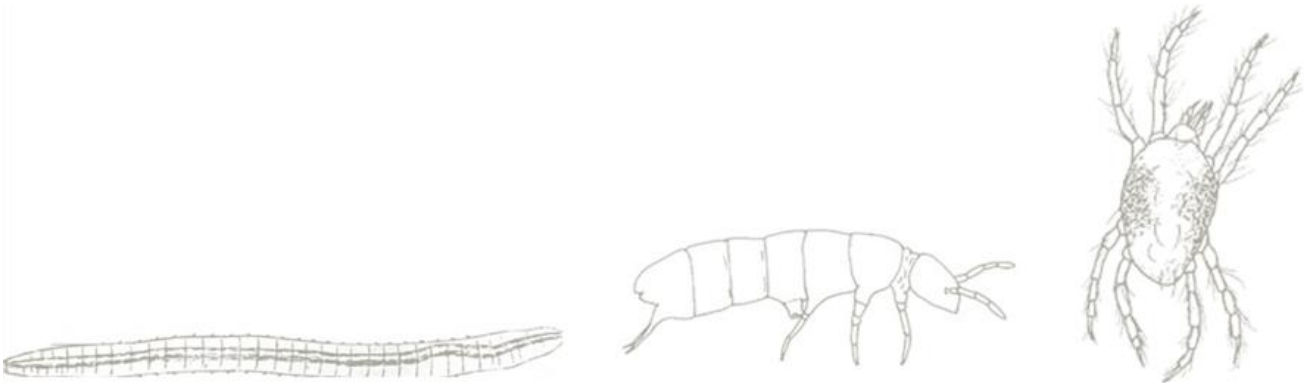
DEPARTAMENTO DAS CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE COIMBRA

Prediction of salinisation effects on soil ecosystems due to climate changes



Carla Sofia Pereira

2014

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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 Sousa (Universidade de Coimbra) e da Doutora Sónia Chelinho (Universidade de Coimbra).

Carla Sofia Pereira

2014

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Resumo

O aumento da temperatura média potencia efeitos como a perda de stocks de gelo e icebergues e o aumento do nível do mar. Os efeitos negativos da salinisação dos solos tendem a aumentar devido ao aumento do nível do mar e consequente intrusão salina. Os impactos da exposição à água do mar (constituída principalmente por cloreto de sódio – NaCl) no compartimento solo são desconhecidos. O presente estudo tem por objectivos (1) avaliar os efeitos da salinisação em invertebrados de solo, (2) avaliar a possibilidade do uso de NaCl como indicador dos efeitos da água do mar na fauna de solo, (3) avaliar a capacidade de aclimatação a baixas concentrações de sal de duas espécies de invertebrados de solo e a resistência dos organismos aclimatados quando posteriormente expostos a um gradiente de concentrações do mesmo sal, (4) avaliar os efeitos da salinisação numa comunidade natural de solo devido a intrusão e irrigação salinas. Para cumprir os objectivos (1) e (2), testes padronizados de reprodução foram efectuados usando um solo standard artifical – OECD – e um gradiente de diluições de água do mar ou concentrações de NaCl. A reprodução e mortalidade de três espécies padrão de invertebrados de solo (o colêmbolo *Folsomia candida*, o enquitraideo *Enchytraeus crypticus* e o ácaro *Hypoaspis aculeifer*) foram avaliadas. Os resultados mostraram ausência de efeitos da adição de água do mar ou cloreto de sódio na mortalidade dos adultos. No entanto, na reprodução dos organismos foi observado um aumento da sensibilidade na seguinte ordem: *H. aculeifer* << *E. crypticus* ≈ *F. candida*. Estes resultados são consistentes com as diferentes vias de exposição ao sal: colêmbolos e enquitraideos estão

expostos por ingestão e contacto enquanto que os ácaros estão expostos apenas por ingestão devido à presença de um exoesqueleto rígido e contínuo. Apesar das pequenas diferenças observadas nos valores de EC's calculados, a água do mar e o NaCl provocaram os mesmos efeitos gerais. Estes resultados indicam que o NaCl pode ser usado como indicador dos efeitos da água do mar nos organismos de solo, numa primeira etapa de avaliação de risco. Para avaliar o potencial de aclimatação de duas espécies standard de invertebrados de solo (o colêmbolo *Folsomia candida* e o enquitraideo *Enchytraeus crypticus;* objectivo (3)), os mesmos foram expostos durante 26 semanas, em solo artificial OECD contaminado com uma concentração equivalente ao EC_{20} (concentração que causa 20 % de efeitos na reprodução) calculado previamente. Após este período, os organismos aclimatados foram extraídos e usados num teste padrão de reprodução usando a mesma série de concentrações de sal previamente testadas com organismos não aclimatados. Numa exposição mais prolongada dos colêmbolos ao NaCl, foi observado um efeito drástico na sobrevivência e reprodução, efeito esse superior ao teoricamente esperado (20 %). Pelo contrário, os enquitraideos aclimatados mostraram uma maior tolerância ao NaCl após o tempo de aclimatação, comparativamente aos não aclimatados. Os mecanismos de sobrevivência usados por estes grupos faunais podem explicar a tolerância a níveis baixos de sal, juntamente com uma mudança na população, através da sobrevivência dos fenótipos mais resistentes. Para cumprir o objectivo (4) foram usados Modelos de Ecossistemas Terrestres (TME's) simulando dois cenários de salinisação dos solos, com duas diluições de água do mar: a) intrusão salina, que consistiu na imersão dos últimos 10 cm dos TME's nas diluições de água do mar e rega

com água destilada e b) intrusão salina previamente descrita combinada com rega usando as mesmas diluições de água do mar. Foram estabelecidos três períodos de amostragem de macrofauna, mesofauna de solo e biomassa de plantas de forma a avaliar a comunidade inicial (T0), os efeitos da exposição à água salina (T1) e a possível recuperação das comunidades (T2). No caso dos TME's verificou-se uma elevada variação espacial principalmente considerando a abundância de colêmbolos e ácaros. No entanto, na conductividade mais baixa a abundância de organismos de solo não foi afectada enquanto que no tratamento com a conductividade mais alta, um decréscimo no número de enquitraideos foi observado quando comparado com o controlo. No entanto, os enquitraideos foram capazes de recuperar totalmente a abundância total da população oito semanas após o *terminus* da rega salina, enquanto que para as minhocas e para a biomassa de plantas se verificaram efeitos retardados. Apesar da resistência de algumas espécies de solo a níveis baixos de salinisação, algumas funções de solo podem ser afectadas num cenário real devido à capacidade de algumas espécies evitarem condições salinas. Aliás, efeitos negativos podem ser observados em solos considerados não salinos pois o limite actual (4000 μScm^{-1}) para definir solos salinos, não reflecte o conhecimento existente para a fauna de solo.

Abstract

The increase of global mean temperature is a rising problem due to its potential negative effects such as melting of glaciers and ice caps and sea level rise. Soil salinisation problems are increasing with the rising of the sea level and intrusion of seawater. Impacts of exposure to seawater (mainly constituted by sodium chloride - NaCl) on the soil compartment are still unknown. The present study aimed at (1) evaluating the effects of salinisation on soil invertebrates, (2) assessing the possibility of using NaCl as a surrogate of seawater effects on soil fauna, (3) evaluating the ability of two standard soil invertebrates to acclimate to low concentrations of NaCl, and the resistance of acclimated organisms exposed to a range of concentrations of the same salt, (4) evaluating the effects of salinisation on a soil natural community due to intrusion and irrigation with saltwater. To fulfill aims (1) and (2), standard ecotoxicological reproduction tests were performed using standard artificial OECD soil spiked with serial dilutions of seawater or a gradient of sodium chloride concentrations. The reproduction and mortality of three standard soil invertebrates (the springtail *Folsomia candida*, the enchytraeid *Enchytraeus crypticus* and the predatory mite *Hypoaspis aculeifer*) were evaluated. Results showed no effects of seawater and sodium chloride addition in adult mortality. However, for reproduction, an increased sensitivity was observed in the following order: *H. aculeifer* << *E. crypticus* ≈ *F. candida*. These results are consistent with the different ways of exposure: springtails and enchytraeids are exposed by ingestion and contact while mites are only exposed by ingestion due to a continuous and thick exoskeleton. Although the small differences observed in

the calculated EC's values, seawater and NaCl had the same overall effects. To assess the acclimation ability of two standard soil invertebrates (the springtail *Folsomia candida* and the enchytraeid *Enchytraeus crypticus;* aim (3)), the organisms were exposed to artificial OECD soil spiked with the equivalent EC_{20} concentration previously derived for NaCl exposure, during 26 weeks (acclimation). After that period, acclimated organisms were extracted and exposed to a gradient of NaCl concentrations already tested with unacclimated organisms. In a long-term exposure of springtails to NaCl, a drastic effect on survival and reproduction of the population, more than theoretically expected (20%), was observed. On the contrary, acclimated enchytraeids showed a higher tolerance to NaCl after the exposure period than the unacclimated organisms. The survival mechanisms used in these faunal groups to desiccation may explain the tolerance to low salt concentrations alongside with a change in the population with the survival of the resistant phenotypes. To fulfill aim (4), Terrestrial Model Ecosystems (TME) were used simulating two soil salinisation scenarios with two dilutions of natural seawater: 1) saltwater intrusion by immersing the lower 10 cm of the TME's in the dilutions of seawater and surface irrigation with distilled water and 2) saltwater intrusion described earlier plus surface irrigation with the dilutions of seawater. Three sampling periods of soil macrofauna, mesofauna and plant biomass were established in order to evaluate the initial community (T0), effects of saltwater exposure (T1) and possible community recovery (T2). TME's showed some associated variability mainly when considering springtail and mite abundance. However, at the lowest conductivity values the abundance of soil organisms was not affected while for treatments with highest conductivity, a lower number of enchytraeids were

found when compared with the control. Nevertheless, enchytraeids were able to recover their population after the eight weeks of recovery while earthworms and plant biomass showed delayed effects in this sampling period. Despite the resistance of some soil species to low salinisation levels, some soil functions can be affected at field scale in a real scenario due to the ability of some species to avoid highly saline places. Besides, adverse effects can be observed on soils not consider saline since the actual limit (4000 μScm^{-1}) does not reflect the existing knowledge considering soil fauna.

Chapter I: GENERAL INTRODUCTION

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General Introduction

I.1. Climate change

"Climate change" is a recent term, firstly used in 1975 alongside with the term "Global warming" in a paper published by Wallace Broecker (Plait, 2014). Despite the increasing population awareness to climate change and its effects, greenhouse gases and ozone-depleting substances like chlorofluorocarbons and chlorinated solvents are still being launched to the atmosphere. Indicators of climate change like the increase of atmospheric water vapour and surface temperature, changes in precipitation, occurrence of extreme climatic events, melting of glaciers, ocean and land ice, and rise of sea level are becoming of more concern every day. The high amount of water vapour in the atmosphere promotes the warming in the troposphere and the cooling in the stratosphere which enhances the ozone loss in the last one. In fact, an increase of 0.85 ºC on combined land and ocean surface temperature was observed between 1880 and 2012 which combined with changes in precipitation enhances extreme events like wet and dry seasons. Extreme events are worsening with the melting of glaciers and ice caps and stocks. Estimated ice losses differ according to the region, but a general decrease on ice sheets was observed in the last decade. Permafrost temperatures are also increasing since the 80's (e.g. 3 and 2 ºC of increase in Alaska and Russian European North). Consequently, sea level rise varied between 1.5 and 1.9 $mmyr^{-1}$ in the last

century and between 2.8 and 3.6 $mmyr^{-1}$ in the last decade. It is expected a sea level rise of 40 to 67 cm until 2100 (IPCC, 2013).

I.1.1. Soil salinisation

Effects of climate change like the melting of ice stocks and glaciers with a consequent sea level rise alongside with water expansion (due to the increase of temperatures; IPCC, 2013) can lead to seawater intrusions (IPCC, 2007). Saltwater can be consequently used for irrigation (Wang and Li, 2012) since the freshwater availability and quality will be reduced leading to salt-affected soils (IPCC, 2007). Salt-affected soils can be defined depending on their composition, i.e., high salt contents constitute saline soils and high sodium content constitutes sodic soils. Some countries around the world already possess a large area of salt-affected soils, ranging from 3 thousand hectares (Austria) to 47325 thousand hectares (Spain). In Portugal 250 thousand hectares of salt-affected soils have been currently reported (Eckelmann et al, 2006).

Soil salinisation is by definition the accumulation of soluble salts in soils affecting its fertility and can occur through natural processes like the increased evapotranspiration (primary salinisation), and/or through human activities, like the increase withdrawals from aquifers (secondary salinisation) (European Soil Portal, 2012). The origin of soil salinisation is highly dependent on the geographic location. While soils of coastal areas are often affected by groundwater withdrawals and extreme events (tsunamis and floodings), soils distant from the coast are usually affected by irrigation with salt water, high

evapotranspiration rates and the impediment of salts washing due to the soil textural characteristics (European Commission, 2005).

At a global scale, the arid and semi-arid parts of Australia, South America, Asia and Europe will be more affected by soil salinisation (European Soil Portal, 2012). More than 77 million hectares of saline soils have been estimated for the whole planet with 43 million hectares derived from secondary salinisation with a tendency to spread at a rate of up to 2 million hectares per year (Abbas et al, 2011). Soil salinisation is a key threat to soils in Europe with the countries near the Mediterranean and Caspian Seas being the most affected (European Soil Portal, 2012) with an increase of 1 million hectares of saline soils estimated in 2002 (Commission of The European Communities, 2002) and the prediction to 2012 are for 3 million hectares (European commission, 2012).

The classification of saline soils depends not only on the amount of salts dissolved, but also on the pH and the exchangeable sodium percentage (ESP). Soils with a pH lower than 8.5, an ESP lower than 15% and an electrical conductivity equal or higher than 4000 μScm^{-1} are considered saline. This last threshold was defined based on effects on plants (Micheli et al, 2002). The available information on the effects of salinisation on the different groups of terrestrial and aquatic organisms is described in Table I.1.

Actually, saline soils are starting to be used in saline agriculture instead of being recovered. This type of agriculture uses saline areas combined with saline irrigation water and tolerant species to achieve a better production. Nevertheless, this is a recent strategy that is still being optimized and the potential of tolerant species production under such conditions and the

usefulness of such species to the human diet still need to be confirmed (Ladeiro, 2012).

Table I.1: Literature review of the effects of salinisation on different organisms.

Environmental Compartment	Organisms	Effects	Reference
Soil	Plants	Changes in plant physiognomy	De Melo et al, 2011
		Decrease on biomass	Conesa et al, 2011
		Insufficient water, mineral nutrients, small organic molecules, proteins and hormones supply	Perez-Alfocea et al, 2011
		Deficient seedling growth	Hardikar et al, 2011
	Soil macrofauna	Avoidance behaviour to saline soils of earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*)	Owojori and Reinecke, 2009
		Reproduction and survival impairment on earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*)	Owojori et al, 2009
	Soil mesofauna	Cessation of reproduction on	Owojori et al, 2009

		springtails (*Folsomia candida*) and enchytraeids (*Enchyraeus crypticus*)	
		Avoidance behaviour to saline conditions dependent on the presence/absence of food by the nematode *Caenorhabditis elegans*	Adachi et al, 2010
	Microorganisms	Changes on the community with an increase on *Archea*	Andronov et al, 2012
		Lower catabolic activity, reduced efficiency of substrate utilization, depression of soil respiration and enzyme activity	Ghollarata and Raiesi. 2007
		Low microbial biomass	Wichern et al, 2006
Water	Global ecosystem	Reduction in aquatic biodiversity	Cañedo-Arguelles et al, 2013
	Macrophytes	Change on the community with a dominance of salt tolerant species, reduction in	Davis et al, 2003

		abundance	
	Zooplankton	Lower numbers of density	Van Meter et al, 2011
	Microinvertebrates	negative effects on growth, development and reproduction	Kefford et al, 2007
	Fishes	absence of the threat-sensitive anti-predator response	Hoover et al, 2013

I.2. Soil salinisation in Portugal

Portugal has a mild Mediterranean climate with mean annual air temperatures varying between 7 and 18 ºC and mean annual precipitation slightly above 900 mm (varying between 500 mm and 3000 mm; Santos et al, 2011). In Portugal an increase in mean air temperature has been observed result of a faster rise of the daily minimum temperature and not due to the rise of daily maximum temperature, as it was expected. A decrease in precipitation was also found in the period between 1931 and 2000 with a worst scenario after 1976. Precipitation data after 1976 shows significant differences between seasons. A higher amount of extreme events have been observed with a higher number of consecutive dry days (risk of droughts) and consecutive rainy days (risk of floodings). Effects of climate change are expected to impact mainly water resources, coastal zones, agriculture, energy, health and biodiversity. The main impacts are effects on water scarcity (due to the precipitation decrease and consequent reduced runoffs and rivers flow affecting also the agriculture

and the hydropower). The sea level rise observed in the 20th century of 10-20 cm along the Portuguese mainland coast, with an estimated sea level rise between 1.3 and 1.7 mmyr^{-1}, will also have important impacts due to coastal erosion, reduction of sediment input, land loss of about 67 % of the coastal zones until 2100 and destruction of coastal ecosystems. In agriculture, the increase of pests, diseases and weeds can cause changes in agricultural crops and lead to loss of biodiversity. With the increase of temperature, the occurrence of fires will increase leading to the introduction of invasive species, avoidance of endemic species and heat-related deaths (Santos et al, 2001).

