DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Climate change effects on *Porcellionides sexfasciatus* (Isopoda) inhabiting metal-contaminated sites

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 Dalila Costa (Universidade de Coimbra)

Nuno Xavier Jesus Capela

2015

Agradecimentos

Por estes dois anos de mestrado e pela ajuda para realizar este trabalho, gostaria de agradecer:

Aos meus orientadores, Professor Doutor José Paulo Sousa e Doutora Dalila Costa pela ajuda e orientação durante este período. Ao Professor por ser o “chefe” com solução para todos os problemas. À Dalila por me ter ajudado ao longo de todo o processo mas acima de tudo por me ter dado espaço para errar, aprender e evoluir.

À professora doutora cristina Canhoto pelos sábios conselhos, que me fez “manter os pés na terra”.

Aos colegas do Laboratório de Ecologia e Ecotoxicologia de Solos, Mathieu, Sónia, Tiago, Henrique, Filipe, Filipa, Sara, Carla, Raimundo, Toni e Joana, pela companhia nos dias de trabalho, pelas risadas, pelas jantaradas e acima de tudo pelo companheirismo e facilidade com que adoptaram um novo membro. Obrigado por serem um grupo heterogéneo mas extremamente funcional.

À minha mãe. Porque apesar de todas as dificuldades, nunca baixou os braços e esforçou-se todos os dias para que os filhos tivessem um futuro que estaria bem longe do seu alcance sem o seu apoio.

À mãe emprestada, Engª Paula Moço. Por ter ajudado o miúdo jardineiro a seguir os seus estudos. Sem a sua ajuda nunca teria chegado tão longe.

Aos primos, pelos bons fins-de-semana e pelo companheirismo ao longo de todos estes anos.

Aos amigos de Coimbra, principalmente à colheita de 2009, à família, aos EMAE, aos da casa do Povo, da Residência, da casa das Formigas e da Fortaleza. Pela amizade, pelas longas conversas, pelos concelhos, pelas parvoíces e por me terem ajudado a evoluir e ser melhor.

Ao Caloiro, porque passou de um afilhado a um amigo incondicional e ao grupo das cenas do mal por serem uns bons “filhos”.

À Sara.

TABLE OF CONTENTS

Resumo

Os isópodes são organismos de solo que ajudam na decomposição da matéria orgânica, desempenhando um papel importante nos ciclos de carbono e de nutrientes. Na natureza, os isópodes estão sujeitos a um conjunto de stresses que influenciam o seu comportamento, distribuição e balanço energético. Um desses stresses, de origem antropogénica, é a contaminação por metais. Populações que vivem sob contaminação de metais precisam de utilizar energia do seu metabolismo para lidar com esse stress, podendo ser mais afectados por stresses adicionais, tais como alterações climáticas. Os cenários de alterações climáticas prevêem um aumento na temperatura e de eventos extremos (cheias / secas). Estas alterações podem levar a mudanças na biodisponibilidade e toxicidade dos metais e a mudanças na resposta dos organismos a contaminantes. Quando expostas à contaminação por metais, as populações de isópodes tendem a evitar a contaminação; se não tiverem sucesso, precisam de desenvolver tolerância a metais, para podere-.m viver e reproduzir-se, através de alterações fenotípicas ou genotípicas.

Para testar os efeitos das alterações climáticas em populações de isópodes expostas a metais, foi estudada uma população de *Porcellionides sexfasciatus*, cujo habitat se localiza perto de uma mina abandonada em São Domingos (Sul de Portugal). Essa população (Santana de Cambas – SC), juntamente com uma população de referência (Lago – LG), foi sujeita a testes de mortalidade e de consumo. No capítulo 2 foi feita uma avaliação do efeito das alterações climáticas na mortalidade e consumo. O capítulo 3 avalia como é que as alterações

climáticas influenciam a tolerância a metais dessas populações, comparando a resposta de ambas quando expostas a um gradiente de cádmio. Os objectivos principais desta dissertação são: (1) avaliar se as populações de isópodes que vivem em locais contaminados por metais são mais sensíveis às alterações climáticas, testando 3 temperaturas (20, 25 and 30°C) e 3 níveis de moisture (20, 40 and 60%) (capítulo 2); (2) estimar se as alterações climáticas afectam a sua tolerância a metais, e se essa tolerância está relacionada com a exposição prévia a metais, testando também 3 temperaturas (20, 25 and 30°C) e 2 níveis de humidade do solo (20 e 40%) (capítulo 3).

No capítulo 2, as populações apenas mostraram diferenças nos tratamentos com humidade de solo a 60%, tendo a população SC uma maior taxa de sobrevivência. No capítulo 3, a população SC foi mais afectada pela subida de temperatura, tornando-se mais susceptível à contaminação por cádmio. Além das diferenças entre populações, foi registada uma resposta comum à temperatura: o seu aumento estimulou maiores taxas de consumo. Foi também registado que a humidade do solo a 60% (ambiente molhado) provocou 100% de mortalidade em 20 dias, enquanto a humidade do solo a 20% (ambiente seco) não afectou a resposta das populações.

Este trabalho permitiu concluir que as alterações climáticas afectam as populações que habitam locais contaminados, com uma redução da tolerância a metais com o aumento de temperatura; afectam a sobrevivência de ambas as populações em caso de chuvas intensas; e promovem as taxas de decomposição dos isópodes com o aumento da temperatura, pelo menos até aos 30ºC.

Abstract

Isopods are soil organisms that help on organic matter decomposition, playing an important role in the carbon and nutrient cycles. In nature, isopods are subjected to several stressors that influence their distribution, ecology, behavior, and physiology (including their energy balance). One of those stressors, from anthropogenic origin, is metal contamination. Populations that live under metal contamination need to use energy from their metabolism to deal with metals, becoming prone to be more affected by additional stressors such as climate variations. With the present climate change scenarios, an increase in temperature and in extreme rainfall events (floods / drought) is predicted. These climate events can change metals' bioavailability and toxicity, and may affect isopods' response to contaminants. When facing metal contamination, isopod populations tend to avoid contamination; if they fail to do it they will need to develop tolerance to metals, in order to live and reproduce, by phenotypic or genotypic alterations.

In order to test the effects of climate change in metal-exposed isopod populations, a *Porcellionides sexfasciatus* population living near an abandoned mine in São Domingos (south of Portugal) was studied. That population (Santana de Cambas - SC), along with a reference one (Lago – LG), were subjected to lethal and sub-lethal tests (feeding). The main goals of this thesis were: (1) to evaluate if isopod populations living under metal contamination are more sensitive to climate change, testing in a full factorial experiment 3 temperature (20, 25 and 30°C) and 3 moisture levels (20, 40 and 60%) (Chapter 2); and (2) to estimate if climate change affects isopods' metal tolerance and if that tolerance is connected with their

previous exposure to metals (testing also in a full factorial design, 3 temperature (20, 25 and 30°C) and 2 moisture levels (20 and 40%) (Chapter 3).

Results from chapter 2 showed differences between populations only at the 60% moisture treatments, with SC having higher survival rates. Besides differences between populations, it was registered a common response to temperature increase, enhancing food consumption rates. Moisture conditions showed that 60% moisture (wet environment) caused 100% mortality after 20 days. Results obtained when testing Cd tolerance (Chapter 3) showed that SC population was more affected by temperature rise than LG, becoming more susceptible to cadmium contamination along temperature rise. In both chapters, the 20% moisture treatment (dry environment) did not affect isopods' response.

This work allowed to conclude that climate change affected isopod populations from metal contaminated sites, with decreased tolerance to cadmium with temperature increment; affected isopod survival in cases of extreme rainfall, and enhanced consumption food rates (and therefore decomposition ability) with temperature increase at least until 30ºC.

Chapter I - General Introduction

I.1 Metal contamination

Organisms are exposed to multiple stressors that influence their distribution, behavior, physiology and abundance. Those stressors could be natural (e.g. climate variations or organic matter availability) or from anthropogenic origin, as metal contamination, resulting from mining and smelting activities that originates high levels of metals on water and soil. The toxicity of those metals depends on metal identity, quantity, bioavailability and the vulnerability of organisms being exposed. Essential metals (e.g. copper and iron) are necessary to organisms' development; however, they may become prejudicial in high concentrations. Non-essential metals (e.g. cadmium and lead), are not used by organisms, been toxic even at low concentrations.

Not all metals present in soil are available for organisms' intake; they need to occur in a bioavailable condition, which is influenced by many factors as pH, redox potential, organic matter, soil type, moisture and temperature (Rieuwerts *et al*., 1998). From these factors, pH is the most influent, while climate conditions as moisture and temperature play a minor role (Rieuwerts *et al*., 1998). Regardless of that, moisture increase positively affects metals' solubility, leading to a higher bioavailability (Hernandez-Soriano & Jimenez-Lopez, 2012), while temperature increase was appointed to be important through alteration of metals kinetics (Spurgeon *et al*., 1997).

In order to evaluate contamination and environmental hazard to organisms, besides metal concentrations, the abiotic conditions should also be accounted for, since they affect metals toxicity and organisms' reaction, and the organism facing the contaminant should be considered (Rieuwerts *et al*., 1998; Violante *et al*., 2010; Anderstanti & Van Gestel, 2013).

