Universidade de Aveiro Departamento de Biologia
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Avaliação dos efeitos combinados de stressores químicos e naturais no isópode terrestre *Porcellionides pruinosus*

Single and joint effects of natural and chemical stressors to the terrestrial isopod *Porcellionides pruinosus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar e co-orientação do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

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Ecotoxicologia, alterações climáticas, pesticidas, combinação de stressores, modelo de ação independente, ecossistemas do solo, isópodes terrestres

resumo

A contaminação ambiental e as alterações climáticas são duas das mais sérias ameaças aos ecossistemas edáficos. A agricultura é atualmente uma prática altamente otimizada da qual a aplicação de vários pesticidas é uma componente essencial. Apesar da sua importância, o uso de pesticidas pode implicar elevados custos ambientais, particularmente quando em misturas. Não obstante, no contexto de condições ambientais desfavoráveis estas misturas podem ainda revestir-se de uma relevância acrescida. Estudos recentes têm demonstrado que a ocorrência de stressores naturais, como a temperatura, humidade do solo ou radiação ultravioleta, pode influenciar a toxicidade de pesticidas, o que tem suscitado uma crescente apreensão relativamente à eficácias das avaliações de risco ambiental. Esta preocupação prende-se principalmente pelo facto de estes estudos não terem em conta a complexidade das interações entre múltiplos stressores. De forma a contribuir para este debate, com a presente tese pretendeu-se avaliar os efeitos individuais e combinados de stressores naturais e pesticidas no isópode terrestre *Porcellionides pruinosus*. A primeira fase do estudo consistiu na avaliação dos efeitos individuais de vários factores abióticos (temperatura, humidade do solo e radiação ultravioleta) na performance de *P. pruinosus* usando vários parâmetros como a sobrevivência, parâmetros alimentares, atividade locomotora e evitamento de condições adversas. Os resultados demonstram claramente a importância que estes stressores podem assumir na performance dos organismos e enfatizam a urgência de serem tidos em conta em avaliações de risco ambiental. De seguida foram estudados os efeitos da radiação ultravioleta recorrendo a um conjunto de biomarcadores relacionados com diferentes processos, no qual ficou patente a influência do meio de exposição e da idade dos organismos na vulnerabilidade a este stressor. Uma abordagem semelhante foi desenvolvida para avaliar os efeitos individuais e combinados dos pesticidas clorpirifos e mancozebe em *P. pruinosus*. Os adultos e juvenis expostos pareceram exibir diferentes padrões de resposta aos pesticidas relativamente ao balanço energético e custos metabólicos. Finalmente foi avaliada a influência da temperatura e da humidade do solo na toxicidade desta mistura binária. No que diz respeito à sobrevivência dos isópodes, os pesticidas mostraram ser influenciados de maneira oposta pela temperatura. Enquanto a toxicidade de clorpirifos pareceu aumentar com o aumento da temperatura, os efeitos do mancozebe foram mais proeminentes a temperaturas baixas. Pelo contrário, a humidade do solo não mostrou influenciar significativamente a mortalidade causada pelos pesticidas. No que diz respeito aos parâmetros alimentares foram detectadas várias interações entre ambos os stressores naturais e os pesticidas, apesar de a aditividade de efeitos ter sido o resultado mais frequente.

Em conclusão, os resultados reportados ao longo desta tese reforçam o perigo associado à negligência de stressores naturais, ou mesmo da conjugação de múltiplos stressores em geral, para as avaliações de risco ambiental.

keywords

Ecotoxicology; climate changes; pesticides; multiple stressors; independent action model; soil ecosystems; terrestrial isopods.

abstract

Environmental contamination and climate changes constitute two of the most serious problems affecting soil ecosystems in agricultural fields. Agriculture is nowadays a highly optimized process that strongly relies on the application of multiple pesticides to reduce losses and increase yield production. Although constituting, *per se*, a serious problem to soil biota, pesticide mixtures can assume an even higher relevance in a context of unfavourable environmental conditions. Surprisingly, frameworks currently established for environmental risk assessments keep not considering environmental stressors, such as temperature, soil moisture or UV radiation, as factors liable to influence the susceptibility of organisms to pesticides, or pesticide mixtures, which is raising increasing apprehension regarding their adequacy to actually estimate the risks posed by these compounds to the environment. Albeit the higher attention received on the last few years, the influence of environmental stressors on the behaviour and toxicity of chemical mixtures remains still poorly understood. Aiming to contribute for this discussion, the main goal of the present thesis was to evaluate the single and joint effects of natural stressors and pesticides to the terrestrial isopod *Porcellionides pruinosus*. The first approach consisted on evaluating the effects of several abiotic factors (temperature, soil moisture and UV radiation) on the performance of *P. pruinosus* using several endpoints: survival, feeding parameters, locomotor activity and avoidance behaviour. Results showed that these stressors might indeed affect *P. pruinosus* at relevant environmental conditions, thus suggesting the relevance of their consideration in ecotoxicological assays. At next, a multiple biomarker approach was used to have a closer insight into the pathways of damage of UV radiation and a broad spectrum of processes showed to be involved (i.e. oxidative stress, neurotoxicity, energy). Furthermore, UV effects showed to vary with the environment medium and growth-stage. A similar biomarker approach was employed to assess the single and joint effects of the pesticides chlorpyrifos and mancozeb to *P. pruinosus*. Energy-related biomarkers showed to be the most differentiating parameters since age-classes seemed to respond differently to contamination stress and to have different metabolic costs associated. Finally, the influence of temperature and soil moisture on the toxicity of pesticide mixtures was evaluated using survival and feeding parameters as endpoints. Pesticide-induced mortality was found to be oppositely affected by temperature, either in single or mixture treatments. Whereas chlorpyrifos acute toxicity was raised under higher temperatures the toxicity of mancozeb was more prominent at lower temperatures. By the opposite, soil moisture showed no effects on the pesticide-induced mortality of isopods. Contrary to survival, both temperature and soil moisture showed to

interact with pesticides to influence isopods' feeding parameters. Nonetheless, was however the most common pattern.

In brief, findings reported on this thesis demonstrated why the negligence of natural stressors, or multiple stressors in general, is not a good solution for risk assessment frameworks.

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CHAPTER 1: General Introduction

1.1. Soil ecosystems in agricultural fields

1.1.1. Soil as a resource to humankind: from gathering to farming

Although being fairly recent from a humankind evolution point of view (only about 10,000 years ago) (Feynman & Ruzmaikin 2007), the human transition from hunting and gathering to an agricultural way of subsistence constituted, perhaps, the most important landmark that would determine its successful establishment worldwide (Gepts 2001; Dow et al. 2005). By providing surplus food supplies, agriculture was largely responsible for the sudden civilizational development verified in the subsequent ages, featured by unprecedented population growths and remarkable technological advances (Gepts 2001; Nortcliff 2002; Haber 2007; Winterhalder & Kennett 2014). In other words, agriculture opened the way to the emergence of modern societies, whose development was grounded in and leveraged by this activity. Alongside, this transition also became of pivotal importance on the following history of earth, since it constituted one of the first significant episodes of serious human dominance over natural terrestrial ecosystems, that would lead to drastic transformations on their structure and functioning, particularly in soil (McNeill 2004; Haber 2007; Vitousek et al. 2008).

If prior to agriculture the human dependence on soil was already absolute, although indirect, it became even stronger and rather more obvious with the further advent of this activity (McNeill 2004). In fact, the close relationship historically established between humans and soil cannot be exclusively restricted to the agriculture-related services, since several other ecological and non-ecological assets have also long been of great socio-economic value (i.e. source of raw materials, human protection, gene reservoir, physical basis to human activities, cultural heritage) (Van Straalen 2002; Blum 2005; Kefeli & Blum 2011). Services related with biomass production have, nonetheless, been central since agriculture quickly became the main source of food and fiber for almost all the human societies worldwide. The pressure imposed by a growing population aiming for higher life quality standards led, in first place, to massive conversions of natural ecosystems to agricultural fields and, more recently, to the intensification of agriculture regimes (Matson 1998; Tilman 2001; Tilman et al. 2002; Trewavas & Trewavas 2002; Haber 2007). The land area currently used in agriculture is estimated in 24% to 38% of all earth's ecosystems, or about 50% if excluding the outrightly non-arable areas (Swinton et al. 2007). Therefore, a considerable part of some of the most productive terrestrial

ecosystems worldwide was already modified with this purpose, what is, *per se*, clearly indicative of the prominence achieved by this activity in modern societies and emphasizes the widespread nature of this issue. With such a higher amount of land area occupied by agroecosystems, it is not surprising that a considerable number of species has become strongly dependent on these habitats (Tscharntke et al. 2005). For instance, according to the European Environment Agency, about 50% of all European species are known to, at least partly, inhabit agricultural areas (Stoate et al. 2009). Considering the central role of edaphic ecosystems on the ecological processes working at a landscape level (Coleman et al. 1992), it seems critical to analyse the habitat quality of these soils in agroecosystems and evaluate whether these are properly functioning so they can deliver the environmental services required to support the overall communities. Moreover, since the role of soil communities on the services provided by this compartment is still not completely clear (Brussaard 1997; Fitter et al. 2005), a considerable effort should be devoted to understand the susceptibility of soil biota to the conditions found in these amended ecosystems, as well as the causal relationships in soil productivity stemming from these potential imbalances.

Soil is nowadays starting to be regarded as a non-renewable resource, whose quality can be quickly impoverished by overexploitation and mismanagement, leading to degradation levels virtually impossible to revert (Nortcliff 2002; Haber 2007; Kefeli & Blum 2011). Nevertheless, extensive gaps of knowledge must still be fulfilled in almost every fields of soil science, from structure to functioning.

1.1.2. Soil health and functioning in agroecosystems

Soil is the central element of terrestrial ecosystems. Besides providing the mechanical support, it is still involved on the regulation and partition of water resources, and storage and recycle of nutrients and trace elements (Brussaard 1997; Lal 1997; Burger & Kelting 1999; Seybold et al. 1999; Nortcliff 2002; Blum 2005). Furthermore, it also confers protection to soil biota by acting as a buffer for adverse environmental conditions or filtering, immobilizing, degrading and detoxifying chemical contaminants (Lal 1997; Seybold et al. 1999; Nortcliff 2002; Burauel & Baßmann 2005). The main factor contributing for the poor understanding of soil ecosystems' functioning is probably the intrinsic complexity that features this compartment (Coleman et al. 1992; Ettema & Wardle 2002; Kibblewhite et al. 2008). By being positioned on the interface between the lithosphere, atmosphere, hydrosphere, and biosphere, soils are normally subject to a

group of forces operating on each of these physical systems, conferring them a dynamic and multiphase character, with an exceedingly high spatial and temporal heterogeneity (Ettema & Wardle 2002; Lavelle & Spain 2003; Bardgett 2005). In fact, soil results from the interaction of multiple factors such as climate, organisms, parent material, topography and also time (Coleman et al. 1992; Kibblewhite et al. 2008). All of these can partly contribute for the major ecosystem processes, from primary production to decomposition and nutrient cycling (Coleman et al. 2004). A hierarchical model was, nevertheless, proposed to explain the weight of such elements in the processes of soil, where broadly operating factors tend to constrain those acting more restrictedly (Lavelle 1996). Climate is thought to be the most prominent factor operating at larger spatial and temporal scales, followed by the edaphic factors and finally by biological sources, predominantly the primary producers and then by the remaining organisms (Lavelle 1996). However, this hierarchy is not strict since the factors working at lower scales can always exert direct or indirect influence in some of those operating broadly (Lavelle 1996). Moreover, the human impacts must also be included since they can easily become the chief factor above all others, inclusively, by affecting all the remaining factors (Lavelle 1996). This is particularly true for agricultural soils since these constitute amended ecosystems whose natural processes have been more or less altered (Seybold et al. 1999). In a landscape perspective, agricultural cropping systems can still be considered as an ecological unit that is subject to the typical forces that shape natural ecosystems (Conway 1987; Ferro et al. 1987; Swinton et al. 2007). The main difference to natural ecosystems is that the basic processes in agroecosystems are often overlaid by agricultural ones (Conway 1987). Unlike natural disturbances, however, that mostly consist on temporary transformations followed by more or less lasting recovery periods, human-induced alterations can hardly become a revitalizing agent since they generally lead to further degradation (Rapport & Whitford 1999). Healthy ecosystems are able to accommodate periodic disturbances without serious damages, but once a critical limit is reached, they lose their bearing capacity, the degradation evolves quickly, and the effects can be long-lasting (Lal 1997; Costanza & Mageau 1999; Seybold et al. 1999). Consisting mostly in monocultures and seldom reaching a “natural” level of ecological stability (Ferro et al. 1987), agroecosystems are generally featured by low rates of energy and nutrient cycling and are often not persistent enough to encompass solid biotic relationships, above- and below-ground, that would provide them a higher resistance and resilience to external perturbations (Pimentel & Edwards 1982). It is thus unlikely that under such circumstances these systems manage to keep their functional and structural integrity,

necessary to ensure a sustainable soil quality (Lal 1997), i.e. “the capacity (…) to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health” (Doran 2002).