Portuguese territory has a maritime borderline with areas of land elevation close to the sea level that are more exposed to soil salinisation (Fig. I.1). In fact, a study developed in the groundwater wells of Costa da Caparica (Lisbon) showed seawater intrusions up to 5 % presenting a conductivity value of 6290 μScm^{-1} (Ferreira, 2012). The South of Portugal seems to be more affected due the arid/semi-arid climate (high summer temperatures and scarce rainfall), inadequate leaching of the salts and high evapotranspiration rates (Gonçalves et al, 2006). Around the Alqueva dam, more than 60 % of the area presents a medium to elevated risk of soil salinisation problems (Mendes and Carvalho, 2009).

Fig. I.1: Land elevation in relation to sea level, highlighting the most vulnerable areas to salinisation (source: http://www.globalwarmingart.com/wiki/Special:SeaLevel).

I.3. Soil protection entities and policies

Soil is a limited resource and may be affected by several threats: erosion, organic matter decline, contamination, salinisation, compaction, soil biodiversity loss, sealing, landslides and floodings (Commission of the European Communities, 2006). Soil protection has been addressed in technical reports and proposals of different entities. For example, FAO related soil threats (including salinisation) with agriculture and food scarcity (FAO, 2014). Also, in Europe technical reports on soil protection and climate changes have been published by the European Commission (European Commission, 2008) and by

the Intergovernmental Panel on Climate Change, being the last one written and published in 2013/2014 (IPCC, 2014).

The IPCC reports have two components: a bibliographic review incorporating all the available information on climate change, its driving forces and observed effects combined with models that predict the different possible scenarios and their consequences on ecosystems (IPCC, 2014).

The technical reports and the “Soil Atlas of Europe” published by the European Commission are more focused on describing salinisation processes, its effects on the ecosystem and identify some of the major affected regions (European Commission, 2005 and European Commission, 2012). The estimated cost of soil salinisation varies between 158 and 321 million euros per year (European Commission, 2012), thus, the need for the identification and management of saline soils. The proposal for a “Soil Framework Directive” (Commission of the European Communities, 2006) includes soil salinisation as one of the eight causes of soil degradation in Europe. In this document, the identification of saline soils is proposed to be done considering soil typological unit, soil texture, soil hydraulic properties, irrigation areas, chemical properties of irrigated water and type of irrigation techniques, groundwater information and climate. The proposal aims to implement risk reduction targets and programs after the identification of the areas at risk and its driving forces (Commission of the European Communities, 2006). This proposal was pending for nine years with many discussions on the subject. In May 2014, the proposal was withdraw but the Commission of the European Communities is still committed to the cause. The next step is to evaluate the best strategy to achieve soil protection (European Commission, 2014)

I.4. Aims and thesis structure

I.4.1. Aims

The present study aimed to evaluate the effects of salinisation due to sea level rise on soil ecosystems, contributing to reduce uncertainties inherent to risk assessment of soil salinisation and to promote consistent biodiversity protection/conservation policies. More specifically the present study had four main objectives:

(1) to evaluate the effects of both NaCl and seawater on standard soil invertebrate species,

(2) to assess the ability of two soil invertebrate species to acclimate to low concentrations of salt,

(3) to predict the effects of salinisation on soil ecosystems and its recovery potential, using terrestrial model ecosystems (TME's) and a natural soil community,

(4) to assess safety levels for salinity to soil ecosystems.

I.4.2. Thesis structure

To fulfil the aims described earlier, the experiments were performed in three main steps. Together with this introductory chapter and a final discussion, they constitute the five sections of this thesis:

(1) An overall introduction to climate change and soil salinisation (Chapter I)

(2) Assessment of the different sensitivity of three standard soil invertebrate species (*Folsomia candida*, *Enchytraeus crypticus* and *Hypoaspis aculeifer*) to

soil salinisation using both seawater and sodium chloride, the latter used as a possible surrogate of seawater (Chapter II)

(3) Evaluation of the acclimation potential of two standard soil invertebrate species (*Folsomia candida* and *Enchytraeus crypticus*) by exposing them to soils spiked with low concentrations of salt and then using the acclimated organisms in reproduction tests (Chapter III)

(4) Assessment of soil salinisation effects and the recovery potential of a natural community using TME's and simulating both saline intrusion and irrigation (Chapter IV)

(5) A general discussion presenting the most important findings of the present study and also upcoming work on soil salinisation effects (Chapter V)

I.5. Experiments conducted and expected results

In order to evaluate how different salinity levels affect mortality and reproduction rates of soil organisms, standard reproduction tests were performed using three standard soil invertebrate species (the springtail *Folsomia candida*, the enchytraeid *Enchytraeus crypticus* and the mite *Hypoaspis aculeifer*) in standard artificial OECD soil spiked with a gradient of salt concentrations (sodium chloride – NaCl) and a gradient of seawater dilutions. Within this first aim, the possibility of using sodium chloride as a surrogate of seawater was also assessed. Effect concentrations (EC's) of 20 % and 50 % on reproduction were estimated. It was expected that species exposed mainly via soil pore water, like soft-bodied organisms, would be more sensitive to salinity. Furthermore, since salt water has a large percentage of

NaCl, it was expected that latter would act as surrogate of the former (Chapter II).

In Chapter III, the acclimation potential of two standard soil invertebrate species was assessed. They were kept for 26 weeks in standard artificial OECD soil spiked with the concentration corresponding to the EC_{20} value previously estimated. It was expected that after this time, only the resistant individuals would be able to acclimate and reproduce (more tolerant offspring). After that incubation time, the acclimated organisms were used in a standard reproduction test to evaluate their reproductive output. It was expected that acclimated organisms would be more resistant against salt contamination than unacclimated ones (Chapter III).

Terrestrial Model Ecosystems (TME's) simulating both the seawater intrusion and irrigation for 6 weeks were used to fulfill the last aim. In this last part of the work, negative effects of salinity were expected mainly in the highest seawater dilutions in all groups sampled (especially the soft-bodied organisms) except for the nematodes (since they are known to be very resistant to sodium chloride). After the recovery period, it was expected that the abundance of the groups sampled would be similar to the control (Chapter IV).

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Chapter II: EFFECTS OF SOIL SALINISATION ON SOIL ORGANISMS

Chapter II

Effects of soil salinisation on soil organisms

II.1. Abstract

The increase of global mean temperature is rising serious concerns worldwide due to its potential negative effects such as droughts and melting of glaciers and ice caps. Expected impacts on soil compartment include floodings, seawater intrusions and subsequent use of saltwater for irrigation, with unknown effects on soil ecosystems and their inhabitants. The present study aimed at evaluating the effects of salinisation on soil ecosystems due to sea level rise and assessing the possibility of using sodium chloride as a surrogate of seawater effects on soil fauna. The reproduction and mortality of three standard soil invertebrates (the springtail *Folsomia candida*, the enchytraeid *Enchytraeus crypticus* and the predatory mite *Hypoaspis aculeifer*) in standard artificial OECD soil spiked with serial dilutions of seawater or a gradient of sodium chloride were evaluated according to standard guidelines. Results showed no effects of seawater and sodium chloride addition in adult mortality. However, for reproduction, an increased sensitivity was observed in the following order: *H. aculeifer* << *E. crypticus* ≈ *F. candida*. For springtails and enchytraeids the results obtained were similar (the EC_{50s} in seawater were 857 μScm^{-1} and 1449 μScm^{-1}, respectively for *F. candida* and *E. crypticus*; and with NaCl were 986.5 μScm^{-1} and 883.5 μScm^{-1}, respectively for *F. candida* and *E. crypticus*). These results are consistent with the different ways of exposure: springtails and enchytraeids are exposed by ingestion and contact while mites

are only exposed by ingestion due to a continuous and thick exoskeleton. Although the small differences observed in the calculated EC's values, seawater and NaCl had the same overall effects with a lower sensitivity of mites and a similar sensitivity of springtails and enchytraeids. In a test screening phase, NaCl could be used as a surrogate of seawater effects on soil fauna. In the long term, the lack of resistance of soil species to salinity may negatively affect the ecosystem services provided by soil invertebrates. These adverse effects can be observed on soils not consider saline since the actual limit (4000 μScm^{-1}) does not reflect the existing knowledge considering soil fauna.

II.2. Introduction

Over the past years, the increase of global mean temperatures is causing the decrease of the snow cover and of the ice stocks. These events have originated a rising of the sea level (IPCC, 2007a). It increased from 1.5 to 1.7 mm/year, observed in the last century, to 3 mm/year in the last decade (IPCC, 2013). The prediction is between 40 and 62 cm until 2100 (IPCC, 2013) augmenting the risk of drought and flooding events (IPCC, 2007b). At a global scale, the most affected regions will be the arid and semi-arid parts of Australia, South America, Asia and Europe (European Soil Portal, 2012). In Europe, countries near the Mediterranean and Caspian Seas have been the most affected (European Soil Portal, 2012) with an increase of 1 million hectares of saline soils in 2002 (Commission of The European Communities, 2002) and an estimated 3 million hectares in 2012 (European commission, 2012). In Spain, about 3% of the 3.5 million hectares of irrigated land is affected by soil salinisation limiting the local

agriculture (Van-Camp et al, 2004). Along with Spain, France, Greece, Italy and Portugal (among others) have extensions of 250, 3.5, 450, 25 thousands hectares of saline soils, respectively (Eckelmann et al, 2006). The rise of sea level will lead to soil salinisation mainly due to seawater (constituted by free ions of sodium – 31 % - and chloride - 55%; Wiesenburg and Little, 1987-1988) intrusions (IPCC, 2007b) and irrigation with saltwater (Wang and Li, 2012) since the freshwater availability and quality will be reduced (IPCC, 2007b). The accumulation of soluble salts on soils negatively affects their fertility (European Soil Portal, 2012) and can occur through natural processes like the increased evapotranspiration (primary salinisation) and/or induced by human activities like the increase withdraws from aquifers (secondary salinisation) (European Soil Portal, 2012). The classification of saline soils depends not only on the amounts of salts dissolved, but also on the pH and the exchangeable sodium percentage (ESP). Soils with a pH lower than 8.5 and ESP lower than 15% are considered saline when the electrical conductivity (EC) is equal or higher than 4000 μScm^{-1} (Micheli et al, 2002). Crops yields can be further affected due to the increase of pest species and/or diseases, risk of disappearance of less resistant species to salinity, with potential loss of biodiversity (IPCC, 2007b).

Terrestrial and aquatic communities are differently affected by salinisation but all suffer a change on their species abundance and diversity with a dominance of salt-tolerant species (Davis et al, 2003, Andronov et al, 2012). Despite this, some species are known to tolerate salinity due to special morphological or physiological traits. The spider *Arctosa fulvolineate* and the beetle *Merizodus soledadinus* can accumulate amino acids which originate an increase in the osmolality of body fluids (Foucreau et al, 2012; Hidalgo et al, 2013), while

different species of amphipods can regulate the internal concentration of salts by their release in the urine (hypo-osmotic or isosmotic urine; Morritt, 1988).

Despite the existing knowledge on the impacts of salinity on coastal ecosystems in freshwater and plant species (James et al, 2003), soil organisms and their responses have been neglected. From the few studies conducted, the most studied soil fauna groups are earthworms and nematodes. Besides the avoidance behavior to natural saline soils by earthworms (Owojori and Reinecke, 2009) and the complex relationship between nematodes and salt (with tolerance of *Caenorhabditis elegans* to salt in the presence of food and avoidance in its absence) (Adachi et al, 2010), effects in a long-term experiment are only described for earthworms. Owojori et al (2009) exposed two earthworm species (*Eisenia fetida* and *Aporrectodea caliginosa*) to a natural saline soil with electrical conductivities (EC) between 0.08 and 1.62 dSm^{-1} and found significant effects on growth, mortality and reproduction. Both species showed a higher sensitivity when considering reproduction (with the production of cocoons only in the control). In the same study, reproduction of springtails (*Folsomia candida*) and enchytraeids (*Enchytraeus doerjesi*) were also evaluated with significant effects on reproduction. Complete cessation of reproduction was observed for springtails at 1620 μScm^{-1} and for enchytraeids at 1310 μScm^{-1} and above.

Effects like those reported above can originate an impairment in soil functioning due to the relevant role that soil fauna has on key ecological processes like organic matter decomposition, nutrient cycling and maintenance of soil structure (Lavelle et al, 2006). Therefore it is essential to better comprehend salinisation impacts on soil fauna, to better perceive the effects of this stressor on soil functions underlying key ecosystems services.

The present study had three main objectives: 1) to evaluate the effects of exposure to sodium chloride (NaCl) and seawater on reproduction of three standard soil test-species; 2) to evaluate the use of NaCl as a surrogate of exposure of soil organisms to seawater and 3) to derive safety levels of salinity to soil fauna. In order to fulfill these aims, standard reproduction tests using three standard soil invertebrates (the springtail *Folsomia candida*, the ecnhytraeid *Enchytraeus crypticus* and the mite *Hypoaspis aculeifer*) were performed using artificial OECD soil spiked with a gradient of concentrations of salt (sodium chloride) or a gradient of seawater dilutions, the later with equivalent conductivities to the former.

II.3. Materials and Methods

II.3.1. Test soil and concentration range for NaCl and seawater

The artificial OECD soil, used in all assays, was prepared mixing 5 % of air dried and sieved sphagnum peat, 20 % of kaolin clay and 75 % of quartz sand. The pH was adjusted with $CaCO_3$ to pH 6.0±0.5 (OECD-226, 2008). In order to evaluate the use of sodium chloride (NaCl) as a surrogate of seawater, two sets of tests were performed. A gradient of NaCl concentrations or seawater dilutions was used as shown in Table II.1. NaCl concentrations were prepared diluting a stock solution of sodium chloride (Merck KGaA, 64271 Darmstadt, Germany) in distilled water. Seawater was collected from Praia da Barra in Aveiro, Portugal, filtered through cellulose nitrate membranes (0.20 µm) and kept at 4ºC until used. The NaCl gradient was prepared using a multiplication factor of 1.37 starting on 0.5 gKg^{-1} dry weight (DW) and ending on 4.5 gKg^{-1} DW

(Table II.1). The range of concentrations was performed based on the reproduction tests performed by Owojori et al (2009) for springtails and enchytraeids. A different concentration range was used for the mite test due to the lower sensitivity observed in this species in the range finding test performed earlier (were no effects were observed on concentrations of 0.5, 0.9, 1.8, 3.3 and 4.5 gKg^{-1} of NaCl DW). The seawater dilutions were prepared mixing seawater with distilled water. The concentrations of NaCl and the seawater dilutions were prepared in order to obtain an equivalent range of conductivities. In case of mite tests, only five seawater dilutions were prepared since the equivalent conductivities of the last three NaCl concentrations were higher than the conductivity of the soil when mixed with pure seawater (Table II.1).

II.3.2. Test Organisms

All test organisms were obtained from cultures maintained in the Soil Ecology and Ecotoxicology Laboratory from the Department of Life Sciences, University of Coimbra, Portugal. Springtails and mites were fed three times per week after the change of the culture medium. Mites were fed with “cheese mites” (*Tyrophagus putrescentiae*) while springtails were fed with dry yeast (ISO-11267, 1999; OECD-226, 2008). Enchytraeids were fed once a week and were maintained in agar plates (ISO-16387, 2003). All cultures were maintained at 20±2ºC with a 16:8 (light:dark) photoperiod.

