence the reproduction of the organism (Kaneda and Kaneko 2002). Also, reproduction is a robust endpoint for effect assessment and representative for ecological risk (Amorim et al. 2012). This parameter integrates the possible long term effects of the contaminant with possible changes which may occur in the species future reproduction capacities (Cortet et al. 1999). The main disadvantage of the reproduction test with soil species is that we cannot observe directly the reproduction, and cannot see the juvenile mortality and hatching success (Hopkins 1997).

Pesticides and other contaminants are tested in organisms before they receive a license for use by humans. Thus, increased environmental awareness has led to the introduction of tests for non-target organisms (Hopkins 1997). Thereby, numerous ecotoxicological tests are used for aquatic environment (using bacteria, protozoa, algae and animals) and tests on terrestrial organisms. A standard test of mortality on the earthworm *Eisenia fetida* (OECD 1984) has been standardized, enchytraeids (*Enchytraeus* sp., *Cognettia* sp.) and reproduction  of collembolans have been the most widely used groups because of their ease of culture and relatively short generation times (Fountain and Hopkin 2005). Plant germination and microbial activity are other organisms that we must consider when we are trying to access de toxicity of a compound. The results of those tests may be used for decision making in various pesticide evaluation procedures (Van Straalen and Van Rijn 1998).

For environmental pollution studies, the use of invertebrates bring us some advantages, including the fact of those organisms are the most widely distributed living organisms on Earth, have short life cycle, high reproduction rates and are sensitive to pollutants (Cattaneo et al. 2009).

## 1.4 – Aim of the study

Considering the above, this study aims to study how abiotic factors will change the toxicity of chemicals and how will they interfere with organisms' physiology and balance. For that we have chosen as test species the soil-dwelling collembolan *Folsomia candida* and the test-chemical carbaryl, using a combined exposure with different abiotic conditions, and study their joint effects on *F. candida* reproductive effort, survival, egg production and the capacity of eggs to hatch. For that single and combined exposures were carried out with carbaryl and two different natural stressors: moisture and UV radiation.

## 1.5 – Carbaryl

Carbaryl (1-naphthyl *N*-methylcarbamate; commercial name, Sevin) is one of the world's most commonly used broad-spectrum pesticides, functioning as insecticide, acaricide, molluscicide, and ectoparasiticide (R.A. Relyea and N. Mills 2001) and has been used for about 30 years, acting through contact and ingestion, controlling some chewing and sucking pests on fruit and vegetables crops. The study of this compound is important because is one of the major active ingredients of many commercially available insecticides. (Hardersen and Wratten 1997; WHO 1994; Tsogas et al. 2006).

Carbaryl and other carbamates act by disrupting the normal function of organisms' nervous system, inhibiting acetylcholinesterase which is an enzyme that transmits impulses through the central nervous system in insects, mammals and other species, controlling basics bodily functions like breathing, blood flow and digestion (Rick A. Relyea and Nathan Mills 2001).

Mainly its wide use is due to its intrinsic characteristics as low persistence in the environment and a more readily intake by soils with high organic content (rather than sandy soils), especially when bacteria communities are present. With "good agricultural practices", dissipation is rapid, as carbaryl has a half-time of 8 days to 1 month under normal conditions and the rate of decomposition is more rapid under hot climatic conditions (WHO 1994).

In terms of environmental levels and human exposure, food represents the major source of carbaryl intake for the general population. However, their presence in total dietary samples is relatively low, ranging from trace amounts to 0.05 mg/kg. Symptoms of carbaryl poisoning in exposed people include stinging eyes, wheezing, sweating, and nausea (WHO 1994).

Carbaryl is not likely to represent a risk of acute mortality to birds and ranges from slightly to highly toxic to several species of fish. This chemical ranges from moderately to very highly toxic to marine invertebrates, such as shrimp and oysters and it is very highly toxic to aquatic invertebrates such as freshwater shrimps and stoneflies and honey bees

(Lima et al. 2011; Hardersen and Wratten 1997; Tsogas et al. 2006; Rick A. Relyea and Nathan Mills 2001; LIMA et al. 2010). According to this, before applying carbaryl, we must consider the potential effects on birds, aquatic life, and non-target insects because it possibly affects those organisms.

## 1.6 –Test-organism - *Folsomia candida*

*Folsomia candida* is a member of the order Collembola, a widespread arthropod and is considered as an important stimulant of decomposition. This soil dwelling Collembola is a parthenogenic species which is distributed worldwide (Fountain and Hopkin 2005; Petersen and Luxton 1982; Tully et al. 2006). However, it often occurs in very high numbers in sites rich in humus and organic matter. Springtails play an important role in the soil food chain because they prepare organic matter for the mineralization by bacteria and also serve as prey for other soil animals.

*Folsomia candida* is considered a tramp species and we don´t know exactly where its original biogeographical location but there are many records of its presence in caves and mines. This unpigmented springtail is 1.5 to 3 mm in length at maturity, have a white or yellowish colour and can be exposed to contaminants via the soil, food, gas, pore water, contaminated leaf surfaces and topical application onto the individual (Hopkins 1997; Fountain and Hopkin 2005). However, the most toxic route of exposure is the contact with contaminated water in soil (pore water). Food may directly change the body growth and population growth of the species. Its diet is mainly composed of litter, fungi and bacteria (Crouau et al. 1999), and their feeding behavior directly influences microbial activity and biomass in the soil ecosystem (Kaneda and Kaneko 2002).

*Folsomia candida* is used on ecotoxicological bioassays because of its widespread distribution, the extensive knowledge of culturing, have short life cycles and also because acute and reproductive toxicity tests using this species had been established and standardized (Greenslade and Vaughan 2003; ISO 1999; OECD 2009). Therefore, according with everything that was presented, a study of the impact of the agricultural practices – including pesticides use - on populations of this organism seems extremely important.

## 1.7 - Relevance of the study

Nowadays, the extensive use of pesticides in agricultural activities leads to a contamination of soil and consequently affecting biota community. Contamination problems are often characterized by complex mixtures of chemicals belonging to the same or to different compound classes and mixtures between chemicals and abiotic factors (Loureiro et al. 2009). Being a representative pesticide, carbaryl is one of the most used pesticides in agriculture, and its presence on the field can influence the survival and reproduction of soil organisms. Usually the evaluations of deleterious effects due to contamination are based on single exposures under controlled/optimum conditions. But in the environment, organisms are exposed to chemical mixtures and to a vast combination of natural stressors and chemicals. Knowing that, the importance of this dissertation is to evaluate how abiotic stressors like moisture and UV radiation will affect the toxicity of carbaryl, mimicking possible real scenarios. Therefore, we can assess more realistically the real influence of this chemical in environment.

## 1.8- Organization of the thesis

The present thesis is organized in four chapters. The second and third chapters are structured as scientific papers, describing some experiments and results.

**Chapter 1** provides an introduction of the thematic of soil pollution, assessment and the potential influence of abiotic factors to soil organisms.

**Chapter 2** - Single exposure effects of the chemical compound carbaryl and the natural stressors soil moisture and UV radiation.

**Chapter 3** - Combined exposure of *Folsomia candida* to carbaryl and natural stressors (moisture and UV radiation).

**Chapter 4** – Provides a short discussion and some conclusions of the work.

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# Chapter 2

# 2. Chemical and abiotic stress effects to *Folsomia candida*: the case study of carbaryl, soil moisture and UV radiation

## 2.1 – Abstract

At the same time that organisms are exposed to chemical contaminants, they can also experience a large range of environmental fluctuations such as drought and flood conditions or even UV radiation increments. In this study we have carried out the exposure of the collembolan *Folsomia candida* to chemical and abiotic stressors, in order to attain for differences in exposure type effects. Carbaryl is one of the most used pesticides worldwide, and we observed that it influences the survival and reproduction of the soil-dwelling collembolan *F. candida*, showing a clear dose response curve. Under drought and flood situations, the organism responded with a decrease on its reproduction capacity and survival, but only when experiencing extreme conditions. In addition *F. candida* was also highly sensitive to UV radiation, affecting its reproduction and survival. Two situations were tested when the organisms were exposed to UV radiation: direct and indirect exposure to the radiation. When radiation was applied directly, at the highest UV intensities all exposed collembolans died. Surprisingly, the reproduction was higher at the highest intensities when they received indirect radiation. Also, with our results, we proved the negative importance of soil compaction on the soil fauna regarding the protection it can also provide to this kind of organisms, when evaluating UV radiation effects. Furthermore, UV radiation influenced the capacity of egg's hatching. At high intensities of UV, the number of eggs hatched was much lower

than in control situations. Both natural and chemical stressor affected survival and reproduction of *F. candida*.

## 2.2 - Introduction

In the last decades, anthropogenic factors affected the ecosystem sustainability which leads to a reduction on biodiversity, increasing the risk for environmental and human health (Loureiro et al. 2006). Anthropogenic contamination is usually related to industrial, agricultural and urban activities. However, these anthropogenic factors are not the only source of stress to the environment. Organisms in soil are not under optimal conditions because they are exposed to severe environmental conditions. This stress can influence the behaviour and physiology of organisms, leading even to their death when under extreme conditions (Holmstrup et al. 2010; Laskowski et al. 2010).