1.2. Pesticides in agriculture: pros, cons and future trends

Modern agriculture regimes are strongly featured by an intensive use of plant protection products. Along with the use of better cultivars and new human-made fertilizers, these compounds constitute a key component of the intensive agriculture and are largely responsible for the increasing productivity and cost-effectiveness achieved by the agricultural industry (Matson 1998; Aktar et al. 2009). In fact, pesticides constitute a rapid, effective and economical mean of controlling crop pests and diseases in the short-term (Peshin & Dhawan 2009). In addition to increase the crop yields and aiming at reducing the pest-related losses, pesticides also contributed for decreasing the man- and mechanical-power required, thus allowing to cut costs and partly sparing soils to erosive practices (Ridgway et al. 1978). Furthermore, their increasing application also results from the elevated cosmetic standards, particularly for the higher value crops like fruits and vegetables that are priced according to their external looking (Pimentel et al. 1993; Conway & Pretty 2013).

In spite of these benefits, an increasing controversy has been associated to the application of pesticides in agricultural fields. This disagreement stems from the increasing public perception that some of these compounds can constitute a serious environmental problem. Indeed, overwhelming evidences exist nowadays, indicating the risks posed by pesticides to non-target organisms, including humans, and even their real advantages have been questioned since the extensive increase in pesticide usage was not necessarily followed by significant reductions in crop losses (Pimentel et al. 1993; Yudelman et al. 1998; Carvalho 2006). Although initially very effective in reducing pest infestations, soon became clear that the use of pesticides as a first-line defense against these organisms would later have its efficiency decreased by a set of feedback mechanisms expected to neutralize them or even lead to more virulent outbreaks (Lewis et al. 1997; Wilson 2001). These feedback mechanisms are mostly related with the disregard of forces and interactions tying each component of the ecosystem and how the

action on a single link can entail profound effects on the communities (Lewis et al. 1997). Pesticides can, indeed, constitute an important driver of imbalances since they potentially disrupt the biological systems of the agroecosystems, leading to changes in their communities' stable state (Pimentel & Edwards 1982; Giller et al. 1997). Contrary to what is established as a good practice, few pesticides have shown to be truly selective for the target group since they generally act by interfering with basic biological processes, affecting a wide range of organisms (Conway & Pretty 2013). In this way, by also affecting pests' natural enemies, pesticides can lead to a vicious situation where further outbreaks are expected and more applications are required (Pimentel & Edwards 1982). The potential removal of competitors is also expected to favor the resurgence of outbreaks as well as to create new issues with secondary pests (Risch 2012). Furthermore, the overuse of these compounds and other mismanagement practices may also have stimulated the target organisms to develop resistance mechanisms making their outbreaks successively harder to control (Dehne & Schönbeck 2012). In overall, this highlighted the shortcomings of pesticide application as the single crop, or even the principal, protection approach and has been triggering the development of new methodologies (Cowan & Gunby 1996; Lewis et al. 1997; Kogan 1998).

The prevention of pesticides' overuse is one of the most important components of the Integrated Pest Management (IPM), an agricultural management concept increasingly followed in developed countries that seems to be, at least partly, responsible for the reductions verified on pesticide application (Zilberman et al. 1991). Instead of using rigid scheduled applications in anticipation of outbreaks, it defends the deferring of spraying events until pest damages reach an economic threshold level of attack (Matthews et al. 2014). If used responsibly, pesticides are likely to bring considerable net benefits, with minimum environmental costs (Tilman 2001; Cooper & Dobson 2007). However, this can only be achieved with highly trained farmworkers since it requires more skills than conventional farming and is thus still far of being widespread, particularly in developing countries where the amount of pesticides used is expected to suffer the biggest rises (Matson 1998). Even if pesticide application trends in developed countries continue showing a solid negative trajectory, there will hardly be room for an overall decrease in the next years/decades given the accelerated development in some of the most populated regions in the world (Yudelman et al. 1998). Moreover, if one consider the interactive effects between the increasing global food demands projected for the near future and the ongoing climatic changes, this assumption seems to be even reinforced (Tilman 2001). The spatial and temporal distribution of crop pests and diseases is largely determined by

climate (Rosenzweig et al. 2001; Anderson et al. 2004). For instance, it can act directly on insect populations, restricting or promoting their growth and movement, or indirectly on the host plants, enhancing or impairing their quality as food item for herbivores (Risch 2012). In both situations, temperature, moisture conditions or wind can become the main trigger for herbivore insect outbreaks (Risch 2012). Likewise, the incidence of pathogens is also known to be boosted by the weather, particularly by temperature and moisture conditions (Gregory et al. 2009). Considering this close connection between these and climate, significant challenges can be foreseen in the future (Rosenzweig 2007; Gregory et al. 2009). It is still not clear how can these changes in climate affect the resilience of agroecosystems to pests and diseases. First of all, climate changes include a rather complex set of events with multiple implications at several levels, still shrouded in a considerable uncertainty. Moreover, trying to predict the effects of climate changes in the multiple biological processes encompassed within the several hierarchical levels of an ecosystem is also a herculean task. However, some evidences exist already that the imbalances caused by climate changes can, in some situations, increment the occurrence of serious pest/pathogen outbreaks (Rosenzweig et al. 2001; Gregory et al. 2009). It is reasonable to expect, given their opportunistic nature, that such disturbances in biological systems might end up favoring these groups, for instance allowing them to increase their vital range and spreading to regions previously safe of crop pests and pathogens (Anderson et al. 2004; Gregory et al. 2009). Again, this suggests that, notwithstanding all the concern regarding the widespread use of pesticides, these compounds will certainly hold a major importance in agriculture in the future, and emphasize the significance of thoroughly evaluating their effects.

1.3. Environmental risk assessment of pesticides

Although constituting a secular practice, it was only after the 1960s that the recognition of environmental risks was associated with the application of chemicals with agricultural purposes. The steady and widespread increase in pesticide usage, in progress since the 1940s, led to the arising of environmental movements that culminate in stark statements, such as Rachel Carson's "Silent Spring" (1962), hence reaching the general public and highlighting the urgency of regulating these practices. In fact, pesticides constitute a particularly unique class of soil contaminants in the sense that they

are actually designed to exert toxic effects but are still intentionally released in the environment (Deneer 2000; Lydy et al. 2004). In this way, in order to provide decision-makers with a sound scientific knowledge that could help them analyzing the costs and benefits deriving from the use of pesticides, the environmental risk assessment (ERA) framework was later introduced as a predictive tool for the environmental risks posed by these agrochemicals. These frameworks constitute nowadays a mandatory step to complete the registration process that any new pesticide has to accomplish before reaching the market in industrialized countries. Grounded on strict toxicological and ecotoxicological requirements, these procedures have elevated the standards for agrochemicals, forcing the chemical industry to conceive new innovative active ingredients that are coincidently more efficient but less hazardous (Dehne & Schönbeck 2012).

Environmental risk assessment (ERA) is the "process of collecting, organizing and analyzing environmental data to estimate the risk of contamination for ecosystems" (Jensen & Mesman 2006). These frameworks basically consist of comparing the predicted concentration of a chemical in the environment (PEC) with the concentration that causes no adverse effects on the majority of organisms (PNEC) (Van Leeuwen et al. 1996). In this way, ERA aims to provide a sound scientific ground for the subsequent risk management process (Van Leeuwen et al. 1996). Three main components are included in ERA: i) hazard identification; ii) analysis of the risk; and iii) risk characterization (Norton et al. 1992). Hazard identification, or problem formulation, includes the planning and scoping processes that will allow the identification of environmental assets to be protected (Norton et al. 1992; Finizio & Villa 2002). The analysis of risk consists on the technical evaluation of data gathered during the previous phase and includes two components: the characterization of the exposure that allows the derivation of PEC and the characterization of the effects by which PNEC is calculated (Norton et al. 1992; Van Leeuwen & Vermeire 2007). A tiered approach is generally adopted for both these components since simple and inexpensive tests are first performed and further refined, if necessary, with more comprehensive approaches (Bradbury 1995; Van Leeuwen & Vermeire 2007; Suter 2008). This last phase must comprise a set of considerations regarding the uncertainties detected during the course of the assessment since they must be accommodated by using safety (uncertainty) factors (Norton et al. 1992; Chapman et al. 1998). The quality of the data used in these ERA procedures is undoubtedly a fundamental element influencing the accuracy of the resultant assessments, and ultimately affecting the sustainability of the management practices.

1.4. Ecotoxicological assessment of pesticides in soil

1.4.1. Role of toxicity testing in environmental risk assessments of soil ecosystems

Laboratory toxicity testing is, therefore, a fundamental constituent of the ERA procedures, either for prognostic or diagnostic approaches (van Gestel 2012). Although being highly relevant from an ecological perspective, the cause-effect relationships from field inventories are always hard to identify since the ascription of any observed community changes to a certain factor is often complicated by the huge amount of other variables that usually co-vary with each other (Van Straalen & Verhoef 1997). Moreover, they are costly and time-consuming so it is often a better solution to replace a chemical for which the first tier was inconclusive, for another that showed to be harmless in this stage, than undertaking the whole tiered process (Van Leeuwen & Vermeire 2007). Toxicity assays can provide an insight into the bioavailability of contaminants and this is the primary factor determining the biological effects, rather than total concentration (Harmsen 2007). This is, indeed, particularly relevant for soil since in this compartment chemicals are specially prone to processes such as adsorption, desorption, dissolution, diffusion, dispersion and convection that might substantially affect the amount that is able to interact with the organisms (Katayama et al. 2010). Total concentrations of pesticides in soil can be divided in three fractions: i) the readily available fraction that included pesticides dissolved in soil solution; ii) a potentially available fraction which includes the amount of pesticide that is not readily available but might become through biochemical reactions such as decomposition of organic matter or other dissolutive processes; an unavailable fraction that is very tightly bound to soil matrix and may be considered inert (Harmsen & Rulkens 2005; Wightwick et al. 2010) Finally, toxicity tests can also offer the possibility of investigating the consequences of the exposure to contaminants whose biological effects are poorly understood, making them a good starting point to characterize the nature of a xenobiotic (Environment Agency 2003).

Of utmost importance for the enhancement of ecotoxicological assessments has been the development of standard guidelines. By harmonizing protocols, they constituted a leap towards the comparability, reproducibility and validity of different experiments performed in different laboratories around the world, thus helping on the setting of quality criteria for toxicity testing.

