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Soil salinization as a stress factor for soil fauna

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor José Paulo Filipe Afonso de Sousa (Universidade de Coimbra) e do Professora Doutora Ruth Maria de Oliveira Pereira (Universidade de Aveiro)

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Table of contents

Chapter III - The combined effect of NaCl and a commonly used pesticide (Lambda-cyalothrin) in the reproduction of *Folsomia candida*

Chapter IV – General Discussion

Resumo

A existência de condições desfavoráveis no solo (p.e. um agente químico) pode influenciar a presença de organismos de solo ou parâmetros do ciclo de vida tais como a reprodução da fauna do solo num local específico. Logo, a resposta de evitamento e o sucesso reprodutivo de organismos em locais contaminados pode ser utilizada como uma primeira ferramenta de avaliação de risco ecológico, já que respostas negativas de evitamento ou reprodução significam que deverá haver algum contaminante no solo testado. Neste trabalho abordou-se o problema da salinização dos solos, numa tentativa de avaliar o stress causado pela mesma nos invertebrados de solo, e o seu efeito conjunto com diferentes percentagens de matéria orgânica no solo, assim como com um pesticida frequentemente utilizado (lambda-cyalotrina). O objectivo final deste projecto era desenvolver modelos utilizando Modelação Linear Generalizada (GLM) que permitam prever valores de evitamento e reprodução quando um solo está contaminado com NaCl, e no caso específico de se encontrar também contaminado com o pesticida utilizado. Para alcançar o objectivo utilizaram-se três espécies de invertebrados de solo nos testes: *Eisenia andrei*, *Folsomia candida* e *Enchytraeus crypticus*. *Folsomia candida* e *Eisenia andrei* foram utilizados para os testes de evitamento, enquanto que para os testes de reprodução foram utilizados *Folsomia candida* e *Enchytraeus crypticus*. Os resultados obtidos pelos testes mostram uma efeito negativo claro da salinidade quer na resposta de evitamento quer na reprodução, e uma influência negatica do pesticida na reprodução de *Folsomia candida*. Os modelos obtidos explicam a maior parte da variável de resposta e podem vir a ser ferramentas robustas para prever valores de reprodução e evitamento em solos salinos.

Abstract

The existence of unfavorable conditions in soil (e.g. a chemical stressor) may influence the presence of soil organisms or life cycle parameters such as reproduction of soil fauna in a particular site. Therefore avoidance response and reproductive output of organisms to contaminated sites can be used as an early screening assessment of ecological risk, since a negative response on avoidance or reproduction means that there must be some contaminant in the tested soil. In this work the problem of soil salinization is addressed, in an attempt to evaluate the stress caused by soil salinization to soil invertebrates, and its combined effect with different organic matter percentages in the soil and also with a commonly used pesticide (lambda-cyalothrin). The final goal of this project was to develop predictive models using Generalized Linear Modeling (GLMs) that would allow to calculate a predicted value for avoidance response and reproductive output when a soil is contaminated with NaCl, and in the specific case of contamination by both NaCl and the pesticide. To achieve this, three species of soil invertebrates were used in the tests: *Eisenia andrei*, *Folsomia candida* and *Enchytraeus crypticus*. *Folsomia candida* and *Eisenia andrei* were used for the avoidance tests while for the reproduction tests *Folsomia candida* and *Enchytraeus crypticus* were selected. The results provided by the tests show a clear negative effect of salinity in both avoidance behavior and reproduction output, and a negative influence of the pesticide in the reproduction of *Folsomia candida.* The GLM models obtained explain most of the response variability and can become powerful tools for predicting avoidance and reproduction values in a saline soil.

Chapter I General Introduction

Chapter I – General Introduction

I.1 – Ecological Risk Assessment and the integration of avoidance and reproduction tests

In recent years Ecology has become a major science all around the world, with the growing concern about the preservation and sustainability of different ecosystems. The human activities have a very important economic role (e.g. industry, agriculture, fishing), and the influence of these activities in both terrestrial and marine ecosystems compel to a sustainable management of these systems. The most affected and contaminated sites are a result of anthropogenic activities, therefore the sustainability of these systems, in the case of this work, the soil systems, depends on a suitable management of the environmental problems. This implies the application of ecological risk assessment (ERA) procedures. The estimation of the effects of chemicals in the environment is a part of a risk assessment and it could be based on historical and ongoing (retrospective risk assessment) or on future activities (prospective risk assessment) (Weeks et al., 2004).

As proved by some authors (e.g., Natal da Luz, 2008) both avoidance and reproduction tests provide very reliable results as early screening assessments (avoidance tests) or tier 2 ecological risk assessment (reproduction tests), of the risk derived by soil contamination. Soil salinization, the problem addressed in this work, is no exception to this, thus the choice of avoidance and reproduction tests to assess the effect of NaCl as a stress factor for soil invertebrates (Owojori [2], 2008).

I.2 – Soil Salinization

The problem of accumulation of salts in soil comprises three different aspects: Salinization (Saline soils), which consists in the accumulation of soluble salts of Sodium, Magnesium and Calcium in the upper soil horizons; Sodification (Sodic soils), which consists in the increase on the content of exchangeable Sodium in the soil; and potential salinization, which is the risk of salt accumulation in the superficial horizons of the soil. There are also two types of salinization: Primary and Secondary salinization. Primary salinization is the salt accumulation by natural processes (physical or chemical weathering of saline deposits and aftermost transport), whereas secondary salinization is caused by inadequate human behavior (bad irrigation and draining techniques, abusive use of fertilizers and sewage sludges with high saline content (Tóth, 2008).

There are some alarming facts about soil salinization that suggest we should take this problem seriously into account. Salinization affects 1 to 3 million hectares in the E.U. (Szabolcs, 1996), mainly in the Mediterranean and Pannonian regions (100.000 ha in Portugal), it is a serious form of soil degradation and a cause of desertification, and together with sodification, it is one of the most threatening processes to the quality, hence functionality, of European soils (Van-Camp, 2004). The potential salinization and already affected areas are displayed in Figure 1. As shown the most affected areas are the Pannonian and the Mediterranean regions, and in Portugal, many coastal areas are greatly affected by salinization.

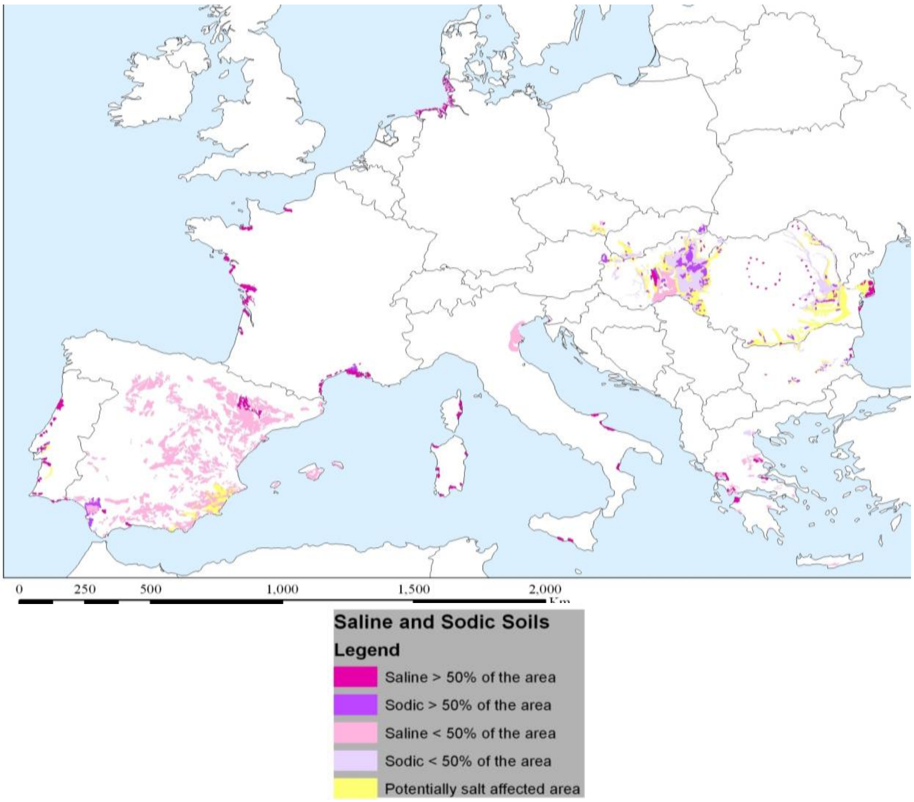

Figure 1 – Map of soil salinization in Europe

(eusoils.jrc.ec.europa.eu/library/themes/Salinization/)

The causes of salinization vary, being the irrigation of areas with low rainfall, high evapotraspiration rate and soil texture preventing the washing of the salts, the main factors that cause soil salinization, since the salt accumulates in the superficial soil layers(Van-Camp, 2004). In addition to these factors, irrigation with water containing a high saline content contributes for aggravating the problem. In coastal areas the problem can be caused by the rising of groundwater level due to climate changes and intrusion of salt water and the irrigation with water containing a high salt content (Van-Camp, 2004).