Currently, due to its low toxicity to mammals and its relatively short lifetime in the environment, carbaryl is one of the world's most commonly used, broad-spectrum pesticides with many applications, including agricultural practices, forestry activities, in wetlands and applications on domestic animals to control parasites (R.A. Relyea and N. Mills 2001).

Organisms can experience a large range of environmental fluctuations such as temperature changes, drought and flood conditions or even UV radiation increments. Alterations in moisture and UV radiation are two of the most important factors that which in their extremes may lead to physiological stress in the organism.

Stratospheric ozone depletion is one of the global environmental issues of the twentieth century. Decreased ozone results in increased ultraviolet transmission, which can affect the health of plants, animals and humans (Solomon 1999; Lavola et al. 1997). One of the major causes for that is the release of chlorofluorocarbon (CFC) compounds that when in contact with the stratosphere, destroy the ozone molecule. As a consequence, the amount of UV radiation that reaches the earth is higher than in normal conditions and has been increasing among recent years (Ribeiro et al. 2011).

The presence or absence of water in soil is one of the major factors that we have to take into account, because contaminants can dissolve in water, changing its concentration in soil, and altering their bioavaiability. By its known that soil moisture can impair organisms' health status, but usually only drought conditions are accounted as influencing ecosystems' functioning. There may be different reasons for a reduced drought tolerance when organisms are simultaneously under the influence of chemicals. One possibility consists on a physiological water-conserving mechanism that can also be affected by the toxicant, reducing the synthesis of glucose and myoinositol - crucial for the tolerance of desiccation (Sørensen and Holmstrup 2005). Other possibility consists on the reduction of drought tolerance due the effects of chemicals on the fluidity and function of cell membranes (Sørensen and Holmstrup 2005). To our knowledge only few studies look for effects of flood scenarios on soil functioning or directly on soil organisms (e.g. Lima et al. 2011)

The aim of this study was to assess the single effects of two major natural stressors - UV radiation and the presence or absence of water in soil - on the soil-dwelling *F. candida* and the effects of a chemical – carbaryl – on the same organism. For that, endpoints like survival, reproduction effort and egg production were used.

## 2.3 – Material and methods

### 2.3.1 – Test species and soil

All experiments were carried out using the natural standard soil LUFA 2.2 from Speyer, Germany (Lokke and 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, maxim water holding capacity of 55% (weight per volume) and a cation exchange capacity of 11.2 cmol/kg.

The soil-dwelling organism *Folsomia candida* was kept in laboratory and maintained at dark and a constant temperature of 20 ± 2 $^{o}$C. The collembolans were maintained in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 9:1. Once a week, granulated dry yeast was added as food in small amounts on two sides of the culture.

### 2.3.2 - Experimental design for the *F. candida* reproduction test

Tests were performed in accordance to the OECD 232 guideline (OECD 2009). The *Folsomia candida* reproduction test consists of exposing juveniles to contaminated soil and comparing the rate of reproduction with that of animals placed in non-contaminated control soil. Organisms are placed inside the experimental pots in contact with the contaminated soil / control for 28 days at 20±2 $^{o}$C with a 16/8 h photoperiod. The methodology used for counting juveniles and adults consisted on the addition of water to soil replicates and, as organisms can stand at the water surface tension, it is possible to count them. For that, each replicate was photographed and adults and juveniles counted using SigmaScan Pro5.

The experience was started with 10 synchronized springtails with the same age (10-12 days). These animals were obtained by allowing adults to lay eggs during two days. After this period, the adults were removed and the eggs present in the containers hatched about two weeks later. Animals were transferred from the hatching boxes to the experimental pots using a suction device.

### 2.3.3 - Test substance

Carbaryl (CAS No 63-25-2) was purchased from Sigmae-Aldrich Ltd. (99.8% purity). The stock solution was prepared using acetone due to its low solubility in water, and applied to pre-moistened soil 24 hours before the start of the experience for acetone evaporation.

Organisms were exposed to different concentrations of carbaryl (1, 4, 7 mg/Kg) and two controls (a water control and a acetone control), according to the *F. candida* reproduction test (OECD 2009). For each concentration and control, five replicates were performed. The chemical exposure test was carried out in a controlled room temperature at 20±2 $^{o}$C and a 16/8 h photoperiod. After 28 days, the adults and juveniles were counted to record mortality and reproduction of the organisms exposed to carbaryl.

### 2.3.4 – UV Radiation experiments

All UV radiation exposures were performed inside a room where the temperature (20± 2$^{o}$C) and photoperiod (16/8 h) were controlled. The UV light was provided by a UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) and it was placed 30 cm above the vials and clear cellulose acetate sheets (0,003mm) were used to cut-off UV-C range wavelengths. These cellulose acetate sheets were previously UV irradiated for 12 h before used in the experiments to minimize differences in UV radiation intensity that passes the cellulose acetate sheets. Table 1 presents the times of exposure to the UV radiation and the equivalent intensities for each time of exposure, in mW $m^{-2}$ $nm^{-1}$. For all intensities, a UV dose was calculated, taking into account the time of exposure that the organisms are subjected, following the formula:

$$\mathrm{UV\ dose\ (Joules/m^2)} = \frac{\mathrm{Intensity\ (mW.m^{-2}.nm^{-1}) \times Time\ of\ exposure\ (s)}}{1000}$$

As *Folsomia candida* is a soil-dwelling arthropod, experiences were performed and adapted to understand the effects of UV radiation on this organism. Experiment were carried out using the natural standard soil LUFA 2.2, which would enable animals to hide on the soil matrix and behave normally, but also  using a mixture of plaster of Paris and activated charcoal (9:1, mixed with approximately equal volume of distilled water), where UV exposure could not be avoided. In addition, soil compaction was also tested as an additional stressor that would unable collembolan to avoid UV radiation.

The following experiments were performed:

1) **Mortality test** – 30 Juveniles (10-12 days) and adults (22 days) were exposed to different intensities of UV radiation to accesses the influence of the stressor on the mortality of the organism. At the same time, a control experiment was performed without any UV radiation, inside a room where the temperature (20±2$^{o}$C) and photoperiod (16/8 h) were controlled. This experimental set up was carried out in soil and plaster.
2) **Compaction test** – To understand the interaction between the collembolan and the soil matrix, and to mimic a more realistic exposure than the plaster substrate, where organism could not hide on the soil matrix an experiment was also performed exposing springtails (adults and juveniles with the same age of the mortality test) to Lufa 2.2 soil that was previously watered till 100% water holding capacity (WHC), suffering latter on a slight compaction after water evaporation.
3) **Reproduction after exposure** – Experiments were carried out with 30 adults (22 days) and 30 juveniles (10-12 days) to study the influence of UV pre-exposure on the reproduction of *F. candida*; the parameter recorded was the number of eggs laid by collembolans pre exposed to UV radiation. This experimental set up was carried out in soil and plaster.
4) **Direct exposure of eggs** - Experiments were carried out with 50 eggs with the same days previously laid by collembolans under control conditions. After exposure, the number of eggs that hatched was counted. This experimental set up was carried out in soil and plaster.

Mortality and reproduction were recorded in three periods of time after exposure (24h, 48h and 72h). This experiment was carried out because in our previously tests (data not shown) we found that the UV radiation affected organisms during 3 days after exposure. In addition, organisms were not affected at the same time, and 3 days were considered sufficient to reach a total mortality on our tests at the highest UV intensity.

The intensities of UV used on our tests are relevant because their consistent with the radiation of tropical zones because we want to study the extremes, confronting the organisms with extreme situations.

**Table 1** - Time of exposure to ultraviolet radiation (h) and its correspondent UV intensities (mW $m^{-2}$ $nm^{-1}$) transmitted by the UV lamp and equivalent UV doses for each time interval.

| Time of exposure (h) | UV intensity (mW $m^{-2}$ $nm^{-1}$) | UV dose (Joules/$m^2$ ) |
|---|---|---|
| **1** | 4108.3 | 14790 |
| **2** | 3589.1 | 25841.6 |
| **3** | 3690 | 39852.1 |
| **4** | 3485.7 | 50194.4 |

## 2.3.5 – Flood and drought experiments

Tests with collembolans were adapted from the reproduction and survival protocol (OECD 2009). Collembolans (10 juveniles with 10-12 days) 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), as advised in the OECD guideline (OECD 2009). To control moisture levels during the experiments, soil pots were weighted daily and water replenished at each two days.

### 2.3.6 – Statistical analysis

All statistical analysis were performed using the software package SigmaPlot 11.0, provide by Systat Software Inc. To compare the water control and acetone control, a Student's t test was performed.

One way (ANOVA), followed by Dunnett's test, was used to analyze differences between control and treatments. Whenever data were not normally distributed and to evaluate the differences between groups, a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed, followed by Dunn's method if significant differences were found. $EC_{50}$ values were calculated using a sigmoidal (logistic, 3 parameter) equation.