Table II.1: Range of NaCl concentrations, seawater dilutions (SW) and the corresponding measured conductivity values used in the reproduction tests with *Folsomia candida*, *Enchytraeus crypticus* and *Hypoaspis aculeifer* (NaCl - salt concentrations; Cond - measured conductivity in the solution; SW - seawater dilutions; DW – dry weight.).

Folsomia candida* and *Enchytraeus crypticus	NaCl (g/Kg DW)	0	0.5	0.7	0.9	1.3	1.8	2.4	3.3	4.5
	Cond (µS/cm)	107.4	262	305	372	494	634	825	1057	1415
	SW (%)	0	5	8	10	14	19	25	33	45
	Cond (µS/cm)	134.5	309	388	456	615	742	988	1264	1677
Hypoaspis aculeifer	NaCl (g/Kg DW)	0	1.6	2.6	4.1	6.6	10.5	16.8	26.8	42.9
	Cond (µS/cm)	108.5	629	841	1276	1963	2760	4250	6830	10130
	SW (%)	0	17	24	38	59	91	100	---	---
	Cond (µS/cm)	180.5	729	997	1395	1973	3040	3320	---	---

II.3.3. Reproduction Tests

II.3.3.1. Springtails (*Folsomia candida*)

The tests were performed according to ISO guideline (ISO-11267, 1999). Briefly, ten individuals, between 10 and 12 days old, were placed into vessels with 30 g fresh weight of the test soil. The springtails were fed with dry yeast at the beginning of the test and at the 14th day. After 28 days, the content of each vessel was transferred to a larger vessel and then flooded with water and gently stirred with a spatula. A few drops of dark-blue ink were added to increase contrast of the springtails. The surface of the vessels was then photographed

and the organisms counted using Image Tool software (software available at http://compdent.uthscsa.edu/dig/itdesc.html).

II.3.3.2. Enchytraeids (*Enchytraeus crypticus*)

The enchytraeid reproduction test was performed according to ISO-16387 (2003). Ten individuals were placed in vessels with 20 g dry weight of the test soil and fed once a week with autoclaved rolled oats. After 28 days, 5 ml of ethanol (96%) were added to the vessels and then filled with water (one centimeter above the soil level) and a few drops of Bengal red 1% were added to stain the organisms. The vessels stood for 24h and the enchytraeids were then counted as described by Chelinho et al (2014).

II.3.3.3. Mites (*Hypoaspis aculeifer*)

Reproduction tests with mites were performed according to OECD-226 (2008) in vessels placing ten females with an age of 28-35 days in 20 g dry weight of the test soil. The mites were fed at the beginning and twice a week with cheese mites (*Tyrophagus putrescentiae*) during 14 days. After those 14 days, the mites were extracted using a Macfadyen high-gradient extractor and counted under a stereomicroscope.

All tests described before were maintained at 20±2ºC with a photoperiod of 16:8 (light:dark).

II.3.4. Data analysis

A one way analysis of variance (ANOVA) was used to test differences between the control and the different concentrations/dilutions of NaCl or seawater followed by post hoc comparisons (Dunnett's test) in Statistica 7.0 (http://www.statsoft.com/). ANOVA assumptions, normality and homoscedasticity, were checked using Shapiro-Wilk's and Levene's tests, respectively. Whenever these assumptions were not fulfilled, data were transformed (using logarithmic, square root and exponential transformations according to the type of data). Effect concentrations (EC's) causing 20 and 50 % reduction in reproduction were calculated for each test species for both NaCl and seawater tests using non-linear regression models, according to Environment Canada (2007). For comparison purposes, values are expressed in μScm^{-1}.

II.4. Results

The validity criteria defined for each test by the respective guideline were fulfilled. For springtails (ISO-11267, 1999) adult survival and number of offspring in the control averaged 93 % and 345 respectively, with the latter having a coefficient of variation (CV) of 16 % for NaCl. For the test using seawater, springtails showed an adult survival, number of offspring and respective CV of 90 %, 433 and 20 %. For enchytraeids (ISO-16387, 2003) average number of individuals and corresponding CV were 712 individuals and 7 %, respectively. When testing seawater, enchytraeids showed an average number of individuals and respective CV of 519 and 29 %. For mites (OECD-

226, 2008) the mean female survival was 96 % and the mean number of juveniles produced in the control was 235, with a CV of 11 % for NaCl. For seawater the values of the same parameters were 94 %, 160 and 28 %, respectively. Furthermore, no relevant adult mortality was observed in any of the concentrations tested on the six tests (NaCl: average adult survival of 91 % and 99 % for springtails and mites, respectively; seawater: average adult survival 93 % and 91 % for springtails and mites, respectively). The last concentration of NaCl tested with mites presented a significant reduction on adult survival, but this concentration had conductivity higher than the soil mixed with pure seawater.

II.4.1. Effects of exposure to NaCl

The three species tested showed different responses to a 28-day NaCl exposure period (Fig. II.1). For springtails and enchytraeids, an increase of the number of juveniles in the first concentrations in relation to the control was observed although not statistically significant (One Way ANOVA, Dunnett test, $p>0.05$). Enchytraeids, at concentrations higher than 0.7 gKg^{-1} NaCl DW (295 μScm^{-1}), showed a gradual decrease on the number of juveniles, only statistically significant at concentrations higher than 1.8 gKg^{-1} NaCl DW (616.5 μScm^{-1}; One Way ANOVA, Dunnett test, $p<0.05$; Fig. II.1A). On the other hand, for *F. candida* the number of juveniles only decreased after 1.3 gKg-1 NaCl DW (493.5 μScm^{-1}) and it was only statistically significant at concentrations higher than 3.3 gKg^{-1} NACl DW (1091 μScm^{-1}; One Way ANOVA, Dunnett test, $p<0.05$; Fig. II.1B). Mites showed similar number of juveniles in relation to the control until 10.5 gKg^{-1} NaCl DW (2740 μScm^{-1}). Only in the last three

concentrations (with conductivity values higher than the soil mixed with pure seawater) a statistically significant decrease on the number of juveniles was observed (One Way ANOVA, Dunnett test, $p<0.05$; Fig. II.1C).

Despite the different responses to the salt concentrations, enchytraeids and springtails showed similar sensitivities with EC_{50s} of 883.5 ± 99.2 μScm^{-1} and 986.5 ± 202.6 μScm^{-1}, respectively; and EC_{20s} of 562.2 ± 133.6 μScm^{-1} and 797.7 ± 162.1 μScm^{-1}, respectively. Mites were the least sensitive with an EC_{50} of 6027.6 ± 350.3 μScm^{-1} and EC_{20} of 4570.4 ± 452.8 μScm^{-1} (Table II.2).

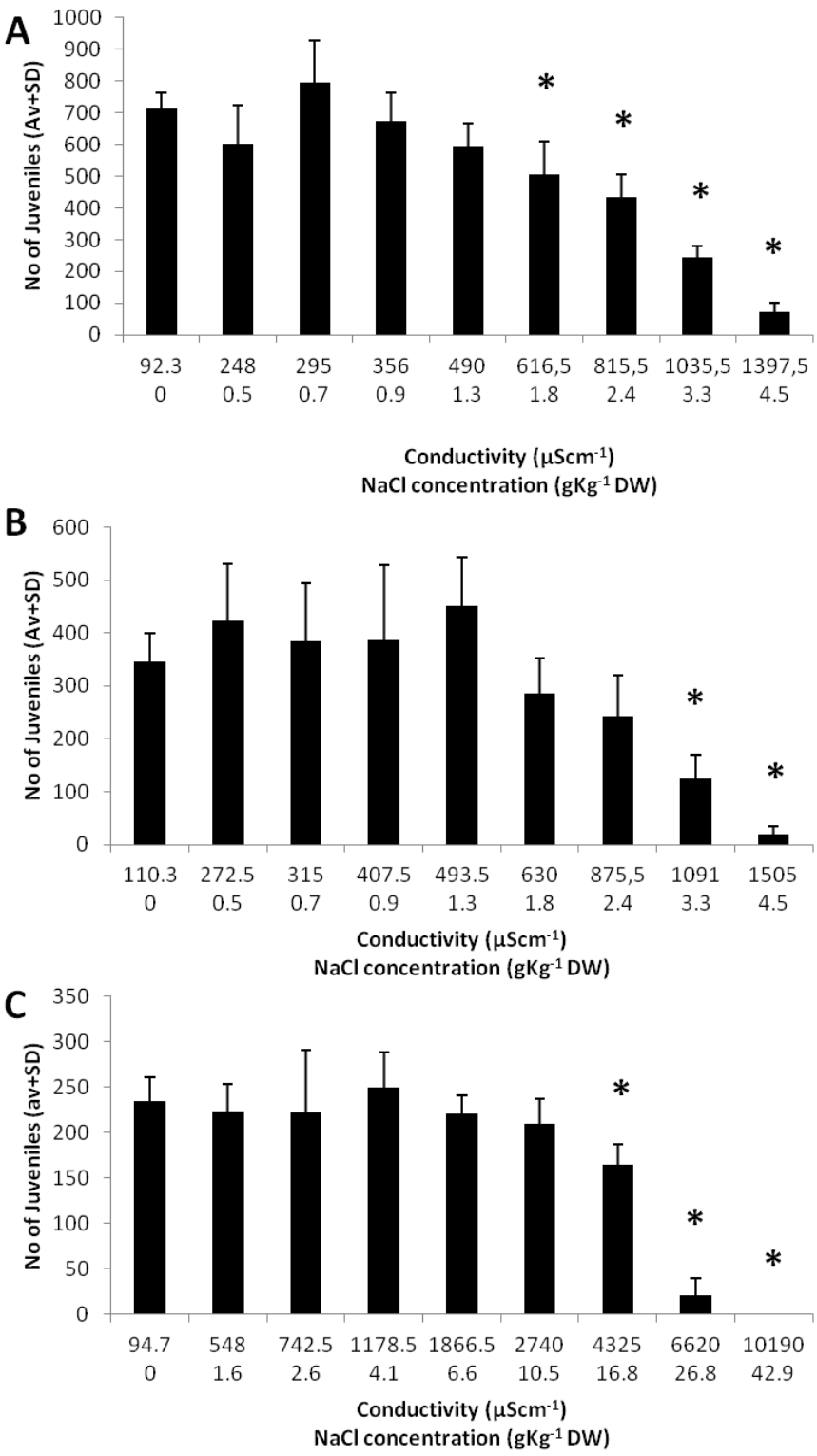

Fig. II.1: Mean number of juveniles (+ standard deviation) of *Enchytraeus crypticus* (A), *Folsomia candida* (B) and *Hypoaspis aculeifer* (C) when exposed to artificial soil spiked with a range of NaCl concentrations (conductivity values

shown on the x axis are means of the conductivity values measured at the beginning and at the end of the test). * mean statistically different from control, One Way ANOVA, Dunnett test, $p<0.05$.

II.4.2. Effects of exposure to seawater

When exposed to seawater, the dose-response was irregular. Enchytraeids showed a different reproduction response along the first six dilutions tested (until 25 % SW; 985 μScm^{-1}), although closer to the control. Only at dilutions equal or higher than 33 % SW (1293.5 μScm^{-1}) a statistically significant decrease on the number of juveniles was observed (One Way ANOVA, Dunnett test, $p<0.05$; Fig. II.2A). Different reproduction outputs were also found in springtails until 19 % SW (746 μScm^{-1}). At dilutions equal or higher than 25 % SW (985 μScm^{-1}) a statistically significant decrease on the number of juveniles was observed (One Way ANOVA, Dunnett test, $p<0.05$; Fig. II.2B). Mites did not show a clear dose-response and no statistically significant effects of seawater were found (One Way ANOVA, Dunnett test, $p>0.05$; Fig. II.2C).

The toxicity parameters derived for the seawater exposure revealed larger differences between enchytraeids and springtails than those obtained for NaCl exposure (Table II.2) with EC_{50s} of 1449.0 ± 322.2 μScm^{-1} and 857.0 ± 264.4 μScm^{-1}; and EC_{20s} of 1083.3 ± 252.3 μScm^{-1} and 511.1 ± 318.2 μScm^{-1} respectively for *E. crypticus* and *F. candida*.

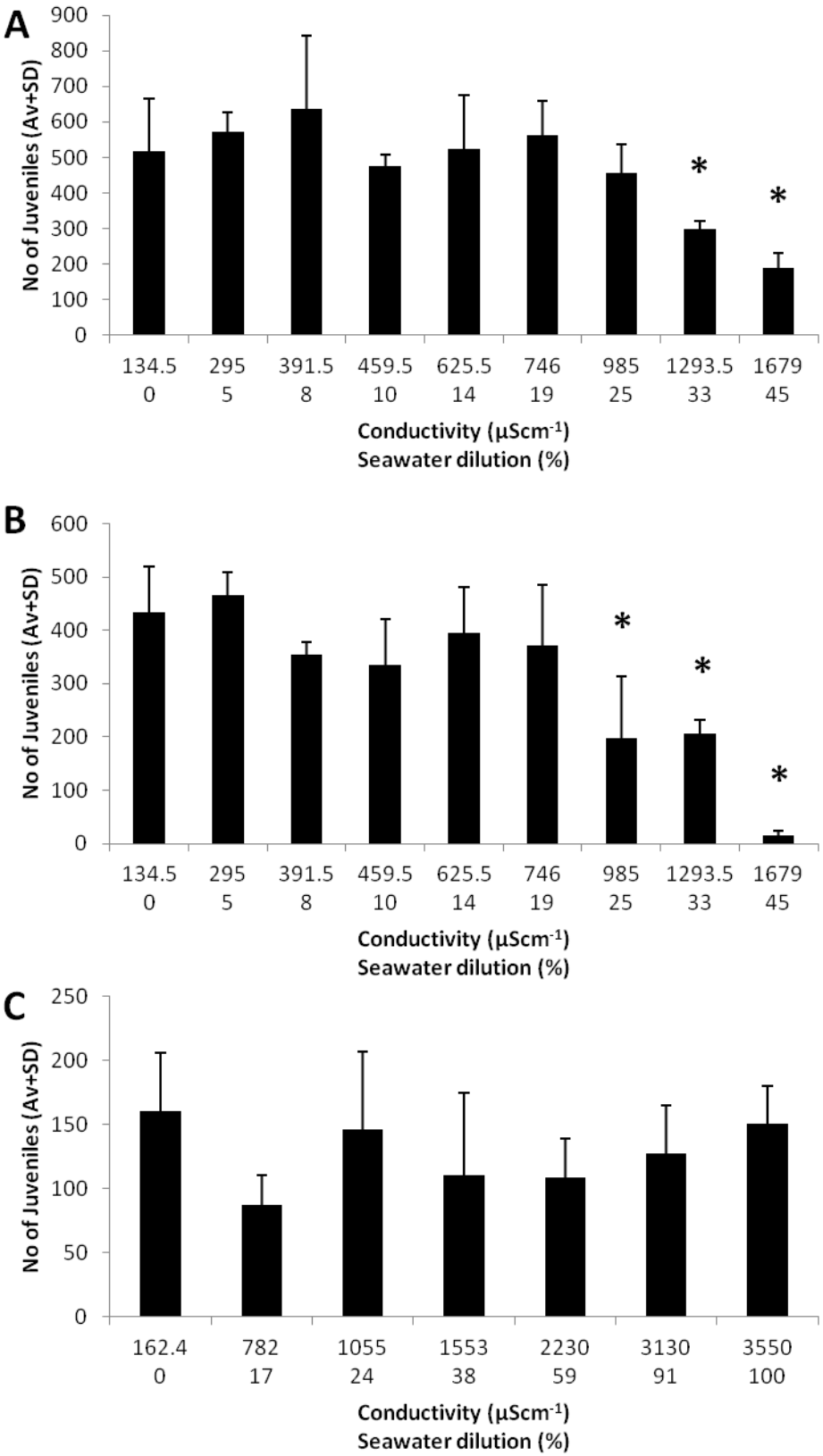

Fig. II.2: Mean number of juveniles (+ standard deviation) of *Enchytraeus crypticus* (A), *Folsomia candida* (B) and *Hypoaspis aculeifer* (C) when exposed

to artificial soil spiked with a range of seawater dilutions (conductivity values shown on the x axis are means of the conductivity values measured at the beginning and at the end of the test). * mean statistically different from control, One Way ANOVA, Dunnett test, $p<0.05$.

Table II.2: EC (Effect Conductivity) values calculated for the three tested species (*Folsomia candida*, *Enchytraeus crypticus* and *Hypoaspis aculeifer*) after exposure to sodium chloride and seawater in artificial soil. Data in brackets refer to upper and lower confidence limits (n. d. – not determined).