Despite the youth of soil ecotoxicology as a research field, it has been since its inception, featured by a quick developmental pace. This situation has been simultaneously promoted and complicated by the complex nature of this heterogeneous compartment, since it was soon realized that an insight into the effects of xenobiotics in soil, could hardly be achieved without using a battery of tests with different surrogate organisms. Several toxicity tests have, hence, been developed, with a multiplicity of procedures and endpoints and using multiple species. Van Gestel (2012) suggested that in order to get a comprehensive understanding, three criteria must be fulfilled regarding the pool of species included: i) they must include several species belonging to different functional and taxonomic groups, different life-histories, and also differing on their exposure routes; ii) the responses measured in such surrogate species must be relevant for the protection of soil populations and communities; iii) all surrogate species must offer the possibility of being performed in the same test medium.

1.4.2. Use of soil invertebrates in ecotoxicological studies

As regards to soil fauna, particular attention has been devoted lately to the use of soil invertebrates for toxicity testing. Soil invertebrates comprise a taxonomically and functionally diverse group of organisms that "live during an essential part of their life cycle in soil" and embodies varied sizes, morphologies, physiologies, life histories, feeding strategies and more (Donker et al. 1994). Along with this diversity, they also play several critical roles on soil functioning, either being fundamental links in the edaphic trophic chains, promoting processes like nutrient cycling or primary production, or in some situations, as ecosystem engineers (Lavelle 1996; Brussaard 1997). Furthermore, they are known to be severely affected by human activities, having long been suggested as a good bioindicator of soil ecosystems' health (Lavelle 1996; Paoletti et al. 1996).

Within the context of the project SECOFASE (**S**ublethal **E**ffects of **C**hemicals **o**n **Fa**una in the **S**oil **E**cosystem), Løkke and van Gestel (1998) compiled and organized the state-of-the-art information regarding the available procedures for toxicity testing with soil invertebrates. These included enchytraeids, earthworms, mites, collembolans, staphylinids, millipedes, isopods, and nematodes. Even today, most of these groups lack standard guidelines for toxicity testing but their use increased considerably. More recently, van Gestel (2012) reviewed once again the standard guidelines for toxicity testing using soil invertebrates and suggested that, although more complete, they continue not being

balanced since they keep showing a clear under-representation of arthropods when compared to the real importance and diversity of this group in the field.

A considerable effort has been invested over the last years by soil ecotoxicologists in order to provide a sounder basis for the risk assessment of xenobiotics in soil. These included the use of a diverse assemblage of model organisms, the incorporation of new protocols focusing on multiple organization levels, and the optimization of the existing so they can become more comprehensive.

1.4.3. Endpoints at the individual/population level

Following the principles long established for aquatic ecotoxicology, survival was the first endpoint used to evaluate the effects of xenobiotics on soil invertebrates. Indeed, the OECD guideline describing the acute toxicity test with earthworms (OECD 1984) was the first standardized approach conceived to take part of ERA frameworks. Nevertheless, despite constituting a valuable tool for screening chemical toxicity, soon became clear that neither the survival endpoint alone nor this classical acute exposure were the most sensitive or relevant approaches, respectively, for being included in risk assessments (van Gestel et al. 1992).

The use of chronic tests has, hence, been suggested as an alternative to acute toxicity assays. By extending the exposure, it is possible to have a more accurate insight into the time-dependent effects of the xenobiotic on the population dynamics and provides the opportunity of including the assessment of sublethal parameters (Loureiro et al. 2005). In addition to survival, sublethal parameters such as reproduction or growth, have been suggested to be a relevant measurement of the individual performance and are expected to indicate earlier effects at the population level (van Gestel et al. 1992; Römbke & Moser 2002; Fountain & Hopkin 2005; Loureiro et al. 2006).

Behavior has also been increasingly proposed as a suitable endpoint for evaluating the effects of chemicals on soil invertebrates. Although the almost unlimited possibilities arising from the fact that toxicants are liable to affect virtually all behavioral responses, a thorough knowledge is required so they can be properly used in toxicity assessments (Desneux et al. 2007). In this way, the narrow knowledge on species' behavioral ecology is still an important obstacle to the wider use of behavior as an endpoint (Dell'Omo 2002). Notwithstanding, several toxicity tests were already described in literature, where simple behavioral patterns were successfully used as indicators of exposure to toxicants, either by directly indicating toxicity effects or conversely by showing

an adaptive response of the organisms (Dell'Omo 2002). Avoidance response tests constituted, perhaps, the most successful use of behavioral features in toxicity assessments, since it was effectively tested with multiple edaphic invertebrates (Slimak 1997; Hund-Rinke & Wiechering 2001; da Luz et al. 2004; Loureiro et al. 2005; Novais et al. 2010), and even led to the creation of standard guidelines for some of those (ISO 2005; ISO 2008). These avoidance tests are short-term assays, based on the rationale that organisms are able to perceive chemical contaminants, selecting an environment that is as suitable as possible. Feeding behavior also proved to be successful when evaluating the effects of soil and food contamination (Loureiro et al. 2006; Desneux et al. 2007). This endpoint becomes particularly relevant when using saprophytic organisms since a great deal of their ecological role, such as the involvement on organic matter turnover and the recycle of nutrients, is exactly related with their feeding activity (Drobne 1997; Loureiro et al. 2006; Tourinho et al. 2013). Likewise, locomotion activity has also been suggested to be a relevant fitness indication in any organism that greatly depends on its movements to find food, reproduce, and avoid predators thus providing a linkage between individual and population stress (Bayley et al. 1997; Engenheiro et al. 2005; Bednarska et al. 2010).

1.4.4. Infraorganismal endpoints: the biomarker approach

Notwithstanding the effectiveness of toxicity tests based on organism- and population-level endpoints for environmental management and regulatory purposes, these tools fail on providing important information regarding the underlying processes responsible for the observed effects (Hyne & Maher 2003). Considerable benefits can, hence, be obtained if in addition to this approach, the modes of toxic action are also investigated.

In this regard, the use of biomarkers has been one of the most important approaches in ecotoxicology studies in the last decades. Initially conceived in the context of medicine and human toxicology, the biomarker approach quickly drew the attention of ecotoxicologists, seduced by the perspective of being able to evaluate or monitor the ecosystems' health only by measuring simple indicative responses that were simultaneously sensitive, early warning and easy to obtain. The term *biomarker* was defined by van Gestel and van Brummelen (1996) as "any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism". These responses may include

biochemical, physiological, histological and morphological measurements known to be related with the organisms' fitness (van Gestel & Van Brummelen 1996). This definition is quite useful since it can clearly illustrate the main rationale behind this approach: to be an early indication of the exposure to sub-lethal levels of a stressor, thus helping to anticipate further effects at higher levels of organization possibly arising from longer and/or more severe exposures.

Despite the modest track record of using biomarkers for assessing the effects of agrochemicals on soil biota, particularly if compared with aquatic species (Sanchez-Hernandez 2011), there are multiple protocols nowadays established that have been successfully employed with a considerable amount of different soil species (Ferreira et al. 2010; Santos et al. 2010a; Calisi et al. 2011; Novais et al. 2011; Oliveira et al. 2014). Several of them consist in enzymatic assays and stress proteins.

When it comes to assessing the effects of pesticides, one of the most widely used biomarkers has certainly been the inhibition of cholinesterases (ChE). This method was developed as a specific biomarker of exposure to organophosphorus and carbamate pesticides since the inhibition of cholinesterases constitute, indeed, the primary target of these compounds (Guilhermino et al. 1998). ChE are better known for regulating the acetylcholine-mediated activity in cholinergic synapses and neuromuscular junctions (Grisaru et al. 1999). By hydrolyzing acetylcholine (ACh), ChE is able to dissociate this neurotransmitter from the receptors, hence terminating the cholinergic transmission (Soreq & Seidman 2001). When exposed to ChE-inhibiting compounds, organisms may overly accumulate the neurotransmitter ACh in the synapses, which will consequently lead to an overstimulation of cholinergic receptors (Grisaru et al. 1999; Soreq & Seidman 2001). Given the wide distribution of this cholinergic system, the effects of ChE inhibition are known to entail severe symptoms mainly related with hyperexcitation, respiratory problems, loss of neuromotor faculties and eventually death (Roex et al. 2003; Aluigi et al. 2005). The pervasive importance of this enzyme across a wide range of evolutionarily diverse animals (Grisaru et al. 1999; Soreq & Seidman 2001) is a clear example of how the lack selectivity of pesticides can affect non-target organisms and emphasizes the importance of its evaluation.

Gluthathione-*S*-transferase (GST) is a group of drug metabolizing enzymes that has also been widely used as a biomarker for assessing the effects of pesticides in different organisms (Booth & O'Halloran 2001; Olsen et al. 2001; Domingues et al. 2009; Santos et al. 2010a; Ferreira et al. 2015). GST is involved on the phase II of pesticides biotransformation (Lagadic et al. 1994; Xu et al. 2005). It catalyzes the conjugation of

reduced gluthathione (GSH) with several compounds provided with an electrophilic center, making the subsequent GSH conjugation products less toxic, more water-soluble, and easier to excrete from cells (Pickett & Lu 1989; Callaghan et al. 2002; Domingues et al. 2009). Moreover, despite not being directly involved in free radicals scavenging, it is also known to play an important role against oxidative stress events (Pickett & Lu 1989; Carbone et al. 2003; Terada 2005).

Oxidative stress is another important mode of action of several xenobiotics, including some pesticides (Sohn et al. 2004; Jager et al. 2007). Despite being an essential element in most of the biochemical pathways of aerobic organisms, oxygen is also liable to be partially reduced assuming configurations that are potentially toxic, such as reactive oxygen species (ROS) (Davies 2000; Regoli et al. 2002). ROS can induce oxidative damage to biomolecules leading to a wide array of pathologies. Organisms are provided with a complex antioxidant protection system that relies on both enzymatic and non-enzymatic compounds, acting synergistically against free radicals (Kono & Fridovich 1982; Rikans & Hornbrook 1997). This system is normally in equilibrium with the endogenous production of ROS but when submitted to some prooxidant agent, an imbalance may occur (Sies 1997). Several parameters can be measured as biomarker of oxidative stress. One can directly assess the rate of peroxidation in membrane lipids (LPO) or evaluate the activity and content of, respectively, enzymatic and non-enzymatic antioxidants. Amongst enzymatic compounds, the most widely used are catalase (CAT), superoxide dismutase (SOD), and gluthathione peroxidase (GPx) whilst non-enzymatic antioxidants include reduced gluthathione, vitamin E (α-tocopherol), vitamin C (ascorbic acid), and others.

Another sub-organismal approach that has been increasingly used in ecotoxicology is the measurement of the energy budget. This approach is grounded on the assumption that any exposure to a stressor might potentially lead to metabolic changes in the organism. Having this in mind, De Coen and Janssen (2003) developed the cellular energy allocation (CEA) index. This biomarker reflects the energy status of an organism at the cellular level and results of the integration of the total energy reserves available and the energy consumption, measured as the mitochondrial electron transport activity. The benefit of using the energetics is that they are closely linked to virtually all life-history traits and can consequently provide an insight into the overall fitness of the organisms (De Coen & Janssen 2003).

Given the extensive amount of data gathered when employing a multiple biomarker study, a critical next step to fully optimize the potential of this approach, is to

integrate all the information. Having this in mind, Beliaeff and Burgeot (2002) conceived the Integrated Biomarker Response (IBR). This index was originally conceived to optimize the use of biomarkers in field studies but it also proved to be very useful for laboratory tests (Morgado et al. 2013). By integrating all the parameters assessed, it provides an overview of the effects registered for the different treatments and enables the follow-up of their toxicity patterns throughout the study period (Beliaeff & Burgeot 2002).