## 2.4 – Results and discussion

### 2.4.1 – Carbaryl exposures

There were no significant differences on the reproduction output and survival between the water control and acetone control. Therefore the solvent control data was used to compare the results obtained for all carbaryl treatments. Collembolans survival and reproduction were significantly affected by carbaryl exposure (ANOVA, $p \leq 0.05$, Figure 1). At higher concentrations of carbaryl (4 and 7 mg/Kg) significant differences were found on survival, compared with the control. Only at the highest concentration of carbaryl, at 7 mg/Kg of soil, the reproduction was significantly affected. A dose response curve upon *F. candida* exposure to carbaryl was observed. The $EC_{50}$ and $LC_{50}$ of that experiment were 5.1 and 5.4 mg/Kg, respectively. As mortality at higher concentrations was very representative, the results of the production of juveniles, at those concentrations, must be regarded carefully.

How far we can conclude, no experiments were published testing the effects of carbaryl on *Folsomia candida* but we can compare our results with results obtained with other species. For example, using the earthworm *Eisenia andrei*, in terms of survival, the $LC_{50}$ was 53.3 mg/Kg after 7 days of exposure and 45.5 mg/Kg after 14 days. For the same species, the biomass (weight loss), was not significantly influenced at the concentrations used (Lima et al. 2011). On the same study, the plant species *Brassica rapa* and *Triticum aestivum* were also exposed to carbaryl, showing a dose-response pattern and where similar responses for length and biomass weight were observed upon carbaryl exposure. Plant biomass production growth and emergency were adversely affected and the severity of the response was directly related to increasing carbaryl concentrations. Therefore *F. candida* showed to be more sensitive to carbaryl than other species also exposed to carbaryl on soil (plants and earthworms). We must compare these data carefully due to the time of exposure that our organism was exposed (28 days) and the exposure of other cited organisms (7 and 14 days).

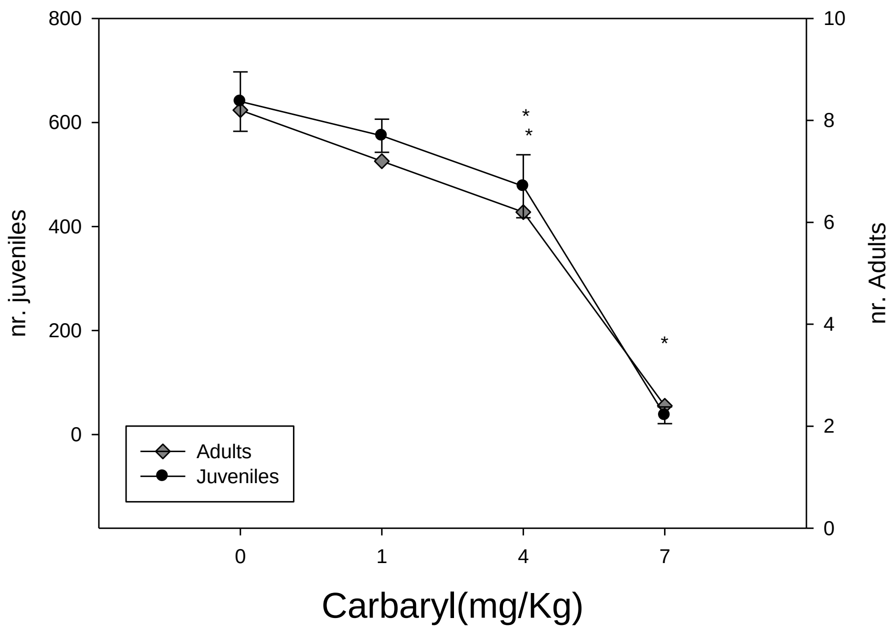


Figure 3 - Effect of carbaryl on *Folsomia candida* survival and reproduction after 28 days of exposure. Data is expressed as mean values and standard error (* $P<0.05$, Dunnett's method)

## 2.4.2 – Flood and drought experiments

The moisture experiments were divided in two experimental setup groups: drought stress evaluation (10 to 40% WHC) and flood stress evaluation (80 to 120% WHC). Both experiments were compared to a control situation, at 60% WHC. In terms of drought stress, the $LC_{50}$ was 15.89% WHC.

Regarding reproduction, when exposed to drought stress (Fig.2 (B)), organisms showed a lower reproduction capacity when exposed to situations of drought stress. The $EC_{50}$ was 28.29 WHC and comparing with the $LC_{50}$, we can conclude that the survival was more affected than reproduction, showing a different pattern between reproduction and survival when organisms are exposed to drought stress. This was expected, according to previous studies (Bayley and Holmstrup 1999) suggesting that *Folsomia candida* can tolerate, at an certain point, situations of drought.

Collembolans were firstly considered organisms with no physiological or metabolic means to prevent water loss, being incapable of acclimating to drought, and migrating to deeper soil layers (Harrisson et al. 1991). On the other hand, there are studies suggesting that *Folsomia candida* is well adapted to the desiccating forces likely to occur in the root zone during drought (Bayley et al. 2001). This organism can actively increase the osmolality of its body fluids in response to drought, regaining water lost from initial drought exposure by passive water vapor absorption, and thus remain active on these conditions. *F. candida* can tolerate these conditions due to the ability of synthesis of high concentrations of glucose and myo-inositol contributing to the increased body fluid osmolality (Bayley et al. 2001).

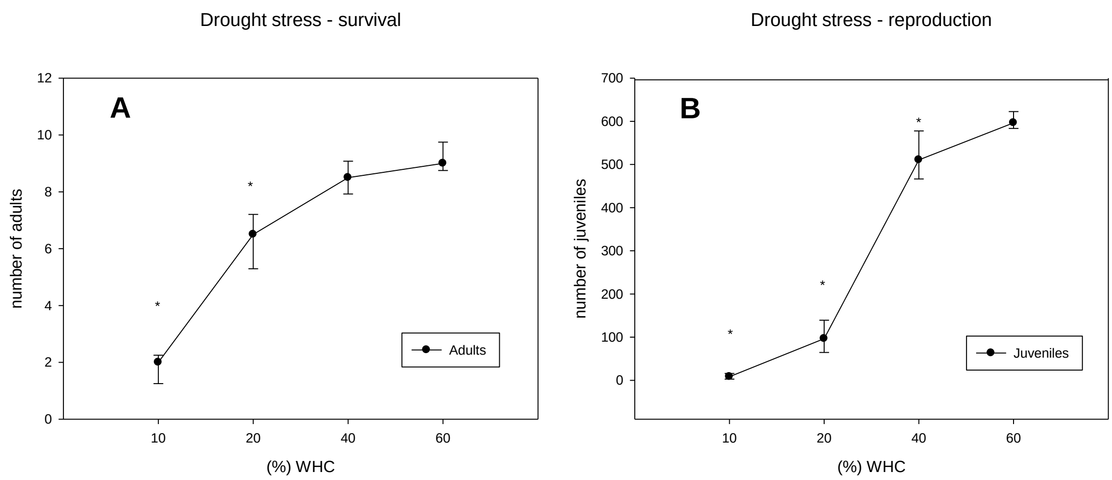


Figure 4 –Effect on drought situation on *Folsomia candida* survival (A) and reproduction (B) after 28 days of exposure (*p ≤ 0,05 Dunnett's method, compared to the control).

When the organism is exposed to flood situations, we were confronted with the same pattern, with an increase of the mortality and decrease of reproduction in highest stress conditions.

In terms of survival, the $EC_{50}$ was 75.47 % WHC and $LC_{50}$ was 100.21 %) WHC, showing that its reproduction capacity was affected at levels of stress that we did not observe in terms of survival. We observed significant differences in reproduction at 80, 100 and 120% of WHC and survival was only significantly affected at 120% of WHC. Observing the obtained results (Fig. 3), we can conclude that in high presence of water, collembolans of that species are affected in their life traits, with a decrease of number of juveniles and even survival. The flood stress is often ignored but is has already been discussed that it may also induce stress to soil organisms (Lima et al. 2011). From these results, it is observed the inability of *F. candida*, which is not adapted to flood, to reproduce and survive at situations where water is present in extreme situations and also the differences between this survival and reproduction under a 28 day of exposure.