		Conductivity NaCl (μScm^{-1})	**Conductivity Seawater (μScm^{-1})**
E. crypticus	EC20	562.2 (428.6 – 695.8)	1083.3 (828.9 – 1337.7)
	EC50	883.5 (785.2 – 982.7)	1449.0 (1126.8 – 1771.2)
F. candida	EC20	797.7 (635.6 – 959.8)	511.1 (192.9 – 829.3)
	EC50	986.5 (783.9 – 1189.1)	857.0 (604.7 – 1109.2)
H. aculeifer	EC20	4570.4 (4117.5 – 5023.2)	n. d.
	EC50	6027.6 (5677.4 – 6377.9)	n. d.

II.5. Discussion

II.5.1. Effects of exposure to NaCl and seawater

Mites were the least sensitive organisms with EC_{50s} and EC_{20s} about six times higher than springtails and enchytraeids when exposed to NaCl (Table

II.2). Mites were also the least sensitive organisms, with no significant reproduction impairment in all seawater dilutions tested. Springtails and enchytraeids showed similar sensitivities, especially when considering exposure to NaCl (Table II.2).

Terrestrial invertebrates are able to osmoregulate due to different mechanisms: 1) reduced permeability of the “skin”, varying from an almost “waterproof” to a very permeable cuticle; 2) excretion done by specialized organs and gut; 3) cellular regulation; 4) uptake systems; and 5) reduction of the water loss in respiration (Willmer, 2006). The high tolerance of mites to the substances tested in this study might be due to their morphological features since they present a continuous exoskeleton (OConnor, 2003) with a rigid dorsal shield in the case of the tested species (Jänsch et al, 2005), that may provide them a lower exposure to substances present in soil pore water, such as salts. In fact, it has been described in other mite species that the cuticle plays an important role in osmoregulation and protection against pathogens (Cook et al, 2008). Osmoregulation in *Hypoaspis aculeifer* is not well studied, but the presence of specialised organs in osmoregulation have been described in other mite species like the presence of coxal glands (Bayartogtokh and Chatterjee, 2010) and sclerotized rings in the cuticle (Witalinski et al, 2002). The regulation of the hemolymph osmolality by the production of aminoacids (Foucreau et al, 2012; Hidalgo et al, 2013) and the regulation of the presence of salt in their bodies by its release in the urine (Morritt, 1988) has also been described in other organisms with a rigid exoskeleton. The presence of a similar or even one or more of these mechanisms in the tested species could have facilitated its survival and reproduction under saline conditions.

The similar sensitivity of springtails and enchytraeids was not expected since enchytraeids are soft-bodied organisms and thus, theoretically, should be more exposed to salt via soil pore water, while springtails possess an exoskeleton that would offer a higher protection against harmful substances present in soil pore water (Peijnemburg et al, 2012). Although not described for the tested enchytraeid species, it is known that some annelid species are able to survive in saline conditions by (1) releasing an hypotonic urine and (2) due to an impermeabilization of their membranes (Generlich and Giere, 1996), physiological avoidance mechanisms that could explain the lower sensitivity (than expected) of enchytraeids. In fact, another species of enchytraeids (*Enchytraeus albidus*) has shown a high tolerance to salinity since the transport of active amino-acids and sugars is done through an ATP-ase-dependent Na-gradient which is activated in the presence of sodium and inactivated in its absence (Siebers and Bulnheim, 1977). The accumulation of these substances, adjusting the fluid osmolarity helps the enchytraeids to survive in saline conditions (Patricio Silva et al, 2013). Theoretically, springtails should have been more tolerant than enchytraeids which was not observed in the present study. The fluid exchange in springtails occurs by drinking and via the ventral tube (collophore; Hopkin, 1997), and can be increased by the fact that springtails have their bodies segmented causing a higher contact with the soil pore water in the thinner part of the exoskeleton. Furthermore, their higher sensitivity than expected can be also related with dehydration since it has been described the reduction of water uptake by the ventral tube with the increase of salinity (Eisenbeis, 1982).

Some data are available for the effects of soil salinisation to invertebrate species. Owojori et al (2009) found statistically significant effects on the reproduction of enchytraeids (*Enchytraeus doerjesi*) and springtails (*Folsomia candida*) using a natural saline soil for electrical conductivities starting at 1030 µScm $^{-1}$. In fact, springtails and enchytraeids showed negative effects on similar conductivity values when looking to the results of seawater exposure obtained in the present study (1293.5 for enchytraeids and 985.0 µScm $^{-1}$ for springtails). When comparing the results obtained by Owojori et al (2009) – 1030 µScm $^{-1}$ - with the ones obtained in the present study for NaCl exposure, for springtails significant effects were found at similar conductivity values (1091.0 µScm $^{-1}$ in the present study) but enchytraeids showed a higher sensitivity (616.5 µScm $^{-1}$ in the present study). The different sensitivities can be due to the presence of other substances (not only sodium chloride) on the seawater composition that may have affected differently the test-species. Owojori et al (2009) also found no reproduction at conductivity values of 1620 µScm $^{-1}$ and 1310 µScm $^{-1}$ for springtails and enchytraeids, respectively, although testing a natural saline soil.

II.5.2. NaCl as a surrogate for seawater effects on soil communities

Sodium chloride, as a major constituent of seawater (Wiesenburg and Little, 1987-88), should be a good surrogate of seawater effects in soil organisms. In fact, for springtails, both substances showed similar effects with differences of about 15 % between both EC_{50s}. Despite this, enchytraeids were slightly more sensitive to sodium chloride than to seawater with about 40 % of variation between both EC_{50s}. Although the small differences described in this study for the test species, NaCl and seawater generally caused the same overall effects

consisting in a lower and similar tolerance of springtails and enchytraeids and a higher tolerance of mites. Despite this, the use of sodium chloride as a surrogate of seawater exposure can lead to an overestimation of the toxic potential of the later to some soil fauna groups, and therefore its use is more advisable in a screening phase, when evaluating the overall effects of seawater.

II.5.3. Safety levels for salinity to soil communities

Soils are considered saline when they present an electrical conductivity of or above 4000 μScm^{-1} (Micheli et al, 2002). In fact, mites were only affected by salt deposition at those conductivity values (statistically significant effects on reproduction starting from 4325 μScm^{-1}). In opposition, springtails and enchytraeids were already affected by NaCl and seawater at conductivities lower than 4000 μScm^{-1} (the maximum value tested was 1679 μScm^{-1} for seawater and 1505 μScm^{-1} for NaCl). The results described in the present study were obtained using an artificial soil and would much probably be quite different when using a natural saline soil. However, it has been already described zero reproduction of enchytraeids and springtails in conductivity values under 2000 μScm^{-1} (Owojori et al, 2009) using a natural saline soil.

The actual limit to define saline soils was obtained taking into consideration effects on plants (Micheli et al, 2002). However, as explained above, soils presenting those conductivity values would already affect soil fauna. Integrating the information that already exists about salinisation effects on soil fauna, and since this group is essential to the maintenance of soil ecological functions, the limit to define saline soils should probably be changed. In order to define a more accurate value, more tests should be performed using, for example, field

collected soils and field collected species, community tests, and tests under different conditions, especially to test the interactions between abiotic factors (e.g. different conductivity values, temperature, humidity).

II.6. Conclusions

Seawater and sodium chloride exposure did not affect the mortality of the tested species but differently affected their reproduction. Mites were the least sensitive organisms while springtails and enchytraeids showed similar sensitivity. The same overall effects were obtained with both substances (seawater and NaCl) but a slightly higher sensitivity was found for enchytraeids to NaCl exposure. Seawater composition could be the explanation for the different sensitivity observed. Nowadays, the limit posed to define a saline soil – 4000 μScm^{-1}, may not reflect the knowledge that already exists for effects of salt exposure to soil organisms. Indeed, in the present study, at conductivity values below that threshold value, soil invertebrates were negatively affected. Therefore, harmful consequences for soil ecosystem inhabitants are expected in a real exposure scenario.

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Chapter III: SALT TOLERANCE ON SOIL ORGANISMS: CAN THEY ACCLIMATE TO LOW SODIUM CHLORIDE LEVELS?

Chapter III:

Salt tolerance on soil organisms: can they acclimate to low sodium chloride levels?

III.1. Abstract

Soil salinisation is a emerging problem mainly due to the sea level rise and its effects (droughts, floodings, intrusion of saltwater and consequent use for irrigation). Impacts of exposure to seawater (mainly constituted by sodium chloride - NaCl) on the soil compartment are still largely unknown. Some soil species are able to avoid and even acclimate to extreme conditions resulting in under or overestimation of risk when the effects assessment is done through standard one generation tests. The main objectives of this study were to evaluate the ability of two standard soil invertebrates to acclimate to low concentrations of NaCl, and to assess the changes in resistance of acclimated organisms exposed to a range of concentrations of the same salt. The springtail *Folsomia candida* and the enchytraeid *Enchytraeus crypticus* were exposed to artificial OECD soil spiked with the equivalent EC_{20} concentration previously derived for NaCl exposure (797.7 μscm^{-1} for springtails and 562.2 μScm^{-1} for enchytraeids), during 26 weeks (acclimation period). After that period, acclimated organisms were extracted and exposed to a gradient of NaCl concentrations already tested with unacclimated organisms. Results showed that a long-term exposure of springtails to NaCl had a drastic effect on survival and reproduction of the population, more than theoretically expected (20%). On the contrary, acclimated enchytraeids showed a higher tolerance to NaCl after

the exposure period, presenting higher $EC_{50's}$ (1250.2 μScm^{-1}) and $EC_{20's}$ (1079.6 μScm^{-1}) than the unacclimated organisms. The survival mechanisms used in these faunal groups to desiccation may explain the tolerance to low salt concentrations alongside with a change in the population with the survival of the resistant phenotypes.

III.2. Introduction

Earth has been suffering an intensification of extreme events associated to climate change due to anthropogenic causes. Nevertheless, even if the anthropogenic influence stopped, some climate extremes would still exist (IPCC, 2012). These extreme events, such as long drought periods, have been facilitating the de-icing of the snow covers and ice stocks that along with water expansion is causing the rise of the sea level (IPCC, 2007a), predicted to be between 40 and 62 cm until 2100 (IPCC, 2013). The freshwater availability and quality will be consequently reduced leading to seawater intrusions (IPCC, 2007b) and consequent irrigation with it (Wang and Li, 2012). The deposition of soluble salts on soil affecting its fertility, named soil salinisation, is one of the key threats to soil in Europe (European Soil Portal, 2012). Countries like Portugal, Spain, France, Greece and Italy have already considerable areas of saline soils (25, 840, 250, 3.5 and 450 thousands of hectares, respectively) (Eckelmann et al, 2006). Soil salinisation can be associated with different factors depending on the geographic location. Soils of coastal areas are more affected by groundwater withdrawals and extreme events like tsunamis and floodings while soils distant from the coast are more affected by irrigation with

salt water, with higher evapotranspiration rates and the impediment of salts washing due to the soil textural characteristics (European Commission, 2005). Effects of soil salinisation on crop yields include the increase of pest species and/or diseases and the disappearance of less resistant species with potential loss of biodiversity (IPCC, 2007b). Consequences of these events on soil fauna have been assessed mainly with earthworms through avoidance (Owojori and Reinecke, 2009) and reproduction tests (Owojori et al, 2009). Negative effects on survival and reproduction were also found by Owojori et al (2009) for springtails (*Folsomia candida*) and enchytraeids (*Enchytraeus albidus*) using a natural saline soil. Negative effects on reproduction of springtails (*Folsomia candida*) and enchytraeids (*Enchytraeus crypticus*) were also found in Chapter II.

The acclimation to low concentrations of salt, although associated with costs for the organisms, has already been assessed for aquatic organisms such as bivalve species and crabs (Dunlop et al, 2005; Piller et al, 1995), bacteria (Los et al, 2013) and plants like strawberries (Orsini et al, 2012) which presented a higher tolerance to salt due to changes on their physiognomy. Although taking more time than standardised guidelines, assessing acclimation has proved to determine high accuracy limits of potential risk of salinity (Filippov, 1998).

Acclimation processes in soil ecosystems have been studied mainly for temperature. For example, springtails, one of the most abundant and widespread terrestrial arthropod groups (Hilligsoe and Holmstrup, 2003), can tolerate extreme environments like freezing and desiccation conditions using 4 different mechanisms: 1) loss of body water to the surrounding environment in order to reach the melting point of their internal organs (Bahrndorff et al, 2007);

2) absorption of water vapour; 3) reduction of water loss increasing the hemolymph osmolality (by the abnormal increased production of sugars and polyols) (Hilligsoe and Holmstrup, 2003) and 4) production of chemicals turning the cell membranes fully functional by changes in their fluidity and functionality (Sorensen and Holmstrup, 2005). Besides these mechanisms, springtails have the ability to osmoregulate regulating the uptake of water and salts - drinking or through the ventral tube (Hopkin, 1997). Soft bodied soil organisms such as enchytraeids (Kobeticová et al, 2008), also possess strategies (some of them common to other invertebrates) that allow them to survive in extreme environments: 1) migration to deeper and moister microhabitats, 2) population survival in the most desiccation-tolerant cocoon stage (Briones et al, 2010) and 3) ability to hibernate in the adult stage (Maraldo and Holmstrup, 2010). Osmoregulation processes may also be activated like the accumulation of amino acids and sugars through the activation by the presence of sodium of the ATP-ase-dependent Na-gradient (responsible of these substances through the cuticle; Siebers and Bulnheim, 1977).

In a real case scenario of soil salinisation, some species when exposed to a stressor such as salt will probably avoid it. This behavior has been previously described for earthworms exposed to lower concentrations than the ones causing effects on reproduction (Owojori and Reinecke, 2009), thus leading to an underestimation of the ecological risk. In the other hand, other species could acclimate to a low concentration of salt as it has been already described for aquatic invertebrate species (Piller et al, 1995), bacteria (Los et al, 2013) and plants (Orsini et al, 2012) and therefore ecological risk may be overestimated.

The main objective of the present work was to assess the capability of two soil invertebrate species (*Folsomia candida* and *Enchytraeus crypticus*) to acclimate to low concentrations of sodium chloride and to evaluate the resistance of the acclimated organisms after the exposure to a gradient of NaCl concentrations. In order to fulfill these aims, the two test species were exposed to a low concentration of salt for 26 weeks after which only the survival of the resistant phenotypes was expected. After the acclimation period, the acclimated organisms were used in a standard reproduction test where a higher tolerance of the acclimated organisms was expected in relation to the unacclimated organisms.

III.3. Materials and Methods

III.3.1. Test soil and test species

The artificial OECD soil was used for all assays and was prepared according to OECD-226 (2008).

Test organisms were obtained from cultures maintained in the Soil Ecology and Ecotoxicology Laboratory, Department of Life Sciences, University of Coimbra, Portugal, as previously described in Chapter II. Mites (*Hypoaspis aculeifer*) were excluded from this work since they showed to be the less sensitive species in the standard ecotoxicological tests carried out previously, as effects were only observed at NaCl concentrations with conductivity values higher than the ones presented by soil mixed with pure seawater (see Chapter II).

III.3.2. Test-procedures

III.3.2.1. Acclimation assays

For each test species, two boxes were filled with 1500 g dry weight (DW) of artificial OECD soil. In the first, the soil was mixed with distilled water (further referred as “Control Box”) and in the second with sodium chloride (NaCl) concentration corresponding to the EC_{20} (effects concentration causing 20 % of inhibition in reproduction) previously derived for each species (further referred as “EC_{20} Box”; Table III.1). The acclimation assay took 26 weeks (more or less eight generations), and for both test-species, boxes were maintained at 20±2 ºC, with a photoperiod of 16:8 light:dark and fed once a week with yeast and rolled oats (autoclaved) for springtails and enchytraeids, respectively. For both test species, the solutions used in each EC_{20} box were obtained mixing the corresponding concentration of NaCl (Merck KGaA, 64271 Darmstadt, Germany) in distilled water - 0.99 gKg^{-1} and 1.99 gKg^{-1} for enchytraeids and springtails, respectively. Initial parameters for both species are described in Table III.1.

III.3.2.1.1. Springtails (*Folsomia candida*)

Eight hundred adults were selected from culture-boxes and divided between the “Control box” and the “EC_{20} box” (400 adults in each). After the acclimation period, the content of each box was divided in 10 portions and the springtails were extracted in a Macfadyen high-gradient extractor at 35 ºC during five days. During the extraction period and in a daily basis, the extracted springtails were collected in Falcon tubes lines with two centimeters layer of culture medium (ISO-16387, 1999) and transferred into culture boxes (11.5 cm Ø x 4 cm

containing between 1 or 2 cm layer of culture medium; ISO-11267, 1999) to prevent desiccation.