1.5. Multiple stressors

1.5.1. Implications for ERA of pesticides

Despite the great leap forward that was the development of standard guidelines for ecotoxicity testing, the adequacy of this approach as the mainstay of the ERA frameworks has been increasingly questioned (Hertzberg & MacDonell 2002). This criticism has been mainly prompted by the growing awareness that such standard studies might not be representative of the real detrimental effects associated to the release of xenobiotics in the field (van Gestel & van Diepen 1997a; Laskowski et al. 2010; Bednarska et al. 2013). As previously stated, a major part of the effects characterization component in ERA frameworks is based on laboratory assays. Here, organisms of a model species are exposed to a range of concentrations of a single compound, and any other factor is kept at near-optimal conditions (Holmstrup et al. 2010). In nature, however, and particularly in agroecosystems, organisms hardly experience optimal or constant conditions. First of all, they are continuously subject to considerable fluctuations in environmental conditions or resources (van Gestel & van Diepen 1997b). Furthermore, it becomes equally unlikely that organisms in agricultural fields are exposed to a single pollutant at a time (Santos et al. 2011a). Growing evidences have shown that by neglecting the concurrent effects of the simultaneous exposure to multiple stressors, these single-tests may lead to under- or over-estimations of the risk. Either of these situations is undesirable from the perspective of an ecosystems services management since they may often imply, respectively, elevated environmental and/or economic costs (Holmstrup et al. 2010). Risk assessments generally tend to have a precautionary nature so the possible underestimations caused by the non-accounting of these factors in the ERA frameworks are generally counterweighed by the application of safety factors (uncertainty factors). There is, however, a considerable doubt around these assumptions and they should preferably be avoided. In this way, new

approaches seem to be required so the risk assessments can be provided with pragmatic and cost-effective ways of integrating the information regarding the effects of multiple stressors in soil agroecosystems.

1.5.2. Pesticide mixtures

The exposure to a single agrochemical is indeed an unlikely event for soil biota in agrecosystems. First of all, in order to handle with the multiple kinds of pests and pathogens, the vast majority of crops require the application of more than just one type of pesticide over the growing cycle. These pesticides are often simultaneously applied, or during consecutive days. Even for the scheduled prophylactic applications, characteristic of broad spectrum pesticides, there is a real possibility that several active ingredients end up being coincidently applied, or that it occurs with very short intervals such that the recovery from the previous pesticide is not completely undertaken on the occasion of the next one. Moreover, exposure to mixtures can also be the consequence of pesticide drift, whenever different crops happen to be intermingled across the landscape, from any specific strategy of crop protection (i.e. reduction of pest resistance, reinforcement of protection, etc.), or even just result from the commercial formulations that frequently include several active ingredients (Bruce et al. 1980). It is therefore surprising that in Europe, for instance, only in very specific situations mixtures of chemicals are addressed in ERA, but never for soil compartment (Syberg et al. 2009). There are nowadays far too many active ingredients in the market, as well as many new ones to be included at any time (Yudelman et al. 1998). Given the impracticality of evaluating every possible mixture, a critical step to reasonably include the assessment of mixture toxicity effects in the ERA is to make a selection or prioritization according to the toxicity and likelihood of occurrence (Syberg et al. 2009). It has been suggested that this can be achieved through the knowledge of the mechanistic aspects of their toxicity since it can strongly influence how chemicals behave in mixtures (Escher & Hermens, 2002). A less peaceful issue is, however, the attempt of predicting the toxicity of chemical mixtures based only on the knowledge of the toxicity mechanisms of its constituents (Borgert et al. 2004; Cedergreen et al. 2008). Borgert et al. (2004) alerted to the risks of assigning any behavior to a mixture only by knowing the mechanisms of toxicity since an adequately detailed mechanistic information is still rather unusual for the majority of pesticides. This could lead to a disregard of some possible alternative modes of action that arise at different concentrations or ratios in the mixture or even any temporal relationships that require experimental confirmation (Borgert et al. 2004).

Two main approaches have been used to assess the effects of chemical mixtures in ecotoxicology: the concentration addition (CA) (Loewe & Muischnek 1926) and independent action (IA) models (Bliss 1939). Primarily developed with pharmacological purposes, these theoretical models have also been effectively used to assess the effects of mixtures in ecosystems' health (Lydy et al. 2004; Loureiro et al. 2009; Santos et al. 2010b). Although they both assume no interaction (or *additivity*) between the mixture constituents, they are fundamentally incompatible in some of their assumptions (Kortenkamp & Altenburger 1998). CA is mostly used for mixtures whose constituents have similar modes of action and assumes that each chemical "can be replaced totally or in part by an equal fraction of an equi-effective concentration of another" (Kortenkamp & Altenburger 1998). Hence, it implies a concentration-based summation of all the toxicities, scaled to reflect their relative weight on the effects of the mixture (Loureiro et al. 2010). For a mixture of *n* chemicals, CA can be expressed as:

$$\sum_{i=1}^{n} \frac{\mathrm{c}_i}{\mathrm{EC}x_i} = 1 \qquad (1)$$

where, c_i is the concentration of the chemical *i* and ECx_i is the effect concentration of the chemical *i* that produces *x*% of the effect when individually applied.

By the opposite, the IA model has been mostly used as a reference model for mixtures whose constituents have different modes of action and "measures the joint probability of individual sensitivity to the compounds in the mixture assuming that the chemical mechanisms are fully independent" (Martin et al. 2009). Contrary to CA that assumes every single chemical in the mixture as partly contributing to the overall effects, the IA model states that compounds present at concentrations lower than the effect thresholds will not contribute to the mixtures' joint effects (Kortenkamp & Altenburger 1998). For quantal responses, the IA model can be mathematically expressed as:

$$\mathrm{Y} = \prod_{i=1}^{n} \mathrm{q}i(\mathrm{c}i) \qquad (2)$$

where Y is the biological response, c_i is the concentration of chemical *i* in the mixture and $q_i(c_i)$ represents the probability of non-response. In order to be used with a continuous data set, a maximum value must be included (assumed to be the control). Hence, it can be expressed as:

$$Y = \mu_{max} \prod_{i=1}^{n} qi(ci) \quad (3)$$

where, μ_{max} represents the control response for the endpoint assessed.

Synergistic interactions between pesticides are, generally, those stemming a higher apprehension among risk managers and general public. However, it is fair to say that they are not frequent when regards to pesticide mixtures. Literature generally shows that additivity and less-than-additivity are the most commonly found situations when assessing the combined behavior of these compounds (Syberg et al. 2009). Notwithstanding, some situations were already described in literature, including some rather commonly used active ingredients (Lydy & Linck 2003; Santos et al. 2010b), In general, synergistic interactions can be expected for chemicals whose modes of action happen to be complementary, either on the toxicokinetic or in the toxicodynamic phase (Lydy et al. 2004). These can include, for instance, chemicals that enhance the uptake or transportation of each other to the target site, or chemicals that interfere with the metabolization mechanisms that normally work in the organisms to deal with the other chemical (Andersen & Dennison 2004; Lydy et al. 2004; Syberg et al. 2009).

It must be noted, however, that even when no interactions occur, the simple addition of effects may lead to consequences that were not clearly anticipated by single toxicity assays. Mixture toxicity research is normally biased towards the seek of synergistic interactions and frequently neglects the ecological relevance of additive effects (Silva et al. 2002). In this regard, Silva et al. (2002) reported the joint-toxicity of eight chemicals applied at very low doses to result in significant estrogenic effects, even though no synergism was detected. This seems to be a clear example of the inappropriateness of current risk assessments to deal with mixtures. The safeguarding of the precautionary principle is often only achieved by using arbitrary safety factors, which entails a significant uncertainty and low predictive capability.

1.5.3. Effects of natural stressors in pesticides toxicity

Since the perception amongst ecotoxicologists, that the environmental conditions can influence the toxicity of several xenobiotics, a considerable effort has been devoted to explore the effects of several natural stressors in a wide number of pesticides and using a multiplicity of model organisms (Demon & Eijsackers 1985; Everts et al. 1991; Puurtinen & Martikainen 1997; Zaga et al. 1998; Bridges & Boone 2003; Sørensen & Holmstrup 2005; Skovlund et al. 2006; Bednarska & Laskowski 2009; Bindesbøl et al. 2009; Lima et al. 2011; Ribeiro et al. 2011; Knillmann et al. 2013; Cardoso et al. 2014; Lima et al. 2014). The comprehension of such joint effects assumes an even higher relevance in the present context of climate changes since they can significantly influence the maximum threshold levels accepted for environmental contamination. This situation, together with the increasing ubiquity of pesticides application, suggests that a higher probability of interaction should be expected from now on and emphasizes the importance of evaluating their simultaneous effects. Most of this attention has, however, been focused on the aquatic compartment whilst in soil such relationships are substantially less understood. Among the environmental factors thought to influence the toxicity of pesticides to soil organisms, temperature and soil moisture have been, perhaps, those who gathered more attention (Sørensen & Holmstrup 2005; Skovlund et al. 2006; Bednarska et al. 2009; Bindesbøl et al. 2009; Lima et al. 2011). The interactive effects of ultraviolet radiation (UVR) and pesticides have also been assessed in terrestrial organisms but seldom in soil invertebrates (Cardoso et al. 2014). All these natural stressors have, in some situations, been suggested to enhance or decrease the toxicity of pesticides in multiple ways. By acting directly on the physicochemical properties of the pesticides, they can change these compounds' adsorption, desorption, volatilization and/or degradation rates (Arnold & Briggs 1990). This can lead to drastic changes in the bioavailability of the chemical, hence affecting its uptake by the organisms. In fact, pesticides possess a set of features, such as a high lability and mobility, which makes them particularly relevant to assess in different conditions. A clear example of that are the products of their transformation or degradation since they can originate metabolites that are more or less toxic than the parental compound (Guven et al. 1999; Easton et al. 2001; Sinclair & Boxall 2003; Giordano et al. 2007). In this way, any factor that promotes or slows the pace of these processes is expected to have a direct influence on a pesticides' toxicity. Furthermore, environmental factors may also act on the organisms, either by affecting their fitness and consequently their sensitivity to pesticides, or by influencing the uptake, metabolism, and detoxification processes (Maraldo et al. 2006; Noyes et al. 2009). The biggest problem of predicting the

effects of natural stressors in pesticides toxicity is the extraordinary case-specificity found in these interactions and the virtually unlimited scenarios to be assessed. Each pesticides' toxicity may vary significantly with the environmental conditions and lead to markedly different patterns since it will ultimately result of the complex weighting between the properties of the chemical, the organisms, and the magnitude of stressors involved (Holmstrup et al. 2010). Regardless of the growing attention that these issues have received lately, several gaps of knowledge must still be fulfilled. For instance, studies involving more than binary combinations of natural and chemical stressors are still scarce, despite being, definitely, the most usual circumstance in nature (Laskowski et al. 2010). However, the interaction between toxicants may lead to different outcomes when occurring under different environmental conditions, as shown by Bednarska et al. (2009), by exposing carabid beetles to chlorpyrifos and nickel.

1.6. Aim of the thesis

In view of the foregoing, considerable challenges can be foreseen as regards to the future management of soil health in agroecosystems. One of the toughest will probably be the maintenance of the edaphic biodiversity, with particular attention for some key groups that are known to have a disproportionate importance in soil functioning. Aiming to contribute for this topic, the main goal of this thesis was to evaluate the influence of multiple natural stressors on the joint toxicity of two widely used pesticides, chlorpyrifos (CPF) and mancozeb (MCZ), using *Porcellionides pruinosus* as a model species. *Porcellionides pruinosus* is a synanthropic and widely distributed terrestrial isopod that has been frequently used in soil ecotoxicology experiments (Loureiro et al. 2002; Ferreira et al. 2010; Santos et al. 2010b; Morgado et al. 2013; Tourinho et al. 2013; Silva et al. 2014; Ferreira et al. 2015). In fact, terrestrial isopods fulfil several criteria that make them a good choice when regards to assessing the effects of soil contamination (Drobne 1997). First of all, they can constitute the dominant component of the arthropod macrodecomposer guild in several temperate habitats (Paoletti & Hassall 1999). Furthermore, they are important regulators of the edaphic ecosystems because of their deep involvement in several functional processes like litter fragmentation, breakdown of organic matter (Wieser 1978), nutrient turnover, enhancement of microbial community (Kautz & Topp 2000), or even pesticide degradation (Loureiro et al. 2002; Ferreira et al.