I.2 Climate change

Climate change is an issue debated worldwide, driven by natural causes such as solar irradiance, but mainly by human causes: the huge increment on greenhouse gas emissions as carbon dioxide (280 ppm in the pre-industrial age to 379 ppm[3] in 2005), methane (715 ppb in the pre-industrial age to 1774 ppb in 2005) and nitrous oxide (270 ppb in the pre-industrial age to 319 ppb in 2005) (IPCC, 2007). Those causes brought an increase in temperature: from 1901 to 2012 the global mean surface temperature increased 0.89°C; however, from 1951 to 2012 the increase was about 0.72°C (IPCC, 2013), showing a direct relationship with the rise on greenhouse gas emissions.

Temperature increase leads to a raise of ice melting rates and sea level (1.8 mm per year over 1961-2003 but about 3.1 mm per year over 1993-2003) and changes in precipitations patterns (longer droughts since 1970s in tropics and subtropics; increased frequency of heavy precipitation events (IPCC, 2007)). With this data, from the last 100 years, it was possible to see changes in temperature, precipitation, wind patterns and extreme weather events as drought and heavy precipitation. Moreover, the use of models enables the calculation and prediction of future climate events: in IPCC report from 2013, an increase on the surface global mean temperature from 1.4°C to 5.8°C until 2100 was projected. This warming will possibly exceed the ecosystems' resilience, due to climate disturbances (e.g. flooding, drought, wildfires), aggravated by other global changes as landscape use, pollution and habitat fragmentation (IPCC, 2007). This would lead to shifts in species geographical ranges, potentially having negative consequences for biodiversity.

In Portugal, an increase from 3°C to 7°C in the summer season, in the next 100 years, is expected. On top of that, it is also expected an increase in the frequency of heat waves, a reduction of 20% to 40% in rainfall leading to drought, and also an increase of rainfall during winter (Portuguese Environment Agency, 2010).

I.3 Climate influence on metal toxicity

All climate predictions show continued increases in temperature and extreme events. But then, how will this climate conditions influence metal toxicity and organisms' response?

Rainfall modifies soil moisture. A higher percentage of soil moisture means an increase in metals solubility, leading to a higher bioavailability of metals (Hernandez-Soriano & Jimenez-Lopez, 2012). So, with higher percentage of water in soil, we expect higher toxicity from metals. As for soil organisms, moisture affects their distribution: low humidity during summer months can lead to higher desiccation, leading to organisms' death or migration. Moreover, it can affect the amount of time organisms spend on feeding in exchange to shelter (Dias *et al*., 2012). So, soil drought leads to changes in their abundance and decomposition rates (Pflug & Wolters, 2001), jeopardizing the nutrients' cycle. As for metal exposure and moisture variation, no evidence was found showing that metals' exposure increases drought induced mortality (Sørensen & Holmstrup, 2005).

Temperature affects soil organisms' response when exposed to metals. An increase of 10ºC can double biochemical reactions, and enhance metabolic rates (Luoma, 1983). Changes in metabolic rates affect organisms' growth (Abdel-Lateif *et al*., 1998; Lukkari *et al*., 2006), food consumption and oxygen consumption (Khan *et al*., 2007) (table I.1). The main question is how the presence of metals influences these variations.

Abdel-Lateif *et al*. (1998) tested 4 different temperatures (12, 16, 20, 24ºC) on isopod growth rate. As expected, isopods subjected to higher temperatures had higher growth rates. Additionally, they also tested four cadmium concentrations and verified that in the highest ones (0.14 and 0.30 µmol Cd/ g dry litter) the growth induced by temperature increase was inhibited due to metals. This condition was also registered by Lukkari *et al*. (2006), in which earthworms subjected to 18ºC, showed the same growth in the control soil and the lowest concentration (a mixture of 79/ 139 mg of Cu/ Zn per kg soil). But then again, in the highest metal concentration (a mixture of 178/ 311 mg of Cu/ Zn per kg soil), growth was negatively affected. Spurgeon *et al*. (1997), using *Eisenia fetida* exposed to a temperature of 15ºC, registered a higher medium lethal concentration (LC_{50}) than at 25ºC, suggesting an increase of zinc toxicity with increasing temperature. These studies showed that temperature increase leads to higher metal toxicity.

Table I.1: Ecotoxicological studies with soil organisms testing metals EC_{50} (growth and reproduction) and LC_{50} (survival) response with temperature variation. All studies were made with laboratory populations.

Reference	**Organism**	**Measured features**	**Contamination**		**Temp. (°C)**	**EC_{50} / LC_{50} (µg / g)**
Abdel Lateif *et al*., 1998	*Porcellio scaber* (isopod)	Growth rate	Food Cadmium		12	27.9
					16	51.4
					20	38.6
					24	30.4
Donker *et al*., 1997	*Porcellio scaber* (isopod)	Growth rate	Food Zinc		12	3.4
					16	3.5
					20	2.2
					24	2.6
Spurgeon *et al*., 1996	*Eisenia fetida* (earthworm)	Survival	Soil Zinc		15	1598
					20	1235
					25	1131
Voua Otomo *et al*., 2013	*Enchytraeus doerjesi* (potworm)	Survival	Soil	Cadmium	15	293
					20	262
					25	232
				Zinc	15	420
					20	518
					25	>640
		Reproduction		Cadmium	15	24
					20	35
					25	17
				Zinc	15	41
					20	112
					25	112
Sandifer & Hopkin, 1997	*Folsomia candida* (collembolan)	Reproduction	Soil	Cadmium	15	540
					20	590
				Copper	15	640
					20	700
				Lead	15	1570
					20	2970
				Zinc	15	590
					20	900

However, the increase in metabolic rate with temperature (Luoma, 1983) can affect both metal uptake and excretion (Heugens *et al*., 2001). Therefore, metal's bioaccumulation may or may not change, meaning that organisms' susceptibility to metals wouldn't be affected by temperature raise, since it would create a null effect. Nevertheless, organisms do not exhibit the same assimilation/ excretion rates. A study carried out by Janssen and Bergema (1991) revealed different responses by two different arthropod species when exposed to an increase in temperature. While the collembolan *Orchesella cincta* increased both metals' uptake and excretion, leading to an internal equilibrium, the mite *Platynothrus peltifer* increased its uptake but no differences were found on excretion rates. These two examples show that there are organisms more suitable to stress tolerance than others. Another explanation is based on the organisms' tolerance to temperature, since they have a thermal tolerance range, outside of which they became more vulnerable to temperature variations and to additional stressors as metal toxicity (Pörtner, 2001; Heugens *et al*., 2001; Khan *et al*., 2007). Khan *et al*. (2007) demonstrated that oxygen consumption in earthworms was lowest at 10-12ºC and increased until 18ºC, after which it decreased again, evidencing that the metabolism increased with temperature until reaching a critical limit (in this case 18 ºC) above which organisms decreased their activity, being more susceptible to additional stressors.

Although the majority of studies points to a direct relation between temperature increase and metals toxicity, there are data pointing the other way (e.g. Voua Otomo *et al*., 2013; Sandifer & Hopkin, 1997). While cadmium LC_{50}

decreased with temperature increase, zinc's LC_{50} increased along with temperature (Voua Otomo *et al*., 2013). This could be explained because Zn, as opposed to Cd, is an essential metal. Additionally, Sandifer & Hopkin (1997), despite showing that lead medium effect concentration (EC_{50}) increased from 15ºC to 20ºC, cadmium, copper and zinc EC_{50s} were the almost the same at both temperatures. This could be explained by the organisms' (*Folsomia candida*) mechanisms to handle metals (e.g. improvement of metals excretion rates). However, at 25ºC, it was not possible to calculate any EC_{50} because organisms' thermal tolerance is achieved becoming more susceptible to additional stressors.

The majority of the studies points to an increment of metals toxicity with temperature – rise of metabolic rates with temperature. Nonetheless, once again, that condition varies with metal identity and with the organism.

I.4 Organisms' response to stressors

When exposed to stressors, populations can suffer powerful selective forces, altering populations' genetic variability, intergenerational transfer, adaptability and survival of future generations (Fox, 1995). In order to survive, populations may adjust by phenotypic plasticity or adapt by genetic changes leading to populations' evolution (Gienapp *et al*., 2008).

Acclimation processes lie in plastic traits, which help organisms to tolerate a stress until a limit is reached. Based on the assumption that organisms have a limited budget of energy to live, they will use that energy for survival and reproduction. But living under constant stress, they will reach a point where they need to compromise their main objectives, since a slice of their energy budget is deviated to detoxification (Stone, 2001; and references therein). Based on this assumption, populations that inhabit metal contaminated sites (SC) would be more susceptible to other stressors since part of their energy expenditure is already being used to tolerate metal contamination.

Additionally, if the stressor is too strong, it could lead to populations' extermination or to adaptation, in which the most tolerant to that stressor will survive. Theoretically, these populations adapted to live under metal contamination, will tolerate higher contamination than others that live in uncontaminated sites. Lukkari *et al*. (2006) shows that earthworms with chronic exposure to metal contaminated soil tolerate higher soil metal concentrations than reference populations. Nonetheless, populations' adaptation to metal contamination could lead to reduced fitness in the absence of metals – loss of genetic variability through bottlenecks and inbreeding (Ribeiro & Lopes, 2013). Moe *et al*. (2013) classified individuals as toxicant-tolerant or climate-tolerant. When exposed to toxicants, the ones that can survive in those conditions will be selected, leading to a decrease of climate-tolerant individuals on that population: meaning that the adaptation to one stressor could lead to loss of fitness regarding other stressors.