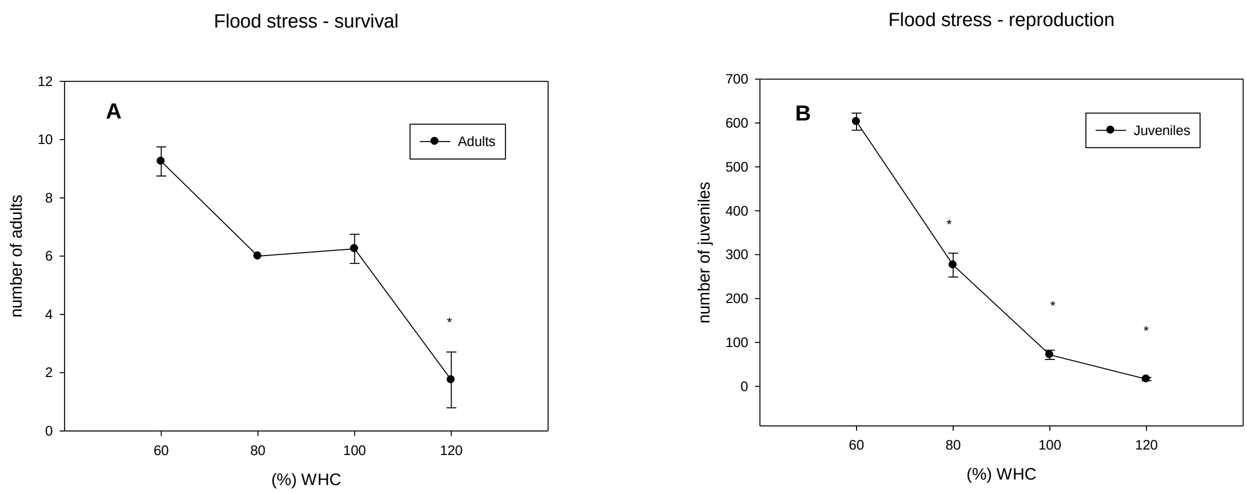


Figure 3 – (A) Effect of flood situation on *Folsomia candida* survival after 28 days of exposure (*p ≤ 0,05 Dunn's test, compared to the control). (B) Effect of flood situation on *F.candida* reproduction after 28 days of exposure (*p ≤ 0,05 Dunnett´s test, compared to the control).

In conclusion, we can observe that the best conditions for survival and reproduction of these organisms are at 60% WHC. This conclusion is not fully consistent with the guidelines of standard tests using *F. candida*, where the use of soils between

40% and 60% WHC is a standard procedure (ISO 1999; OECD 2009). According to our findings, the reproduction was significantly affected at 40% of WHC. According with field studies in zones under influence of inundation and extreme drought with other collembolan species (e.g. Marx 2008), flood constitutes an eminent impact for the collembolan community of their habitat, but the continued presence of all dominant species from the control samples on flood locals shows a good adaptation to flooding conditions. However, after the long-term drought, very strong consequences for the total species composition could be registered.

## 2.4.3 – UV radiation experiments

All experiments involving UV radiation were performed using two exposures: plaster and real soil, as discussed on material and methods. The first approach, in terms of UV radiation effects on *F. candida*, was to discover how the organism reacts to UV radiation when inserted on these two ways of exposure, i.e. with the possibility to escape and without. After the test, the organisms were observed every day during three days. The results of the exposure of *F. candida* to UV radiation on plaster (fig. 4 (A)) were very conclusive, showing the high sensibility of *F.candida* to different intensities of UV radiation, when directly exposed. Comparing with fig. 4 (B), where collembolans were exposed in soil, where they can partially avoid the UV radiation, the survival was much higher comparing to plaster. These results were consistent with our expectations, due to that the possible ability of collembolans avoiding UV radiation, passing to the inner layer of the soil. An interesting conclusion is that the effect of UV radiation on collembolans was not immediate, because the number of dead animals increased with time after exposure. The observations stopped at 72h because animals, at the highest intensity of

UV, were all dead. Although, the majority of the organisms were not dead at the first observation in all UV intensities and we observed that they moved slowly and in an uncoordinated form.

The experiments showed on fig. 4 (A) and fig. 4 (B) were performed with adults with 22 days. The same tests were carried out with 10-12 days juveniles and the same results were obtained (data not shown).

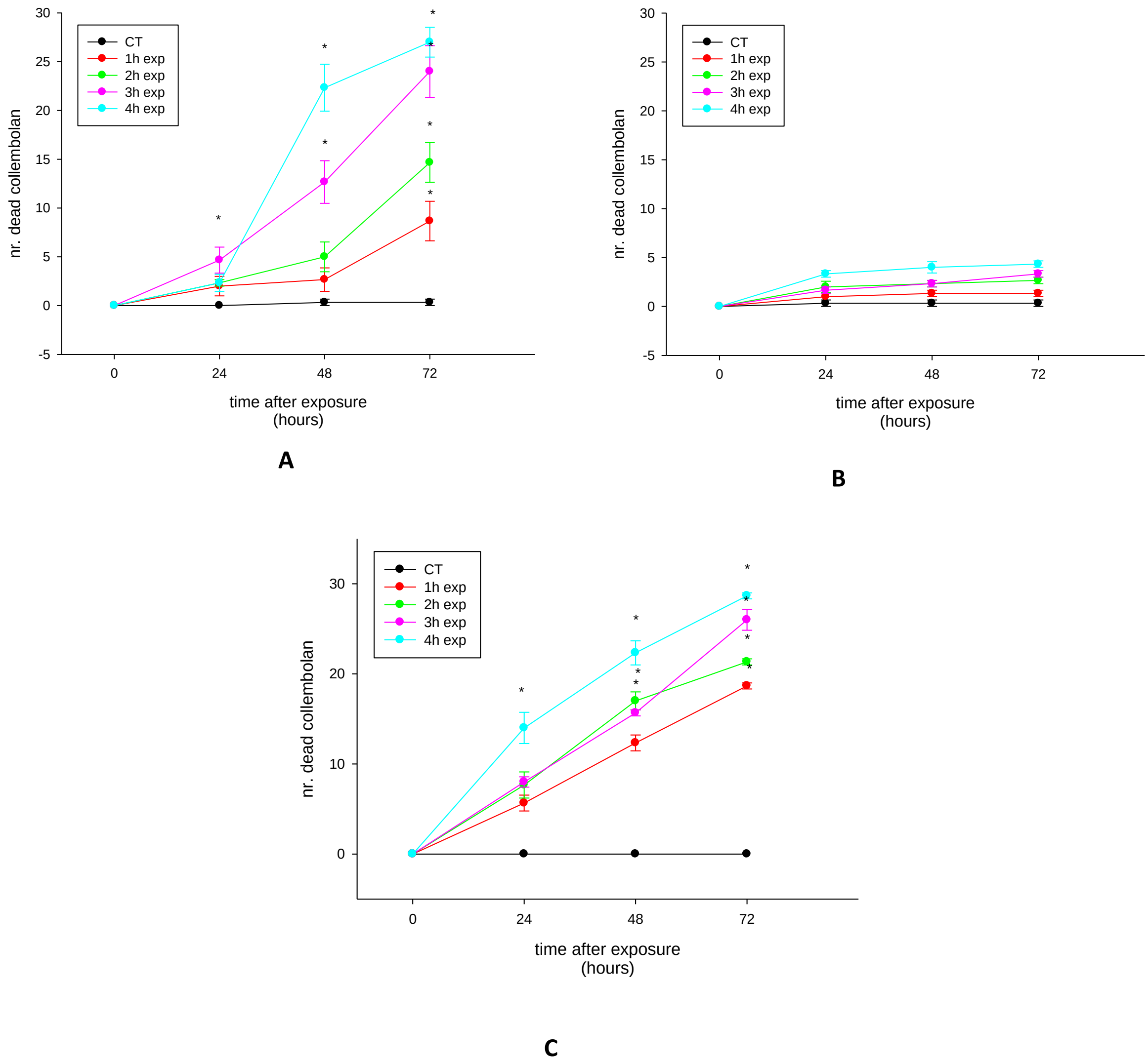


Figure 4 – Mortality of *Folsomia candida* after 24, 48 and 72h of direct UV radiation exposure on plaster (A), Lufa 2.2 soil (B) and compacted Lufa 2.2 soil (C). Four different intensities were studied, according with the table 1. (*p ≤ 0,05 Dunnett's test, compared with the control (no exposure to UV radiation).

Once again, we observe the protection that soil gives to organisms, preventing the direct and harmful influence of UV radiation. To evaluate in a more realistic scenario that a good physical condition of soil is also of major importance, we exposed also organisms to a compacted soil. We found that soil compaction is one of the most important factors that we have to take into account when studying the effects of UV radiation on soil organisms. The response of the organisms was similar to the one upon plaster exposure (Fig. 4 (C)). Compaction is regarded as one of the most serious environmental problems caused by conventional agriculture, it is the most difficult type of degradation to locate and rationalize, principally as it can show no evident marks on the soil surface (Hamza and Anderson 2005). It has been shown that compaction can alter the soil structure and were quantified how changes in habitable pore space affected the abundance of some collembolan species (Larsen et al. 2004). Besides of the greater exposure to UV radiation, experiments from field showed negative correlations between collembolan abundance and compaction (Larsen et al. 2004; Dittmer and Schrader 2000; Heisler and Kaiser 1995).

Beyond the influence of UV radiation on survival, we observed an influence on eggs directly exposed to UV. Figure 5 represents the number of eggs hatched after directly exposure to UV radiation. We can observe that when eggs are directly exposed to UV radiation, in plaster, we have significant differences between all the treatments, comparing to the control, where a decrease of number of hatched eggs was registered with the increase of UV radiation will induce. Comparing to eggs laid in real soil, where they did not receive direct influence of UV radiation, we did not find significant differences between the different intensities of UV, comparing with the control (Fig. 6).