There are also factors that determine the accumulation of salts in the soil: the source of the salt; the transporting agents (water, wind); limited vertical or horizontal drainage conditions; the driving force for the movement of the solution [relief (surface runoff), hydraulic gradient (groundwater flow), suction (capillary transport) or concentration gradient (diffusion)]; negative water balance (evapotraspiration greater than precipitation) (Van-Camp, 2004).

The accumulation of salt in the soil has many negative effects to soil fertility, as it can prevent, limit, or disturb the normal metabolism of plants and soil organisms(Lippi et al. 2000; Yuan et al. 2007; Owojori [2] 2008). Salinization can cause cell plasmolysis and also have a toxic or caustic action (Robidoux, 2001). It can also decrease plant available water and cause plant stress and cause soil flocculation(Ramoliya et al. 2004; Kadukova and Kalogerakis 2007).

I.3 – Published studies concerning the effects of soil salinization (alone and in interaction with other contaminants) to soil invertebrates

Only recently soil salinization has become a concern regarding its effects on soil invertebrates. As a result, there are only a few published studies that evaluate the effects of salt in the life cycle parameters of soil organisms (Table I). These referenced studies showed that the reproduction of these organisms is greatly affected by salt from conductivities of 1030µS/cm and above (Owojori [2] 2008), which is far lower than the conductivities that classify a soil as safe for plants [4000µS/cm and above (Sumner 1995)] and below the 2000µS/cm that is considered to be the threshold below which a soil is classified as non saline for plants (Schoeneberger et al. 2002). Salinization can also have a cumulative effect with zinc in *Eisenia fetida*, increasing the bioavailability of the metal and increasing its toxic effect on the earthworms (Owojori [4], 2008). The interaction between salinity and copper was also studied but in this case there was apparently no addictive effect of salinity to the toxicity of copper (Owojori [5], 2008).

Table I – what is done until now concerning the organisms used in our tests and the effects of NaCl

Avoidance	Reproduction	Interactions with othe contaminants
In OECD and natural soils using *Eisenia fetida* and *Aporrectodea caligionsa* (Owojori [3], 2009)	In natural soils using *Eisenia fetida*, *Folsomia candida*, *Enchytraeus doerjesi* and *Aporrectodea caliginosa* (Owojori [2], 2009)	Interaction with zinc znd copper using *Eisenia fetida* (Owojori [1], 2008; Owojori [4], 2008)

I.4 – Objectives

The general purpose of this study was to assess the effects of salinity as a stress factor for soil fauna and to complete some missing information on the avoidance behavior of *Folsomia candida* and *Eisenia andrei* in response to NaCl contamination. The general objectives can be sub-divided as followed:

1. Assess the effect of salinity and organic matter percentage in the soil in the avoidance behavior of *Folsomia candida* and *Eisenia Andrei*;
2. Assess the effect of salinity and organic matter percentage in the soil in the reproduction of *Folsomia candida* and *Enchytraeus crypticus*;
3. Develop predictive models for the avoidance behavior of *Folsomia candida* and *Eisenia Andrei* and for the reproductive output of *Folsomia candida* and *Enchytraeus crypticus*;
4. Assess the combined effects of salinity and a commonly used agricultural pesticide (Lambda-cyalothrin) in the reproduction of *Folsomia candida*;
5. Develop a predictive model for the combined effect of salinity and Lambda-cyalothrin in the reproduction of *Folsomia candida*.

I.5 – General experimental procedures adopted

I.5.1 – Test organisms

In this study earthworms, enchytraeids and collembolans were used as test organisms. Earthworms are widely used test-organisms in terrestrial ecotoxicology, mostly because they play a crucial role in the soil ecosystem, but also due to their sensitivity to chemicals and their ability to be cultivated in the laboratory. They play an important role on the soil turnover and incorporation of decaying matter, enhancing decomposition and mineralization. Their burrowing activities allow the formation of soil aggregates, which improve the structure and water holding capacity of the soil and stimulates soil aeration and drainage (Karaca, 2011). The species used in this test was *Eisenia andrei* (Oligochaeta: Lumbricidae), due to his relatively short life cycle and handling ease.

Regarding collembolans, the species *Folsomia candida* (Collembola: Isotomidae) (used in this study) is the most used species in laboratory tests with this group of organisms. Usually collembolans have a particular importance in soil communities due to the high population densities usually present in all soil types (Hopkin, 1997). They act as catalysts of litter fragmentation and organic matter decomposition, carrying an important role in the soil system. The collembolan *Folsomia candida* is a blind and non-pigmented species with highly sensible antennas which gives it the ability to detect contaminants in the environment (Hopkin, 1997). The most characteristic in its morphology is a highly developed furca in the posterior region of the abdomen that renders it great locomotive capacities which provides a good avoidance response to adverse conditions. These characteristics allied to the fact that Folsomia candida is easy to maintain in the laboratory and has a short generation time, makes it a good species for ecotoxicological tests (Domene et al, 2011).

As for enchytraeids, the model species used in our tests was *Enchytraeus crypticus (Oligochaeta: Enchytraeidae).* Enchytraeids have a similar ecological role as earthworms and are also easy to keep and grow in laboratory conditions. They are also highly sensitive to chemicals and as a result they were chosen to perform one of the reproduction tests (Jänsch et al, 2005, Chelinho et al, 2011).

I.5.2 – Experiments conducted

In order to achieve the first objective, two-chamber laboratory avoidance tests with earthworms and collembolans were performed using OECD soil with 1% and 5% of organic matter (peat) and four different NaCl concentrations (0, 1, 2 and 4 g/Kg). All possible combinations were tested in order to verify the avoidance responses of these organisms and how they were correlated with NaCl, organic matter, and the interaction between both factors.

Reproduction tests were also performed during this phase of the work, to accomplish the second objective,(in this case with collembolan and enchytraeids), using OECD soil with 1%, 2.5% and 5% organic matter (peat) and the same four different NaCl concentrations (0, 1, 2, 4 g/Kg). These tests were performed to evaluate the reproductive output of these species in the presence of different salt contents and also different organic matter percentages and the interaction between these two factors.

For the final part of the work (and to achieve the fourth objective) we intended to assess the combined effects of salinization and a commonly used agricultural pesticide (Lambda-cyalothrin) in the reproduction of Collembolans. Therefore, reproduction tests with seven pesticide concentrations (0, 0.1, 0.25, 0.65, 1.5, 4 and 10 mg/Kg) and 3 NaCl concentrations (0, 1 and 2 g/Kg) were performed in OECD soil with 5% organic matter. All possible combinations (total of 21) were tested to evaluate the effect of the pesticide in the reproduction of collembolans according to different levels of NaCl.

The results obtained with all these tests were used to generate predictive models for each of the test parameters evaluated using Generalized Linear Modeling (GLM), in order to accomplish objectives 3 and 5.

I.6 – Thesis structure

This dissertation is organized in 4 chapters, including this one. Chapters II and III are based on papers under preparation and attain to accomplish the objectives of this work. Chapter II attains objectives 1, 2 and 3 and chapter III attains the objectives 4 and 5. Chapter IV includes a general discussion of the results obtained in chapters II and III.

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Chapter II Avoidance and reproduction tests with *Folsomia candida*, *Eisenia andrei* and *Enchytraeus crypticus* to access the effects of soil salinization

II - Avoidance and reproduction tests with *Folsomia candida*, *Eisenia andrei* and *Enchytraeus crypticus* to access the effects of soil salinization

II.1 Abstract

Addressing the growing problem of soil salinization in Mediterranean and Pannonian areas we ran a series of avoidance and reproduction tests with three different species (*F. candida*, *E. Andrei* and *E. crypticus*). The objective of these tests was to see how salinity affects the avoidance and reproductive behaviors of these species (avoidance of *F. candida* and *E. andrei* and reproduction of *F. candida* and *E. crypticus*), and also to evaluate how soil organic matter content would influence these responses. With the results we wanted to build predictive models using Generalized Linear Modeling, that would allow us no only to predict these responses in the presence of soil salinization, but also to be used to remove the effect of NaCl as a confounding factor in tests with natural soils with other contaminants. The results showed a clear avoidance behavior of *F. candida* and *E. andrei* to increasingly higher NaCl contents and also a reduction in the reproductive output of *F. candida* and *E. crypticus* as an effect of NaCl, with no reproduction in the highest NaCl content except for one combination with *E. crypticus*. The organic matter content of the soils did not have a big influence in the results, showing that NaCl is the major stressor in these cases. The models obtained for avoidance tests were less accurate than for reproduction tests, being the best model the one for the reproduction of collembolan , explaining 77% of the results, suggesting that collembolan reproduction is the most reliable test among these you should use if you want to assess soil salinization.

II.2 Introduction

Soil salinization has been a growing problem in recent years mainly in the Pannonian and Mediterranean regions, representing a serious form of soil degradation and desertification (Tóth, 2008).

Soil salinization is, together with sodification, one of the most threatening processes to the quality, hence the functionality of European soils. These phenomena are usually related to irrigated areas with low rainfall, high evapotraspiration rates or soil texture that prevents the washing of the salts, which accumulate in the superficial soil layers (Sumner, 1995). In coastal areas the irrigation with water with high saline content contributes to aggravate the problem (Tóth, 2008).