To summarize, organisms that face metal contamination can avoid or tolerate that stress. To tolerate metals they need to develop mechanisms (phenotypic, genotypic or both) that are energy expendable, leading to less energy to deal with other stressors, compromising these populations' sustainability.

I.5 Model organism

The model organism used in this work was the terrestrial isopod *Porcellionides sexfasciatus.* Isopods are widely used in ecotoxicological tests because they fulfill the eligibility criteria – small size, high abundance, simple identification, background knowledge on their biology and being responsive to sub-lethal concentrations of toxicants (Drobne, 1997), ubiquitous distribution, and easy maintenance in laboratory.

These organisms inhabit the soil/ litter compartment. Soils contain about 2/3 of the total organic carbon in terrestrial systems (Dias *et al*., 2012), and isopods play an important role in the carbon cycle. They are comminutors, feeding mainly on organic matter, changing the soil cover in a physical, chemical and biological way, stimulating the decomposition of organic matter by soil micro-organisms (Dias *et al*., 2012), thus playing a key role in nutrient cycling.

It has been explained how metals and temperature affect soil organisms in general. As for isopods, they have the distinguishing capacity of hiperaccumulating metals in the hepatopancreas, which acts as a detoxification mechanism, being by far the most important storage organ of metals (Hopkin & Martin, 1982; Jones & Hopkin, 1998; Mazzei *et al*., 2014). If the hepatopancreas becomes saturated, cell disruption occurs and metals are released to the lumen, leading to isopods death

(Mazzei *et al*., 2014). Besides, they have the ability to distinguish and avoid contaminated food, increasing their tolerance to metals (Zidar *et al*., 2005; Odendaal & Reinecke, 1999). To increase their tolerance even more, it has been proved that isopods that live under chronic stress by metals reproduce earlier and increase their reproductive allocation (energy deposited in reproduction that is measured by the weight of the brood in comparison to mothers' weight), having more individuals per brood, but smaller than the ones from a reference site (Donker *et al*., 1993).

Along with decreasing body size due to metal contamination, an universal decrease in body size as a response to climate warming was noticed (Gardner *et al*., 2011). As claimed before, an increase in temperature increases ectothermic metabolism, leading to higher metabolic rates and, in the majority of cases, to increasing metal toxicity. As isopods are sensitive to desiccation, during dry summers they will spend more time sheltering and less time eating, compromising their survival (Dias *et al*., 2012). In addition to sheltering (as a defense mechanism), they have an aggregation behavior that can diminish the effects of temperature increase (Hassal *et al*., 2010).

I.6. Aims and thesis structure

I.6.1. Aims

The main goal of this thesis was to investigate how climate change will affect terrestrial isopod *Porcellionides sexfasciatus'* populations living under metal contamination. To fulfill the main goal two specific objectives were established:

(1) To evaluate the effects of temperature and moisture, according to predicted climatic scenarios, in a chronically metal-exposed and a reference populations. The main question here was if a background of exposure to metal contamination leads to an increased susceptibility to additional stress factors such as temperature and moisture increase. This study will help evaluate the response of soil organisms, exposed to metal contamination, to additional stressors and to understand the effects of climate change on soil processes underlying key ecosystem services.

It's expected that the metal-exposed population will be more affected by climate change: if they are adapted to metal contamination, and that adaptation led to fitness loss regarding other stressors, it could be expected less intra-specific genetic diversity leading to less tolerance; if metal tolerance is achieved by plastic traits, a lower available energy to deal with climate change is expected.

(2) To estimate the effects of climate change on metal tolerance of a chronically metal-exposed and reference populations. This study will increase the knowledge on the effects of multiple stressors and their interactions, and the knowledge on metal-exposed field populations' response to stressors.

For both populations, a decrease on metal tolerance with temperature and moisture increase is predicted due to increased metal toxicity; nonetheless, higher metal tolerance from the metal-exposed population in the control treatment is expected, assuming previous metal tolerance development for inhabiting a metal-contaminated site. Also, lower tolerance to climate changes in the metal-exposed population, taking into account the assumed lower capacity to handle additional stressors, is expected.

I.6.2. Thesis structure

This study, on the influence of climate change and metal contamination on a metal-exposed population, will contribute to find isopods’ response to future climate conditions and to evaluate their metal tolerance variation in those conditions, considering their history of metal contamination.

To achieve the described aims, multiple stressor experiments, divided into 2 chapters, were developed. Along with a general introductory chapter and a final discussion there are a total of 4 chapters:

(1) A general introduction on metal contamination, climate change, isopods and their interaction (chapter I).
(2) An estimation of the effects of climate change on a metal-exposed population of *Porcellionides sexfasciatus* (Chapter II).
(3) An evaluation of metal tolerance of a metal-exposed population of *Porcellionides sexfasciatus* under future climatic scenarios (Chapter III).
(4) A general discussion with the major conclusions of this work and a brief analysis on the work that can be done to improve these findings (Chapter IV).

Chapter II - Climate change effects on *Porcellionides sexfasciatus* populations living in metal contaminated sites

II.1. Abstract

Isopods play an important role in organic matter decomposition, helping in the carbon and nutrients cycles. Climate change may lead to changes in decomposition processes since increases in temperature and frequency of extreme events are predicted, potentially modifying isopods behavior (including feeding behavior) and distribution. Additionally, isopods inhabiting metal contaminated sites need to deviate energy to deal with that stress, compromising their fitness. Our goal was to assess how climate change affects isopods populations living under metal contamination. To evaluate these populations' response, a population from a metal contaminated site (SC) along with an uncontaminated population (LG) were subjected to a survival and feeding test, being exposed to different climate conditions (temperature: 20°C, 25°C, 30°C; soil moisture: 20%, 40%, 60%) accordingly with climate change predictions. It was found that none of the populations could survive in moisture levels higher than 60%. Also, the metal-exposed population had higher food consumption rates than the control population at 20°C, but that difference was not found at higher temperatures. Those results allowed to conclude that SC populations are less susceptive to wet environments (60% moisture), and both populations are not affected by dry environments (20% moisture) at least within the tested time. Additionally, it is proposed that the SC population can originate a higher organic matter fragmentation rate due to their higher food consumption to cover its needs of more energy to deal with metals body burden and both populations were affected by temperature increase, leading to higher decomposition rates, potentially altering the carbon and nutrients cycles.

II.2. Introduction

Isopods are well known organisms, with a ubiquitous distribution and a notorious importance on the carbon and nutrient cycles: they feed on organic matter, shredding it to help the activity of microorganisms during decomposition (Dias *et al*., 2012; Godet *et al*., 2011). Being ubiquitous organisms they can be exposed to several environmental stressors, such as extreme temperature and moisture conditions, and pollutants. According to IPCC reports (IPCC, 2013), the last 3 decades have been increasingly warmer. This increase in the global mean surface temperature is highly connected with the industrial age and the intensification of greenhouse gas emissions. Based on these data, an increment of 1.4°C to 5.8°C until 2100 in the global mean surface temperature and an increase of the frequency of extreme events (as drought and floods) is predicted (IPCC, 2013).

Those changes in climate will directly affect isopod populations, which could alter their contribution to carbon and nutrient cycles. Since they live in direct contact with the soil solution phase, they depend on soil moisture to control internal water content. Therefore, soil moisture is responsible for their distribution and abundance, mainly through their capacity to handle desiccation (Dias *et al*., 2013). Moreover, water content is also temperature dependent, since higher temperatures lead to higher water losses (Warburg *et al*., 1965). Additionally, isopods are ectothermic, depending on outer temperatures to regulate their body temperature, with a direct influence on ectothermic metabolic pathways, such as excretion (Janssen & Bergema, 1991), consumption (Rombke *et al*., 2011), and growth rates

(Donker *et al*., 1998; Abdel-Lateif *et al*., 1998) as well as in several reproductive traits (Sandifer & Hopkin, 1997; Voua Otomo *et al*., 2013).

Additionally, some populations are also exposed to pollutants from anthropogenic origin such as metal contamination. In terrestrial ecosystems, metals accumulate both in soil and litter compartments, resulting in a continuous exposure for soil organisms, with both paths potentially having an additive effect (e.g. Vijver *et al*., 2006).

Climate change may affect metal bioavailability and therefore their toxicity. It's considered that temperature affects bioavailability by altering metal kinetics, favoring uptake by soil organisms (Spurgeon *et al*., 1997). As for soil moisture increase, it positively affects metal solubility, leading also to a higher bioavailability (Hernandez-Soriano & Jimenez-Lopez, 2012).

Theoretically, besides bioavailability increase, populations inhabiting metal contaminated sites will be more susceptible to climate variations than reference ones, since chronic exposure to high metal contamination can increase their susceptibility to additional stressors (Stone *et al*., 2001).

The main goal of this study was to infer how having a history of metal exposure affects isopods response to climate change. Based on future climate scenarios, the effect of temperature and moisture on survival and feeding activity of two isopod populations (one from a metal contaminated site and other from a reference site) was evaluated.

It's expected that the metal-exposed population (SC) will be more affected by climate change because their previous exposition to metals could have led to fitness loss regarding other stressors and/ or because they are already deviating energy to deal with metal contamination, leaving less energy to deal with climate change.