Table III.1: Soil parameters and NaCl concentrations, with the corresponding initial (beginning of the experiment) and final (after 26 weeks) conductivity values, for both test-species in the acclimation assay.

	EC_{20} previously calculated	**NaCl concentration (gKg^{-1})**	**Moisture (%)**	**pH**	**Initial Conductivity (μScm^{-1})**	**Final Conductivity (μScm^{-1})**
E. crypticus Control Box	562.2 μScm^{-1}	0	34.4	6.36	181.1	205
E. crypticus EC_{20} Box		0.99	36.1	6.29	450.0	561
F. candida Control Box	797.7 μScm^{-1}	0	34.4	6.38	158.2	217
F. candida EC_{20} Box		1.99	34.2	6.30	791.0	803

III.3.2.1.2. Enchytraeids (*Enchytraeus crypticus*)

Eight hundred enchytraeids with a visible clitellum were selected from culture boxes and divided between the “Control box” and the “EC_{20} box” (400 in each). After 26 weeks, the enchytraeids were handpicked from the “Control box” and the “EC_{20} box” and used in a reproduction test exposing the organisms to the same range of NaCl concentrations used in Chapter II (see next section).

III.3.3. Exposure to NaCl after the acclimation period

An enchytraeid reproduction test was performed according to ISO-16387 (2003) and using a gradient of NaCl concentrations: 0; 0.5; 0.7; 0.9; 1.3; 1.8; 2.4; 3.3 and 4.5 gKg^{-1} DW. Ten acclimated adults were placed in each test vessel with 20g DW of the test soil. They were fed once a week with rolled oats (autoclaved). The test was kept at 20±2ºC and photoperiod of 16:8 (light:dark). After 28 days, five milliliters of ethanol (96%) were added to the vessels that were then filled with water (one centimeter above the soil) and a few drops of Bengal red 1% were added to stain the organisms (Chelinho et al, 2014). The vessels stood for 24h to colorful the enchytraeids that were then counted.

The reproduction test using the acclimated springtails was not possible since an insufficient number of organisms were retrieved from the "EC_{20} box".

III.3.4. Data analysis

A one-way analysis of variance (ANOVA) was used to test differences between the control and the different concentrations on the number of juveniles produced by the acclimated organisms from both boxes (control and EC_{20}) followed by post hoc comparisons (Dunnett's test) in Statistica 7.0 (http://www.statsoft.com/). ANOVA assumptions, normality and homoscedascity, were checked using Shapiro-Wilk's and Bartlet's tests, respectively. Effect concentrations (EC's) 20 % and 50 % were calculated using non-linear regression models after the guideline published by Environment Canada (EC, 2007).

III.4. Results

III.4.1. Acclimation

For both test-species, in both "control" as well as in "EC_{20}" boxes, the soil parameters were maintained during the exposure period, although with a small increase of the conductivity values (Table III.1).

The "acclimated" *E. crypticus* recovered from each box were more than enough to use in the subsequent standard reproduction test. However, the same was not observed for springtails. In the "Control box", a large number of "acclimated" *F. candida* was recovered. In the "EC_{20} box" only 20 adults were recovered. Since the number of adults recovered was not sufficient to prepare a reproduction test, they were kept under controlled conditions (temperature of 20±2 ºC and photoperiod of 16:8 light:dark) in order to test the first generation of juveniles. As for several months they were unable to reproduce, the planed reproduction test was cancelled.

III.4.2. Exposure of *Enchytraeus crypticus* to NaCl after the acclimation period

The validity criteria for the ecotoxicological tests with acclimated enchytraeids were fulfilled (ISO-16387, 2003). For enchytraeids from the "Control box", average number of juveniles and corresponding CV were 1053 individuals and 14 %, respectively. The enchytraeids from the "EC_{20} box" showed an average number of juveniles and respective CV of 1101 and 24 %.

The reproduction of enchytraeids extracted from the "Control" and "EC_{20} boxes" showed different patterns (Fig. III.1B and C) in relation to the results

obtained with unacclimated organisms (Fig. III.1A – results obtained from Chapter II). In these last organisms, a continuous and statistically significant reduction on the number of juveniles was observed at the NaCl concentration of 1.8 gKg^{-1} (616.5 μScm^{-1}; One Way ANOVA, Dunnett test, $p<0.05$). The enchytraeids extracted from the "Control box" did not show a clear dose-response. Statistically significant effects were found at conductivity values of 436.5 μScm^{-1} (One Way ANOVA, Dunnett test, $p<0.05$). No statistically significant differences were found between the NaCl concentrations and the control with enchytraeids from the "Control box" except for the highest concentration tested (conductivity of 1484 μScm^{-1}, One Way ANOVA, Dunnett test, $p<0.05$). The different sensitivities of these organisms were reflected in the derived $EC_{50's}$ and $EC_{20's}$, whose values are summarised in Table III.2. The enchytraeids extracted from the "Control box" showed lower values (EC_{50} of 578.1 μScm^{-1} and EC_{20} of 317.0 μScm^{-1}) if compared with the enchytraeids extracted from the "EC_{20} box" (EC_{50} of 1250.2 μScm^{-1} and EC_{20} of 1079.6 μScm^{-1}).

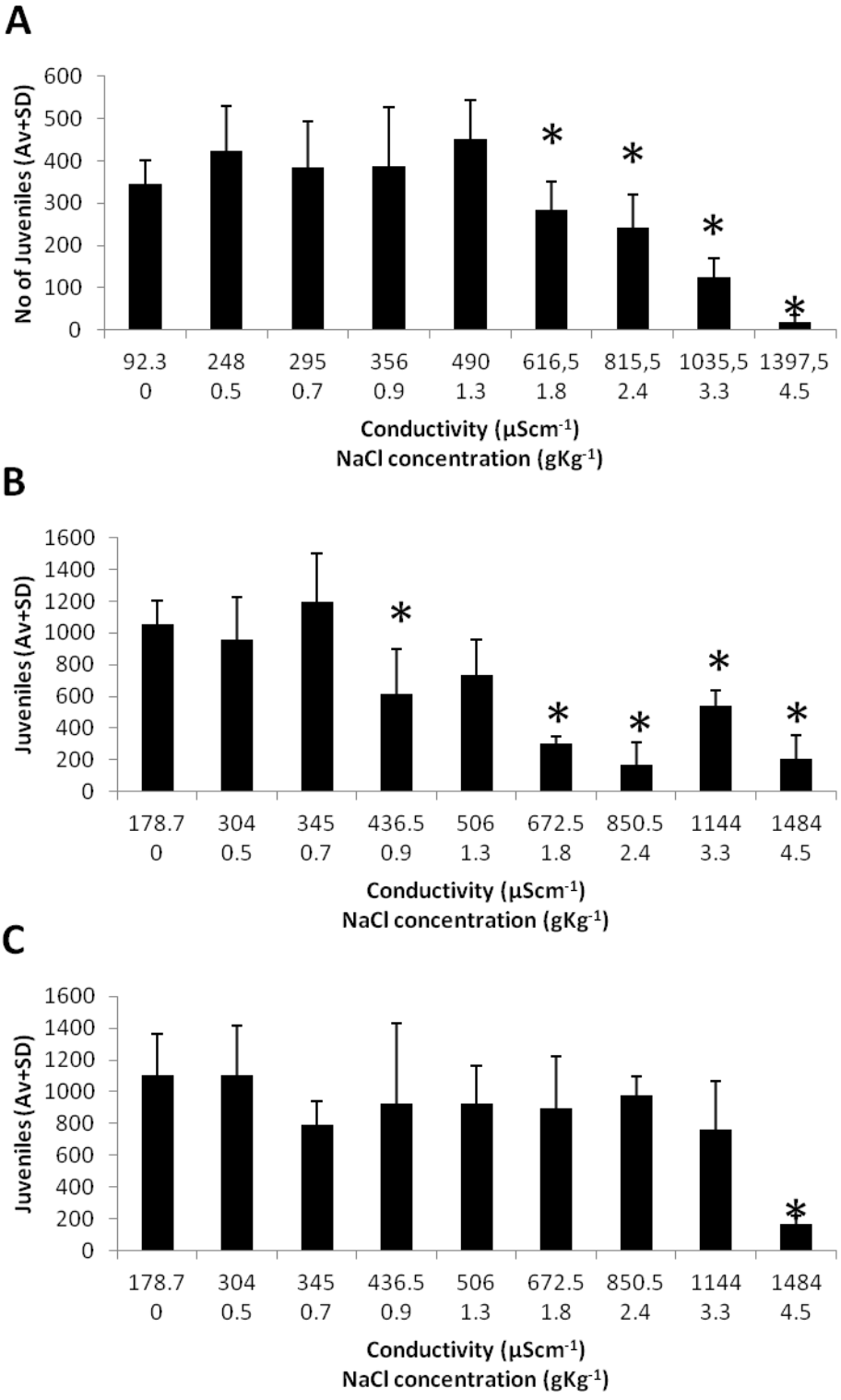

Fig. III.1: Mean number of juveniles (+ standard deviation) of *Enchytraeus crypticus* exposed to OECD artificial soil spiked with NaCl concentrations using unacclimated organisms (A; results from Chapter II) acclimated organisms to a

NaCl concentration equivalent to the EC_{20} for reproduction previously derived ("EC_{20} box", B) or to uncontaminated OECD soil ("Control box", C; see section Materials and Methods). Values shown on the *xx'* axis are means of the soil conductivity values at the beginning and at the end of the test. * mean statistically different from control, $p<0.05$, One Way ANOVA, Dunnett test.

Table 2: EC (effect conductivity) values calculated for *Enchyraeus crypticus* for unacclimated organisms (data from Chapter II) and after an acclimation period of 26 weeks to artificial OECD soil spiked with a concentration of NaCl equivalent to the EC_{20} previously calculated (EC_{20} box) or to artificial soil OECD soil mixed with distilled water (Control box).

	EC_{20} (μScm^{-1})	**EC_{50} (μScm^{-1})**
Unacclimated organisms	562.2 (428.6 – 695.8)	883.5 (785.2 – 982.7)
Control Box	317.0 (93.3 – 540.6)	578.1 (357.9 – 798.2)
EC_{20} Box	1079.6 (801.3 – 1357.9)	1250.2 (942.0 – 1558.5)

III.5. Discussion

III.5.1. Acclimation

Acclimation processes are related with phenotypic plasticity and environmental variation (Hasegawa, 2013). In fact, phenotypic plasticity plays an important role in the climate change effects on soil fauna being responsible for the way the individuals react to stress with the survival of the resistant

phenotypes inside the population (Chown et al, 2007). Acclimation depends, also, on habitat conditions (frequency, duration and repeatability of the stress) and flexibility of the physiological characteristics (van Dooremalen et al, 2013). In the present study, the use of a low concentration of salt, representative of soil salinisation, aimed to induce changes in the population, although avoiding its extinction. The exposure to saline soil was the same for the two species tested (26 weeks) and at concentrations expected to cause 20 % of reduction in reproduction were used. Enchytraeids were retrieved from both boxes in a sufficient number to perform a standard reproduction test. On the contrary, springtails in a prolonged exposure could not maintain their population density since it was observed a reduction of around 95% in the organisms extracted from the "EC_{20} box", if compared with the number of initially introduced organisms. Besides, even after the exposure event, springtails retrieved from the "EC_{20} box" were not able to reproduce unlike the ones retrieved from the "Control box" (which survived and reproduced for several months). Springtails population was almost extinct in a prolonged exposure which can be due to the low phenotypic plasticity. Thus, the result previously obtained in the standard reproduction test with unacclimated individuals (using only one generation), might be an overestimation of the 20 % or 50 % effect concentrations in relation to multi generational and/or prolonged exposure assays. In a real case scenario of soil salinisation due to accumulation of NaCl salt, and looking to the results obtained in the present study, enchytraeids could acclimate to a small increase of soil salinity. In opposition, springtails, whenever possible, would most probably avoid such conditions referred before or would drastically decrease their population. It has been described for both faunal groups strategies that

allow them to survive under extreme conditions like high/low temperatures and drought conditions like the selection of moist microhabitats (Briones et al, 2010 and Elnitsky et al, 2008). Springtails also show other strategies as the absorption of water vapour, reduction of water loss increasing the hemolymph osmolality (Hilligsoe and Holmstrup, 2003) and production of chemicals turning the cell membranes fully functional (Sorensen and Holmstrup, 2005). Enchytraeids also have other strategies like the release a hypotonic urine, impermeabilization of the membranes (although not yet described for this species; Generlich and Giere, 1996), survival in the most desiccation-tolerant cocoon stage (Briones et al, 2010) and hibernation in the adult stage (Maraldo and Holmstrup, 2010). Some of these strategies alongside with the capacity to osmoregulate due to the accumulation of amino acids and sugars increasing the body fluids osmolality (Siebers and Bulnheim, 1977) could have been used by enchytraeids from the "EC_{20} box" to survive during the exposure period. For the equivalent treatment, salinity could have compromised some of the strategies used by springtails alongside with some dehydration effects due to the low water uptake (already described in springtails in saline conditions; Eisenbeis, 1982). To better understand the ecotoxicological and physiological effects of NaCl on soil organisms more studies should be performed enlarging the range of test-species and using natural soils, representative of coastal areas, as test-substrates. Also, increasing the ecological realism, with the evaluation of the effects of NaCl or, even more realistic, seawater, exposure in natural soil communities, including a high number of species, with different sensitivities and ecological relationships, is desirable. Standard one generation tests may not be enough in risk assessment as shown by the results obtained in the present

study. Thus, prolonged exposures, preferentially including multi generations, should be included in the standard ecotoxicological battery tests.

III.5.2. Exposure of *Enchytraeus crypticus* to NaCl after the acclimation period

Theoretically, after an exposure period to a stressor, the surviving organisms would be more resistant to it than unacclimated ones. In fact, for enchytraeids this was observed. The salt acclimated enchytraeids were more resistant showing higher $EC_{50's}$ (1250.2 μScm^{-1}) than the ones in the control box (578.1 μScm^{-1}) and the unacclimated ones (883.5 μScm^{-1}). Unexpectedly, the individuals in the "Control box" showed a higher sensitivity to NaCl than the unacclimated ones. The laboratory conditions used in the acclimation assay were equal to the ones used in the standard reproduction tests and the range of conductivity values used in the standard reproduction tests with unacclimated organisms and acclimated ones (from both boxes) were similar. A possible explanation for this higher sensitivity could be the overpopulation on the "Control box" and the consequent different access to food from the organisms. Even if this happened, it should also occur in the "EC_{20} box" since a high number of organisms were retrieved in both boxes.

In relation to the organisms that were extracted from the "EC_{20} box", the higher EC's values obtained (Table III.2) can be due to the survival and reproduction of the organisms with strategies cited above. All the results obtained in the present study suggests that only the resistant individuals survived and reproduced after a prolonged exposure to salt as it was expected. The use of these strategies should have a higher cost to the individuals as

described for other species (Dunlop et al, 2005) and it would be interesting to perform more studies evaluating the long-term viability and energy expenditure processes of the acclimated organisms. It would also be important to assess the differences in life expectancy between acclimated and unacclimated organisms.

III.6. Conclusions

The acclimation to the EC_{20} concentration of NaCl was only observed for *E. crypticus* and not for *F. candida* with drastic effects on survival and reproduction of the latter, more than theoretically expected (20 %). Springtails were more sensitive than expected with a reduction of about 95 % of the population in the "EC_{20} box" which in a real case scenario of a prolonged exposure to a low concentration of salt can limit their survival. Sensitivity of the surviving organisms could not be assessed since they were not sufficient to perform a standard ecotoxicological test. After an exposure period of 26 weeks to a low concentration of NaCl (equivalent to the EC_{20} previously derived for *E. crypticus*), enchytraeids extracted from the "EC_{20} box" were less sensitive to a range of NaCl concentrations than unacclimated organisms.

The acclimation test described here tried to reduce the under/overestimation of the results obtained using standard one generation tests. The data obtained in the present study suggest that, for NaCl exposure in artificial OECD soil, the results obtained through standard one generation tests could be overestimations of the real risk to *E. crypticus*, as acclimated organisms were clearly less sensitive than non-acclimated ones. In opposition, an underestimation of the real risk to *F. candida* might occur, due to the low

survival of organisms extracted from the "EC_{20} box". In a real scenario of NaCl accumulation on soil, different responses such as the movement to more favourable habitat conditions could be observed. Standard guidelines are very useful when assessing the effect of a stressor in a determined species but, to increase the ecological realism, new approaches, such as testing multi generational assays (prolonged exposure scenarios) and soil communities from natural soils should be taken into consideration.