2015). Since a great deal of their ecological role is mostly related with the activity of feeding, a particular focus was placed on the feeding-related endpoints.

CPF is an organophosphate insecticide, used to control Coleoptera, Diptera, Homoptera and Lepidoptera, both in soil or foliage, and has the inhibition of acetylcholinesterase as the main mode of action (Schreck et al. 2008). MCZ is a dithiocarbamate fungicide, classified as a Multi-Site Action compound (Gullino et al. 2010), that is frequently applied against a wide spectrum of fungal diseases (Cycoń et al. 2010). Actually, MCZ must be considered as a pro-fungicide since it is not fungicidal itself. It breaks down, when exposed to water, releasing ethylene bisisothiocyanate sulfide (EBIS), that is further converted into ethylene bisisothiocyanate (EBI). These metabolites are both active toxicants, thought to interfere with fungi enzymes containing sulphydryl groups (Gullino et al. 2010).

Before studying the joint effects of multiple stressors, this thesis will first focus on the individual effects of several ubiquitous abiotic factors to *P. pruinosus*. Abiotic factors constitute one of the most important drivers shaping terrestrial ecosystems (Dunson & Travis 1991) but are frequently neglected by risk assessment procedures. With this work it is intended to highlight the influence of these stressors on the performance of soil organisms in order to understand the effects of subtle and drastic changes and foresee the implications of global environmental changes.

1.7. Outline of the thesis

This thesis will be divided in 7 chapters whose first, Chapter 1, is the current General Introduction, Chapters 2 to 6 constitute the description of the experimental component of this thesis, and finally Chapter 7 comprises the General Discussion of the results found.

Chapter 2 is entitled “Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*” and describes the effects of temperature, soil moisture and UV radiation on the performance of the isopods. Several organismal- and population-level endpoint were used in this chapter.

Chapter 3 is entitled “Environmental- and growth stage-related differences in the susceptibility of terrestrial isopods to UV radiation” and includes the assessment of the pathways of damage caused by UV radiation on the terrestrial isopods. It also evaluates differences in the susceptibility to UV radiation that could be related to the environment of exposure or the growth stage.

Chapter 4 is entitled “Joint toxicity of chlorpyrifos and mancozeb to the terrestrial isopod *Porcellionides pruinosus*: a multiple biomarker approach” and describes the use of multiple biomarkers and energy-related parameters to assess the effects of the pesticides chlorpyrifos and mancozeb, as well as their mixture at different doses and ratios, to *Porcellionides pruinosus*. It also compares the susceptibility of adults and juveniles.

Chapter 5 is entitled “Temperature induces different pesticide mixture effects on the terrestrial isopod *Porcellionides pruinosus*” and aims to assess the effects of temperature on the toxicity and behavior of a mixture of chlorpyrifos and mancozeb in *Porcellionides pruinosus*. Endpoints assessed include survival and feeding parameters, namely consumption ratio and biomass gain/loss.

Chapter 6 is entitled “Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*” and aims to assess the effects of soil moisture on the toxicity and behavior of a mixture of pesticides in *Porcellionides pruinosus*. Endpoints assessed include survival and feeding parameters, namely consumption ratio and biomass gain/loss.

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CHAPTER 2: Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*

Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*

2.1. Abstract

Abiotic factors constitute one of the most important drivers shaping soil ecosystems. Although being a strongly buffered environment, soil's heterogeneous nature combined with the limited mobility of its organisms can make them highly sensitive to unfavourable conditions. In a context of global environmental changes a thorough knowledge of these factors is a critical element to understand the implications of these events. In this study we evaluated the influence of temperature, soil moisture and UV radiation on the performance of the terrestrial isopod *Porcellionides pruinosus* using several endpoints: survival, locomotor activity, feeding parameters and avoidance behaviour. The results showed that abiotic factors might affect this species at relevant environmental conditions and therefore emphasized the need of being considered in ecotoxicological assays and further on risk assessment. At the range assessed, temperature did not affected survival but showed marked effects on sublethal endpoints. The feeding parameters and locomotor activity showed a right-shifted response with a gradual temperature-induced increase in performance until reaching optimum temperature and abruptly declining thereafter. On the contrary, soil moisture was found to significantly affect isopods' survival but the effects on the feeding parameters were not clear. Isopods exhibited a clear preference for intermediate soil moisture values tending to avoid overly dry or wet conditions. Nonetheless, when it comes to avoidance behaviour, isopods showed to be more sensitive to dry environments where higher percentages of avoidance were found. UV radiation showed to affect survival, body weight and locomotor performance. The use of several endpoints related to different traits allowed us to have an insight into several physiological and behavioural responses.

Keywords: Climate changes; terrestrial isopods; temperature; soil moisture, UV radiation, locomotion, feeding

2.2. Introduction

Abiotic factors have long been acknowledged as one of the most important drivers shaping edaphic ecosystems. They have, traditionally, been considered to define the pool of species physiologically capable of being present in a certain habitat, thus creating the basepoint from where biotic relationships will act (Dunson & Travis 1991). Abiotic factors can indeed become the dominant element that operates at broader scales and are known to exert strong influence on soil organisms' performance (Bardgett 2002; Lavelle & Spain 2003). Soil is a rather complex compartment and, although being strongly buffered, it is still featured by marked spatial and temporal heterogeneity (Ettema & Wardle 2002). These conditions, along with the limited mobility of most soil organisms, can make some of them particularly susceptible to unfavourable environmental conditions (Briones et al. 1997). Considering the deep involvement of soil fauna in most ecosystems processes (Stork & Eggleton 1992; Lavelle et al. 2006), it is important to understand how abiotic factors affect their performance, as well as the potential consequences arising on soil functioning.

The relevance of assessing the effects of abiotic factors becomes even higher in the present context of global environmental changes. The expected rises in temperature are likely to influence hydrologic systems, leading to changes in soil moisture (Ragab & Prudhomme 2002; Weltzin et al. 2003). Furthermore, in addition to the gradual changes of mean climate patterns, of paramount importance are also the future perspectives of increasing frequency and severity of extreme weather events given their unpredictable nature and disruptive potential on ecosystems already debilitated by changes in soil quality (Huszar et al. 1999). In addition, increasing intensities of ultraviolet radiation are reaching Earth's surface, as a consequence of ozone layer depletion, introducing extra stress to ecosystems (Mintzer 1992).

Among soil-dwelling biota, macrofauna and particularly those living on the surface layer, are likely to be more sensitive to environmental changes since they are less protected by soil buffer properties (see Lavelle & Spain 2003). Terrestrial isopods constitute a widespread group of surface-living saprophytic detritivores that play a key role on soil ecosystems, mainly through the consumption of leaf litter and by improving nutrient cycling (Drobne 1997; Loureiro et al. 2002). Despite present in most climatic zones (Sutton et al. 1980) and often as the dominant component of the arthropod macrodecomposer guild (Paoletti & Hassall 1999), this group is still considered poorly adapted physiologically to terrestrial life (Sutton et al. 1980). Thus, such limitations, along

with their major ecological importance, make them important candidates to have in mind when the purpose is to evaluate the impacts of global changes in edaphic ecosystems' processes.

Most of the assessments of biological responses to environment-related factors have been mainly focused on critical limits. Nevertheless, sub-optimal conditions are the most usual situation in nature and might also entail strong consequences in organisms' performance, ultimately affecting their contribution to the delivery of ecosystems services (Bednarska et al. 2010)**.** Moreover, given the complexity of biological systems, a whole-picture view is only possible by using multiple endpoints related to different traits and their choice must fall upon those highly sensitive and closely related to both the fitness of the organisms and the role they play in the ecosystem. Besides survival analysis or the critical limits, locomotor activity is also relevant as a fitness indication in any organism that depends on its movements to find food, reproduce, and avoid predators (Bayley et al. 1997). Likewise, the ability to consume, assimilate and allocate food is an essential trait and any occurrence decreasing these processes may seriously impair organism's condition (Loureiro et al. 2006). Furthermore, since isopods' faecal production is known to enhance the turnover of organic matter, egestion may also be considered an ecologically relevant parameter to assess the effects of any stressor in breakdown processes (Loureiro et al. 2006).

In this work, we assessed the effects of temperature, soil moisture, and UVR on the survival, locomotor performance, feeding parameters and avoidance behaviour of the terrestrial isopod *Porcellionides pruinosus*.

2.3. Material and methods

2.3.1. Test organisms and soil

In this experiment, the terrestrial isopod *Porcellionides pruinosus* was used as test-species. These animals were hand-collected in a horse manure heap and maintained in laboratory cultures at 20 ^{o}C (±2 ^{o}C), 16:8 (light:dark) photoperiod, with soil adjusted to a moisture content of 60% and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Only adult animals (15 - 25 mg wet weight) were used in these assays, excluding moulting animals, those with any visible problem and pregnant females. No gender differentiation was done.

The natural certified loamy sand soil LUFA 2.2 (LUFA Speyer) was used in all the experiments. The properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, water holding capacity (WHC) = 41.8 ± 3.0 (g/100 g), texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

2.3.2. Test designs

In order to cover a range of temperatures that can characterize temperate/Mediterranean climates, the following treatments were selected for the temperature experiment: 5 ^{o}C, 10 ^{o}C, 15 ^{o}C, 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, and 35 ^{o}C, with soil moisture always adjusted to 60% WHC. Control temperature was assumed to be 20 ^{o}C since this is the condition commonly used for similar ecotoxicological tests performed with terrestrial isopods or other soil species (Loureiro et al. 2009). Concerning soil moisture experiment, soil was adjusted to the following water contents: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% of the soil WHC and left in a room with controlled temperature at 20 ^{o}C ± 1 ^{o}C. Fifty per cent of soil WHC was assumed to be the control treatment for soil moisture because it is the value normally used in soil ecotoxicology experiments (Loureiro et al. 2009). Regarding the UVR experiment, organisms were kept at 20 ^{o}C in a temperature-controlled room and daily irradiated for 2 hours with a UV lamp (Spectraline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm). In order to cut the UV-C wavelengths, the lamp was covered with a clear cellulose acetate sheet (0.003 mm, Grafix plastics, USA) that had been previously irradiated during 12h to stabilize its permeability to UVR. Three UVR treatments were assessed, UV-L (Low UV intensity = 182.13 mW.cm^{-2}/ UV daily dose = 1311.30 J.m^{-2}), UV-M (Median UV intensity = 265.97 mW.cm^{-2}/ UV daily dose = 1914.97 J.m^{-2}) and UV-H (High UV intensity = 393.26 mW.cm^{-2}/ UV daily dose = 2831.50 J.m^{-2}), plus an additional set of non-irradiated organisms used as control. UV-L, UV-M and UV-H correspond, respectively, to UV indexes of 7.29, 10.64 and 15.73. Daily doses applied can easily be registered in Portugal between January and March (TEMIS 2013). This range of intensities was obtained by disposing the test isopods to different distances to the UV lamp and was measured using a spectro-radiometer connected to a monochromator and analysed with BenWin+ software (Bentham Instruments, Reading, UK). The intensities were corrected using the weighting factors of the CIE reference action spectrum for erythema in human skin (McKinlay & Diffey 1987). These corrected intensities (I_{eff}) were used to calculate the corrected UV doses (UVD_{eff}) using equation (1).

$$UVD\ (\mathrm{Jcm^{-2}}) = \frac{I_{eff}\ (\mathrm{mWcm^{-2}})\ \mathrm{x}\ time\ of\ exposure\ (\mathrm{s})}{1000} \quad (1)$$

2.3.3. Experimental set up

Temperature experiment followed a different protocol from the remaining. For assessing survival three replicates per treatment were used, each one consisting in a box with 10 isopods. Moisture content was initially adjusted (60% WHC) and controlled throughout the experiment by adding distilled water every two days. Mortality was checked periodically and finally registered at day 14.