II.3. Materials and methods

II.3.1. Model organism

Tests were conducted with *Porcellionides sexfasciatus* from two populations: a population with a history of metal exposure and a reference one. Sampling was made by hand, collecting approximately 600 individuals at each site. Isopods were taken to the laboratory and kept in boxes with soil from their respective sampling site in a climate-controlled room at 20°C ± 2°C with a photoperiod of 16h light: 8h dark. They were fed with alder leaves (*Alnus glutinosa*) for at least one month before being used in survival and feeding experiments. Female isopods were placed in individual containers to make sure only non-pregnant females were used.

II.3.2. Study site

Populations were collected at Mina de São Domingos, an abandoned cupric-pyrite mine with high levels of metals in soil as well as in litter, located in south Portugal. The metal-exposed population collection site (Santana de Cambas_SC_37º37’56’’N, 7º31’06’’W), 5Km from the mine pit, receives metal inputs mainly through wind. The reference population was collected in a nearby place called Lago (LG_37º40’55’’N, 7º30’54’’W), with no metal contamination. Soil and litter metal concentrations, from both sampling sites are present in Table II.1 (data from Costa *et al*., 2013). Soil and litter were collected at both sites to measure organic matter content (soil was combusted at 500°C for 6h), soil pH (with KCl 1M 1:6v/v), water holding capacity (ISO DIS 11268-2), soil texture (according to Gee & Bauder, 1986), cation exchange capacity (Tedesco *et al.*, 1995) and to perform the survival test.

Table II.1. Soil and litter total metal concentrations (µg/ g) from both sampling sites (SC = Santana de Cambas; LG = Lago). Data from Costa *et al*. (2013).

	Soil		**Litter**	
Metals	**SC**	**LG**	**SC**	**LG**
Cu	933	34	302	22
Fe	94413	54663	50935	31068
Zn	320	77	283	64
Cd	3.1	<2.8	4	<2.8
Pb	3276	54	1192	<45

II.3.3. Test conditions

For both survival and feeding experiments, isopods were individually placed in boxes with 8 cm diameter, 4.5 cm height; the lid of the boxes were perforated to allow air exchange. Tests were performed in temperature-controlled cameras with a photoperiod of 16h light: 8h dark. Three different temperatures (20ºC [control], 25ºC and 30ºC) and three different soil moisture contents (20%, 40% [control] and 60% of the water holding capacity) were tested. A total of 9 treatments (3 temperatures; 3 moistures) were setup, in which isopods were subjected to a combination of temperature and moisture conditions. For each treatment, 20 replicates (10 males and 10 females) per population were setup. Soil moisture was adjusted using distilled water by weighting the boxes every other day.

For the survival experiment 40 g of the population respective soil were placed in each test container. Soil was previously defaunated by two freeze/thawing cycles, and sieved with a 5 mm mesh sieve. No food was offered; therefore isopods fed only on soil organic matter. Each container was checked every day for dead individuals, for a total of 32 days.

For the feeding experiment, instead of soil, 30 g of pure sand were placed in each container to avoid having organic matter in the test medium. In this experiment, only two moisture conditions (20% and 40%) were tested. Isopods were fed with alder leaf discs (made with a cork borer with 16 mm diameter). The substrate was renewed every week to remove fecal pellets, and fresh food was added. To determine food consumption, alder leaf discs' dry weight was measured at the beginning and at the end of every week; also, isopods were weighted at the beginning of the experiment, which lasted for 28 days.

II.3.4. Data analysis

Survival data was fitted to an exponential model to calculate medium lethal time (LT_{50}):

*Mortality=a*EXP(LOG((a-a*0.5-b*0.5)/a)*(Days/c))+b)*

Treatments were compared using the likelihood ratio test. Food consumption rate was calculated as:

Consumption = dry leaf consumption / 28 days / isopod initial weight.

Due to lack of homogeneity of variance, consumption values were transformed into ranks and a Friedman test was performed. Treatments were compared by post-hoc analysis, with Wilcoxon signed-rank tests, using a False Discovery Rate (FDR) correction with the Benjamini–Hochberg method (Benjamini & Hochberg, 1995) to obtain a realistic p value ($p < 0.031$). All analyses were performed using the *Statistica 7* software (StatSoft, Inc., 2004).

II.4. Results

II.4.1. Soil and litter analysis

Cation exchange capacity (CEC), pH, organic matter content (OM), water holding capacity (WHC) values, as well as soil texture were similar between collection sites (table II.2).

Table II.2: Values for cation exchange capacity (CEC), pH, organic matter (OM, %), water holding capacity (WHC, %) and soil texture from both sampling sites (SC = Santana de Cambas; LG = Lago).

	SC	**LG**
CEC	13.32	8.67
pH	6.2	6.8
OM	9.8	7.8
WHC	47.9	42.3
Texture	sandy loam	sandy loam

II.4.2. Survival experiment

During the experiment, treatments with 20% and 40% soil moisture content did not originate 50% mortality of the isopods. Therefore, it was not possible to calculate LT_{50} values for these treatments. At 60% moisture level, 100% mortality was achieved before 20 days allowing LT_{50} values to be calculated. For all treatments allowing the calculation of LT_{50} values, the model used explained more than 98% of the data variability. LT_{50} values were significantly higher for SC population in all temperatures tested (table II.3). Additionally, analysing individually each population for moisture and temperature, no differences on moisture conditions were found. As for temperature, LT_{50s} from SC population increased from 20°C (1.9 days) to 30°C (3.45 days) with the 25°C treatment showing a higher LT_{50} value (5.16 days). At LG, LT_{50s} were not significantly different (Fig. II.1).

Table II.3: LT_{50s} (days) and confidence intervals, calculated by fitting data into an exponential model for *Porcellionides sexfasciatus'* survival test with moisture and temperature treatments (SC = Santana de Cambas; LG = Lago). The test last for 32 days.

Pop.	**Moist. (%)**	**20ºC**	**25ºC**	**30ºC**
LG	20	>32	>32	>32
SC	20	>32	>32	>32
LG	40	>32	>32	>32
SC	40	>32	>32	>32
LG	60	0.38 (0.35-0.40)^{a,1}	0.46 (0.36-0.55)^{a,1}	0.31 (0.29-0.33)^{a,1}
SC	60	1.9 (1.52-2.27)^{b,1}	5.16 (3.92-6.40)^{b,2}	3.45 (2.93-3.97)^{b,3}

^{a,b} Significant differences between populations at each temperature; ^{1,2,3} Significant differences between temperatures within the same population.

II.4.3. Feeding experiment

Isopods from both populations presented different initial weight values (Kruskall–Wallis H test, H=5.99, $p<0.02$), with individuals from Santana de Cambas being lighter (15.61 ± 0.34 mg) than those from Lago (17.58 ± 0.49 mg).

There were statistical differences between treatments ($X^2(11) = 50.0598$, $p < 0.001$). Differences between populations were only found at 20°C/40% WHC treatment: the population from Santana de Cambas presented higher consumption rates than the one from Lago ($Z = 2.438$, $p < 0.02$) (table II.4).

Regarding intra-population analyses, at the two moisture conditions tested, no differences were found. Nonetheless, both populations present a significantly lower consumption rate at 20°C when compared with the other two temperatures tested at both moisture regimes. Furthermore, the population from Lago had significantly higher consumption rates at 30°C than at 25°C, at 20% moisture.

Table II.4: Food consumption rates per treatment with respective standard deviation, for the two populations of *Porcellionides sexfasciatus* (SC = Santana de Cambas; LG = Lago). The test lasted for 28 days. Food consumption rates were calculated by grams of food eaten by day per isopod initial weight.

	SC		**LG**	
	20%	40%	20%	40%
20°C	0.036 ± 0.014^{1}	$0.037 \pm 0.014^{a,1}$	0.033 ± 0.009^{1}	$0.027 \pm 0.013^{b,1}$
25°C	0.054 ± 0.010^{2}	0.053 ± 0.016^{2}	0.050 ± 0.013^{2}	0.050 ± 0.011^{2}
30°C	0.064 ± 0.022^{2}	0.067 ± 0.019^{2}	0.073 ± 0.019^{3}	0.068 ± 0.018^{2}

[a,b] Significant differences between populations.

[1,2,3] Significant differences between temperatures within the same population and moisture condition.

At almost all moisture conditions, populations' consumption rates increased with temperature, showing a higher increase between 20°C and 25°C than between 25°C and 30°C (Fig.II.2).

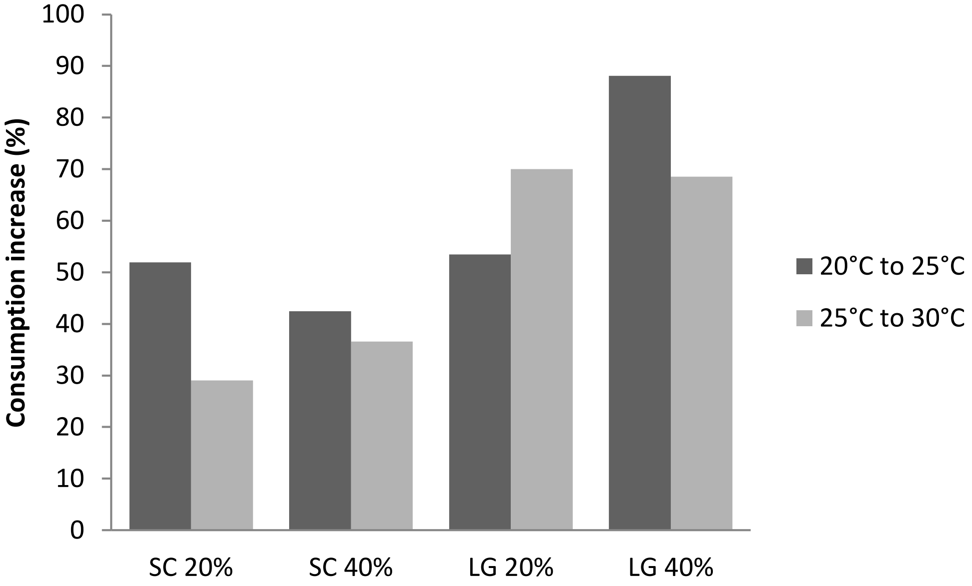

Figure II.2: Food consumption increase (%) along a gradient of temperature for both populations (SC = Santana de Cambas; LG = Lago) and moisture conditions. At almost all conditions, food consumption increase is higher from 20°C to 25°C than from 25°C to 30°C. Consumption was calculated by food eaten per day by isopod initial weight. The test last for 28 days.