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Chapter IV:

PREDICTION OF SALINISATION EFFECTS ON SOIL ECOSYSTEMS USING TERRESTRIAL MODEL ECOSYSTEMS

Chapter IV:

Prediction of salinisation effects on soil ecosystems using Terrestrial Model Ecosystems

IV.1. Abstract

Soil salinisation problems are increasing with the rising of the sea level (due to the melting of glaciers and ice caps and to water expansion) and intrusion of seawater. In agricultural fields of coastal areas, both intrusion and irrigation with saltwater can threaten crop yields and the effects of these actions to soil fauna are still unknown. The aim of this study was to evaluate the effects of salinisation on soil ecosystems due to saline intrusion and irrigation with saltwater. To fulfill this goal, Terrestrial Model Ecosystems (TME) were used to simulate two soil salinisation scenarios with two dilutions of natural seawater (6000 and 18000 μScm^{-1}): 1) saltwater intrusion by immersing the lower 10 cm of the TME's in the dilutions of seawater and surface irrigation with distilled water and 2) saltwater intrusion described earlier plus surface irrigation with the dilutions of seawater. The control consisted in the immersion of the lower 10 cm of the TME's in distilled water and surface irrigation also with distilled water. Three sampling periods of soil mesofauna, macrofauna and plant biomass were established (T0, T1 and T2). The acclimation period took two weeks (after TME's extraction) during which all soil cores were under control conditions (T0). The effects of saltwater intrusion and irrigation were measured after six weeks of exposure (T1). At this point, and during eight weeks, the remaining soil cores were again submitted to irrigation with distilled water and saline intrusion to

measure population's recovery (T2). Results showed immediate effects on springtail, mite and enchytraeids abundance, especially in treatments with both saline intrusion and irrigation and in the highest conductivity level tested. No immediate effects were observed for nematodes, earthworms and plants. After the cessation of saline irrigation, the abundance of the above mentioned soil fauna groups fully recovered, and some delayed effects were observed in earthworm abundance and plant biomass, especially at the higher soil conductivity level tested. Since the observed effects occurred at soil conductivity values much lower than the threshold values to define a saline soil, the data obtained with this study enhanced the urgency in updating this threshold values, with the risk that even for soils that are currently considered "not saline" may be observed an impairment in some soil processes, and associated ecosystem services, mediated by soil organisms.

IV.2. Introduction

Global mean temperature increase and consequent water expansion along with de-icing of snow covers and ice stocks are facilitating the sea level rise. This has severe effects on groundwater, potentiating its salinisation (IPCC, 2013). Between 2002 and 2003, Bablani and Soomro (2006) recorded the seawater influence on groundwater of 18 % on areas of southern Pakistan far from the coast and 92 % in coastal areas. Between 2003 and 2004, in the same areas a high seawater influence on coastal zones groundwater (77 %) was still observed, while zones far from the coast were less affected (17 %). Ferreira (2012) sampled water wells at Almada (Lisboa, Portugal) and found a 5 % of

seawater intrusion which corresponded to an electrical conductivity of 6290 μScm^{-1}. Seawater intrusions (IPCC, 2007) and the consequent use of this saline water for irrigation is an emerging problem due to the scarcity and quality of freshwater leading to the accumulation of salts on soils (salt-affected soils; IPCC, 2007). Soil salinisation (accumulation of soluble salts to the extent that affects its fertility) can occur through natural processes like the increased evapotranspiration (primary salinisation) and/or induced by human activities like the increase withdraws from aquifers (secondary salinisation) (European Soil Portal, 2012). It depends on the geographic location, being soils of coastal areas more affected by groundwater withdrawals and extreme events (tsunamis and floodings; European Commission, 2005). Saline soils possess an electrical conductivity of 4000 μScm^{-1} (pH lower than 8.5 and exchangeable sodium percentage lower than 15 %; Micheli et al, 2002). Globally, the most affected regions of soil salinisation are the arid and semi-arid parts of Australia, South America, Asia and Europe (European Soil Portal, 2012) with an estimated 77 million hectares of saline soils (being 43 million hectares due to secondary salinisation). It is estimated that soil salinisation will continue spreading at a rate of 2 million hectares per year (Abbas et al, 2011). Inside Europe, an increase of 2 million hectares was observed between 2002 and 2012 (from 1 million hectares in 2002; Commission of the European Communities, 2002; to 3 million hectares in 2012; European Commission, 2012). Soil salinisation is considered a key threat in Europe with the most affected regions being the ones around the Mediterranean and Caspian Seas (European Soil Portal, 2012). Salinisation effects on agriculture include changes on productivity, risk to less tolerant

species and increase of plant pests and/or diseases. These effects will lead to loss of biodiversity (IPCC, 2007).

Soil organisms are essential to the normal functioning of the soil ecosystems since they intervene in key ecological processes like organic matter decomposition, nutrient cycling and maintenance of soil structure (Lavelle et al, 2006). Salinisation effects are not well studied on soil organisms. A few studies have been conducted using standard tests in which the authors found negative effects of saline conditions on reproduction of two earthworm, one springtail and one enchytraeids species (Owojori et al, 2009) and also the avoidance behavior of earthworms to such conditions (Owojori and Reinecke, 2009). Nematodes were also studied but taking into consideration the neurologic pathways that control de avoidance behavior to salt since their response seems to be controlled by the presence/absence of food (Adachi et al, 2010). Salinisation tolerance has been described for some soil inhabitants like: the spider *Arctosa fulvolineate* and the beetle *Merizodus soledadinus* by the accumulation of amino acids which increase the body fluids osmolality (Foucreau et al, 2012 and Hidalgo et al, 2013). Also different species of amphipods can regulate their internal salt level by its release in different concentrations in the urine (hypo-osmotic or isosmotic urine; Morritt, 1988). In Chapter II, negative effects were observed on the reproduction of springtails and enchytraeids while mites were not affected. Nevertheless, in an acclimation assay described in Chapter III, the springtail species (*F. candida*) was not able to acclimate to low concentrations of salt (salt concentration that theoretically induces a 20 % of reduction on the offspring production) and in fact, a reduction of population density of about 95 % was observed. Despite this, the enchytraeid species tested (*E. crypticus*) was

able to acclimate and in fact the acclimated individuals were more tolerant than the unacclimated ones. In both chapters, a need to use more realistic approaches and techniques was mentioned in order to evaluate salinisation effects and possible recovery of the soil populations.

Reproducible and controlled semi-field set-ups called Terrestrial Model Ecosystems (TME's; Moser et al, 2004) are being used to assess the effects of contaminants on soil communities since a long-term stability of the soil system is provided, allowing not only to evaluate effects but also the communities recovery potential (Scholz-Starke et al, 2013). This method can be used outdoors and also indoors (TME's under controlled conditions are normally used for 16 weeks but they can be kept for up to 1 year) and it can also be used in a variety of ecosystems like crop areas, grasslands or forests. Soil communities of springtails, mites, enchytraeids, nematodes and plants showed to be constant within 1 year using this semi-field tool. The earthworm community tends to occur in lower numbers than other populations (like microarthropods) in some ecosystems. TME's have been used to test effects of insecticides or fungicides compounds under various conditions (including indoor and outdoor experiments, different concentrations and experimental periods) on the communities described earlier and also zinc effects on nematodes (Förster et al, 2004; Förster et al, 2006; Knacker et al, 2004; Moser et al, 2004a; Moser et al, 2004 b; Smit et al, 2002). In the usage of this methodology, a pre-incubation period is advisable in order to allow the stabilization of the communities inside the TME's. Although the suitability of this method on testing the effects of a stressor on soil communities, a high spatial variability (mimicking the one found in the field) have been described for microarthropods and microbial

communities (Sousa et al, 2004). This aspect can sometimes impair the detection of differences when comparing the different treatments to the control. Another limitation of this study is the existing difficulty on choosing the maximum time period and also on assessing the time necessary to detect effects and recovery for the different endpoints (Schäffer et al, 2007).

By using terrestrial model ecosystems (TME) from an undisturbed area, the present study aimed: (1) to evaluate the effects of saline intrusion and saline intrusion combined with irrigation with saline water on communities of soil meso and macrofauna groups in a natural agricultural soil, (2) to assess the recovery potential of those soil communities after the cessation of saline irrigation. To fulfill these aims, total abundance of springtails, mites, nematodes, enchytraeids and earthworms and plant biomass were sampled in two sampling periods. Saline intrusion was simulated alone or combined with saline irrigation to assess salinisation effects. In order to assess the community recovery, the saline irrigation was stopped and replaced by distilled water irrigation. Direct effects will depend on the mode of exposure and on the life form of the organisms. Negative effects are expected on springtail, enchytraeid and earthworm abundances while mites, nematodes and plants should not be affected. Nevertheless, a recovery of the affected populations is expected.

IV.3. Materials and Methods

IV.3.1. Collection of soil cores

Soil cores (total number of 31) were collected in an undisturbed area at the Agricultural School in Coimbra, Portugal. Prior to the collection, the existing

cover vegetation was cut approximately to 5 cm high. The collection was performed in an area of approximate 10 m^2 in December of 2013 (winter). In the area the groundwater level is very close to the soil surface and the soil presents a sandy-loam texture (sand - 32.81 %, silt - 32.49 %, clay - 16.12 %), pH of 7.2 (measured in 1 M KCl) and 3.2 % of organic matter. TME's were extracted using a stainless steel extractor and the intact soil core was encapsulated in a high density polyethylene tube with 16.5 cm internal diameter and 40 cm in length. After the extraction, the TME's were transported to a controlled room (temperature of 20±2 ºC, photoperiod 16:8 light:dark and humidity of 60±3 %) were the experiment was maintained. They were placed in buckets lined with a layer of marbles to improve the contact between soil and intrusion water.

IV.3.2. Test-design

After the collection of the TME's, all soil cores were kept under controlled conditions and immersed in distilled water with irrigation using distilled water for two weeks. After this period (acclimation), a sampling period was performed (T0) to assess the initial communities condition on the TME's. To assess soil salinisation effects on soil communities, the saline intrusion with or without saline irrigation were performed after T0 and for six weeks (sampling period – T1). After T1, saline irrigation was stopped and replaced by normal irrigation with distilled water. Community recovery was assessed after eight weeks (T2). As advised by Schäffer et al (2007), the total length of the test was 16 weeks. The test-design is summarised in Table IV.1.

Table IV.1: Test-design including the different treatments used, their durations and replicates. (IIDW – intrusion and irrigation with distilled water; SIDWI – saline intrusion combined with irrigation using distilled water; SISI – saline intrusion combined with saline irrigation; SIDWI' – saline intrusion combined with irrigation using distilled water after the cessation of the saline irrigation)

	Initial community	**Saltwater exposure phase**			**Recovery phase**		
Sampling period	T0	T1			T2		
Duration (weeks)	2	6			8		
Treatments	IIDW (ct)	IIDW (ct)	SIDWI and SISI		IIDW (ct)	SIDWI'	
Seawater dilutions (%)	0	0	8	31	0	8	31
Corresponding solution conductivity values (µScm^{-1})	0.009	0.009	6000	18000	0.009	6000	18000
Replicates	6	5	4	4	4	4	4
Endpoints measured	Microarthropods, nematodes, enchytraeids and earthworms.	Microarthropods, nematodes, enchytraeids, earthworms and plants.					
Test conditions	20 ± 2 ºC; 16:8, light:dark.						

IV.3.2.1. Test treatments

The total number of 31 soil cores collected was divided into four treatments: (IIDW) intrusion and irrigation with distilled water (control); (SIDWI) saline intrusion and irrigation with distilled water; (SISI) saline intrusion and saline

irrigation; and (SIDWI') saline intrusion and irrigation with distilled water (after the cessation of saline irrigation). Saline intrusion and/or saline irrigation were simulated using two seawater dilutions (8 and 31 %) with conductivity values of 6000 and 18000 μScm^{-1} (Table IV.1).

All treatments were submitted to an intrusion (saline or with distilled water). This design was decided in order to simulate soils of coastal areas in which the groundwater is close the soil surface. In a real case scenario, the farmers would use this water (saline or not) to irrigate their fields, thus the treatment SISI. The last treatment SIDWI' was used in order to assess the community recovery if the saline irrigation would stop. Saline intrusion was not stopped since it would be impossible to replace the saline groundwater.

In each TME, the conductivity value of the water for irrigation and intrusion was the same. Ferreira (2012) assessed the conductivity values of water wells in Almada (Portugal) in which the maximum value recorded was 6000 μScm^{-1} (corresponding to 5 % of seawater intrusion). Thus the choice of this value to the lowest conductivity value tested. Looking to the results obtained in chapters II and III of the present thesis, a solution conductivity value of 6000 μScm^{-1}, and corresponding conductivity values measured in the soil (Table IV.2) would not affect soil communities. So, there was a need to use a higher conductivity value. After adding a series of seawater dilutions on the test soil (on laboratory), the value of 31 % dilution was determined since the conductivity value measured on soil was similar to the EC_{50} for reproduction derived earlier (800 μScm^{-1}).

IV.3.3. Sampling procedure

IV.3.3.1. Plants

Only the aerial part of the plants was sampled. The plant material was cut in T1 and T2 and transported to the Soil Ecology and Ecotoxicology Laboratory (Coimbra, Portugal). The samples were dried at 60ºC for 24h and weighted.

IV.3.3.2. Microarthropods and enchytraeids

For each sampling period and per TME two soil cores with five centimeters in diameter and height were collected. One of the soil cores was placed in a Macfadyen extractor for seven days at 45 ºC to extract microarthropods. The extracted organisms were kept in ethanol 80 % until being sorted under a stereomicroscope at 400x of magnification. The second soil core was used for enchytraeids extraction. The soil was mixed with ethanol 96 % (in a ratio of 5 ml of ethanol *per* 25 g of soil fresh weight – FW), filled with water until all the soil was submersed, and stained with a few drops of rose Bengal (to color the enchytraeids). The enchytraeids were then counted under a stereomicroscope at 400x of magnification.

IV.3.3.3. Nematodes

For each sampling period and per TME three soil cores of one centimeter in diameter and five centimeters in height were collected. The nematodes were extracted using the tray method (McSorley, 2000). The nematode concentrated extract was kept at 4 ºC, for a maximum of 14 days, during which were counted under an inverted microscope (100 and 200 magnification) according to Goodey (1963).

IV.3.3.4. Earthworms

After the sampling of all the organism groups described earlier, the remaining soil was searched for earthworms. After counting, the animals were preserved in ethanol 96 %.

IV.3.3.5. Soil conductivity values

Soil conductivity values were determined for three different parts of each TME. Samples from 0 to 5 cm, 5 to 30 cm, and 30 to 40 cm were collected. Five grams fresh weight of each sample were mixed with distilled water and the conductivity was measured using a conductivimeter. The collection at three different depths was performed in order to evaluate the influence of the intrusion mostly in the last layer (30-40 cm) combined with the influence of irrigation mostly in the superficial layer (0-5 cm). The intermediate layer was sampled in order to assess the water movement inside the TME.

IV.3.4. Data analysis

The results here presented and consequent analysis was performed using the total abundance of mites and springtails, and the number of organisms per gram of soil (fresh weight - FW) for enchytraeids and nematodes. The results were compared through One Way ANOVA. ANOVA assumptions, normality and homoscedasticity, were analysed using Shapiro-Wilks and Bartlet's tests, respectively. Whenever these assumptions were not fulfilled, data was transformed using the logarithmic transformation. In order to assess effects of seawater exposure, comparisons between the different treatments (SIDWI and SISI) and the control of the sampling period T1 was performed using One Way

ANOVA through Statistica 7.0 (http://www.statsoft.com/) tools. To assess the community recovery, comparisons between treatment SIDWI' and the control in T2 were investigated through One Way ANOVA in Statistica 7.0 tools.