To the date of this work, not many studies have been conducted regarding the effects of soil salinization in soil invertebrates (only the recent work of Owojori and collaborators). The avoidance response of *Eisenia Andrei* and *Aporrectodea caliginosa* regarding NaCl contamination has been assessed by Owojori et al. (2008) in natural and OECD soils (With EC50 values for artificial soil of 667mg/Kg NaCl for *A. caliginosa* and 1164 mg/Kg for *E. fetida*; the EC50 for the natural soils were 260µS/cm for *A. caliginosa* and 560µS/cm for *E. fetida*), showing a greater sensitivity for NaCl in both soils for *A. caligionsa*. The NaCl concentrations used in the OECD soil were 0, 0.5, 1, 2, and 4 g/Kg NaCl. The first significant response for *A. caliginosa* was to 1g/Kg NaCl and 2g/Kg for *E. fetida*. As for reproduction tests with salinity, there are some studies for *Eisenia Andrei*, *Folsomia candida* and *Enchytraeus doerjesi* in natural soils (Owojori [2], 2008). The results obtained by these researchers showed no reproduction in *E. fetida* except in the control soils and a significant reduction in the reproductive output of F. candida and E. doerjesi at electrical conductivities from 1030µS/cm onward. These results show that the sensitivity for NaCl content in the soil is much bigger in soil invertebrates that in plants, since a soil with electrical conductivity bellow 2000µS/cm is considered non-saline (Schoeneberger et al. 2002) and also considered safe for plants if the conductivity is bellow 4000µS/cm (Sumner 1995).

The main objective of this work is to assess the possible impairment of the habitat function of soils under different levels of salinization and in combination with different contents of soil organic matter. To attain this objective we provide test results on avoidance behavior of *Folsomia candida* and Eisenia andrei towards saline soils. Avoidance tests are simple to perform and only take 48 hours, making them a useful and quick tool to access the intrinsic ecotoxic potential of a contaminated soil (Natal-da-Luz [1], 2008; Natal-da-Luz [2], 2008). In this case, we believe that it can also act as effective early screening tools to access the quality of the soil due to the sensibility to salt by these organisms and ability to avoid contaminated soils.

Moreover, to further evaluate the effects of NaCl on the reproductive output of soil organisms we performed reproduction tests with Folsomia candida and Enchytraeus crypticus exposed to saline soils. From the data obtained we develop GLM based models that will allow predicting avoidance response and reproductive output of those species due to soils salinization.

II.3 Materials and methods

II.3.1 Test organisms and culture conditions

In these experiments three different soil organisms were used: *Eisenia andrei* (Oligochaeta: Lumbricidae), *Folsomia candida* (Collembola: Isotomidae) and *Enchytraeus crypticus* (Oligochaeta: *Enchytraeidae).* These organisms were selected not only because they are sensitive to chemicals, thus being commonly used in laboratory tests with polluted soils, but also because their locomotor abilities enables them to avoid unfavorable environments (important for avoidance tests – exception made for the *E. crypticus*) and they reproduce considerably in a short period of time under normal conditions (important for reproduction tests).

The species used in the experiments originated from laboratory cultures maintained at a constant temperature of 20 ± 2°C with a photoperiod of 16h light, 8h dark. Earthworms were kept in plastic containers with a mixture of horse manure and potting soil as substrate. This mixture was wetted periodically to maintain the moisture content between 40 and 60% of the water holding capacity (WHC). Animals were fed twice a month with yeast oats, previously wet and microwaved before adding to the soil in culture boxes. The individuals used in the tests were more than one month old with an average weight of 233±55mg.

Springtails were cultured in plastic containers lined with a 8:1 mixture of plaster and activated charcoal. A small amount of granulated dry yeast was added as a food source once a week to avoid spoilage by fungi. Moulded food was removed when detected. All F. candida used in the tests were 10 to 12 days old coming from synchronized cultures.

Enchytraeids were culture in plastic containers in reference natural soil previously defaunated and wetted periodically to keep the moisture constant. The animals were fed with porridge dry flakes once a week and in the day previous to the beginning of the tests to lure the animals to the surface of the soil making them easier to extract.

II.3.2 Test soils

All tests were performed in OECD soil made using variable proportions of 5mm sieved peat, fine sand and kaoline clay. After mixing these components in the respective proportions, soil pH was corrected to vary between 5.5 to 6.5 using CaCO3. The different soils and contaminations used are represented in Table II. In the avoidance tests we used soils with 1 and 5% of organic matter (peat) while in the reproduction tests we used soils with 1, 2.5 and 5% of organic matter. The salinity was classified into 3 levels, Low Salinity (Ls), Medium Salinity (Ms) and High Salinity (Hs), corresponding to 1, 2 and 4 g of NaCl per Kg of soil, respectively. The NaCl contamination was made by using a previously prepared diluted solution of NaCl in deionised water contining the necessary salt content for that particular treatment. The pH was measured in plastic containers, by weighting 5g of the soil to be analyzed, and adding 25mL of a KCl solution at 1M. Afterwards the misture was stirred using a magnetic stirrer for 5 minutes and laid to rest for 2 hours before reading the pH with the pH meter. The WHC was measured using plastic tubes with filter paper covering one of the ends. The filter paper was moistened with deionised water and the tubes were weighted. Then the tubes were filled with soil, leaving approximately 1cm free from the top. The tubes were put in a bigger container filled with deionised water so that the soil would absorb the maximum water it could and left in there overnight. The tubes were weighted again with the soaked soil and then put into a lab oven at 105ºC for 15 hours and, after that, the tubes with the dry soil were weighted. To calculate the WHC the difference between the weight of the wet and dry soil are used to calculate the % of water the soil can hold. Also at the beginning and end of the test, salinity and conductivity were measured by a similar process as the one described for pH, but using deionised water instead of a KCl solution, and the results were read using a portable conductivimeter. Humidity was calculated as well by taking 10g of each contaminated soil and weighting them, and weighting again after drying at 105ºC for 12 hours.

Table II – Summary of the test soils and the NaCl contaminations used in each test

		Organic matter (%)	NaCl (g/Kg)
Avoidance tests	*Folsomia candida*	1 and 5	0, 1, 2 and 4
	Eisenia andrei	1 and 5	0, 1, 2 and 4
Reproduction tests	*Folsomia candida*	1, 2.5 and 5	0, 1, 2 and 4
	Enchytraeus crypticus	1, 2.5 and 5	0, 1, 2 and 4

II.3.3 Experimental procedure

Avoidance tests with *Eisenia andrei*.

These assays were based on the ISO (International Organization for Standardization) Draft No 17512 (ISO, 2005) with some modifications. Plastic boxes (20 cm length, 12 cm width and 5 cm height) were used. Each box was divided into two equal sections with a plastic card, and approximately 300g wet weight of a particular soil combination (percentage of organic matter and salt concentration) were placed into one of the two sections; the other section was filled with the same amount of a different soil combination.

All possible combinations between salinity and percentage of organic matter were tested (23 in total, including the controls), each with 5 replicates. After placing the prepared soils into the containers the card divider was removed. Ten earthworms, previously washed and wiped dry (using absorbent paper), were placed on the middle line of each test container. To prevent the worms from escaping the test containers, these were covered with a transparent lid containing some holes made to facilitate air circulation. The test containers were incubated for 48h at 20±2°C with a photoperiod of 16h light, 8h dark. After this period the two sections of each test box were again divided, the soils from each side were emptied onto separated trays, and the number of worms in each test soil counted. In the eventual case of a worm being found under the midline, it would be considered to be in the section where the anterior segments were. Soil pH, salinity, conductivity and humidity were measured at the beginning and at the end of the experiment for each combination tested.

We also included dual control tests for each control soil with different percentage of organic matter, meaning that each box of the controls had the same soil in both sides. This was not only to assess mortality but also to verify the assumption that in an avoidance test we should get an even distribution of individuals among the two sections of the test container when the same soil type is present on both.

Avoidance test with *Folsomia candida*.

The tests were performed under the ISO guideline ISO/FDIS 17512-2 (ISO, 2011 - currently under final stage of publication)

Cylindrical plastic containers (diameter: 7cm, height:6cm) were used in the tests. The procedure was similar to the described for earthworms. Thirty grams wet weight of soil were placed in each half of the container. The same 23 possible combinations were tested with five replicates each. After the removal of the divider 20 individuals of F. candida were placed onto the soil in the midline of each test container. The individuals were checked for any signs of damage before placing onto the containers to reduce mortality and avoid systematic errors. An extra container without individuals was prepared for each combination and used to pH, salinity, conductivity and humidity measures at the end of the test. To prevent the springtails from escaping the containers were closed with a transparent lid. Test containers were maintained at 20±2°C with a photoperiod of 16h light, 8h dark for 48h. At the end of the test period, both soils were carefully separated with a plastic divider, each soil was emptied into a small vessel. These were filled with water and, after a few drops of ink and gently stirring, the animals floating on the water surface were counted. As for earthworms, dual control tests were also performed, having the same objectives.

Reproduction tests with *Folsomia candida*.