II.5. Discussion

Before we conducted the experiments, females had an isolation period to avoid the use of pregnant ones; based on the assumption that organisms live under a controlled energy budget, females would use energy for reproduction leaving less energy available for survival purposes (Calow, 1991). Also, the use of only one isopod per box was to avoid aggregation behavior, employed by the isopods to escape desiccation (Hassal *et al*., 2010), to account for each isopod as a replicate, and to avoid competition.

The survival experiment showed differences between populations in the treatments with 60% WHC and between temperatures in the SC population. Populations' differences were not in accordance with the expected higher susceptibility of the metal-exposed population to additional stressors. On the contrary, the SC population showed higher tolerance to 60% WHC condition than the LG population. Maybe, the mechanisms providing metal tolerance could be the same that offers flooding tolerance.

As for temperature differences within SC, it could be expected higher survival in the treatments with higher temperatures, because it would diminish soil moisture with higher temperature increase. But soil moisture did not present more than a 5% variation in all treatments.

Despite the differences between populations, this data allowed us to conclude that isopods' survival is strongly affected at 60% WHC regardless of their origin. Isopods perform gas exchanges through internal lungs that are located in the ventral surface and through the integument (Schmidt & Wägele, 2001). When subjected to high moisture conditions (soil slightly soaked), isopods can face hypoxia (Schmitz & Harrison, 2004) compromising survival. In natural conditions, in case of extreme events as massive rainfall periods, isopod populations could be extremely reduced. Based on these results, (100% mortality after 20 days), the feeding test and the tests made in chapter III were not performed with the 60% WHC treatment.

At 20% moisture, as well as in the control (40% moisture), none of the populations achieved 50% mortality, demonstrating that a shortage of water is not prejudicial to *Porcellionides sexfasciatus.* Nonetheless, we could hypothesize that differences would be found with a prolonged test. Since isopods from SC are smaller than LG, they may be more affected by dry environments because isopods with less body surface area have less desiccation resistance (e.g. Dias *et al*., 2013).

Food consumption was measured by food eaten per day, taking into account the isopods initial weight, to reduce size influence on food consumption. However, we only found differences between the reference and the metal-exposed population at the control treatment (20°C: 40%), in which SC had higher consumption than LG. This was expected considering energy needs: theoretically, populations inhabiting metal contaminated sites, in order to handle toxicants, need more energy (Calow, 1991). Nonetheless, the higher consumption from SC seems to be masked by the temperature increase: temperature has a heavy influence in ectothermic metabolism, influencing metabolic rates (Pörtner, 2001) leading to higher food consumption rates with temperature rise (Rombke *et al*., 2011; Jager *et al*., 2003).

In this study, the increase in consumption observed from 20°C to 25°C is higher than from 25°C to 30°C, allowing us to assume that consumption rates will increase until a maximum value. To find that limit it would be necessary to test more temperatures. The substantial increase in consumption with temperature seen in this study agrees with Rombke *et al*. (2011) that tested feeding behaviors along a temperature gradient. *Porcellio scaber* increased its consumption by almost 100% from 20°C to 28°C; however, the same was not observed in *P. pruinosus*, showing that the temperature-consumption relation is species-specific.

In this work, the survival test allowed a large-scale view, finding that climatic variations, such as high moisture conditions (60%), affect populations' perseverance; the feeding test helped to find how temperature increase affects carbon and nutrient cycles, by positively influencing organic matter consumption.

However, in natural conditions, isopods are subjected to metal contamination from both soil and litter (Drobne, 1997), instead of uncontaminated soil and food as in this experiment. In nature, isopods need to find a relation between food consumption and survival due to an increase in metals body burden with exposure time (Vijver *et al*., 2006). However, the use of food from their collection site would not allow for a direct comparison between tests.

Even so, it may be concluded that extreme events as intensive rainfall periods can have a strong negative effect on isopods survival, and that temperature increase is beneficial for isopods' mediated organic matter decomposition, enhancing the carbon and nutrients cycles at least until 30ºC.

Chapter III - Climate change effects on cadmium tolerance of *Porcellionides sexfasciatus* populations living under metal contamination

III.1. Abstract

Terrestrial isopods play an important role in organic matter decomposition. Isopods living under metal contamination can either avoid metals or tolerate them. Metal tolerance can be achieved by phenotypic or genotypic changes. Supposedly, metal-exposedpopulations will develop metal tolerance and be more tolerant to metals than populations without a metal exposure history; such tolerance can be affected by climatic variations. Also, increases in soil moisture and temperature lead to an increment of metal toxicity. This chapter evaluated cadmium tolerance of *Porcellionides sexfasciatus* (terrestrial isopod) populations in different climate change scenarios. The goal was to find how climate change affects isopods' metal tolerance and if that tolerance is related to isopods' background. It was found that cadmium toxicity increases with temperature in the metal-exposed population, showing that previous exposure to metals leads to higher susceptibility to other stressors; also, the metal-exposed population did not develop higher tolerance to metals than the reference population.

III.2. Introduction

Terrestrial isopods are soil organisms responsible for the fragmentation of organic matter, acting as catalysts for microbial communities during decomposition (Drobne, 1997). Thus, they play an important role on carbon and nutrient cycles (Drobne, 1997; Dias *et al*., 2012). Besides, they are good models for ecotoxicological tests because of their wide distribution, simple identification, and background knowledge (Drobne, 1997). They are also recognized as bioindicators of metal pollution (Dallinger *et al*., 1992; Paoletti & Hassal, 1999) for their capacity to store metals in the hepatopancreas (Hopkin & Martin, 1982).

In nature, isopods are exposed to stressors as temperature, moisture, pH, organic matter availability, UV exposition and metal contamination (anthropogenic origin). When living under metal contamination, isopods can avoid or limit their exposure to metals (Zidar *et al*., 2005). If they fail to avoid exposure, they need to tolerate metals at the expense of their development, disturbing physiological processes and leading to changes in populations (Callow, 1991). Those changes can be phenotypic, genetic or both (Gienapp *et al*., 2008). Phenotypic tolerance is achieved by changes in plastic traits (phenotypic plasticity), physiological changes (acclimation) or by maternal effects. Genetic adaptation is based mainly on the survival of the fittest through selection of the most tolerant to metal contamination. This can lead to a reduction of genetic variability and, in some cases, to loss of fitness of the population as a consequence of energy re-allocation to detoxification processes (Ribeiro & Lopes, 2013).

In metal-contaminated sites, metal exposure occurs by two pathways: soil and litter. Since isopods feed on organic matter, it acts as the most direct source of contamination (Vijver *et al*., 2006; Godet *et al*., 2011). Thus, metal exposure can affect food consumption, changing decomposition rates, affecting nutrients and carbon cycles (Farkas *et al*., 1996; Drobne & Hopkin, 1994). This makes food consumption a relevant endpoint when studying the effects of metal contamination on isopods' role in nature (Costa *et al*., 2013). Non-essential metals, such as cadmium (Cd) present a huge risk for those populations; organisms' metal uptake is driven by consumption of contaminated litter, and through passive adsorption by body wall (Spurgeon *et al*., 1997; Pejnenburg et al., 2012). Since Cd it's not used in any physiological process, isopods accumulate it along exposure time (Vijver *et al*., 2006), creating granules in the hepatopancreas. Moreover, it is believed that the energy needed for excretion would be higher than the energy used to store it (Hopkin & Martin, 1982). The lack of mechanisms for cadmium detoxification leads to cadmium body burden increase; when the maximum accumulation capacity is achieved, cell disruption occurs and cadmium is released into the lumen (Mazzei *et al*., 2014) leading to isopods death.

Despite metal concentrations, organisms' metal toxicity change with time, derived from changes in natural conditions (e.g. pH, temperature, moisture), that affect metals' bioavailability and organisms' sensitivity to toxic chemicals (Spurgeon *et al*., 1997). Therefore, it's important to find out how those changes affect organisms tolerance to metals. According to IPCC reports (IPCC 2013), there has been a huge increase in the global mean surface temperature over the last century, wherein the majority of that increment was in the last 60 years (0.72°C of the 0.89°C [1901-2012]). Based in data from previous years, it's predicted an increase in temperature (1.4°C to 5.8°C until 2100) and frequency of extreme events, meaning less but more intensive raining periods, leading to floods and droughts (IPCC, 2013).