IV.4. Results

IV.4.1. Soil conductivity values

An increase in soil conductivity values was observed from IIDW to SIDWI and continuing in SISI at T1 (Table IV.2). As expected, higher values were observed at the bottom (30-40 cm) and middle layers (5-30 cm) on the SIDWI 18000 μScm^{-1} level when compared to the 6000 μScm^{-1} level of the same treatment, indicating an upwards movement of salt by capillarity. The increase in conductivity values on the SISI treatment levels, when compared to the corresponding levels of the SIDWI treatment, especially in the top soil layer (0-5 cm), was also expected due to the simulation with saline water. At T2, the conductivity values from SIDWI' (after the cessation of the saline irrigation) were generally higher than the ones measured in SISI treatment at T1. The exception was the conductivity measured at the top layer (0-5 cm) on the 18000 μScm^{-1} level, where a decrease was observed due to irrigation with distilled water. This could have caused also the leaching of salt to deeper layers as observed by the increase in conductivity values on both in the intermediate (5-30 cm) and the bottom (30-40 cm) layers. Thus leaching phenomenon seems to have occurred on both conductivity levels (Table IV.2).

Table IV.2: Conductivity values of the seawater dilutions and the corresponding conductivity values on the soil (at the three TME's soil depths).

Treatment	Conductivity of the seawater dilution (μScm^{-1})	Soil depth on the TME (cm)	Mean soil conductivity (μScm^{-1})
IIDW	9.3	0-5	102.8
		5-30	115.7
		30-40	31.6
SIDWI	6000	0-5	141.5
		5-30	108.6
		30-40	163.4
	18000	0-5	179.1
		5-30	352.1
		30-40	569.0
SISI	6000	0-5	652.0
		5-30	250.3
		30-40	239.6
	18000	0-5	1628.8
		5-30	565.8
		30-40	529.0
SIDWI'	6000	0-5	805.5
		5-30	453.5
		30-40	322.5
	18000	0-5	1090.0
		5-30	695.8
		30-40	790.8

IV.4.2. Fluctuations of the soil invertebrate groups and plant biomass along the test period

Along the three and half months of the experiment, earthworms and plants increased their total abundance and biomass. For total abundance of earthworm, statistically significant differences were found between the initial community (T0) and the organisms found at the end of the test (T2; One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.1E). Plant biomass recorded at T1 and T2 was also significantly higher than in T0 (One Way ANOVA, Dunnett test,

$p<0.05$; Fig. IV.1F). Springtails and nematodes also showed an increase on total abundance between T1 and T0, followed by a small decrease in T2 (Fig. IV.1A and C). Nevertheless, for springtails these differences were not statistically significant (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.1A) whilst for nematodes differences between total abundance obtained in both sampling periods (T1 and T2) and the initial (T0) were statistically significant (One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.1C). Total abundance on mites showed a gradual decrease along the experimental period although not statistically significant (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.1B). Enchytraeids showed similar values between the initial (T0) and the first sampling period (T1) abundances but a huge decrease in abundance was observed in the last sampling period (statistically significant; One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.1D).

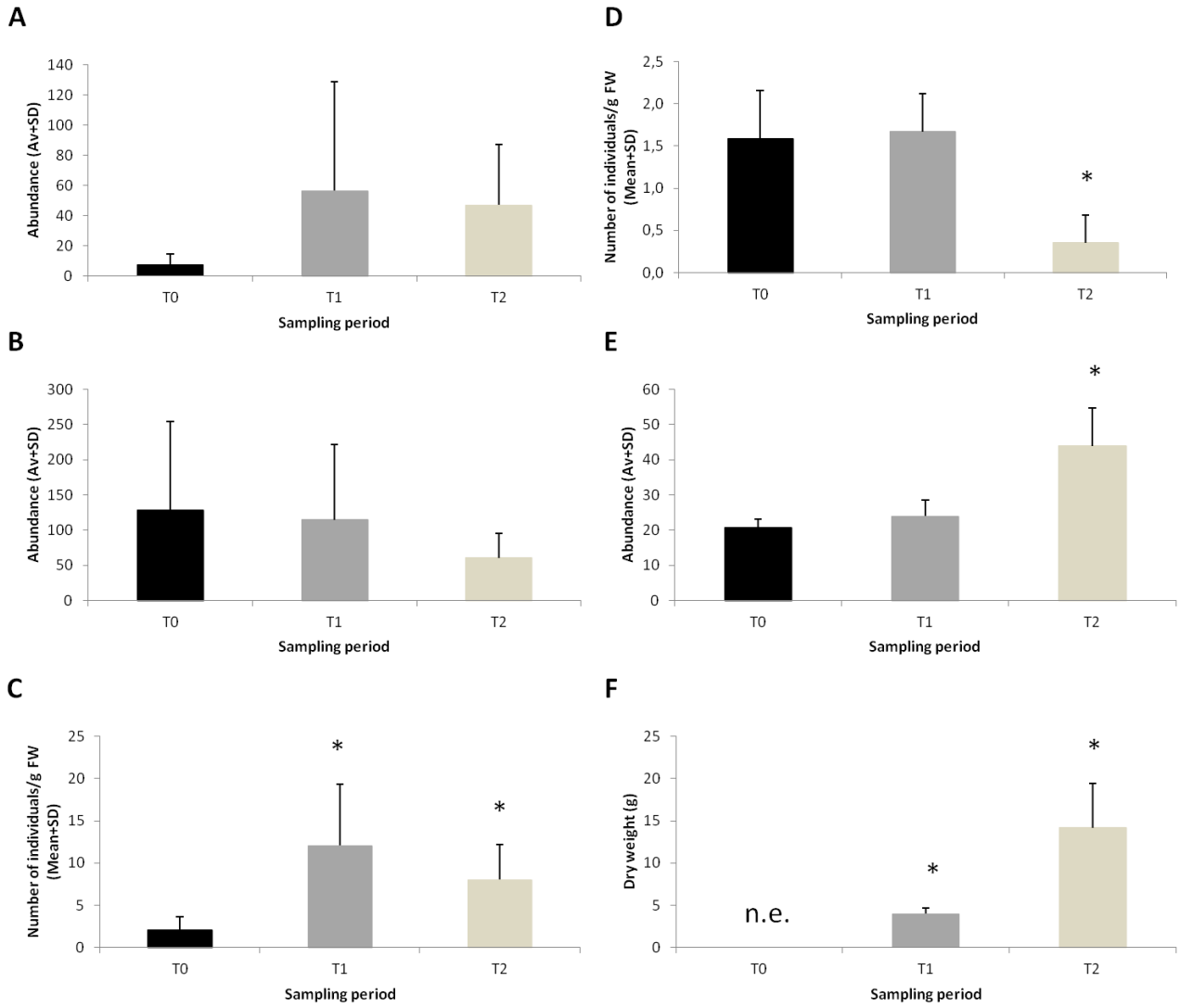

Fig. IV.1: Variation of the total abundance of each sampled group along time at the control treatment (IIDW): mean (+ standard deviation) number of springtails (A), mites (B), nematodes (C), enchytraeids (D), earthworms (E) and dry weight of plants (E) of the beginning of the test (T0), and sampling periods T1 and T2. * - mean statistically different from control in T0, One Way ANOVA, Dunnett test, $p<0.05$; n.e. – not evaluated.

IV.4.3. Effects of soil salinisation on soil fauna groups and plants

No statistically significant differences were found between the control and both treatments for springtails abundance (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.2A). Despite this, a decrease on abundance in relation to the control was observed in both treatments (SIDWI and SISI).

A significant decrease on mites abundance was observed in the TME's under saline intrusion and irrigation with distilled water (treatment SIDWI, One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.2B), but not on the TME's under both saline intrusion and irrigation (treatment SISI, One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.2B).

For nematodes, a similar number was found on the control and on both treatments (SIDWI and SISI). No statistically significant differences were found (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.2C). Also no statistical differences were found for earthworm abundance between the control and both the SIDWI and SISI treatments (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.2E). However, in the later, a small decrease was observed.

A decrease on enchytraeids abundance was observed on both treatments and related with the increase in conductivity values. This decrease was higher on the SISI treatment, where statistically significant differences were found with the control (One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.2D).

Regarding plant biomass, contrasting responses were observed between both conductivity values on both treatments (Fig. IV.2F). Despite this, no statistically significant differences were found between none of the treatments and the control (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.2F).

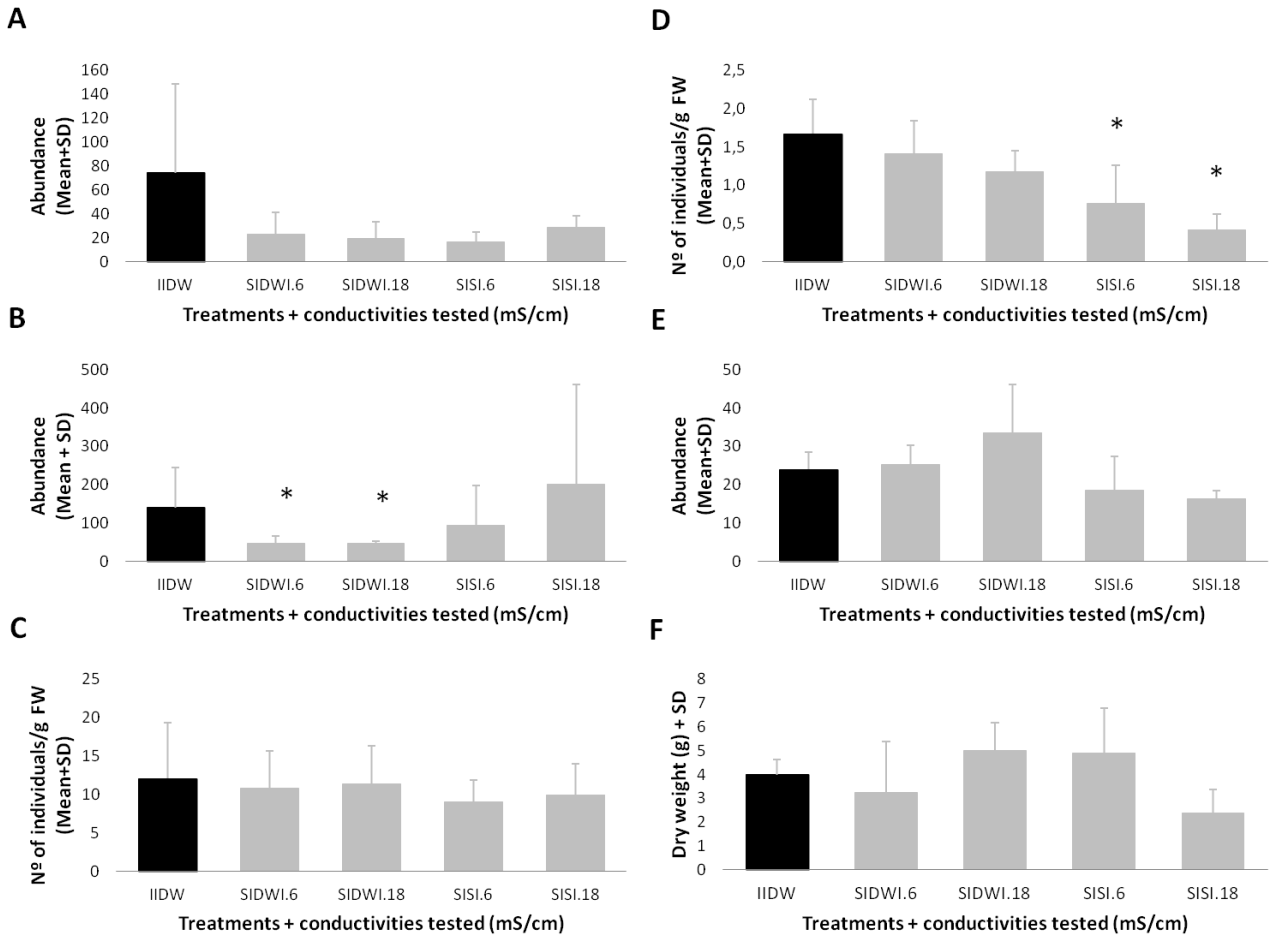

Fig. IV.2: Effects of saltwater exposure: mean number (+ standard deviation) of springtails (A), mites (B), nematodes (C), enchytraeids (D), earthworms (E) and mean dry weight of plants (E) in the control (black bars) and both treatments (grey bars) in the first sampling period (T1). Treatment codes as in Tabe 1. * - mean statistically different from control, One Way ANOVA, Dunnett test, $p<0.05$.

IV.4.4. Recovery potential of the soil organisms after the cessation of saltwater irrigation

In the last sampling period (T2), springtails and mites continue showing high variability and no statistically significant effects were found between the treatment SIDWI' and the control (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.3A and B). Nematodes showed similar values to the control not presenting

any statistically significant effects either (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.3C).

As observed for the nematodes, a general decrease on enchytraeids abundance was observed from T1 to T2, after the cessation of saline irrigation. However, when comparing their abundance of both conductivity levels tested in treatment SIDWI' with the control, no statistically significant differences were found (One Way ANOVA, Dunnett test, $p>0.05$; Fig. IV.3D).

In earthworms, the conductivity levels tested in treatment SIDWI' showed similar values with the ones obtained in treatment SISI (T1). Comparing their total abundance with the one from the control at T2, statistically significant effects were found (One Way ANOVA, Dunnett test, $p<0.05$; Fig. IV.3E).

In general, an increase in plant dry weight was observed between T1 and T2. Nevertheless, a gradual decrease on biomass was observed from the control, to the conductivity level of 6000 μScm^{-1} and continuing in the conductivity level of 18000 μScm^{-1}, the later presenting statistically significant differences in relation to the control (One Way ANOVA, Dunnett test, $p<0.05$; Fig. 3F).

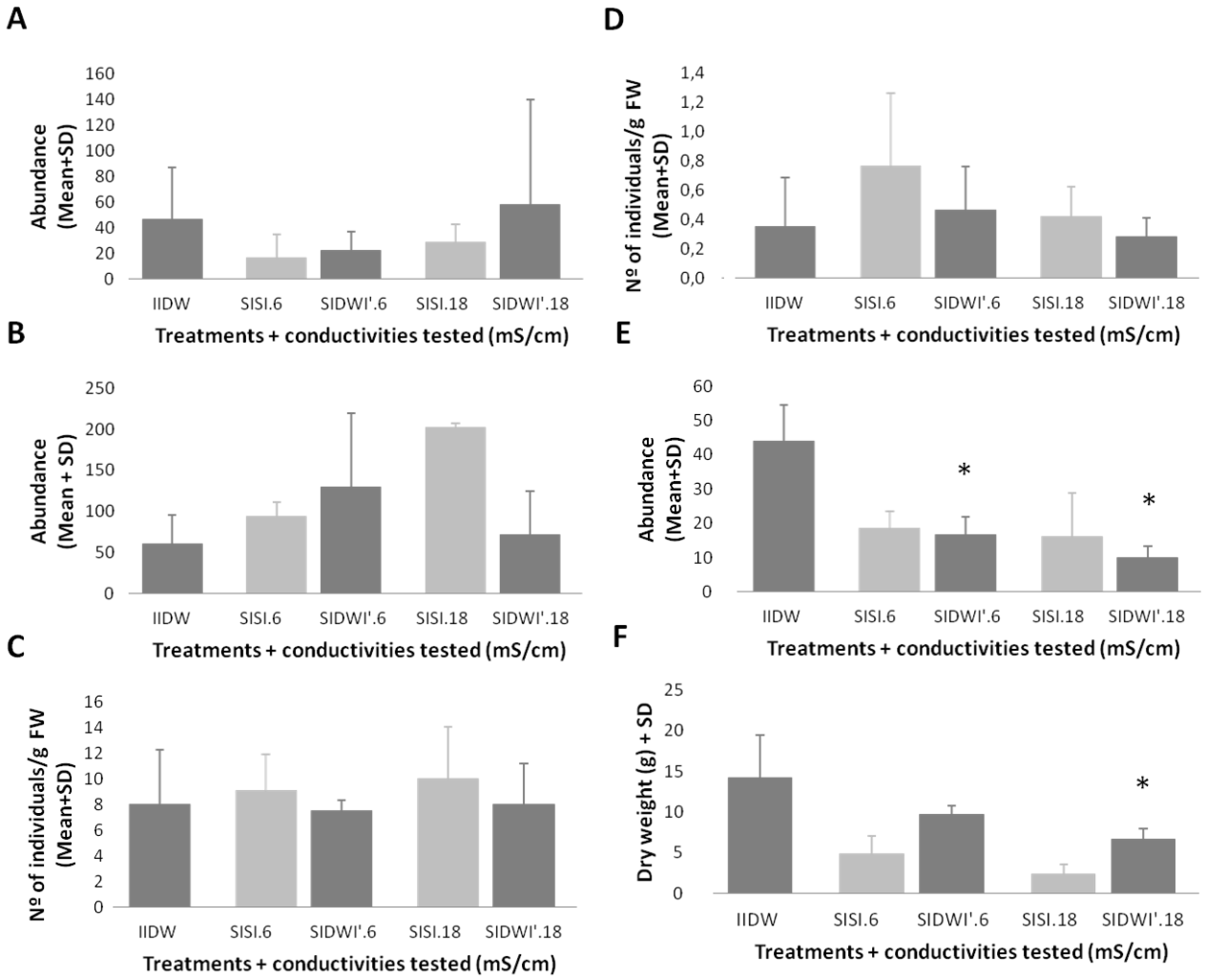

Fig. IV.3: Soil organisms recovery after the cessation of saltwater irrigation: mean number (+ standard deviation) of springtails (A), mites (B), nematodes (C), enchytraeids (D), earthworms (E) and mean dry weight of plants (F) in the treatment SISI from the first sampling period T1 (light grey bars) and control and treatment SIDWI' from the second sampling period T2 (dark grey bars). Treatment codes as in Table IV.1. * - mean statistically different from control, One Way ANOVA, Dunnett test, $p<0.05$.