At day 14th, locomotor activity tests were immediately performed using a protocol adapted from Schuler et al. (2011). After being removed from test boxes, all the surviving isopods for each treatment were pooled together and five of them were randomly selected to use in the locomotor activity test. In this test the time needed by the isopods to run through a narrow 20 cm long racetrack was reported, and the number of stops was also registered. They were placed a few centimetres behind the starting mark of the racetrack and the time started to count when this mark was crossed. Timer was then stopped when they crossed the final mark. However, if an isopod were more than 10 minutes without initially moving, it was discarded and the procedure was repeated with another one. Isopods' runs were video recorded to allow further and more precise analysis.

Feeding tests, for assessing the effects of temperature followed the procedure described by Loureiro et al. (2006) with modifications concerning the type of food. Ten isopods per treatment were initially submitted to a 24h starvation so that their guts could be emptied. After that, body weight was registered and they were individually placed inside a chamber made of two plastic vessels, one placed within the other. Lower vessels had a thin layer of water saturated plaster to help maintaining humidity whilst upper vessels had a mesh bottom that allowed faeces to be collected in the lower vessel and avoiding therefore coprophagy. Dried alder leaves cut into small disks (Ø 10 mm) were used as food items. Disks were weighted before and after the experiment in order to precisely control the total amount consumed by isopods. In the beginning, 4 disks were supplied to each animal (± 40 mg dw). More disks were added if necessary. After 14 days, isopods were subjected to another starvation process and finally reweighted. Isopods'

feces and the remains of leaf disks were recovered from the lower and upper vessels, respectively, dried for 48h at 60 ^{o}C and also weighted. Isopods consumption, assimilation and egestion ratios and assimilation efficiencies were calculated as follows:

$$CR = (W_{Li} - W_{Lf}) / W_{isop} \quad (2)$$

$$AR = [(W_{Li} - W_{Lf}) - F] / W_{isop} \quad (3)$$

$$AE = [(W_{Li} - W_{Lf}) - F] / (W_{Li} - W_{Lf}) * 100 \quad (4)$$

$$ER = F / W_{isop} \quad (5)$$

where, dw - dry weight; W_{Li} - initial leaf weight (mg dw); W_{Lf} —final leaf weight (mg fw); W_{isop} - initial isopod weight (mg dw); CR - Consumption ratio (mg leaf/mg isopod); AR - assimilation ratio (mg leaf/mg isopod); F – faeces (mg); AE - assimilation efficiency (%); ER - Egestion ratio (mg faeces/mg isopod).

A different protocol had to be used to perform the feeding test with different soil moistures, since it indeed required to be conducted in soil. In this way, it was decided to combine all the endpoints in one only experiment by adapting the feeding protocol under a soil exposure. Isopods were also submitted to the same starvation period and then weighted before being exposed in individual boxes. There were 10 replicates per treatment, each one consisting of 1 individual. A similar amount of previously weighted disks of alder leaves was included in each box. Soil moisture was readjusted every other day by adding the necessary amount of distilled water and mortality was also daily checked. The starvation process was repeated in the end of the experiment and they were reweighted. Likewise, the dry weight of disks was also determined. Given the impossibility of recovering isopods' feces from soil, it was only possible to calculate CR (see above) and biomass gain/loss (6).

$$\text{Biomass gain/loss} = [(W_{isop} - W_{isop\,f}) / W_{isop}] \times 100 \quad (6)$$

where, W_{isop} - initial isopod weight (mg dw) and $W_{isop\,f}$ – final isopod weight.

After the experiment, five to ten isopods were randomly selected from each treatment and used to assess locomotion, as described before. It was decided to follow this protocol in the UVR experiment as well because previous attempts of assessing the effects of this stressor in *P. pruinosus* showed that the media of exposure must be taken into account (Morgado et al. 2013).

2.3.4. Statistical analysis

When possible, 50% mortality (LD_{50}), and 50% effect doses (ED_{50}) were derived for every endpoint and for each stressor by fitting experimental data to the most adequate known function. For factors such as temperature, and soil moisture, whose assumed control conditions are in the middle of the range assessed, these values were calculated by splitting the range on above and below control and then fitting data separately for each one of them. A Probit regression scheme was used to calculate the conditions that cause 50% mortality (LD_{50}). A 3-parameter sigmoidal logistic function was used to calculate the ED_{50} for the feeding parameters. A Weibull function was also used in the temperature experiment to assess the conditions at which a maximum feeding performance was reached since it was observed not to be in control. A Mantel-Cox log-rank test was used to test whether there are differences between survival times of different treatments (Bewick et al. 2004). Regarding sublethal parameters, one-way ANOVAs were used to test differences between treatments. When significant differences were detected, a Holms-Šidak *post-hoc* test was performed to compare all the treatments with the control. If data failed on showing a normal distribution, a non-parametric Krushkal-Wallis' test was performed followed by a Dunn's *post-hoc* test. Fisher's exact test was used to test the significance of avoidance responses in the avoidance behaviour tests, one-tailed to assess control *versus* treatment and two-tailed for assessing control *versus* control situations (Natal da Luz et al. 2004; Santos et al. 2011b). LD_{50} calculations were performed on Priprobit 1.63, and the remaining statistical analysis was performed with the Sigmaplot statistic pack (SigmaPlot 12.0 statistic pack; Systat Software, Inc., San Jose, CA, USA) or GraphPad Prism 6 statistical pack (GraphPad Software, La Jolla, CA, USA).

2.4. Results

2.4.1. Temperature

No mortality was registered between 10 °C and 25 °C during the 14 days period (Figure 2.1). Mortality only occurred close to the extremes of the temperature range, though never reaching 50% of the isopods in any treatment. The analysis of variance also found no significant results when comparing the isopods' survival between temperature treatments. Lower and upper LD_{50} were found to be 4.88 °C (CI: 4.56 °C - 5.20 °C) and 36.31 °C (CI: 34.19 °C – 42.17 °C), respectively. Both of them were out of the temperature range tested in this experiment, though being really close to both limits.

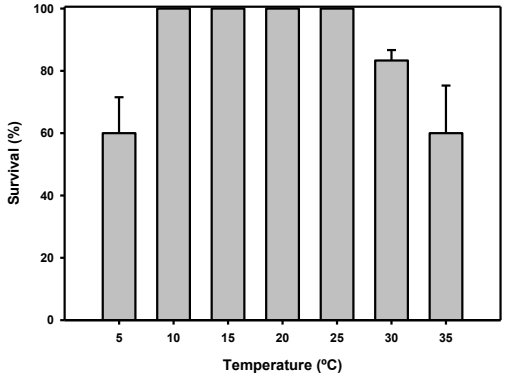

Figure 2.1 – Percentage of survival of *Porcellionides pruinosus* exposed for 14 days in LUFA 2.2 soil to a range of temperatures between 5 °C and 35 °C. Data are expressed as mean (± standard error).

Temperature was found to significantly influence isopods' locomotor performance (One-way ANOVA, $F_{6,30}$=2.981, p=0.021) (Figure 2.2). When compared with control, organisms kept for 14 days at 35 °C took significantly more time to run through the 20 cm track. Isopods kept at 5 °C, 15 °C and 30 °C also seemed to be slower than those kept at 20 °C but no significant differences were found. Significant differences were also found for the number of stops per run (One-way ANOVA, $F_{6,30}$=3.655, p=0.008) with isopods taken from 5 °C and 30 °C stopping significantly more than those taken from 20 °C. Isopods exposed to 20 °C and 25 °C showed similar patterns of locomotor activity on both parameters.

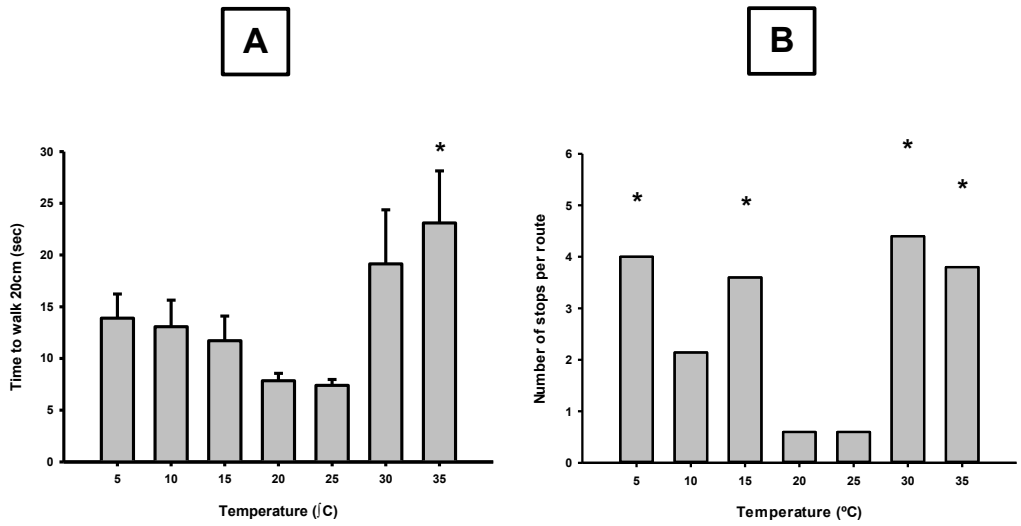

Figure 2.2 – Locomotor perfomance of *Porcellionides pruinosus* after 14 days exposure in LUFA 2.2 soil to different temperatures ranging from 5 to 35 ^{o}C: (a) maximum speed; (b) number of stops during a 20cm racetrack. Data are expressed as mean (± standard error). One-way ANOVA, Holms-Šidak, p<0.05.

When compared to the previous exposure made in soil, similar mortality was found in the feeding experiment with lower and upper LD_{50} values registered at 5.32 ^{o}C (CI: -3.80 ^{o}C – 7.94 ^{o}C) and 37.61 ^{o}C (confidence intervals not determined), respectively. No mortality was observed among control isopods. As regards to the feeding results, temperature was found to influence all the parameters in a similar way. In fact, all the feeding parameters showed to match quite well with each other, as one can see in Figure 2.3. All of them showed to increase with temperature, peaking at 30 ^{o}C and starting to decrease thereafter, even though in most of the situations the isopods kept at 35 ^{o}C still showed a better feeding performance than those kept at 20 ^{o}C.

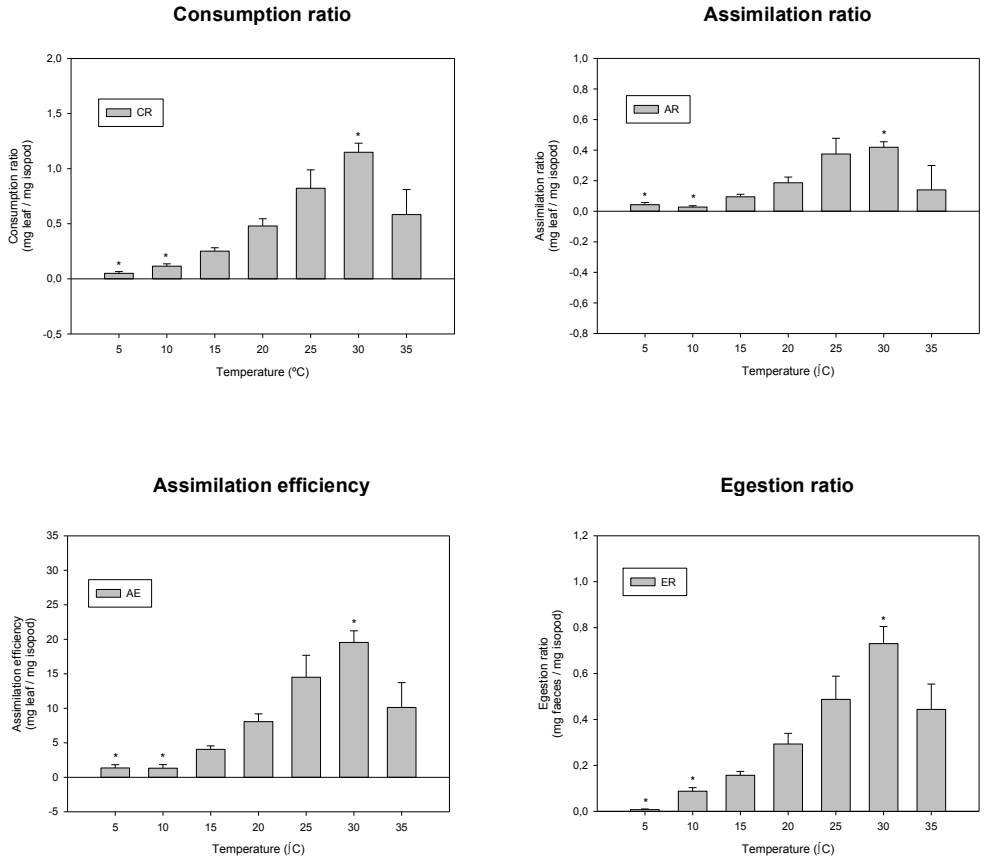

Figure 2.3 – Performance of feeding parameters of *Porcellionides pruinosus* after 14 days exposure in LUFA 2.2 soil to different temperatures ranging from 5 to 35 ^{o}C. Data are expressed as mean (± standard error). One-way ANOVA, Holms-Šidak, p<0.05.