These changes will induce modifications in isopod communities. The higher temperatures are expected to increase isopod metabolic rates, since they are ectothermic, leading to higher food consumption (Rombke *et al*., 2011) and higher growth rates (Abdel-Lateif *et al*., 1998). As for rainfall, it's pointed to be the main reason for soil organisms' distribution (Dias *et al*., 2012), since they depend on moisture to avoid desiccation, and also affect feeding behaviors since organisms spend less time feeding in exchange to shelter (Dias *et al*., 2012). Nonetheless, when inhabiting metal contaminated sites, those responses can diverge, since isopods may need to deviate energy from their physiological processes to deal with metals (Calow, 1991).

Subsequently, this chapter is focused on evaluating the effect of climate change on metal tolerance of two populations of the isopod *Porcellionides sexfasciatus*: a reference one and other with a history of metal exposure. The main goal was to evaluate how climate change affects metal tolerance and to find if the historically metal-exposed population is more affected by additional stressors.

In the control treatment (20°C: 40%) it is expected higher metal tolerance by the metal-exposed population considering their history of metal contamination. With temperature and moisture increase, it is expected higher metal toxicity and thus, a decrease on metal tolerance on both populations. Nonetheless, taking into account the lower capacity to handle additional stressors from the metal-exposed population, lower tolerance of that population is expected.

III.3. Materials and methods

III.3.1.Model organism

Tests were conducted with F1 generation of the isopod *Porcellionides sexfasciatus* from two populations: a population with a history of metal exposure and a reference one. Sampling was made by hand, collecting approximately 600 individuals in each site. Isopods were taken to the lab and kept in a climate-controlled room at 20°C ± 2°C with a photoperiod of 16h light: 8h dark. They were fed with alder leaves (*Alnus glutinosa*) and kept in boxes with soil from their sampling site. F1 generation was born under laboratory conditions and sorted after birth to separate females from males to make sure that only non-pregnant females were used. F1 generation spend at least three months under lab conditions before being used in the following experiments.

III.3.2.Study site

Populations were collected in Mina de São Domingos, an abandoned cupric-pyrite mine with high levels of metals in soil as well as in litter, located in south Portugal. The metal-exposed population collection site (Santana de Cambas_SC_37º37’56’’N, 7º31’06’’W), 5Km from the mine pit, receives metal inputs mainly through wind. The reference population was collected in a place nearby called Lago (LG_37º40’55’’N, 7º30’54’’W), with no metal contamination. Soil and litter metal concentrations, from both sampling sites are present in Table III.1 (data from Costa *et al*., 2013).

Table III.1: Soil and litter total metal concentrations (µg/g) from both collection sites (SC = Santana de Cambas; LG = Lago). Data from Costa *et al*. (2013).

	Soil		**Litter**	
Metals	**SC**	**LG**	**SC**	**LG**
Cu	933	34	302	22
Fe	94413	54663	50935	31068
Zn	320	77	283	64
Cd	3.1	<2.8	4	<2.8
Pb	3276	54	1192	<45

III.3.3. Test conditions

Survival and feeding experiments were developed using cadmium ($CdCl_2.2.5H_2O$, 98% purity, from Fluka) mixed into soil and leaves, respectively. Preliminary tests were performed to obtain the proper Cd concentrations to calculate the medium lethal concentrations (LC_{50s}) and the medium effect concentrations (EC_{50s}).

For both tests, isopods were placed in individual boxes with 8 cm diameter, 4.5 cm height; the lid of the boxes were perforated to allow air exchange. Tests were performed in temperature-controlled cameras with a photoperiod of 16 h light: 8 h dark. Three different temperatures (20ºC, 25ºC and 30ºC) and two different soil moistures (20% and 40% of the water hold capacity) were tested. A total of 6 treatments per concentration (3 temperatures; 2 moistures) were setup, in which isopods were subjected to a combination of temperature and moisture. Soil moisture was adjusted using distilled water by weighting the boxes every other day. For each treatment 6 replicates (3 males and 3 females) were setup per population; a total of 288 isopods of each population were used.

For the survival experiment, in each combination of temperature and moisture, 7 metal concentrations were tested (nominal concentration of 400, 600, 700, 800, 900, 1000, 1300 µg Cd/ g soil dry weight) mixed on Organization for Economic Co-operation and Development (OECD) soil according to OECD-226 (2008). Every day each container was checked and mortality was accounted for; the test ended after 28 days.

For the feeding experiment, alder leaf discs (≈ 16 mm) were contaminated with 7 cadmium concentrations (nominal concentration of 100, 200, 250, 300, 350, 400, 800 µg Cd/ g leaf dry weight). Leaf discs' dry weight was measured; only discs with 15 ± 1 mg were used, being placed in capsules, contaminated with a cadmium solution (1ml) and dried at room temperature for two days. After this process leaf discs were re-weighted and rehydrated. In each container 30 g of sand, one alder leaf disc and one isopod were placed. The medium was renewed every week with new sand and fresh food. The leaves removed after each week were dried and weighted; isopods were weighted at the beginning and at the end of the experiment (after 28 days).

III.3.4. Data analysis

The median lethal concentration (LC_{50}) was calculated from the survival experiment, fitting the data to a Gompertz model:

Survival=maximum response x exp((LOG(0.5)) x (time/LC50)^slope) and LC_{50s} were compared using the likelihood ratio test.

From the feeding test, food consumption rate was calculated as:

Consumption = dry leaf consumption / 28 days / isopod initial weight. Median effective concentration (EC_{50}) was calculated for each treatment by fitting the data to a Gompertz model:

Consumption=*maximum x exp((LOG(0.5)) x (metal conc/EC50)^slope*)

and treatments were compared using the likelihood ratio test. Statistical analyses were performed with *Statistica 7 software* (StatSoft, Inc., 2004).

III.4. Results

III.4.1. Survival experiment

During the experiments, in some climate treatments the lower Cd concentration tested led to 100% mortality making it impossible to calculate any LC_x; thus, in those cases, the LC_{50} was considered to be below 400 µg/ g (Table III.2). There were no differences between populations, and temperature was the major factor affecting LC_{50} values, with a tendency to lower LC_{50} as temperature increases. Intra-population analyses showed differences only in SC population between 20°C and 25°C from both moisture treatments. For all treatments, no differences were found between moisture conditions.

Table III.2.: LC_{50} values and confidence intervals (µg Cd/ g soil), calculated by fitting the data into a Gompertz model for *Porcellionides sexfasciatus'* survival test (SC = Santana de Cambas; LG = Lago). Two moisture conditions and three temperatures were tested under a cadmium gradient (0, 400, 500, 600, 700, 800, 900, 1300 µg Cd/g soil). The test last 28 days.

	SC			**LG**		
	20°C	**25°C**	**30°C**	**20°C**	**25°C**	**30°C**
20%	803 (421-1184)[a]	432 (90-774)[b]	< 400	721 (72-1371)	550 (198-902)	< 400
40%	664 (525-803)[1]	403 (307-500)[2]	< 400	801 (290-1312)	< 400	< 400

[a,b] Significant differences in SC population between temperature in the 20% moisture treatments.

[1,2] Significant differences in SC population between temperature in the 40% moisture treatments.

III.4.2. Feeding experiment

No differences between treatments on food consumption EC_{50} values were observed (table III.3); despite the different climate conditions, both populations showed similar EC_{50s}.

Table III.3.: EC_{50} values and confidence intervals (µg Cd/ g dry leaf), calculated by fitting the data into a Gompertz model for *Porcellionides sexfasciatus'* feeding experiment (SC = Santana de Cambas; LG = Lago). Two moisture conditions and three temperatures were tested under a cadmium gradient (0, 100, 200, 250, 300, 350, 400, 800 µg Cd/ g dry leaf). The test last 28 days.

Population	Moisture	20°C	25°C	30°C
SC	20%	N.C.	210 (130-290)	163 (94-232)
	40%	N.C.	168 (98-238)	135 (62-208)
LG	20%	123 (20-227)	252 (64-440)	222 (172-271)
	40%	252 (134-369)	234 (168-300)	191 (125-257)

N.C.: Not possible to calculate

III.5. Discussion

Some of the studies comparing isopods populations with a history of metal exposure (Donker, 1992; Donker *et al*., 1993; Jones & Hopkin, 1998; Knigge & Khöler, 2000; Godet *et al*., 2012) suggest changes on organisms' physiology driven by metal contamination. However, there is only one study testing metal tolerance (with copper) in *Porcellionides sexfasciatus* populations (Costa *et al*., 2013) in which it was not found evidence of increased copper tolerance by the SC population.

In this study, temperature rise lead to a LC_{50} decrease in the SC population. The increased toxicity with temperature has already been described in other studies: Voua Otomo *et al*. (2013), with a 28-day experiment with *Enchytraeus doerjesi* using cadmium, registered a LC_{50} decrease with increasing temperature from 293 µg/ g at 15°C to 231 µg/ g at 25°C; Cedergreen *et al*. (2013), when studying *Enchytraeus crypticus* also found an increase in the uptake of Cd with time exposure and with temperature increase. The increased toxicity with temperature can be associated with two major factors: metal bioavailability and organisms' metabolic rates. The former has been proposed by the alteration of metals kinetics with temperature increase favoring uptake (Spurgeon *et al*., 1997). Changes in organisms' metabolic rates are a widely accepted explanation since they are ectothermic and depend on outer temperatures to regulate their metabolism (Sandifer & Hopkin, 1997; Abdel-Lateif *et al*., 1998). Higher metabolic rates will lead to higher activity, traducing in higher uptake rates. If excretion rates did not follow this increase, metals will accumulate inside isopods and increase metals body burden. Additionally, Cd is a non-essential metal, not used by organisms, accumulating with time exposure (e.g. Vijver *et al*., 2006).