IV.5. Discussion

IV.5.1. Fluctuations of the soil invertebrates along the test period

In the TME assay performed in the present study it was expected to observe a constant or even an increase on abundance and/or biomass of the organisms sampled (Scholz-Starke et al, 2013). In fact, for springtails and mites, despite the contrasting trend, statistically similar values of abundance were observed in the controls of the three sampling periods. Nevertheless, for these two groups a high variability within replicates at each sampling period was found, which could have masked potential incubation effects. On the other hand, earthworms and plants showed a gradual increase on abundance and biomass, respectively. The TME assay took 3.5 months, during which the soil cores were kept under controlled conditions expected to be optimum to the growth and reproduction of the organisms. Besides, in the case of earthworms and plants, there were no predators, theoretically allowing the growth of their populations over time. On the contrary, nematodes and enchytraeids showed an increase in their populations in T1 and then a decrease between T1 and T2. The scarcity of food or the presence of predators (e.g. mites) could have limited their expansion and so the lower abundance values observed in T2. In the case of enchytraeids, it has been described their sensitivity to the location of food, intra or interspecific attraction or repellency and competition (Moser et al, 2004) which could explain the decrease on the abundance after a certain period of time.

Spatial and temporal variability has been associated to this kind of semi-field tool (TME) for the soil community when testing pesticides application (Schäffer et al, 2007). In the present study, a high spatial variability was found on

microarthropods (springtails and mites) total abundance, but for the other groups sampled (nematodes, enchytraeids, earthworms and plants) that was not observed. Unlike the spatial variability, microarthropods (springtails and mites) showed similar values of total abundance in the controls at all sampling periods not presenting a high temporal variability, maybe masked by the high spatial variability. High temporal variability was observed on the enchytraeids total abundance in which a huge decrease on total abundance was found at the last sampling period. A high temporal variability was also observed for nematodes total abundance with an increase in T1 and a decrease in T2. Earthworms and plants showed a gradual increase over time showing a high temporal variability.

IV.5.2. Effects of soil salinisation on soil organisms

Exposure to saline intrusion or saline intrusion plus saline irrigation, did not cause any visible (and significant) effect on nematodes, earthworms and plants in any of the conductivity levels. Regarding springtails, a visible reduction in abundance was observed in all four treatment levels, despite the absence of statistical significance (due to the high variability found in IIDW – control – treatment). The actual limit to define saline soils – 4000 μScm^{-1} - was defined taking into consideration effects on plants (Micheli et al, 2002). So, it was expected that the conductivity values measured on soil (large majority of values under 1000 μScm^{-1} in T1; Table 2) do not present any hazard for plants. Besides, strategies like the reduction of stomatal density and transpiration were already described in plant species under saline conditions (Orsini et al, 2012). Alongside with plants, no effects were expected on nematodes. It is known that

eggs hatching are not influenced by sodium chloride alongside with other salts in considerable concentrations (Teft et al, 1982) and, in fact, a small percentage of sodium chloride is a part of the perfect solution for eggs hatching (McSorley, 2000) and the exsheathing on nematodes before the parasitic stage (Taylor and Whitlock, 1960). On the other hand, effects of salinity on earthworm reproduction and survival were found by Owojori et al (2009). Two species of earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*) showed no reproduction at conductivity values of 520 μScm^{-1} and higher and effects on survival were found at conductivity values of 920 μScm^{-1} and 1310 μScm^{-1}, respectively for *E. fetida* and *A. caliginosa*. In the present study, no effects on earthworm abundance were found in T1 suggesting no effects on survival and reproduction on the native community of earthworms under study. Owojori et al (2009) also used a springtail species *Folsomia candida*. Salinity effects were not found on survival but were found on reproduction in conductivity values of 1030 μScm^{-1} and above. Results obtained in Chapters II and III of the present thesis show a higher sensitivity of *F. candida* than expected when exposed to a saline stress with an increase of sensitivity in a continued exposure scenario. So, taking into account the sensitivity of *F. candida* to salinity and looking to the conductivity values measured on the TME's, the observed effects on springtail abundance were expected, especially at the highest conductivity level (18000 μScm^{-1}). In fact the decrease in abundance was even observed at the lower conductivity level (6000 μScm^{-1}) of both treatments, indicating that the sensitivity of the natural springtail community could be overestimated when testing *F. candida* only. When testing a community, the range of sensitivities or range of effects gets wider, making the comparison with data obtained from

standard species difficult. In a real case scenario, some species can actively avoid saline conditions, moving to more favourable microhabitats (Elnitsky et al, 2008), or have physiological avoidance mechanisms like absorption of water vapour; reduction of water loss increasing the hemolymph osmolality (by the abnormal increased production of sugars and polyols) (Hilligsoe and Holmstrup, 2003) and production of chemicals turning the cell membranes fully functional by changes in their fluidity and functionality (Sorensen and Holmstrup, 2005). Nevertheless, other species can be more sensitive and, if they dominate the community, a decrease in abundance can be observed.

Mites showed significant differences in the treatment with saline intrusion and irrigation with distilled water but these differences may not be due to the conductivity values measured especially at the top soil layer (0-5 cm). From the results obtained in Chapter II, where an $EC_{50reprod}$ of 6027.6 μScm^{-1} was calculated, mite community was not expected to be influenced at the conductivity values tested in the present study, assuming that the community has a similar sensitivity as *H. aculeifer*. This high tolerance of mites can be due to the presence of a rigid exoskeleton (OConnor, 2003) and/or specialised organs responsible for osmoregulate like the coxal glands (Bayartogtokh and Chatterjee, 2010) and sclerotized rings in the cuticle (Witalinski et al, 2002). Nevertheless, as described for springtails, we can never ruled out any possible variations in sensitivity of different mite species, but most probably the observed effects could have other causes than salinity.

From the results described by Owojori et al (2009) and described in Chapter II, enchytraeids were expected to be affected especially at the higher conductivity levels tested (18000 μScm^{-1}). In the present study, enchytraeids

showed a significant reduction on abundance in the treatment with both saline intrusion and irrigation. This decrease agrees with the EC_{20} and EC_{50} values obtained in Chapter II when compared with the conductivity values measured on both conductivity levels tested at the top soil layer (0-5 cm). The absence of effects on the saline intrusion treatment is due to the low conductivity values measured. Enchytraeids possess osmoregulation mechanisms like the release of hypotonic urine, impermeabilization of their membranes (Generlich and Giere, 1996), transport of active amino-acids and sugars done through an ATP-ase-dependent Na-gradient which is activated in the presence of sodium and inactivated in its absence (Siebers and Bulnheim, 1977). In any case, these mechanisms could have worked in low NaCl concentration but not in high ones, as observed in the upper soil layer of the saline intrusion and saline irrigation treatment (SISI).

IV.5.3. Recovery potential of the soil organisms after the cessation of saltwater irrigation

At T2, after 8 weeks without saline irrigation, no differences of total abundance in relation to the control were found in springtails, mites and enchytraeids. For these three groups, if we compare the evolution in controls (IIDW) between T1 and T2, where a small decrease was observed, with the data on SISI (T1) and SIDWI' (T2) (Fig. IV.3), the effects observed after the exposure at T1 were completely overcome.

On the other hand, total abundance of earthworms and plant biomass showed a decrease in relation to the control, indicating delayed effects of saltwater exposure. This could somehow be in agreement with the increase in

conductivity values particularly on the middle and bottom soil layers reported earlier on the SIDWI' treatments (T2) when compared to the SISI treatments (T1). This delay in effects, particularly in these two organism groups, suggests a potential long-term impairment of the soil functions and services mediated by them.

Ultimately, this study demonstrates a need to update the actual limit to define saline soils, since at conductivity values lower than 4000 μScm^{-1} soil fauna communities and even the plant community showed to be negatively affected.

IV.6. Conclusions

A semi-field tool, Terrestrial Model Ecosystems, was used to assess the effects of soil salinisation on soil communities by saline intrusion combined with irrigation with distilled or saline water. No immediate effects of saline water exposure were observed on plant biomass and earthworm and nematode total abundances. Springtails showed effects on treatments with both saline intrusion and a combination of saline intrusion and saline irrigation, and mites showed effects only on the former treatment. A significant reduction on the abundance of enchytraeids was observed in the treatment of both saline intrusion and irrigation. Results observed after the cessation of saltwater irrigation showed that these groups were able to fully recover from that disturbance. On the other hand, plant biomass and earthworm abundance showed delayed negative effects, despite the absence of saline irrigation.

In a real scenario of soil salinisation due to saline intrusion and the consequent irrigation with saline water, relevant effects can occur even after the cessation of the saline irrigation. The low resistance of some species along with

a low recovery rate could lead to limitations on the provision of some ecosystem services mediated by them. Since the observed immediate or delayed effects were observed at conductivity values lower than 4000 μScm^{-1}, this study demonstrates a need to update the actual limit to define saline soils.

IV.7. References

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Chapter V: GENERAL DISCUSSION AND FINAL REMARKS

Chapter V:

General discussion and final remarks

The sensitivity of soil organisms when facing saline conditions was an understudied subject on the salinisation effects on ecosystems. From the literature, the avoidance behavior and negative effects on reproduction were described for some soil invertebrates (Owojori and Reinecke, 2009; Owojori et al, 2009 and Adachi et al, 2010). But from these studies, no safety levels of salinity to soil inhabitants were derived.

In order to evaluate the sensitivity of three standard soil invertebrate species to soil salinisation (*Folsomia candida*, *Enchytraeus crypticus* and *Hypoaspis aculeifer*), reproduction tests using a gradient of salt concentrations or seawater dilutions were performed (Chapter II). The same overall effects were observed using both salt and seawater: a low sensitivity of *H. aculeifer* and a higher sensitivity of *F. candida* and *E. crypticus*. Nevertheless, *E. crypticus* was slightly more sensitive to NaCl exposure while *F. candida* was slightly more sensitive to seawater exposure as shown by the effect concentration values estimated. The different sensitivities observed for the three standard soil invertebrates used were probably due to the different physiognomic features. *H. aculeifer* possess a continuous exoskeleton (OConnor et al, 2005) with a rigid dorsal shield (Jänsch et al, 2005) that may protect them from substances like salts present on soil pore water. Osmoregulation on the test species is not well studied but could also explain the high tolerance observed. *E. crypticus* showed to be more tolerant than expected, as they are soft-bodied organisms. As for *H. aculeifer*, osmoregulation processes on *E. crypticus* is not well studied. Nevertheless,

other enchytraeid species have shown the release of hypotonic urine, impermeabilization of their membrane (Generlich and Giere, 1996) and the accumulation of amino acids and sugars increasing the hemolymph osmolality by the activation of the ATP-ase-dependent Na-gradient (Siebers and Bulnheim, 1977) as physiological avoidance mechanisms to survive saline conditions. In the other hand, springtails possess an exoskeleton which should provide them protection against substances present in soil pore water such as salts. This higher tolerance was not observed and *F. candida* showed similar EC_x values as *E. crypticus*. This low tolerance of *F. candida* can be due to the segmentation of their body allowing a higher contact with soil and soil pore water, the ingestion of salt water by drinking via ventral tube (Hopkin, 1997) and it can also be a consequence of dehydration due to the low water uptake observed under saline conditions (Eisenbeis, 1982).

Since the overall effects of seawater and NaCl were similar, NaCl was used as a surrogate of seawater effects in testing the acclimation potential of two of the soil invertebrate species (*F. candida* and *E. crypticus*). *H. aculeifer* was not included due to its low sensitivity to salinity. One main objective of this experiment was to determine if there are underestimations or overestimations of the real ecological risk when the assessment of effects is done through standard tests. The results showed that *E. crypticus* was able to acclimate while *F. candida* was not. Besides the ability to acclimate, *E. crypticus* also showed a higher tolerance after the acclimation. The effect concentrations estimated for unacclimated and acclimated organisms reflect the described results. Indeed, a a higher tolerance of acclimated *E. crypticus* to salt was observed if compared to that registered for unacclimated enchytraeids. Contrastingly, a lower

tolerance of acclimated *F. candida* to salt suggested an underestimation of risks when using standard tests. The higher tolerance of *E. crypticus* can be due to the strategies described earlier along with others used to survive during the acclimation period like the survival in the most desiccation-tolerant cocoon stage (Briones et al, 2010). In the other hand, salinity may have disabled the strategies used by springtails causing a high mortality in the acclimation period (decrease on 95 % of the initial population). To complete the results described in the present study, the acclimation assay will be repeated for the springtails using lower salt concentrations (concentrations corresponding to the effect concentration on 5 % and 10 % of the offspring production).

Given the negative effects of salt for soil organisms observed previously, the next step consisted in the evaluation of the effects of seawater exposure and the recovery potential of a natural soil community, with a gain in ecological relevance. A real case of soil salinisation due to saline intrusion and/or irrigation was simulated. Terrestrial Model Ecosystems (TME's) were used and the endpoints measured were the total abundance of enchytraeids, springtails, mites, nematodes and earthworms and plant biomass. Negative effects on total abundance were only found for enchytraeids and springtails but these groups were able to fully recover after the cessation of the saline irrigation. These negative effects were expected taking into consideration the results obtained in chapter II. Also, the results obtained in chapter II indicated the high tolerance of mites to salinity. In this assay, negative effects were found for mite total abundance on the treatment with saline intrusion combined with distilled water irrigation. Nevertheless, a high spatial variability was recorded which could be the reason for these effects. Looking at the results on the literature, earthworms

should be affected since Owojori et al (2009) described negative effects on survival and mortality under saline conditions. In fact, delayed deleterious effects were found after the cessation of the saline irrigation. Delayed negative effects were also observed in plants after the cessation of saline irrigation which was not expected since plants should not be affected at conductivity values lower than 4000 μScm^{-1} (Micheli et al, 2002). Nematodes were not affected, as expected, since this group is known to be highly tolerant to salt, being sodium chloride a component of the optimal medium to study some life cycle parameters in nematodes (Teft et al, 1982).

The actual limit to define saline soils – 4000 μScm^{-1} (Micheli et al, 2002) – may not reflect the existing knowledge for effects of salt exposure to soil organisms. As proven by the present study, soil inhabitants are affected by conductivity values lower than that threshold value. Thus, the impairment of the ecosystem functions provided by the less tolerant species is expected at lower conductivity/salinity values.

V.1. Upcoming work

As a general point of view, the update of the conductivity value to define saline soils is essential. Looking to our results and considering an acceptable risk of 20 % on the population, soils with conductivity values equal or higher than 511.1 μScm^{-1} (the lower EC_{20} estimated) could be considered saline. But, even in soils presenting this conductivity value, the impairment of some soil functions could be observed if soil communities avoid those saline conditions. On the other hand, if some soil species were able to acclimate (as shown for *E. crypticus* in the present study), no impairment would be observed on those

functions. Thus, in order to define an accurate value, more studies should be performed assessing how different species from different taxonomic and trophic levels, would be affected by salt exposure and Hazard Concentration (HC) values (more or less protective, according to specific protection goals) should be calculated by using a SSD approach. Furthermore, to understand how salinity interacts with other abiotic factors (including contaminants) is of paramount importance. These evaluations should be done following traditional test guidelines (i.e., one generation over a short exposure period) on a first tier, but evolving to multi generational assays (with a prolonged exposure), with preference for field species, on a second tier. Finally, calculated HC values should be validated with multispecies, semi-field or field tests under different soil types and environmental conditions.

V.3. References

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