The tests were performed accordingly to the ISO Draft No 11267 (ISO 1999). Cylindrical glass flasks (diameter: 5cm, height: 6cm) were used in the tests. Thirty grams wet weight of each soil from each possible combination between percentage of organic matter and level of salinity (9 in total) plus 3 controls (OECD soil with 1, 2.5 and 5% of organic matter, without NaCl contamination) were placed into each vessel and approximately 2mg of granulated dry yeast were placed and covered with soil to prevent fungi from appearing in the vessels. Then 10 individuals (10-12 days old) were placed into each of the vessels. The vessels were weighted in the beginning of the test for humidity control during the test and covered with a flask lid. During the test the animals were fed once a week and the lids were opened to circulate the air in the vessel and the vessels weighted to maintain the water in the soil constant. The vessels were incubated at 20±2°C during 28 days. After this period the vessels were emptied into plastic containers and filled with water. After ink addition and careful stirring, the floating collembolan were photographed for further counting using Image Tool free software. Soil pH, humidity, salinity and conductivity were measured at the beginning and at the end of the test for each combination.

Reproduction tests with *Enchytraeus crypticus*.

The tests were performed according the ISO draft 16387 (ISO 2003). Cylindrical glass vessels (diameter: 6cm, height: 7 cm) were used in the tests. The same combinations as the collembola reproduction tests were used, with four replicates each. 20g dry weight of each soil was placed into each vessel with 50mg of dry mass rolled yeast oats at the beginning of the test, and 25mg during the rest of the test period. Ten adult individuals, previously checked for signs of damage, were placed into each vessel, and the vessels were closed with a lid. The vessels were weighted at the beginning of the test with the same purpose described in the collembolan reproduction test. The vessels were weighted once a week and the animals were fed twice a week with 25mg dry mass of yeast oats. The vessels incubated at 20±2°C during 28 days. After this period, the vessels were filled with 80% alcohol just above the soil surface to fix the animals and then tainted with 5 drops of Bengal red each. After this the counting was made during the following days using a lab binocular microscope.

As for the previous tests, pH, humidity, salinity and conductivity were measured in the beginning and the end of the test.

II.3.4 Statistical analysis

To detect significant avoidance responses the results obtained in the avoidance and dual tests from both test organisms were analysed by the Fischer exact test. This statistical procedure allows comparison of the distribution of animals in relation to an expected distribution assuming the non-existence of an avoidance response to a determined soil types (the null hypothesis). For the main avoidance tests, a one-tailed test was used; since it is an avoidance response that is being tested, the null hypothesis assumes that half of the individuals are staying in the test soil, meaning that there is no avoidance regarding that soil. For the dual tests a two-tailed test was chosen; in this case the null hypothesis assumes an equal distribution of the organisms on both sides of each test container. The null hypotheses were rejected for a probability equal or lower than 0.05.

The results of the reproduction tests were analyzed using a one-way ANOVA (followed by a Dunnet's test), to assess the possible differences in reproductive behavior between the treatments. Assumptions of normality and homogeneity of variances were evaluated using the Kolmogorof-Smirnov and Barlett tests, respectively. When at least one of the assumptions were not met, ANOVA was done with the Log transformed data.

To develop predictive models for the effects of NaCl (in combination with organic matter) on avoidance behavior and reproduction of the species tested Generalized Linear Modelling (GLM) were applied using the BRODGAR 2.5.6 software (available at www.brodgar.com) and according to the approach described by Chelinho et al. (2011). Before the GLM analysis data exploration was performed to detect possible outliers and colinearity between the variables. In some cases the data exploration included log transformation of the variables. For the avoidance tests a binomial model was adopted using the logit link function and the proportion of individuals in the test soil (calculated using the formula T/N, i.e, the number of individuals in the test soil divided by the total number of individuals) as the response variable. The total number of individuals was used as weight variable. For each combination tested (control soil vs test soil), a quotient for each soil parameter (test soil parameter/control soil parameter) was calculated. A value of $Q<1$ indicates higher values of the soil parameter in the control soil, a $Q=1$ indicate no differences, and a $Q>1$ indicates higher values of the soil parameter in the test soil. A matrix containing all combinations tested and normalized values (Q quotients), expressing differences between the soils, was used as explanatory variables in the models.

For the reproduction tests GLMs were also applied using a Poisson model with the log link function. The number of juveniles was used as response variable and the individual soil parameters as explanatory variables.

II.4 Results

II.4.1 Avoidance test with *Folsomia candida*

The mortality was low in every test soil and inexistent in most of them. In the dual tests there were no statistical differences, meaning that the distribution of the individuals was equal between both sections of the containers ($p>0.05$ for all comparisons tested) (Fig. 1)

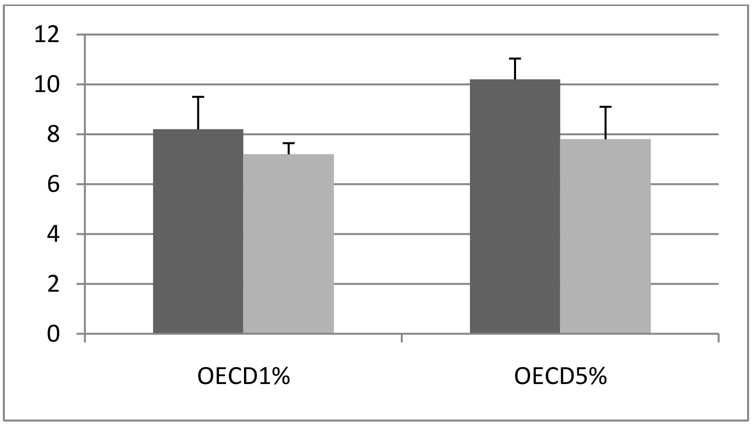

Fig. 1 – Percentage of *Folsomia candida* (average + standard deviation) on each section of the test container on dual control tests for the two soils tested. Left section (dark bars); right section (light bars). OECD1% - OECD soil with 1% of Organic Matter; OECD5% - OECD soil with 5% of Organic Matter. No statistical differences in any of the soils.

We expected differences not only between different salt concentrations but also between the two different OM% (1% and 5%). In Fig.2 the combinations where the organic matter percentage is the same and only NaCl concentrations vary are represented. As observed, only the Ls1-OECD1 and Ms1-OECD1 combinations were not statistically significant. All the other combinations showed a clear avoidance of the soils with a higher NaCl contamination.

As for the combinations between different percentages of organic matter and different NaCl concentrations (Fig. 3) no statistically significant response was observed in the combinations between different organic matter percentages and the same NaCl concentration. However, all the combinations between different organic matter percentages and different NaCl concentrations were statistically significant, with animals always avoiding the higher salt concentration in the combination, independently of the percentage of organic matter present.

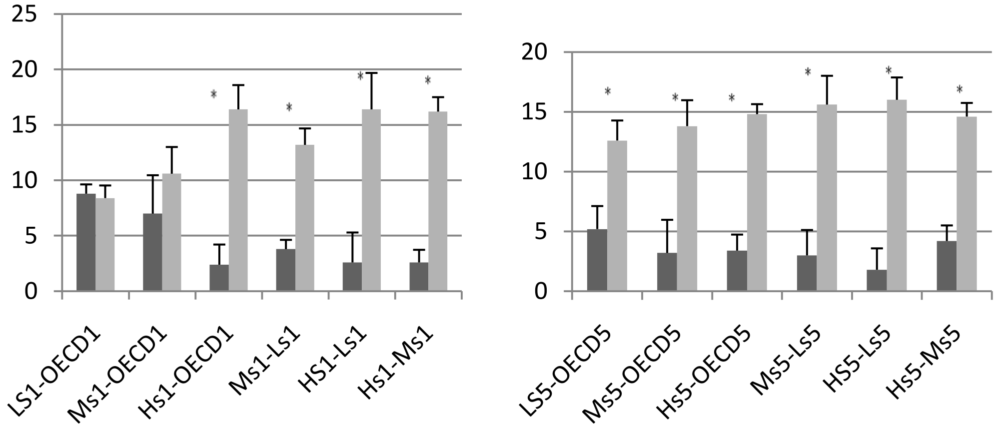

Fig. 2 – Avoidance tests: percentage of *Folsomia candida* (average + standard deviation) in a specific soil combination. Test soil (soil we expect the individuals to avoid, dark bars) tested against the other soils (light bars). Key: Ls – Low salinity (1g/Kg); Ms – Medium salinity (2g/Kg); Hs – High salinity (4g/Kg); OECD – soil without contamination; 1 and 5 – OM% in the soil; * indicates statistical differences ($p<0.05$)

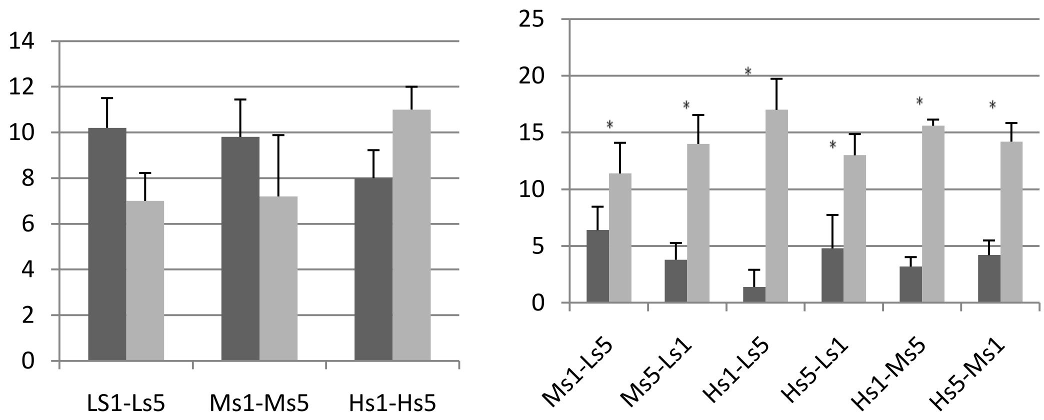

Fig. 3 – Avoidance tests: percentage of *Folsomia candida* (average + standard deviation) in a specific soil combination. Test soil (soil we expect the individuals to avoid, dark bars) tested against the other soils (light bars). Key: Ls – Low salinity (1g/Kg); Ms – Medium salinity (2g/Kg); Hs – High salinity (4g/Kg); OECD – soil without contamination; 1 and 5 – OM% in the soil; * indicates statistical differences (p<0.05)