The similarity of temperature effects among feeding parameters could be clearly evidenced by the comparable ED_{50} values calculated (3-parameter sigmoidal logistic function). The ED_{50} values for the lower temperatures were 14.72 ^{o}C for CR, 15.10 ^{o}C for AR, 15.06 ^{o}C for AE, and 14.46 ^{o}C for ER (Figure 2.4). Regarding higher temperatures, 20 ^{o}C (assumed control) was not the treatment where the highest performance was found so an ED_{50} could not be derived.

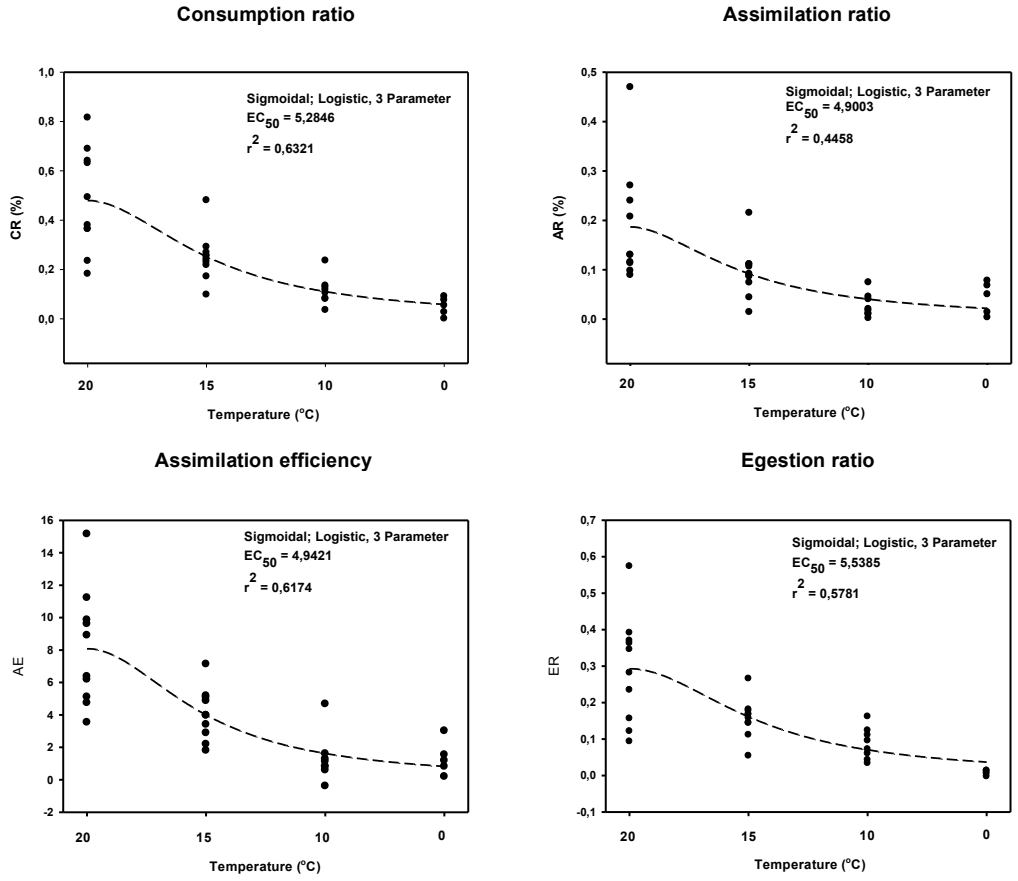

Figure 2.4 – Dose response curves for the performance of feeding parameters of *Porcellionides pruinosus* after 14 days exposure in LUFA 2.2 soil to different temperatures ranging from 5 to 20 ^{o}C. ED_{50} values were calculated using a sigmoidal logistic curve (3 parameters).

The maximum feeding performance was therefore derived by fitting a Weibull function to data and the inflection points were found at 29.66 ^{o}C for CR, 28.30 ^{o}C for AR, 29.65 ^{o}C for AE and 30.38 ^{o}C for ER (figure 2.5). When compared to control, isopods kept at 30 ^{o}C showed to have a higher consumption (Kruskal-Wallis, H=38.677, df=6, p<0.001), assimilation (AR: Kruskal-Wallis, H=38.414, df=6, p<0.001; AE: Kruskal-Wallis, H=38.677, df=6, p<0.001) and egestion performance (Kruskal-Wallis, H=38.677, df=6, p<0.001) whereas, on the contrary, those kept at 5 ^{o}C and 10 ^{o}C presented a decreased performance on the same parameters.

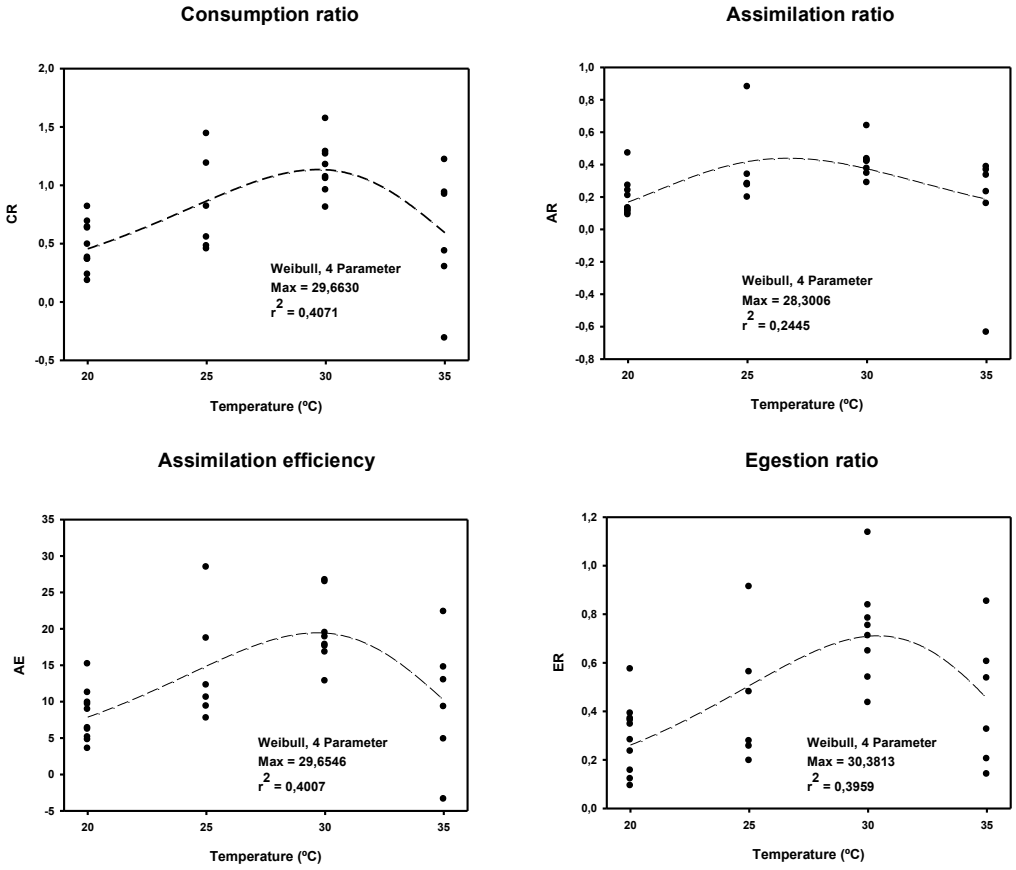

Figure 2.5 – Dose response curves for the performance of feeding parameters of *Porcellionides pruinosus* after 14 days exposure in LUFA 2.2 soil to different temperatures ranging from 20 to 35 ^{o}C. Inflection points were calculated using a Weibull function (4 parameters).

2.4.2. Soil moisture

Contrary to the temperature experiment, the range of soil moisture treatments assessed in this experiment showed to induce strong mortality to *P. pruinosus*. In fact, at day 14th there were no isopods alive at 90% WHC and only 20% managed to survive at 10% WHC (Figure 2.6). The LD_{50} for low moistures in the 14 days period was 15.04% WHC (CI: 9.80% - 19.28% WHC) while for the upper soil moisture regimes the LC_{50} was 80.38% WHC (CI: 79.38% - 81.58% WHC). Survival analysis helped to identify several differences in survival patterns between treatments (Log-rank Mantel-Cox test, χ^2=90.54, df=8, p<0.0001). First of all, survival curves for 90% WHC showed to be significantly

different from any other treatment while for 10% WHC they showed to be different from all except 80% WHC. Although not reaching 50% mortality among the exposed isopods, survival curve was still significantly different from the most favourable treatments where no mortality was registered (50%, 60%, 70% WHC). Finally, no differences were found between 20%, 30%, 40%, 50%, 60%, and 70% WHC as far as survival is concerned.

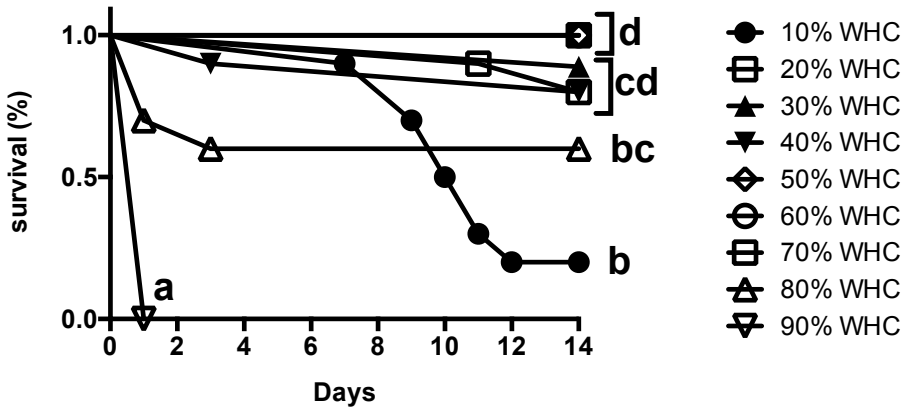

Figure 2.6 – Survival of *Porcellionides pruinosus* after exposure to different soil moistures.

Regarding isopods' locomotor performance, there appeared to be an increasing pattern in isopods' maximum speed from 10% WHC until 50% WHC, which seemed to be the optimum soil moisture for this parameter, starting thereafter to decline again (Figure 2.7a). No significant differences were registered between any treatments (One-way ANOVA, $F_{6,29}$=1.579, p=0.189). Soil moisture values at which a 50% decrease in isopods' performance occurred (i.e. ED_{50} for speed performance) were 27.33% WHC (Four parameter logistic curve, r^2=0.2202) and 54.56% WHC (Four parameter logistic curve, r^2=0.3191), respectively for too dry and too moist environments. Isopods showed to stop significantly more throughout the 20 cm racetrack at 60% WHC than at 20% WHC (Kruskal-Wallis, H=18.125, df=7, p=0.011) (Figure 2.7b). No differences were found among the remaining treatments.