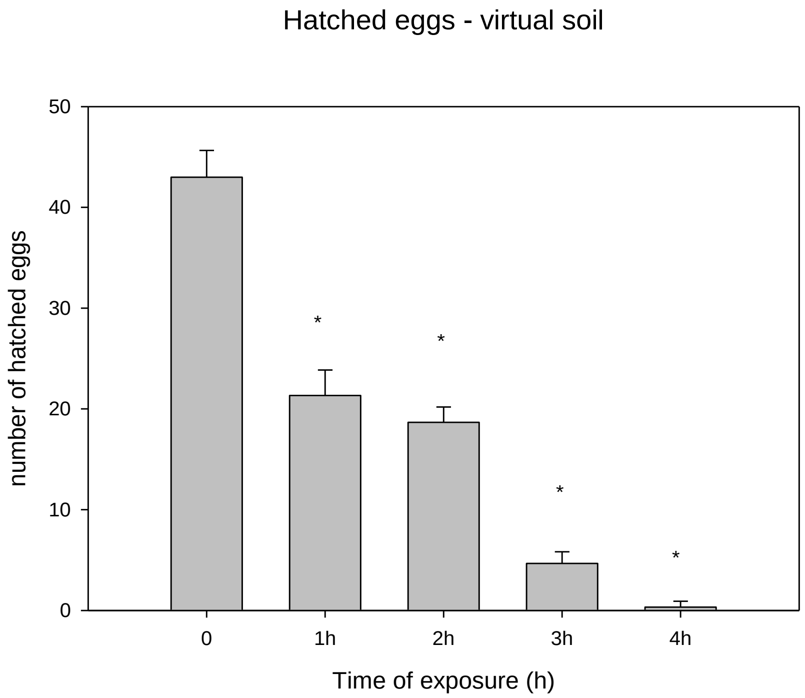


Figure 5 – Number of hatched eggs of *Folsomia candida* directly exposed for 1, 2, 3 and 4 hours to UV radiation in plaster. (*p ≤ 0,05 Dunnett's test, compared with the control ( no exposure to UV radiation – 0 on graph))

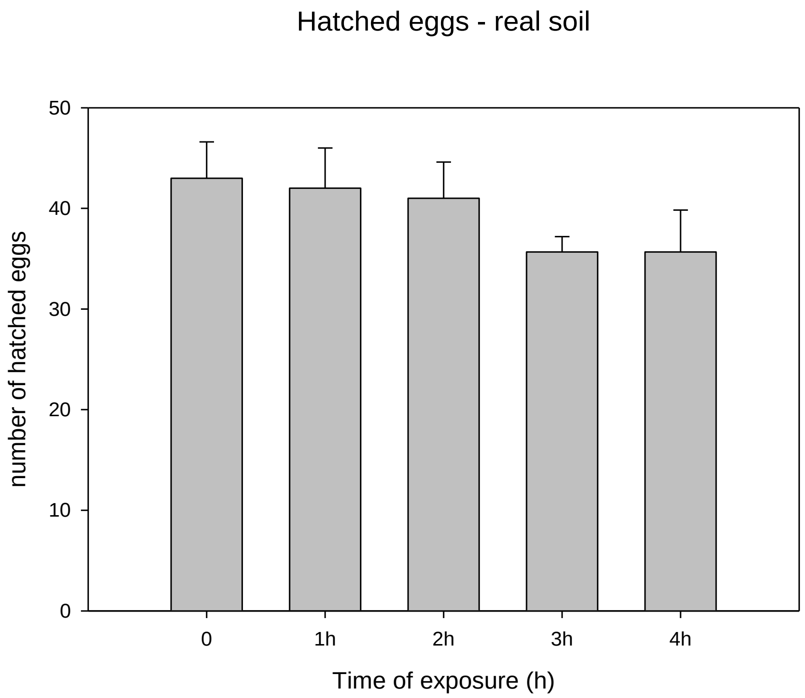


Figure 6 – Number of hatched eggs directly exposed to direct UV radiation in real soil.

To test the influence of UV radiation on the reproductive capacity of *F. candida*, we exposed adult organisms to UV radiation in soil and afterwards animals were removed to regular culture conditions and it was recorded the number of eggs produced. Surprisingly, we found that when the radiation was greater, more eggs were produced by *F. candida* as we can see on Figure 7.

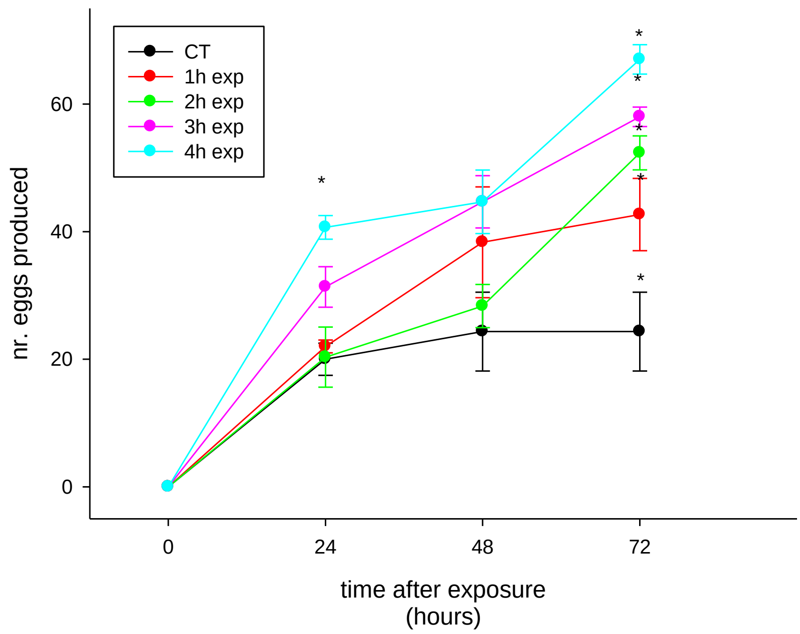


Figure 7– Number of eggs produced by adults of *Folsomia candida* that were previously exposed to UV radiation in Lufa 2.2 soil. (*p ≤ 0,05 Dunnett's test, compared with the control ( no exposition to UV radiation))

Although there was no effect directly on their survival upon exposure on soil, suggesting that they were able to escape from UV radiation, a post-effect could be observed on their reproduction effort. This increase of reproduction under great situations of stress suggests that this organism can act like r – strategist in specific situations. This kind of behavior is present in almost all ecosystems and can be described as early age of maturity, large number of young produced, semelparity, no parental care and a large reproductive effort (Parry 1981; MacArthur and Wilson 1967). In other words, the organism invests all its effort to produce new juveniles when under great stress.

According to our research, no experiments were published exposing *Folsomia candida* to UV radiation. Although, is generally held that terrestrial animals are well protected from the damage caused by UV radiation due to the presence of their exoskeletons, coats or plumage, and/or because they have effective mechanisms for repairing UV damage (Paul and Gwynn-Jones 2003). However, research with amphibians has shown that the embryos and larvae of many amphibians are vulnerable to solar UV-B radiation and adults can be vulnerable to sub lethal UV-B effects (Kiesecker et al. 2001; Ankley et al. 2002; Kats et al. 2000).

We can conclude that UV radiation influence the survival and reproduction of the organisms. Even the eggs directly exposed can suffer damages that can prevent the production of new organisms and may influence the proliferation of species. The effects of the UV radiation may be delayed after exposure with an increasing pattern of damage for 72h when all the organisms were dead on plaster. For more information about the effects that UV causes to collembolans, a genetic approach must be considered, to compare with ecotoxicological endpoints. The protection that soil gives to organisms is one important factor that we have to understand when perform new UV tests on soil organisms.

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# Chapter 3

# 3. Combined exposure of carbaryl and the natural stressors soil moisture and UV radiation to *Folsomia candida.*

## 3.1 – Abstract

The accuracy of ecotoxicological studies is greater when the combined effects of mixtures of chemicals and/or interactions between chemicals and natural stressors are considered. Nowadays, studying tolerance limits in organisms exposed to climatic variations is an important tool. But the presence of chemical compounds can alter these limits, leading to an unexpected and possibly dangerous situation and the chemical toxicity can be altered by abiotic factors too. Carbaryl is one of the most used, broad-spectrum pesticides, and in this study, the soil-dwelling collembolan *Folsomia candida* was exposed to combined stressors: carbaryl and soil moisture and carbaryl and UV radiation. Statistical analyses of the data set were performed using the MixTox tool and were based on the conceptual model of Independent Action (IA) and possible deviations to synergism or antagonism, dose-ratio or dose-level response patterns. Antagonistic responses were obtained when organisms were exposed to carbaryl at drought conditions and under flood situations, a synergistic pattern was observed when carbaryl was the dominant stressor and antagonism when moisture was dominant for the reproduction effort; regarding survival the opposite occurred. A synergistic pattern was observed when carbaryl and UV radiation were combined and as this way, we proved that abiotic factors can alter the toxicity of chemical compounds.

## 3.2 – Introduction

For several decades, chemicals have been released on the ecosystems, leading to a reduction on biodiversity and contaminating the habitats of numerous species. Anthropogenic activities have several impacts on ecosystem sustainability and consequently can disrupt the balance between biota and environment (Lima et al. 2011; Loureiro et al. 2009).