That same significant result was not achieved in LG population mainly due of the huge confidence intervals given by the model and the 100% mortality in the lower concentration tested (400 µg/ g) in three of the six treatments. Nonetheless, at 20% moisture level, from 20°C to 25°C, LG population LC_{50s} were not different, whilst SC LT_{50s} decrease, showing that this population is more affected by an increase in temperature. This confirms our working hypothesis that the history of metal exposure in the SC population compromised their capacity to handle other stressors. These findings are in accordance with Stone *et al*. (2001), in which the carabid beetle *Pterostichus oblongopunctatus* with chronic exposure to metal contamination was more susceptible to additional stressors than reference populations.

In the feeding experiment it was not detected any influence by any of the tested parameters (populations' history, moisture or temperature). Despite the observed tendency of EC_{50} values to decrease with temperature increase, no significant differences were found, mainly because of the huge confidence intervals given by the variability in food consumption rates within each metal concentration.

Also, it was expected that the population from SC had higher metal tolerance than LG due to its history of metal contamination. That expectation would agree with previous studies with populations living under metal contamination: Posthuma (1992), studying *Orchesella cincta* exposed to cadmium, found significantly lower growth reduction in the metal-exposed population than in animals from reference sites; Donker & Bogert (1991) showed decreased consumption and growth in a reference population of *Porcellio scaber* while exposed to cadmium, while isopods from metal contaminated sites were not affected. In both studies the excretion efficiency was pointed has the responsible parameter for increased tolerance. Nonetheless, in this study, both populations had the same tolerance to Cd contamination. Metal-contamination besides species specific is also metal-specific and soil cadmium levels of the contaminated site (3.1 µg/g) were almost the same as in the reference place (considering that those values are near 2.8 µg/g). Since different metals induce different changes in isopods organisms, mainly in hepatopancreas (Mazzei et al., 2014), the pathways to deal with Cd were even for both populations.

Nevertheless, this study provided data on cadmium toxicity of *Porcellionides sexfasciatus* under different environmental conditions. Since the use of isopods has been suggested as bioindicators of environmental hazard of metals (Dallinger *et al*., 1992; Paoletti & Hassal, 1999; Mazzei *et al*., 2014), this study provides important information, since metal contamination is both metal and species-specific, and varies with environmental factors. Therefore, these specie-specific analyses are important before biomonitoring programs (Mazzei *et al*., 2014). Additionally, this multiple-stressor analysis mimics better real conditions, where instead of just one variable affecting a population, multiple variables and its interactions are explored, discovering synergistic or antagonistic effects instead of just considering the sum of individual effects.

From this study, it is concluded that with future climate scenarios, in which a temperature increase is predicted, the toxicity of cadmium is enhanced, being the metal-exposed isopods populations the ones more affected to these climatic changes. As for moisture conditions, a dry environment (20% moisture) does not seem to cause changes in the toxicity of Cd in *Porcellionides sexfasciatus* populations regarding survival or food consumption.

The tests developed in this chapter gave a better understanding on how future changes in climate will influence isopods' tolerance to metals, if that influence is connected with their background and if that background (living in a metal contaminated site) affects their feeding rates, and thus, their role as comminutors affecting litter decomposition and nutrient cycling.

Chapter IV - General discussion

In all climate change scenarios, an increase in temperature is predicted (IPCC, 2013). Consequences of this increase, for ectothermic organisms as isopods, have already been studied, with an increase in metabolic rates with temperature being proposed (Römbke *et al*., 2011). Considering that in the feeding experiment (Cap. II.4.3), the increase in food consumption rates was driven by the increase in metabolic rates, both populations increased food consumption rates with temperature. Furthermore, the raise in temperature was expected to enhance metal toxicity. In the survival test (Cap. III.4.2) that assumption was confirmed with LC_{50} decreasing with temperature (in the SC population, the metal toxicity increased at least 64% [from 403 to 664 µg/ g] from 20°C to 25°C). This same result was not verified in the LG population, showing that a history of metal exposure (SC) increases susceptibility to additional stressors (Stone *et al*., 2001). However, in the feeding test (Cap. III.4.3), despite the slight decrease of EC_{50} with temperature increase, no significant differences were found.

Besides temperature increase, an increase in extreme rainfall events is also predicted (IPCC, 2013). This study showed that high moisture conditions (i.e. 60% of the soil water holding capacity) are extremely prejudicial for isopods' survival and can lead to a large local population reduction (Cap. II.4.2). As for dry environments (i.e. 20% of the soil water holding capacity), they appear to be not harmful for isopods survival or food consumption rates (at least for the test species). These changes in isopods' survival and feeding rates driven by climate variation can compromise (in the case of extreme rainfall) or enhance (with temperature increase) decomposition, leading to changes in isopods' function in ecosystems by modifying carbon and nutrients cycles.

Our experiments go beyond simple species-contaminant analysis, accounting for multiple stressors' interactions. Organisms have different pathways to deal with contaminants, and those contaminants can interact with each other and use the same organisms' uptake routes: leading to additive (Vijver *et al*., 2006; Zidar *et al*., 2009), synergistic (Sørensen & Holsmstrup, 2005) or antagonistic (Odendaal & Reinecke, 2004; Zidar *et al*., 2009) effects and thus, to different tolerance to metals. Therefore, multiple-stressor interactions must be accounted for, in order to a better understanding of the interaction between the organism, abiotic factors and stressors.

Additionally, in order to find more accurate results on metal tolerance (Cap.III.4.3) (decrease EC_{50s} confidence intervals) on the feeding behavior of isopods, a smaller scale on cadmium contamination should be used. Also, the survival and feeding experiments (Cap.III) were developed with artificial soil and food, contrarily to natural conditions, where isopods are exposed to more than one metal which interact with each other. In order to study the real effects of climate change in these populations, field studies would be more suitable than lab ones, to take into account such interactions.

V. References

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

Ardestani, M. M., & Van Gestel, C. M. (2013). Using a toxicokinetics approach to explain the effect of soil pH on cadmium bioavailability to *Folsomia candida*. *Environmental Pollution*, *180*, 122–130.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, 57, 289–300.

Calow, P. (1991). Physiological costs of combating chemical toxicants: ecological implications. *Comparative Biochemistry and Physiology*, *100*, 3–6.

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

Costa, D., Bouchon, D., Van Straalen, N. M., Sousa, J. P., & Ribeiro, R. (2013). Copper tolerance and genetic diversity of *Porcellionides sexfasciatus* (ISOPODA) in a highly contaminated mine habitat. *Environmental Toxicology and Chemistry*, *32*, 884–888.

Dallinger, R., Berger, B., & Birkel, S. (1992). Terrestrial isopods: useful biological indicators of urban metal pollution. *Oecologia*, *89*, 32–41.

Dias, A. T. C., Krab, E. J., Mariën, J., Zimmer, M., Cornelissen, J. H. C., Ellers, J., & Berg, M. P. (2013). Traits underpinning desiccation resistance explain distribution patterns of terrestrial isopods. *Oecologia*, *172*, 667–677.

Dias, N., Hassall, M., & Waite, T. (2012). The influence of microclimate on foraging and sheltering behaviors of terrestrial isopods: Implications for soil carbon dynamics under climate change. *Pedobiologia*, *55*, 137–144.

Donker, M. H. (1992). Energy Reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal-contaminated sites*. Functional Ecology*, 6, 445-454.

Donker, M. H., Abdel-Lateif, H. M., Khalil, M. A., Bayoumi, B. M., & Van Straalen, N. M. (1998). Temperature, physiological time, and zinc toxicity in the isopod *Porcellio scaber*. *Environmental Toxicology and Chemistry*, *17*, 1558–1563.

Donker, M. H., & Bogert, C. G. (1991). Adaptation to cadmium in three populations of the isopod *Porcellio scaber*. *Comparative Biochemistry Physiology C*, 100, 143–146.

Donker, M. H., Zonneveld, C., & Straalen, N. M. (1993). Early reproduction and increased reproductive allocation in metal-adapted populations of the terrestrial isopod *Porcellio scaber. Oecologia*, *96*, 316–323.

Drobne D. (1997). Terrestrial isopods - a good choice for toxicity testing of pollutants in the terrestrial environment. *Environmental Toxicology*, *16*, 1159–1164.

Drobne, D., & Hopkin, S. P. (1994). Ecotoxicological laboratory test for assessing the effects of chemicals on terrestrial isopods. *Bulletin of Environmental Contamination and Toxicology*, *53*, 390–397.

Farkas, S., Hornung, E., & Fischer, E. (1996). Toxicity of copper to *Porcellio scaber Latr*. (Isopoda) under different nutritional status. *Bulletin of Environmental Contamination and Toxicology*, *57*, 582–588.

Fox, G. A. (1995). Tinkering with the tinkerer: Pollution versus evolution. *Environmental Health Perspectives*, *103*, 93–100.

Gardner, J. L., Peters, A., Kearney, M. R., Joseph, L., & Heinsohn, R. (2011). Declining body size: A third universal response to warming? *Trends in Ecology and Evolution*, *26*, 285–291.

Gee, G. W., & Bauder, J. W. (1986). Particle-size analysis. In: KLUTE, A., ed. Methods of soil analysis. Part 1. Physical and mineralogical methods. 2.ed. Madison, American Society of Agronomy, *Soil Science Society of America*, 383-411.