II.4.2 Avoidance tests with *Eisenia andrei*

There was no mortality in all the tested soils. In dual control tests the distribution of the individuals was equal between both sections of the containers, without any significant difference in any of the comparisons made (p>0.05 for all dual tests) (Fig. 4)

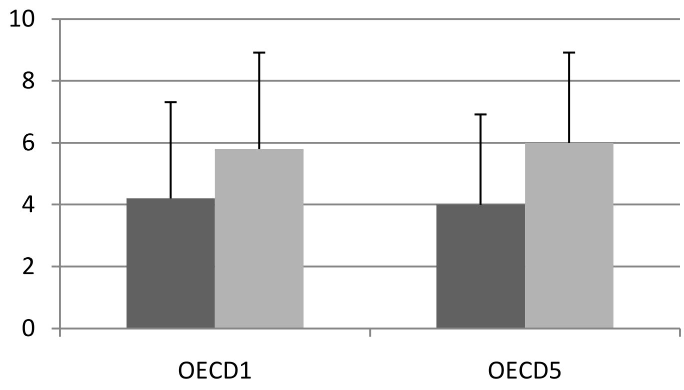

Fig. 4 – Percentage of *Eisenia andrei* (average + standard deviation) on each section of the test container on dual tests for the two soils tested. Left section (dark bars); right section (light bars). OECD1 – OECD soil with 1%OM; OCDE5 – OECD soil with 5%OM. No statistical differences were found in the dual tests.

Earthworms showed a clear avoidance response to NaCl contaminated soils, being more sensitive to salt than collembolans. In most combinations tested an "all or nothing" kind of response, i.e, all the individuals were on one side of the container, was observed. Only in one of the combinations (Ls1-Ls5) there was no statistical difference between the two sides of the container. (Figs. 5 and 6).

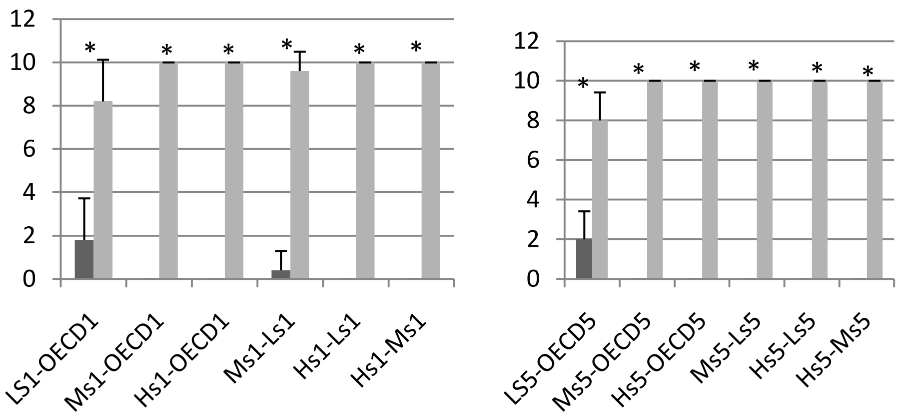

Fig. 5 – Percentage of *Eisenia andrei* (average + standard deviation) on each section of the test container. Test soil (left bar, dark); control soil (right bar, light). Key: Ls – Low salinity (1g/Kg); Ms – Medium salinity (2g/Kg); Hs – High salinity (4g/Kg); OECD – soil without contamination; 1 and 5 – OM% in the soil; * indicates statistical differences ($p<0.05$)

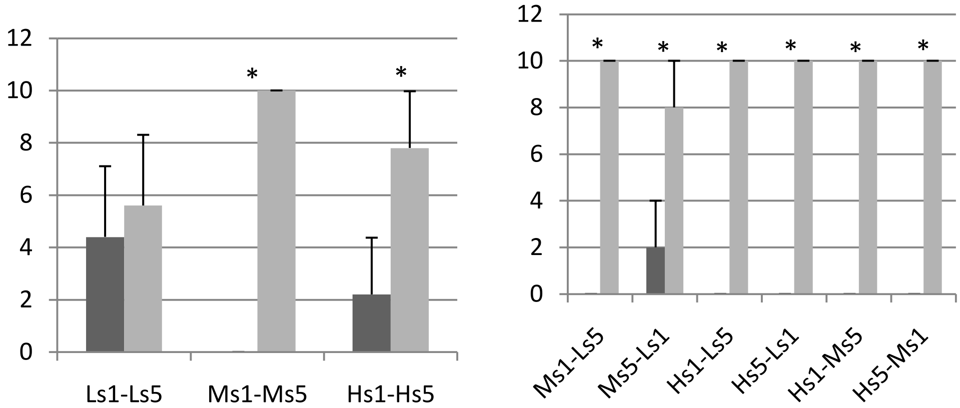

Fig. 6 – Percentage of *Eisenia andrei* (average + standard deviation) on each section of the test container. Test soil (left bar, dark); control soil (right bar, light). Key: Ls – Low salinity (1g/Kg); Ms – Medium salinity (2g/Kg); Hs –

High salinity (4g/Kg); OECD – soil without contamination; 1 and 5 – OM% in the soil; * indicates statistical differences ($p<0.05$)

Reproduction tests

II.4.3 *Folsomia candida* reproduction tests

In all of the tested soils the mortality was low (less than 20%). in the control soils the reproduction was always above 100 juveniles produced and the variation between the replicates was below 30% (11% for the controls with 1% and 2.5% of organic matter and 5% for the control with 5% of organic matter). In the 1% and 2.5% organic matter percentages only the medium and high salinities differed from the respective control soil (Fig. 7), whereas in the 5% organic matter, significant differences were found already in the low salinity. In the High salinity (4g/Kg) there was no reproduction at all, and there was also a difference between the different OM% (higher reproduction rates in lower OM%). (Fig. 7)

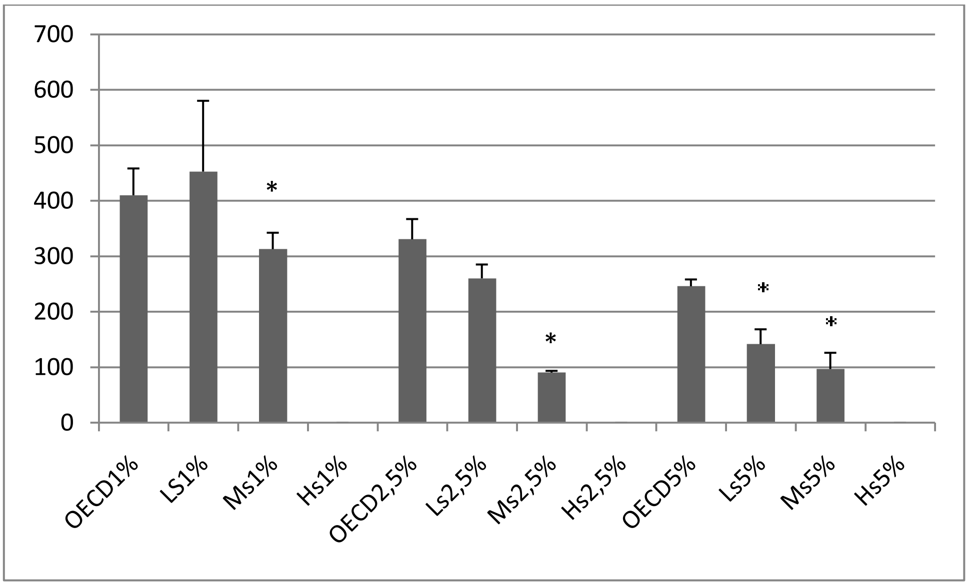

Fig. 7 – Number of juveniles in each soil (average + standard deviation). OECD – no NaCl contamination; Ls – Low salinity (1g/Kg); Ms – Medium Salinity (2g/Kg); Hs – High salinity (4g/Kg); 1%, 2,5% and 5% - OM%. Statistical significant differences with the control soil for the same organic matter% represented by *.

II.4.4 *Enchytraeus crypticus* reproduction tests

No mortality was observed in the tests. All the control soils had more than 25 juveniles and the coefficients of variance were below 50% (5%, 2.4% and 6.8% for the 1%, 2.5% and 5% organic matter soils, respectively). The reproductive output was different from the collembolans, as enchytraeids seem to prefer soils with higher organic matter content, since even reproduced in the High salinity treatment with 5%OM. All the combinations differed from the respective controls. At 2.5% and 5% of organic matter, the reproductive output was higher in the low salinity, relatively to the control. At the higher salinity, only in the soil with 5% of organic matter reproduction was observed (Fig. 8).