Ecotoxicological risk assessment is characterized by studies performed in laboratory conditions where some ecological relevant organisms are exposed to a battery of tests, in order to predict the toxicity of a single compound (Holmstrup et al. 2010). However, these tests are carried out under optimal conditions (moisture, pH, temperature, photoperiod, etc.) and do not represent the real conditions in the field. In their natural environment, organisms are confronted with a variety of stressors, not only chemicals but also natural stressors, leading to a constant exposure to mixtures of pollutants and fluctuations of abiotic exposure conditions (Ferreira et al. 2010). Those additional environmental stressors may alter the effects of chemical contaminants in comparison to the laboratorial conditions, where organisms are exposed to optimal conditions (Holmstrup et al. 2010). Thus, response of soil fauna and flora to chemicals is dependent on the environmental conditions under which they are exposed. However, despite the effort of the scientific community to understand the complexity of environmental mixtures, about 95% of the resources in toxicology were devoted to studies on single chemicals. But the interest for that area is growing between communities, recognizing that exposure to chemical mixtures must be an integral part of protecting public health (Groten 2000), as the combined effects of chemicals and natural stressors presents in nature. If we do not regard the interactions between chemicals and natural stressors, we cannot extrapolate confidently the laboratory results to effects on individuals and populations in the field (Pestana et al. 2009).

Theoretical models based on the two non-interaction concepts for prediction of mixture toxicity, concentration addition (CA) and independent action (IA) are used to predict the expected toxicity of mixtures form the knowledge of the individual toxicity of the mixture compounds (Jonker et al. 2005). Such models have also been transposed to environmental research, in order to predict the effects of chemical mixtures or combinations between natural and chemical stressors (Lima et al. 2011; Ferreira et al. 2010; Ribeiro et al. 2011).

The CA model is based on the idea that chemicals with the same mode of action will act additively, in other words, the summation of the relative toxicities of the individual components in mixture (Ferreira et al. 2008; Loureiro et al. 2009; Jonker et al. 2005). Moreover, the IA model relates to independent modes of action of the mixture components, with no interaction between individual compounds, during exposure, uptake and toxic action (Ferreira et al. 2008).

In real scenarios, both mixtures and combined effects of chemicals and natural stressors may interact and some deviations from the models can also be tested (The IA model was used due to the different mode of action of our stressors). Synergistic patterns – the mixture is more toxic than expected from the toxicity of single compounds or enhancing the probability of effect of one another - and antagonistic patterns - mixture toxicity is lower than expected from the toxicities of single compounds – can be observed (Ferreira et al. 2010; Groten 2000; Ferreira et al. 2008; LIMA et al. 2010; Loureiro et al. 2009). Furthermore, a dose-level response - high dose levels can cause different effects than low dose levels - and a dose ratio response - toxicity of the mixture or combination is mainly caused by one of the components - can be presents.

The aim of this study was to evaluate if natural stressors could change the potential toxicity of single chemical compounds, when combined. For that the soil-dwelling *Folsomia candida* was exposed to carbaryl and two natural stressors – UV radiation and flood and drought conditions and its toxicity evaluated after their combined exposure.

## 3.3 – Material and methods

### 3.3.1 - Test-chemical and test-organisms

Carbaryl (CAS No 63-25-2) was purchased from Sigma-Aldrich Ltd. (99.8% purity). The stock solution was prepared using acetone due to its low solubility in water, and applied to pre-moistened soil 24 hours before the start of the experience for acetone evaporation.

All experiments were carried out using the natural standard soil LUFA 2.2 from Speyer, Germany (Lokke and 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 soil-dwelling organism *Folsomia candida* was kept in laboratory cultures and maintained at dark under constant temperature of 20 ± 2$^{o}$C. The collembolans were maintained in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 9:1. Once a week, granulated dry yeast was added as food in small amounts on two sides of the culture.

### 3.3.2 - Combined exposures

#### 3.3.2.1 – Carbaryl and drought/ flood situations

A chronic test was performed using contaminated soil with carbaryl (1, 4, 7 mg/Kg) and for each concentration of carbaryl, soil moisture was adjusted to 10, 20, 40, 60 (control group), 80, 120% of WHC, simulating drought and flood situations. 10 juveniles with 10 – 12 days were exposed to the above situations, following standard procedure with adaptations (ISO 1999; OECD 2009). After 28 days, the number of adults and juveniles was counted to assess the mortality and reproduction effort of the organisms upon stress of combined exposure of these two stressors.

In order to proceed with statistical analysis using the Mixtox tool and apply the concepts used for combined stressors, we made previously some transformations on the data sets (Jonker et al. 2005). All the data obtained for drought test was transformed, so that with the increase of stress, a diminished response was obtained. For that, the data was transformed as Y=60 – X, where X was the real % of WHC used on that treatment and 60 corresponded to the control situation. On that way, the 10% of WHC (highest dose of stress) was changed to 50 % WHC (60-10) and the 60% WHC (lowest dose of stress) was changed to 0 % WHC.

### 3.3.2.2 – Carbaryl and UV radiation

All the UV exposures were performed inside a room where the temperature (20 ± 2 $^{o}$C) and photoperiod (16/8 h) were controlled. The UV radiation was provided by a UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) and it was placed 30 cm above the vials and clear cellulose acetate sheets (0,003mm) were used to cut-off UV-C range wavelengths. These cellulose acetate sheets were previously UV irradiated for 12 h before it was used in the experiments to minimize differences in UV radiation intensity that passes by the cellulose acetate sheets. Table 1 presents the times of exposure to the UV radiation and the equivalent doses of each time of exposure, in Joules/m$^2$. To assess the real dose of UV that organisms were exposed to, the time of exposure and the intensity of the radiation were taken into account. UV dose (Joules/m$^2$) was obtained by the following equation:

$$UV\ dose\ (\mathrm{Joules/m^2}) = \frac{\mathrm{Intensity\ (mW.\,m^{-2}.\,nm^{-1}) \times Time\ of\ exposure\ (s)}}{1000}$$

In order to study the combined effects of carbaryl and UV radiation, adults were exposed to different UV radiation – during 1, 2, 3 and 4 hours (see table 2) - and simultaneously to several concentrations of carbaryl (1, 4, 7 mg/Kg); a control, without any contamination, was also run. After the simultaneously exposure of carbaryl and UV radiation, the organisms were removed and adults (22 days) and Juveniles (10-12 days) were allowed to lay eggs on plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 9:1 and the number of produced eggs was counted.

The same transformation used for the drought experiment was carried out for the combined exposure of carbaryl and UV radiation. In order to obtain a situation where the highest stress had the lowest response, data was transformed to (highest value of the response) – (real response). That way, at highest doses of stress, the number of eggs laid was lower than in control situations.

### 3.3.2.3 – Statistical analysis

One way (ANOVA), followed by Dunnett's test, was used to analyse differences between control and treatments. Differences between control and solvent control were analyzed using a t-test or a Manne Whitney Rank test when normality failed. The software package SigmaStat was used for that analysis. $EC_{50}$ and $LC_{50}$ values were calculated using a sigmoidal (logistic, 3 parameter) equation.

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 (Jonker et al. 2005), thus comparing the observed toxicity and the expected toxicity of the stressors and also to calculate possible deviations from the two reference models. These deviations are given by quantitative parameters (*a* and *b*) expressing synergism or antagonism (Table 3). The choice of IA to fit the data set from these combinations was mainly based on the assumption of different modes of action of carbaryl and UV radiation, which means that they act in different target sites on the biological systems and/or follow different pathways to cause any observed effect.

**Table 2** - Time of exposure to ultraviolet radiation (h) and its correspondent UV intensities (mW $m^{-2}$ $nm^{-1}$) transmitted by the UV lamp and equivalent UV doses for each time interval.

| Time of exposure (h) | Uv intensity (mW $m^{-2}$ $nm^{-1}$) | UV dose (Joules/$m^2$ ) |
|---|---|---|
| **1** | 4108.3 | 14790 |
| **2** | 3589.1 | 25841.6 |
| **3** | 3690 | 39852.1 |
| **4** | 3485.7 | 50194.4 |

**Table 3** - Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation patterns from concentration addition (CA) and independent action (IA). Adapted from Jonker et al. (2005).