Gienapp, P., Teplitsky, C., Alho, J. S., Mills, J. A., & Merila, J. (2008). Climate change and evolution: disentangling environmental and genetic responses. *Molecular Ecology*, *17*, 167–178.

Godet, J. P., Demuynck, S., Waterlot, C., Lemière, S., Souty-Grosset, C., Scheifler, R., & Pruvot, C. (2011). Growth and metal accumulation in *Porcellio scaber* exposed to poplar litter from Cd-, Pb-, and Zn-contaminated sites. *Ecotoxicology and Environmental Safety*, *74*, 451–458.

Godet, J. P., Demuynck, S., Waterlot, C., Lemière, S., Souty-Grosset, C., Douay, F., & Pruvot, C. (2012). Fluctuating asymmetry analysis on *Porcellio scaber* (Crustacea, Isopoda) populations living under metals-contaminated woody habitats. *Ecological Indicators*, *23*, 130–139.

Hassall, M., Edwards, D., Moss, A., Derhé, M., & Carmenta, R. (2010). Predicting the effect of climate change on aggregation behaviour in four species of terrestrial isopods. *Behaviour*, *147*, 151–164.

Hernandez-Soriano, M. C., & Jimenez-Lopez, J. C. (2012). Effects of soil water content and organic matter addition on the speciation and bioavailability of heavy metals. *Science of the Total Environment*, *423*, 55–61.

Heugens, E. H., Hendriks, A. J., Dekker, T., van Straalen, N. M., & Admiraal, W. (2001). *A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Critical reviews in toxicology* (Vol. 31).

Hopkin, S. P., & Martin, M. H. (1982). The distribution of zinc, cadmium, lead and copper within the woodlouse *Oniscus asellus* (Crustacea, Isopoda). *Oecologia*, *54*, 227–232.

IPCC (2007). Climate Change: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, [M.L. Parry, O.F. Canziani, J.P. Palutikof, P.J. van der Linden and C.E. Hanson, (Eds.)], *Cambridge University Press*, Cambridge, UK.

IPCC (2013). Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. *Cambridge University Press*, Cambridge, United Kingdom and New York, NY, USA.

Jager, T., Fleuren, R. H. L. J., Roelofs, W., & De Groot, A. C. (2003). Feeding activity of the earthworm *Eisenia andrei* in artificial soil. *Soil Biology and Biochemistry*, *35*, 313–322.

Janssen, M. P. M., & Bergema, W. F. (1991). The effect of temperature on cadmium kinetics and oxygen consumption in soil arthropods. *Environmental Toxicology and Chemistry*, *10*, 1493–1501.

Jones, D. T., & Hopkin, S. P. (1998). Reduced survival and body size in the terrestrial isopod *Porcellio scaber* from a metal polluted environment. *Environmental Pollution*, *99*, 215–223.

Khan, M. A. Q., Ahmed, S. A., Salazar, A., Gurumendi, J., Khan, A., & Vargas, M. (2007). Effect of Temperature on Heavy Metal Toxicity to Earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). *Environmental Toxicology*, *22*, 487–494.

Knigge, T., & Köhler, H. R. (2000). Lead impact on nutrition, energy reserves, respiration and stress protein (hsp 70) level in *Porcellio scaber* (Isopoda) populations differently preconditioned in their habitats. *Environmental Pollution*, *108*, 209–217.

Lukkari, T., Teno, S., Väisänen, A., & Haimi, J. (2006). Effects of earthworms on decomposition and metal availability in contaminated soil: Microcosm studies of populations with different exposure histories. *Soil Biology and Biochemistry*, *38*, 359–370.

Luoma, S. N. (1983). Bioavailability of Trace Metals to Aquatic Organisms- A Review. *The Science of the Total Environment*, *28*, 1–22.

Mazzei, V., Longo, G., Brundo, M. V, Sinatra, F., Copat, C., Conti, G. O., & Ferrante, M. (2014). Ecotoxicology and Environmental Safety Bioaccumulation of cadmium and lead and its effects on hepatopancreas morphology in three terrestrial isopod crustacean species. *Ecotoxicology and Environmental Safety*, *110*, 269–279.

Moe, S. J., De Schamphelaere, K., Clements, W. H., Sorensen, M. T., Van den Brink, P. J., & Liess, M. (2013). Combined and interactive effects of global climate change and toxicants on populations and communities. *Environmental Toxicology and Chemistry*, *32*, 49–61.

Odendaal, J. P., & Reinecke, A. J. (2004). Effect of metal mixtures (Cd and Zn) on body weight in terrestrial isopods. *Archives of Environmental Contamination and Toxicology*, *46*, 377–384.

Odendaal, J. P., & Reinecke, A. J. (1999). Short-Term Toxicological Effects of Cadmium on the Woodlouse, *Porcellio laevis* (Crustacea, Isopoda). *Ecotoxicology and Environmental Safety*, *43*, 30–34.

OECD-226 (2008). OECD Guidelines for the testing of chemicals: Predatory mites (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil.

Paoletti, M. G., & Hassall, M. (1999). Woodlice (Isopoda: Oniscidea): Their potential for assessing sustainability and use as bioindicators. *Agriculture, Ecosystems and Environment*, *74*, 157–165.

Peijnenburg, W.; Capri, E.; Kula, C.; Liess, M.; Luttik, R.; Montforts, M.; Nienstedt, K.; Römbke, R.; Sousa, J.P. & Jensen, J. (2012) Evaluation of exposure metrics for effect assessment of soil invertebrates. *Critical Reviews on Environmental Science and Technology*. 42: 1862-1893.

Pflug, A., & Wolters, V. (2001). Influence of drought and litter age on Collembola communities. *European Journal of Soil Biology*, *37*, 305–308.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*, *88*, 137–146.

Portuguese Environmental Agency (2010). Fifth National Communication to the United Nations Framework Convention on Climate Change Second National Communication in the context of the Kyoto Protocol.

Posthuma, L., Hogervorst, R. F., & Van Straalen, N. M. (1992). Adaptation to soil pollution by cadmium excretion in natural populations of *Orchesella cincta* (L.) (Collembola). *Archives of Environmental Contamination and Toxicology*, *22*, 146–156.

Ribeiro, R., & Lopes, I. (2013). Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. *Ecotoxicology*, *22*, 889–99.

Rieuwerts, J. S., Thornton, I., Farago, M. E., & Ashmore, M. R. (1998). Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. *Chemical Speciation and Bioavailability*, *10*, 61–75.

Rombke, T., Rombke, J., & Russell, D. (2011). Effects of temperature increases on the feeding activity of two species of isopods (*Porcellio scaber, Porcellionides pruinosus*) in laboratory tests. *Soil Organisms*, *83*, 211–220.

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

Schmidt, C., & Wägele, J. W. (2001). Morphology and evolution of respiratory structures in the pleopod exopodites of terrestrial isopoda (Crustacea, Isopoda, Oniscidea). *Acta Zoologica*, *82*, 315–330.

Schmitz, A., & Harrison, J. F. (2004). Hypoxic tolerance in air-breathing invertebrates. *Respiratory Physiology and Neurobiology*, *141*, 229–242.

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

Spurgeon, D. J., Tomlin, M. a, & Hopkin, S. P. (1997). Influence of temperature on the toxicity of zinc to the earthworm *Eisenia fetida*. *Bulletin of Environmental Contamination and Toxicology*, *58*, 283–290.

StatSoft, Inc. (2004). STATISTICA (data analysis software system), version 7. www.statsoft.com.

Stone, D., Jepson, P., Kramarz, P., & Laskowski, R. (2001). Time to death response in carabid beetles exposed to multiple stressors along a gradient of heavy metal pollution. *Environmental Pollution*, *113*, 239–244.

Tedesco, M.J.; Gianello, C.; Bissani, C.A. & Volkweiss, S.J. (1995). Análises de Solo, Plantas e Outros Materiais. Porto Alegre: Universidade Federal do Rio Grande do Sul. 174. (Boletim Técnico 5).

Vijver, M. G., Vink, J. P. M., Jager, T., Van Straalen, N. M., Wolterbeek, H. T., & Van Gestel, C. a M. (2006). Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food. *Soil Biology and Biochemistry*, *38*, 1554–1563.

Violante, A, Cozzolino, V., Perelomov, L., Caporale, A. ., & Pigna, M. (2010). Mobility and Bioavailability of Heavy Metals and Metalloids in Soil Environments. *Journal of Soil Science and Plant Nutrition*, *10*, 268–292.

Voua Otomo, P., Reinecke, S. A, & Reinecke, A. J. (2013). Combined effects of metal contamination and temperature on the potworm *Enchytraeus doerjesi* (Oligochaeta). *Journal of Applied Toxicology*, *33*, 1520–1524.

Warburg, M. R. (1965). The Evaporative Water Loss of Three Isopods from Semi-Arid Habitats in South Australia. *Crustaceana*, *9*, 302–308.

Zidar, P., Božič, J., & Štrus, J. (2005). Behavioral response in the terrestrial isopod *Porcellio scaber* (Crustacea) offered a choice of uncontaminated and cadmium-contaminated food. *Ecotoxicology*, 493–502.

Zidar, P., Van Gestel, C. A. M., & Štrus, J. (2009). Single and joint effects of Zn and Cd on *Porcellio scaber* (Crustacea, Isopoda) exposed to artificially contaminated food. *Ecotoxicology and Environmental Safety*, *72*, 2075–2082.