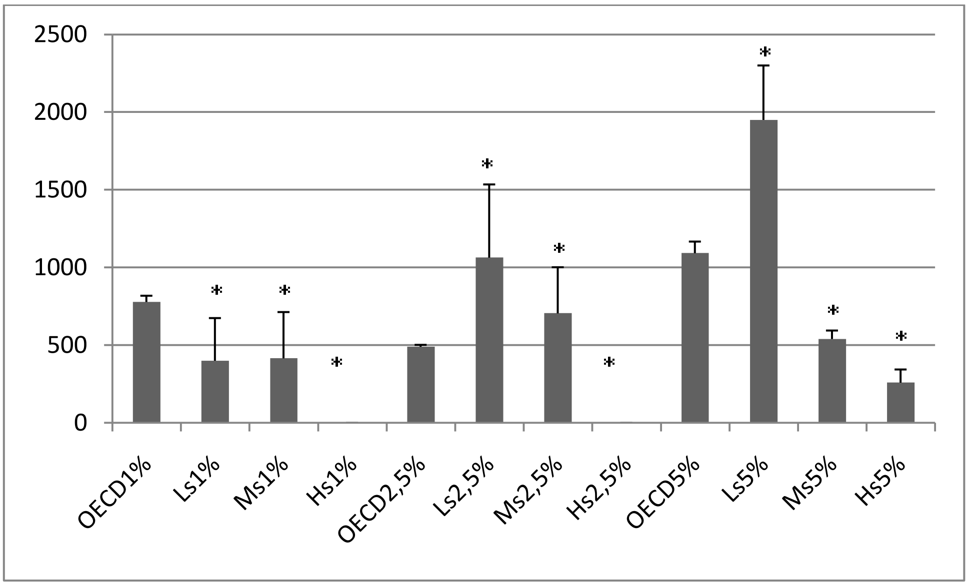

Fig. 8 – Number of juveniles in each soil (average + standard deviation). OECD – no NaCl contamination; Ls – Low salinity (1g/Kg); Ms – Medium Salinity (2g/Kg); Hs – High salinity (4g/Kg); 1%, 2,5% and 5% - OM%. No reproduction in the Hs except for the Hs with 5%OM. Statistical significant differences with the control soil for the same organic matter% represented by *.

II.4.5 Generalized Linear Modeling (GLMs)

Avoidance tests

To develop a model to predict the avoidance behavior of *Folsomia candida* under saline soils with different organic matter contents, a binomial model was adopted and the explanatory variables were log transformed. After data exploration the variables were log transformed. The final model explains 44.8% of the response variable and is given by:

Prop=EXP([-1.8961-10.3772*logQpH-3.1372*logQOM+0.9521*logQCond-39.1441*(logQpH*logQOM)+ 8.2856*(logQcond*logQOM)]/[1+EXP(-1.8961-

10.3772*logQpH-3.1372*logQOM+0.9521*logQCond-

39.1441*(logQpH*logQOM)+ 8.2856*(logQcond*logQOM)])

Where Prop – proportion of individuals in the test soil QpH – Quotient of pH; QOM – Quotient of OM (OM in %); QCond – Quotient of conductivity (conductivity in µS/cm). The significance of the variables is represented in table III.

Table III – Variables used in the model and correspondent p value

Variable	P value
QpH	0.006107
QOM	< 2e-16
QCond	0.000214
QpH:QOM	2.09e-07
QOM:QCond	< 2e-16

To develop a model to predict the avoidance behavior of *Eisenia andrei* under soil with the same conditions, a binomial model was also adopted. The explanatory variables were log transformed. During data exploration, and having observed that a 100% avoidance was observed at the high salinity levels, we have decided to exclude these combination from the model. Therefore, this model only predicts the avoidance response under low and intermediate salinity levels (until 2 g NaCl/Kg soil), assuming that at higher salinity level, avoidance will be 100%. The final model explains 45.5% of the response variable and is given by:

Prop=EXP([-3.8840-17.5776*logQpH-4.6220 *logQOM +0.7521*logQcond +74.8704*(logQpH*logQOM)+ 24.5457*(logQCond*logQOM)]/ [1+EXP(-3.8840-17.5776*logQpH-4.6220 *logQOM +0.7521*logQcond +74.8704*(logQpH*logQOM)+ 24.5457*(logQCond*logQOM)])

Where Prop – proportion of individuals in the test soil QpH – Quotient of pH; QOM – Quotient of OM (OM in %); QCond – Quotient of conductivity (conductivity in µS/cm). The significance of the variables is represented in table IV.

Table IV – Variables used in the model and correspondent p value

Variable	P value
QpH	0.01327
QOM	2.11e-09
QCond	0.19574
QpH:QOM	0.00949
QOM:QCond	8.68e-09

Reproduction tests

To develop a model to predict the reproductive output of *Folsomia candida* under saline soils with different organic matter content, a Poisson model was adopted. The total variation explained by the final model is of 77%. The model is given by:

Juv=EXP[2.232e+01-2.018e-03*Cond-3.673*OM-3.521*pH-9.790e-06*(Cond*OM)
+7.747e-01*(pH*OM)]

Where juv – Number of juveniles in the test soil; Cond – Conductivity (µS/cm); OM – OM% in the soil; pH – soil pH. The significance of the variables is represented in table V.

Table V – Variables used in the model and correspondent p value

Variable	P value
pH	0.000437
OM	0.000280
Cond	1.23e-06
pH:OM	0.000315
OM:Cond	0.935241

To develop the predictive model for the reproductive output of *Enchytraeus crypticus* for soils with the same conditions, a Poisson model was also adopted. Soil pH showed to be collinear with the other variables in this case, so we didn't include it in the model. The final model explains 56% of the response variable and is given by:

Juv=EXP[6.514e+00-1.458e-03*Cond+1.852e-01*OM+4.049e-05*(Cond*OM)]

Where juv – Number of juveniles in the test soil; Cond – Conductivity (µS/cm); OM – OM% in the soil. The significance of the variables is represented in table VI.

Table VI – Variables used in the model and correspondent p value

Variable	P value
Cond	< 2e-16
OM	< 2e-16
Cond:OM	9.09e-06

II.5 Discussion

II.5.1 Avoidance tests

Salt caused a strong avoidance response by both test organisms in most combinations tested, with organic matter content playing a negligible role in the response. The avoidance behavior of earthworms was much more extreme than with collembolan, almost all the time we obtained an “all or nothing” kind of response. This shows that they are highly sensitive to salinity in a first instance, which can be used as an early screening test to salinization. Previous studies have shown that soil parameters like the percentage of organic matter may have an influence in the avoidance behavior of both *Folsomia candida* and *Eisenia andrei* (Natal da Luz, 2008), but in this test NaCl was, without question, the main factor influencing the avoidance behavior.

The GLM model based on the avoidance behavior of the collembolan shows a negative relation between the quotients of pH, OM and the interaction between the two and a positive relation with the Conductivity quotient and the interaction between conductivity and OM (Q=test/control). A positive quocient in the model means that when all the other parameters are kept constant, an increase in the ratio of that parameter will result in more individuals in the test soil, i.e, less avoidance (for example, in the obtained model, the quocient of Conductivity, which was not expected as the results show the with more conductivity (salt content) the avoidance behavior is more evident)

The GLM model based on the avoidance behavior of earthworms shows the same negative relation with the pH and OM quotients and positive relations with conductivity and the interactions (cond:OM and pH:OM). The positive relation with the interaction pH:OM (positive signal in the model) must be a result of the higher sensitivity to different OM% in the soils, since the same parameter for the collembolan avoidance test has a negative signal. Despite this fact the results show that the organic matter has little influence in the avoidance behavior, which is mainly influenced by the NaCl content.

II.5.2 Reproduction tests

Folsomia candida showed a dose response pattern with a significant decrease in the reproductive output with an increase in salinity. This was common in the three organic matter treatments. At higher salinity the complete halt of reproduction was observed. This suggests a good sensitivity of collembolan to salt, and shows that they can be used as a good tool to assess the salinization of a soil. For further tests we suggest leaving out the high salinity since there was no reproduction at all, which is a similar result as the ones obtained by Owojori and collabolators (2008) using natural soils (the reproduction of *F. candida* halted at conductivities of 1620µS/cm which is a higher value than the highest salinity content we used (4g/Kg - 1300µS/cm)). The difference between our results and the ones observed in the aforementioned study is explained by a higher sensitivity of *F. candida* to NaCl in OECD soil than in natural soil (Domene et al, 2011).

The GLM model based on the reproductive output of collembolan explained 77% of the variation, showing a negative relation with all the variables except for the interaction pH:OM. The negative signals in these variables show that a decrease in those parameters will result in a better reproductive output for the collembolan. These results suggest that *Folsomia candida* reproduced better in soils with less organic matter, lower pH and low salinity. Simillar results regarding NaCl were obtained by previous tests (Owojori [2], 2008).

The enchytreids showed a higher sensitivity to the OM%, and, opposite to the collembolan, they reproduced more in the higher organic matter content of the soil, even at the high salinity. These results are not unexpected as several authors have already studied and concluded that different soil parameters can influence the reproduction in oligichaetes (Chelinho et al, 2011; Jänsch et al, 2005). This suggests enchytreids as a more reliable source to assess an eventual interaction between NaCl and organic matter in the soil. Even so, salinity continues to be the major factor affecting the reproduction.