<table>
<tr><th>Deviation pattern</th><th>Parameter a (CA and IA)</th><th>Parameter b (CA)</th><th>Parameter b (IA)</th></tr>
<tr><td>Synergism / Antagonism (S/A)</td><td>$a > 0$ : antagonism<br>$a < 0$ : synergism</td><td colspan="2"></td></tr>
<tr><td rowspan="2">Dose -racio dependent (DR)</td><td>$a > 0$ : antagonism except for those mixture ratios where negative b value indicate synergism</td><td colspan="2">$b_i > 0$ antagonism where the toxicity of the mixture is caused mainly by toxicant i</td></tr>
<tr><td>$a < 0$ : synergism except for those mixture ratios where positive b value indicate antagonism</td><td colspan="2">$b_i < 0$: synergism where the toxicity of the mixture is caused mainly by toxicant $i$</td></tr>
<tr><td rowspan="4">Dose-level dependent (DL)</td><td rowspan="2">$a > 0$: antagonism low dose level and pasynergism high dose level</td><td>$b_{DL} > 1$: change at lower $EC_{50}$ level</td><td>$b_{DL} > 2$: change at lower $EC_{50}$ level</td></tr>
<tr><td>$b_{DL} = 1$: change at $EC_{50}$ level</td><td>$b_{DL} = 2$: change at $EC_{50}$ level</td></tr>
<tr><td rowspan="2">$a < 0$: synergism low dose level and antagonism high dose level</td><td>$0 < b_{DL} < 1$: change at higher $EC_{50}$ level</td><td>$1 < b_{DL} < 2$: change at higher $EC_{50}$ level</td></tr>
<tr><td>$b_{DL} < 1$: No change but the magnitude of S/A is DL dependent</td><td>$b_{DL} < 1$: No change but the magnitude of S/A is effect level dependent</td></tr>
</table>

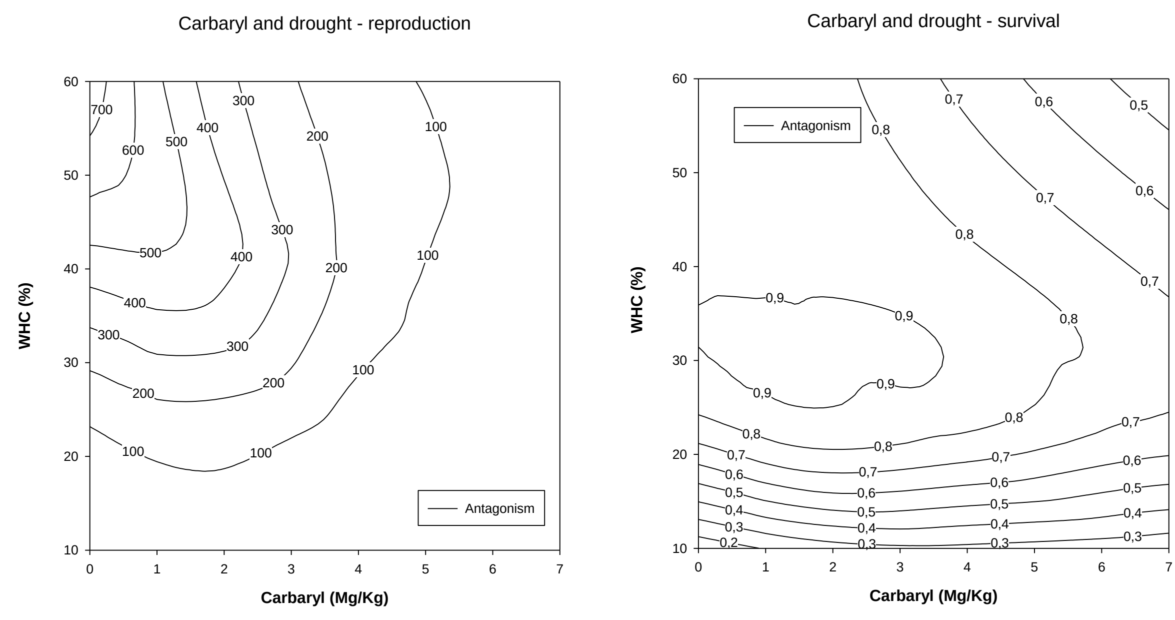
## 3.4 – Results and discussion

Comparing the $EC_{50}$ and $LC_{50}$ values of the studies presented on chapter 1 with the $EC_{50}$ and $LC_{50}$ of this study, there were some changes on toxicity. In the previous experiment $EC_{50}$ and $LC_{50}$ were 5.1 and 5.4 mg/Kg for the single exposure of carbaryl, respectively. However, the $EC_{50}$ of the control of the test of carbaryl at flood situation (60% WHC) was 1.91 mg/Kg, and at drought situation was 1.6 mg/Kg. In terms of mortality, the $LC_{50}$ of the control for carbaryl on flood situation was 6.4 mg/Kg and at drought stress was 6.4 mg/Kg too, which was more similar with the previous study (chapter 1). Considering the combined effects of carbaryl and UV radiation, the $EC_{50}$ for the control for the production of eggs the value was 6.3 mg/Kg. Although the parameter used in both studies was different (nº of juveniles in chapter 1 and nº of eggs produced in this study), they reflect the reproductive effort of collembolan and can be considered similar.

### 3.4.1 – Carbaryl and flood/drought

In order to understand the response of *F. candida* 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. There were effects induced by changes in soil moisture on the survival and reproduction of collembolans, changes in soil water content induced changes in the toxicity of carbaryl, suggesting a potential antagonism at drought situations, in terms of reproduction ($p<0.05$; SS=15457.6; $r^2$=0.980; a=2.39) and survival ($p<0.05$; SS=11; $r^2$=0.801; a=3.36) (Fig.10). This antagonistic pattern was not expected at drought situations, according to studies with

*Eisenia andrei*, revealing a potential synergism between carbaryl and drought situation (Lima et al. 2011). Other studies suggest that soils with low content of water can interact synergistically with chemicals (Holmstrup et al. 2007) and this can be explained by dehydration that occur on the organism, reducing the volume of water within the organism, leading to increasing concentration of the chemical and the risk for toxic damage to occur (Holmstrup et al. 1998). According with some studies (Tsogas et al. 2006; Rick A. Relyea and Nathan Mills 2001), microbial activity is very important in carbaryl decomposition. That microbial community is not present in such large numbers in dry soils leading to maintenance of carbaryl in soil, being available for soil organisms. On the other hand, the main exposure route of chemicals to collembolan is the soil pore water. In this case, it may have happened that the concentration of carbaryl on the soil pore water has decreased, decreasing therefore exposure and toxicity.




**A** **B**

**Figure 10** – A) Combination of drought stress and carbaryl, on reproduction, showing the showing the antagonistic pattern (SS=15457.6; $r^2$=0.980; a=2.39). B) Combination of flood stress and carbaryl, on survival, showing the showing the antagonistic pattern (SS=11; $r^2$=0.801; a=3.36)

At flood situation, reproduction on combined effects of flood conditions and carbaryl in *F. candida* caused a “dose ratio” deviation from the IA model ($p < 0.05$) ($SS=1917.28$; $r2=0.998$; $a= 20.288$; $b= -58.75$) (Fig. 11). An antagonistic pattern was observed when flood stress was dominant, whereas synergism occurred when carbaryl was the dominant stressor (i.e. high doses of carbaryl and low flood stress). Analyzing the survival parameter, combined effects of flood conditions and carbaryl, also caused a “dose ratio” deviation from the IA model ($SS=15.18$; $r2=0.854$; $a= -12.33$; $b= 6.64$.) ($p < 0.05$) but a synergistic pattern was observed when flood stress was dominant, whereas antagonism occurred when carbaryl was dominant. This opposite patterns on different endpoints may be difficult to explain. One could expect that the presence of more water on the soil pores would lead to a decrease on the concentration of carbaryl (by dilution) and therefore inducing a decrease on its toxicity. But this hypothesis does become invalid when looking at survival as a parameter.

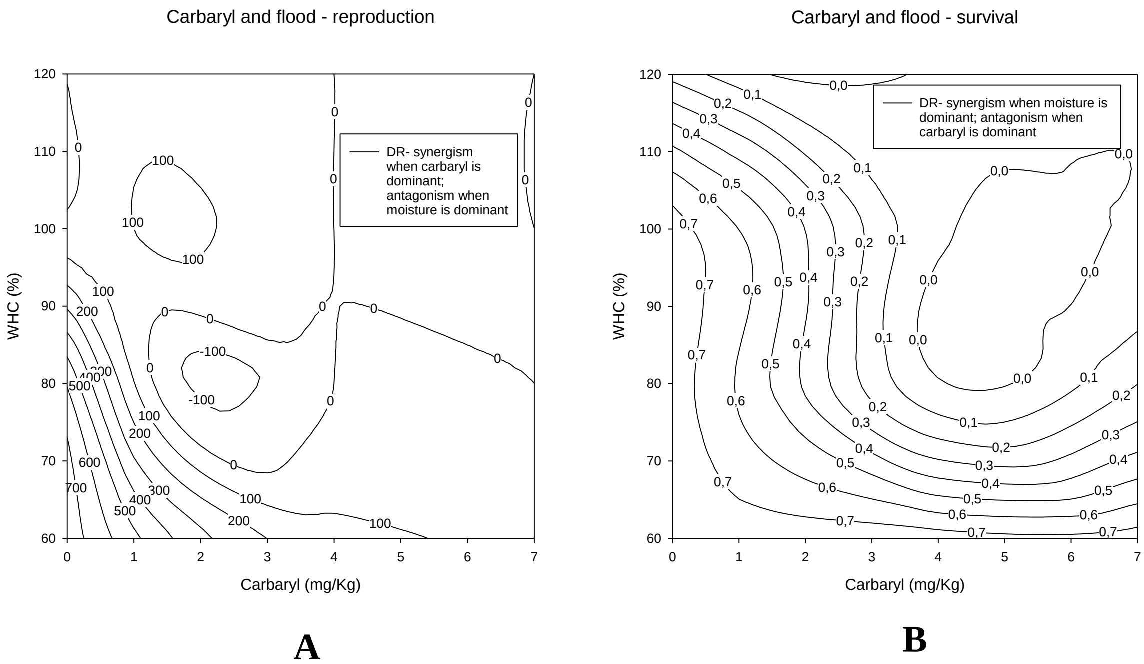



**Figure 11** – A) Combination of flood stress and carbaryl, on reproduction, showing the showing the dose-ratio deviations from the IA conceptual model (SS=1917.28; $r^2$=0.998; a= 20.288; b= -58.75). B) Combination of flood stress and carbaryl, on survival, showing the showing the dose-ratio deviations from the IA conceptual model (SS=15.18; $r^2$=0.854; a= -12.33; b= 6.64)

## 3.4.2 – Carbaryl and UV radiation

Regarding the combined effects of UV radiation and carbaryl, a synergistic pattern was observed (SS=751.47; $r^2$=0.81;5 a= -5.33) (Fig.12). These results must be considered and take into account, but due to the capacity of the organisms to escape from the surface of the soil can mask the obtained results. However, as we concluded on chapter 2, the organisms on real soil are affected by UV radiation, with a higher production of eggs. Nevertheless, a response was obtained from the organisms to the combined exposure and a greater response was expected if the test would be on plaster.