The GLM model based on the reproductive output of enchytreids explained 56% of the variation. The model shows a positive relation with OM and a negative relation with conductivity, meaning that enchytraeids prefer soils with more organic matter and low salinity. The low reproductive output in higher salinities go accordingly to previous studies wich obtained similar results in natural soils (Owojori [2], 2008)

II.6 Conclusions

The results confirm the utility of both earthworm and collembolan avoidance tests as early screening tools for site contamination assessment, as they both show very high sensitivities to NaCl, proving to be good tools for soil salinization assessments.

The reproductive response of collembolan and enchytreids also proved to be very sensible to NaCl, and can also be used as tools for soil salinization assessment.

The GLM models could be improved with the use of more variables and natural soil (the use of artificial OECD soil with few parameters limited the output), so we suggest using these models with caution. To develop better models further investigation should be made, using natural soils, either in normal lab tests similar to these, or in a more advanced way using mesocosms to study the effects in a natural community.

Since the best model of all 4 was the model for collembolan reproduction this could be the most reliable model to use. This means that we can predict most of the reproductive response of *F. candida* in a saline soil with only a few parameters. The models can also be used to assess the effect of salt and remove it as a confounding factor in soils that are contaminated with other contaminants.

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Chapter III The combined effect of NaCl and a commonly used pesticide (Lambda-cyalothrin) in the reproduction of *Folsomia candida*

III - The combined effect of NaCl and a commonly used pesticide (Lambda-cyalothrin) in the reproduction of *Folsomia candida*

III.1 Abstract

In the recent year a growing concern with soil salinization has lead to the need of studying this problem. In addition to salt, most of the times these soils are polluted with agricultural pesticides, such as the one we used for these tests (Lambda-cyalothrin). As an attempt to answer to some of these problems, and to assess the effect of salt alone and remove it as it presents a confounding factor when other contaminants are present, we conducted reproduction tests with *Folsomia candida*. The main objective of this work was to produce a predictive model that would allow to estimate the reproduction of this species when the soil is contaminated with both salt and the pesticide. We ended up with a model that explains 83.8% of the variation, and can become a powerful tool in future assessments.

III.2 Introduction

Soil salinization has become a growing problem in the last years, mainly in Mediterranean and Pannonian regions (Tóth, 2008). It has been shown that soil invertebrates have a higher sensitivity to salt than plants (Owojori [2], 2008) and can be used as early screening tools for ecological risk assessment.

The salinization problem leads to a decrease in soil quality and biodiversity. (http://eusoils.jrc.ec.europa.eu/library/themes/Salinization/) In this work we intended to test the combined effect of NaCl with one of the most common pesticides used, Lambda-cyalothrin, which is a pyrethoid (manmade chemical similar to natural insecticides pyrethins), and assess if the salt content in the soil can increase the effects of the pesticide. This affects the nervous system of insects, causing paralysis and death (NPTN general fact sheet on Lambda-cyalothrin). The half life of this product is around 30 days in lab conditions. Since collembolans are isopods and physiologically similar to insects, and based on previous assessments on the effect of lambda-cyalothrin in isopodes (Jänsch 2005), it is predicted they would be greatly affected by this compound. The objective of this work was to assess the combined effect of salt and lambda-cyalothrin in the reproduction of *Folsomia candida* and to develop a model that would allow us to predict a response.

III.3 Materials and methods

III.3.1 Test organisms and culture conditions

For this reproduction tests we used *Folsomia candida* (Collembola: Isotomidae). This was chosen because we had observed a good response in reproductive behavior to NaCl in previous tests (Chapter II). The species used in the experiments originated from laboratory cultures maintained at a constant temperature of 20 ± 2°C with a photoperiod of 16h light, 8h dark.

F. candida were cultured in plastic containers lined with a 8:1 mixture of plaster and activated charcoal. A small amount of granulated dry yeast was added as a food source once a week to avoid spoilage by fungi. Moulded food was removed when detected. All F. candida used in the tests were 10 to 12 days old coming from synchronized cultures

III.3.2 Test soils

All tests were performed in OECD soil made in the lab using 5mm sieved peat, fine sand and kaoline clay, being the peat responsible for the organic matter percentage of the soil. In these tests we used only 5% organic matter content. The pH of the soil was corrected to be from 5.5 to 6.5 using CaCo3 and the WHC was measured. Also at the beginning and end of the test, salinity and conductivity were measured as well as humidity. The soil parameters were measured as described in Chapter II. The chosen gradients of concentrations were based on a calculated EC50 of 0.86mg/Kg (Brandow, personal communication) of lambda-cyalothrin and the NaCl gradient was based on our previous tests that suggested that we should leave out concentrations higher than 2g/Kg, since up to that concentration we obtain a good reproductive response. The NaCl concentrations used were of 0, 1 and 2 g/Kg and the lambda-cyalothrin concentrations were 0.1, 0.25, 0.65, 1.5, 4, 10 mg/Kg. A solution os NaCl and deionised water was prepared with the necessary NaCl for the tests as well of two stock solutions of lambda-cyalothrin made by diluting the commercial product (25ml with 100g/l of lambda-cyalothrin) 1000x (used for the 4 and 10 mg/Kg concentrations), and the other stock solution by diluting the first stock solution 500x more (for all the other concentrations). These dilutions were necessary because the quantities of pesticide for each concentration were minimal, and it was necessary to increase the volume of the solution so that it would be possible to use the pipettes in the lab, and also to facilitate the dispersion of the pesticide to the totality of the test soils.

III.3.3 experimental procedure

The tests were performed accordingly to the ISO Draft No 11267 (ISO 1999). Cylindrical glass flasks (diameter: 5cm, height: 6cm) were used in the tests. 30g wet weight of each soil (of each possible combination between pesticide and level of salinity, 7 levels of pesticide (0, 0.1, 0.25, 0.65, 1.5, 4, 10 mg/Kg) and 3 of NaCl(0, 1, 2 g/Kg), making a total of 21 treatments were placed into each vessel and approximately 2mg of granulated dry yeast were placed and covered with soil to prevent fugi from appearing in the vessels. Then 10 individuals (10-12 days old) were placed into each of the vessels. The vessels were weighted in the beginning of the test for humidity control during the test and covered with a flask lid. During the test the animals were fed once a week and the lids were opened to circulate the air in the vessel and the vessels weighted to maintain the water in the soil constant. The vessels were incubated at 20±2°C during 28 days. After this period the vessels were emptied into plastic containers and filled with water. After ink addition and careful stirring, the floating collembolan were photographed for further counting using Image Tool free software. pH, humidity, salinity and conductivity were measured at the beginning and at the end of the test for each combination.

III.3.4 Statistical analysis

The results of the reproduction tests were analysed using a one-way ANOVA and post-hoc Dunnet's test to see the differences between treatments using the Statistica 7.0 software (StatSoft, 2004, *available at* www.statsoft.com). An EC50 was calculated for each of the salinity levels to assess the effect of the pesticide according to different NaCl concentrations (calculated using SYSTAT software available at www.systat.com) based on the guidelines provided in the document “Biological test method: test for measuring survival and reproduction of springtails exposed to contaminants in soil” by Environment Canada (2007). The EC50 for the no NaCl content was calculated using a nonlinear logistic model and for the other two salinity levels a nonlinear hormetic-logistic model was used. The models used were:

Logistic model:

model juveniles=(a/(1+(concentr/x)^b))

Hormetic-logistic model:

model juveniles=(t*(1+h*concentr))/(1+((0.5+h*concentr)/(1-0.5))*(concentr/x)^b)

Where: *Y* = value for a measurement endpoint (e.g., number of juveniles)

a = control response

t = control response in the hormetic model

e = base of the natural logarithm

p = % inhibition/100 (e.g., 0.50 for EC50)

C = exposure concentration in test soil

ECp = estimate of effect concentration for a specified percent effect

h = hormetic effect parameter

b = scale parameter

To build a predictive model, we used Generalized Linear Modelling (GLM), using the Brodgar 2.5.6 software (available at www.brodgar.com). The model used was a Poisson model with the log link function. The number of juveniles was the response variable and the soil parameters and contamination parameters the explanatory variables.

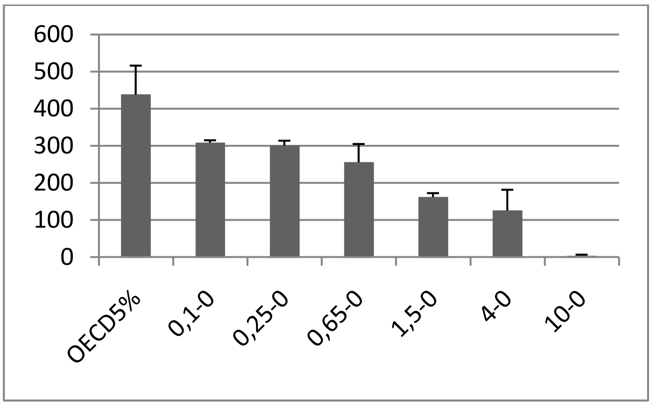
III.4 Results

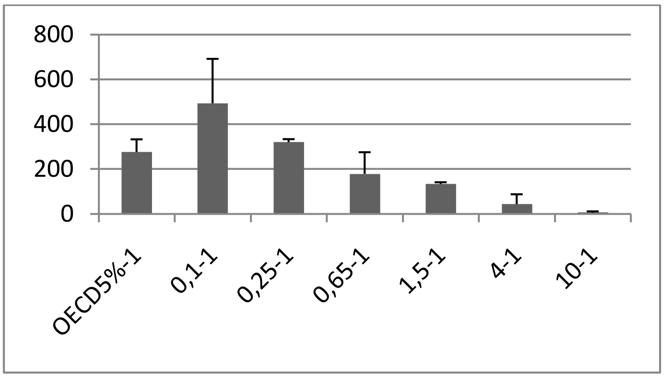
The controls had no mortality; all the controls had more than 100 juveniles and the coefficient of variance was less than 30% (17%) so the test is valid. The mortality was low in every combination, even at the higher contamination with lambda-cyalothrin (10mg/Kg). The reproduction was affected by higher salinities even without pesticide contamination, and in the combinations with high doses of pesticide the reproduction was even more affected. The results are represented in 3 separated graphics according to the NaCl concentration (Fig. 9).

The EC50 for each of the NaCl concentrations (according to the different lambda-cyalothrin concentrations (mg/Kg)) is represented in table VII.

Table VII – EC50 values for each group of NaCl contaminations according to pesticide concentrations in mg/Kg.

Soil group (NaCl concentration)	EC50 (mg/Kg)	Lower and upper limits (95% confidence interval	
OECD5% - 0 g/Kg NaCl	0.65	0.39	1.07
OECD5% - 1 g/KgNaCl	1	0.37	2.7
OECD5% - 2 g/KgNaCl	2.5	1.2	4.3





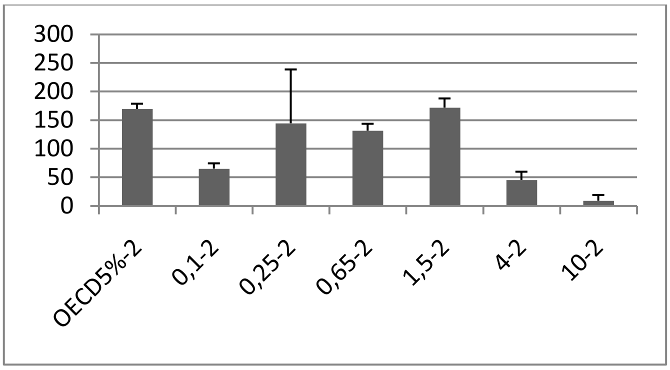


Fig. 9 – number of juveniles in each of the test soils (average + standard deviation). The values on the left are the pesticide contamination, and on the right, the NaCl contamination; OECD – no pesticide. A significant decrease in reproduction occurs from less NaCl to more NaCl (from top graph to bottom graph) and also from less pesticide to more pesticide (from left to right in each graph). The higher contaminations by pesticide (10mg/Kg) showed little to no reproduction, even without NaCl (10-0).

To build the predictive model a Poisson model was used with the log link function. The final model explains 83.8% of the response and is given by:

Juv=EXP(6.072e+00-1.405e-03*Cond-4.512e-01*LC+2.453e-04*(Cond*LC))

Where Juv – Number of juveniles; Cond – Conductivity (μS/cm); LC – Lambda-cyalothrin (mg/Kg). The significance of each variable is represented in table VIII.

Table VIII – Variables used in the model and correspondent p-values

Variable	P-value
Cond	<2e-16
LC	<2e-16
LC:Cond	<2e-16

III.5 Discussion

The results of the reproduction tests suggest that both salinity and lambda-cyalothrin may affect negatively the reproduction of collembolan individually. Looking at the graph (Fig. 9) it seems to exist a bigger inhibition of reproduction in higher salinities and higher pesticide contaminations. In the low pesticide contaminations the NaCl is the main factor affecting the reproduction of the collembolan, but at high pesticide contaminations the pesticide is the main factor, and by looking at the EC50 values, the NaCl seems to have a positive role, decreasing the effect of the pesticide. No other studies with both NaCl and lambda-cyalothrin and the combined effects were found, but similar projects show the potentially devastating effects of this pesticide in isopodes *Porcellionides pruinosus* with a significant effect on mortality at 1mg/Kg lambda-cyalothrin and an EC50 of 0.4mg/Kg for reproduction, in OECD soil (Jänsch 2005) and in *Folsomia candida* with a calculated EC50 for chronic laboratory toxicity with lambda-cyalothrin of 0.09mg/Kg (Jänsch 2006).

The EC50 values obtained for the different NaCl contamination groups suggest a better relative reproductive output as the salt content in the soil increases, in the presence of the higher concentrations of the pesticide. To affect 50% of the reproduction in a soil with higher NaCl concentration (2g/Kg), a higher lambda-cyalotrin concentration is required when compared to a soil with medim NaCl concentration (1g/Kg), suggesting that NaCl may have a positive influence in the presence of this pesticide, reducing its effect on the reproduction of *Folsomia candida*.

By analyzing the GLM model we can see that both salinity and the pesticide have a negative influence in the reproduction, but apparently the interaction between both has a positive influence, which goes accordingly to the previous conclusions taken by analyzing the EC60 values. Probably the bioavailability of the pesticide is reduced by NaCl (in the 10mg/Kg pesticide contamination, more reproduction occurred with higher salinity, even if low relatively to the other combinations). Unfortunately as salinization is a recent concern, it was not possible to find any other similar tests that evaluate the combined effects of these two contaminants, however there are some studies that suggest NaCl in the soil can increase the bioavailability of metals such as zinc and copper (Owojori [], 2008; Owojori [], 2008) and our results suggest that this is an interaction worth of further investigation.

III.6 Conclusions

The reproduction of collembolan is highly affected by soil contaminants such as pesticides and soil salinization, at much lower concentrations that plants start to be affected (Owojori [2] 2008; Jänsch 2005), so these tests may prove to be more accurate in ecological risk assessment, since these organisms are more sensitive to contaminants.

There is an apparent inhibition of the effect of lambda-cyalotrin by NaCl, fact that should be explored in further studies to better understand this relationship (test a more diverse gradient of NaCl to reach better conclusions regarding the interaction).

The model we obtained explains most of the response in reproductive behavior of *F. candida* so we think it may prove a useful tool in similar situations and future studies in pesticide contaminated soils that also suffer with salinization.

Further studies should be made to assess how the salinity affects the availability of pesticides; in our case it seems to reduce the pesticide bioavailability.

II.7 References

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Chapter IV General Discussion

IV – General Discussion

IV.1 – Avoidance tests

The experiments performed with the OECD soils with different organic matter percentages and different NaCl contaminations (Chapter II) showed that both test organisms (earthworms and collembolan) were able to avoid the more saline soils. Earthworms showed a greater sensitivity to both salt and organic matter, which goes accordingly to the data obtained in previous studies (Natal da Luz, 2005; Owojori , 2008). In fact, most of the replicates with earthworms showed an "all or nothing" response, even between Low salinity and control, demonstrating their sensitivity to NaCl. The collembolan didn't have such extreme responses, but still provided reliable data on the avoidance behavior, with clear responses for most of the combinations. The collembolan apparently were not affected by differences between different organic matters only, opposite to earthworms.

The GLM models obtained with these tests didn't explain much of the variance obtained in the results, therefore these models are not as reliable as the reproduction models and should be used with caution, since the responses don't provide a very precise gradient of responses.

IV.2 – Reproduction tests

IV.2.1- Reproduction tests to assess the combined effect of salinity and organic matter

The experiments performed with OECD soil with different combinations of NaCl and organic matter (Chapter II) showed that both organisms (collembolan and enchytraeids) were affected by the different salinities and organic matter percentages, which affected the reproductive output. The collembolans showed a preference for soils with lower organic matter and low salinity, with no reproduction at all in the higher NaCl concentration (4g/Kg). The enchytraeids seemed to be more sensitive to organic matter, as they reproduced better in soils with higher organic matter percentage. As well as the collembolan, enchytraeids had a better reproductive output in lower salinities, but reproduced even in the higher salinity in the soil with the highest percentage of organic matter (5%).

The GLM models obtained with these tests were very satisfying, mainly the collembolan model, which explained 77% of the variation in the reproductive output, so I suggest using this model when predicting the effects of salinization in soil organisms.

IV.2.2 – Reproduction test to assess the combined effect of NaCl and Lambda-cyalothrin

The experiment performed with OECD soil with 5% organic matter, seven pesticide concentrations and 3 NaCl concentrations (Chapter III) and all the possible combinations between them showed some very interesting results. The reproductive output of collembolan is affected by increasing concentrations of pesticide, but when combined with NaCl a higher salinity seems to lower the effect of the pesticide. This should be further investigated using a larger gradient of salinity, to better understand this interaction between NaCl and lambda-cyalothrin in the reproduction of collembolan.

The GLM model obtained with the data from this test explained 83.8% of the variation, showing that the reproductive output is better with lower salinity and pesticide contamination. I suggest the use of this model to predict the reproduction of collembolan in agricultural soils affected by these two contaminants.

IV.3 – References

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