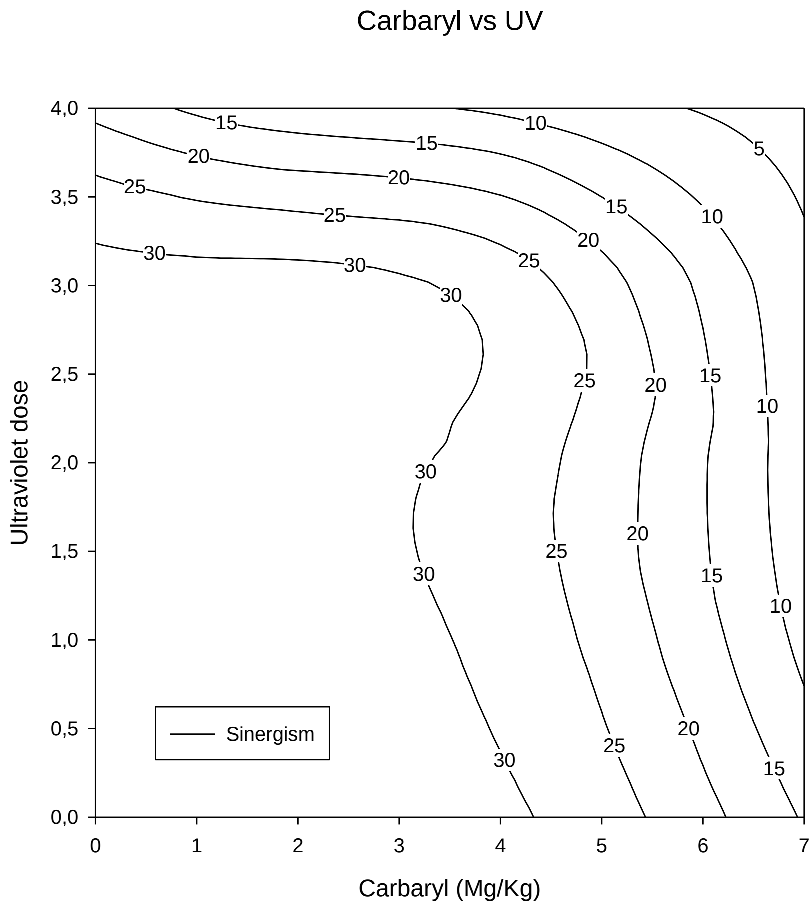



**Figure 12** Dose–response relationship of reproduction of *Folsomia candida* exposed to the combination of ultraviolet radiation and carbaryl, showing the synergistic pattern (SS=751.47; $r^2$=0.81; a= -5.33).

One question that can arise from this study is the potential capacity of UV radiation to degrade chemicals. In our experiments we do not have this kind of information on chemical analysis, but these results showed the synergistic relation between carbaryl and UV radiation. Organisms exposed simultaneously to contaminated soil and UV light laid more eggs than expected from the single exposures of each stressor. Comparing the $EC_{50}$ values of the control (different carbaryl concentrations and absence of UV radiation) and combined situation (different carbaryl concentrations and 4h of UV radiation), the EC50 values were 6.3 mg/Kg and 3.9 mg/Kg, respectively. So, we can observe that a lower concentration of carbaryl is necessary to induces a response when the UV radiation factor is present, comparing to a control situation.

As conclusion, we believe that the interaction between natural and chemical stressors should be considered for the risk assessment of chemicals. Moreover the seasonal pattern of application of pesticides, associated with higher temperatures, drought conditions and possible UV radiation peaks may increase the effects of pesticides on non-target organisms and function as synergistic factors. On the other hand, the combined effects between low temperatures and flood scenarios and released chemicals on environment cannot be disregarded.

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# Chapter 4

# 4- General Discussion and Conclusion

Global warming has become a source of awareness regarding the potential deleterious effects of extreme abiotic factors (e.g., temperature, moisture levels and UV increments) and also their influence on chemicals toxicity (Ferreira et al., 2010). Due to its favorable characteristics, the use of invertebrates for toxicity tests is an important tool to evaluate and predict the effect of single and combined stressors on ecosystems. Within this, the use of *Folsomia candida* as organism for ecotoxicological tests was a right choice due to its importance on ecosystems and favorable conditions of work. More organisms must be tested in order to predict real effect on real scenario (Fountain and Hopkin, 2005, Hopkins, 1997). Within this study it is highlighted the importance of the combined exposure tests, because single exposure tests limit the extrapolation of laboratory results to effects on populations in the field (Pestana et al., 2009).

Our work is based on chronic tests, testing survival and reproduction, with some adaptations as it done for the evaluation on the effects of UV radiation.

Our results showed that *Folsomia candida*, as expected, is sensible to carbaryl, decreasing its survival and reproduction effort with increasing concentrations of chemical, with an $EC_{50}$ of 5.1 mg/Kg of soil. That is a lower $EC_{50}$, when comparing for example, with studies with earthworms were the $EC_{50}$ was 53.3 mg/Kg.

The response of this species to different abiotic conditions is varied. Soil moisture was studied as collembolans are soil-dwelling organisms, and the presence/absence of water in soil has a crucial importance on their behavior and physiological balance. Soil moisture influences survival and reproduction of collembolans, showing that *Folsomia candida* is not well adapted to extreme scenarios of drought and flood. This idea has been reported in few studies, leading to a 60% WHC as optimal conditions for the organism (Bayley and Holmstrup, 1999, Bayley et al., 2001). Soil compaction is one of the major problems that ewe face today, and that factor is very important for soil organisms. Our findings suggested that the soil compaction influences directly and indirectly the life traits of the organism, because soil compaction obligates a soil dwelling organism to live in the surface of the soil, where it is susceptible to several factors that can influence negatively the organism. *Folsomia candida* is much more sensitive to UV radiation when the compaction is present.

Very little information exists on direct effects of UV radiation on terrestrial invertebrates (Leinaas, 2002). Enhanced UV radiation affects structural and functional ecosystem parameters in direct and indirect ways (Verhoef et al., 2000) and have an important role on soil biota. Our results show that at extreme conditions, *Folsomia candida* is highly affected by this stressor, with mortality in all organisms at the highest UV dose. These findings are consistent with previous studies with other collembolan species (Verhoef et al., 2000). Our findings proved that the presence of high doses of UV radiation influences the reproductive behavior of collembolans. However, soil is a major factor of protection for the organisms towards UV radiation, conferring shelter for soil organisms. Altering soil structure by compaction, different responses were obtained when compared to non-compacted soil. In addition, there was an important outcome from the pre-exposure period to UV radiation, where an increase of laid eggs was observed. This is also an indication that although soil prevents acute effects induced by UV radiation (short term exposures), there was a change on egg deposition pattern upon low doses exposures.

The combined effects of chemical and natural stressors are mostly different than the sum of single effects of single stressors.  Our findings confirm the assumption that abiotic factor can alter the toxicity of chemical compounds (Ferreira et al., 2010, Holmstrup et al., 2010, Lima et al., 2011, Loureiro et al., 2009). The combined effects of different moisture situations and carbaryl follow an antagonistic pattern when drought stress was present for both studied endpoints, and a “dose ratio” deviation from the IA model was observed at flood stress and carbaryl. When organism is subject to carbaryl and UV radiation, a synergistic pattern was observed. So, we can prove that the abiotic factors may change the toxicity of chemicals, and mostly of the times, increasing its toxicity. Our findings showed a much lower $EC_{50}$ of a chemical when UV radiation is present in major intensities.

Furthermore, future experiments will be carried out with UV radiation and combined exposures, trying to go beyond the reproduction and survival patterns, analyzing the genetic damage that each single stressor caused to organism. After that, and knowing the genetic damage of combined exposures, we can relate effects at the cellular with those from individual level, and then transpose it also to the population level.

## 4.1 – References


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