Neither consumption ratio nor biomass variation showed to vary significantly with soil moisture (Figure 2.8).

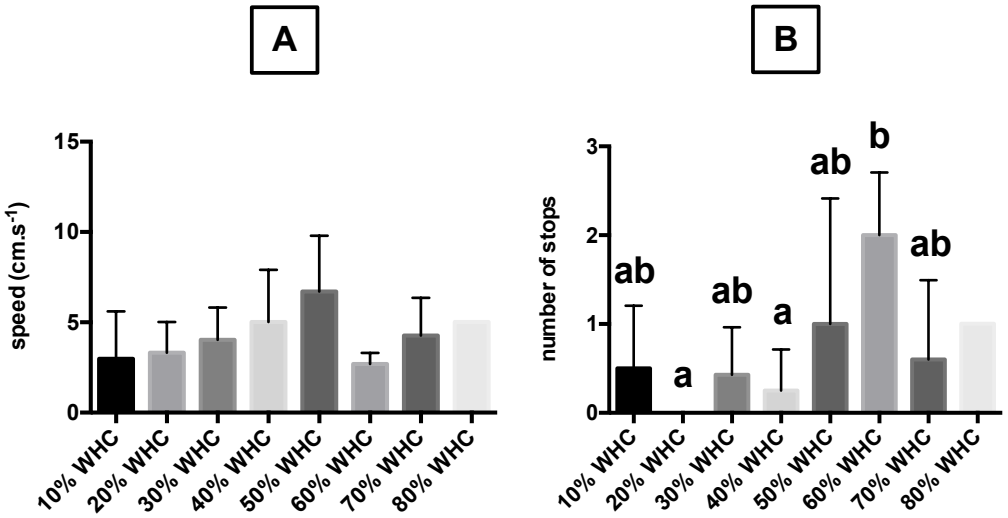

Figure 2.7 – Locomotor perfomance of *Porcellionides pruinosus* after a 14 days exposure to LUFA 2.2 soil adjusted to different soil moistures ranging from 10% to 80% WHC: (a) maximum speed; (b) number of stops during a 20cm racetrack. Different letter denote significant differences between treatments. One-way ANOVA, Holms-Šidak, $p<0.05$.

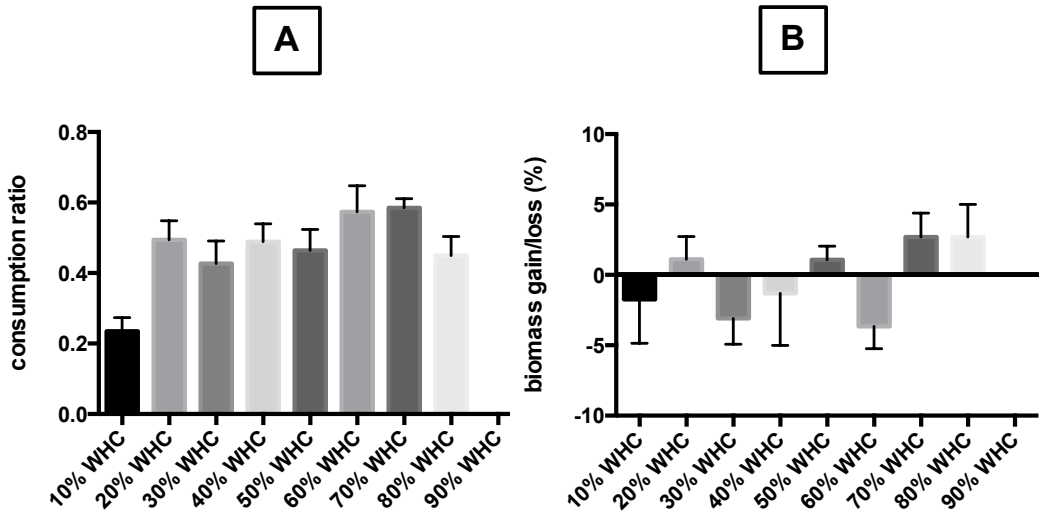

Figure 2.8 – Performance of feeding parameters of *Porcellionides pruinosus* after a 14 days exposure to LUFA 2.2 soil adjusted to different soil moistures ranging from 10% to 80% WHC: (a) consumption ratio; (b) biomass gain/loss. Data are expressed as mean (± standard error). One-way ANOVA, Holms-Šidak, $p<0.05$.

When simultaneously provided with two different soil moisture conditions, isopods almost always selected the side established as control (60% WHC) (Table 2.1). In fact, the only treatment where isopods did not show a significant preference for control side was 40% WHC (Fischers' one-tailed exact test, p=0.225). Significant differences were found for 10% WHC (Fischers' one-tailed exact test, p<0.0001), 20% WHC (Fischers' one-tailed exact test, p<0.0001), 80% WHC (Fischers' one-tailed exact test, p=0.0084), and 100% WHC (Fischers' one-tailed exact test, p<0.0001). The absence of preferences when two control sides were provided (Fischers' two-tailed exact test, p=0.450) seems to indicate that soil moisture was actually the chief factor leading to these results, thus confirming the validity of the above-reported avoidance results.

Table 2.1 – Summary of avoidance behaviour results for several soil moisture treatments (ranging from 10% WHC to 100% WHC) *versus* control moisture (60% WHC) and respective significant differences assessed using Fischer's exact test (one-tailed for control vs treatments and two-tailed for control vs control; α=0.05). Exposures were performed in LUFA 2.2 soil.

Soil moisture	Soil moisture (control)	% avoidance	standard deviation	Fischer's Exact test	
10%	60%	93.75	12.50	p<0.0001	significant
20%	60%	87.50	25.00	p<0.0001	significant
40%	60%	25.00	95.74	p=0.225	non significant
60%	60%	25.00	54.01	p=0.45	non significant
80%	60%	62.50	75.00	p=0.0084	significant
100%	60%	93.75	12.50	p<0.0001	significant

2.4.3. Ultraviolet radiation

In Figure 2.9 are shown the survival curves registered for the different treatments throughout the UVR experiment. Contrary to the UV-exposed treatments, no mortality was registered in control. Curve comparison showed that all the UV-exposed treatments presented a significantly higher mortality than control: UV-L (Log-rank Mantel-Cox test, χ^2=4.724, df=1, p=0.0297), UV-M (Log-rank Mantel-Cox test, χ^2=13.090, df=1, p=0.0003) and UV-H (Log-rank Mantel-Cox test, χ^2=8.104, df=1, p=0.0044). No significant differences were found between UV-M and UV-H (Log-rank Mantel-Cox test, χ^2=1.662, df=1, p=0.1973). The LD_{50} for exposure to UV radiation after 14 days was achieved at 2693.59 $J.m^{-2}$ per day (374.11 $mW.m^{-2}$).

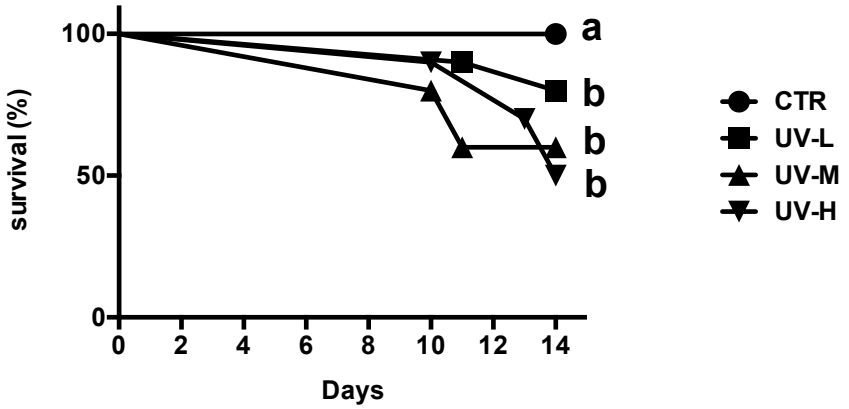

Figure 2.9 – Survival of *Porcellionides pruinosus* after 14 days of exposure to different daily doses of UV radiation.

UV radiation appeared to affect isopods locomotor performance by reducing their maximum speed and increasing the number of stops throughout the 20 cm transect (Figure 2.10). Regarding isopods' speed, although significant differences were found by the analysis of variance (One-way ANOVA, $F_{3,17}$=3.559, p=0.0365), no differences could be identified between treatments using the Holms-Šidak *post-hoc* test. Significant differences were also found for the number of stops (One-way ANOVA, $F_{3,22}$=7.167, p=0.0016). Isopods at UV-M showed to stop significantly more than in unexposed ones (Holms-Šidak, p=0.001). Isopods exposed to the higher UV doses showed no differences, neither to control and UV-L nor UV-M.

A Kruskal-Wallis test followed by a *post-hoc* Dunns' test found no differences in consumption rates between UV exposed treatments and control (Figure 2.11a). On the contrary, biomass variation showed to vary significantly with the exposure to UV radiation (Kruskal-Wallis, H=8.162, df=3, p=0.0428) (Figure 2.11b). Nevertheless, Dunn's *post-hoc* test could only identify significant differences between control isopods and those exposed to intermediate UV doses (Dunn's test, p=0.0293). No valid ED_{50} could be derived for consumption ratio or biomass variation.

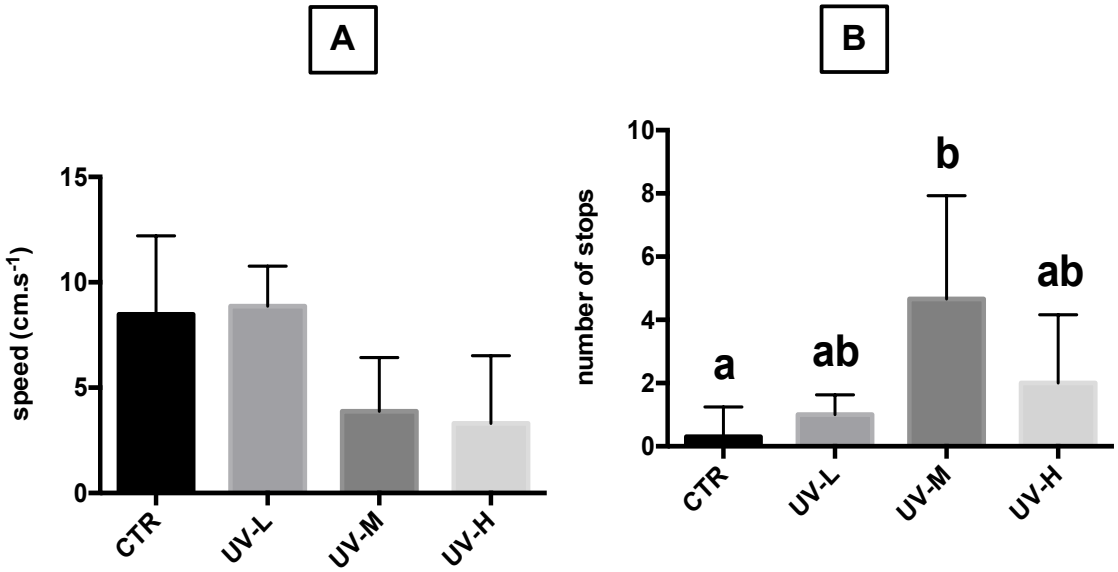

Figure 2.10 – Locomotor perfomance of *Porcellionides pruinosus* after a 14 days exposure to different daily doses of UV radiation in LUFA 2.2 soil: (a) maximum speed; (b) number of stops during a 20cm racetrack. Different letters denote significant differences between treatments (Kruskal-Wallis test, α=0.05).

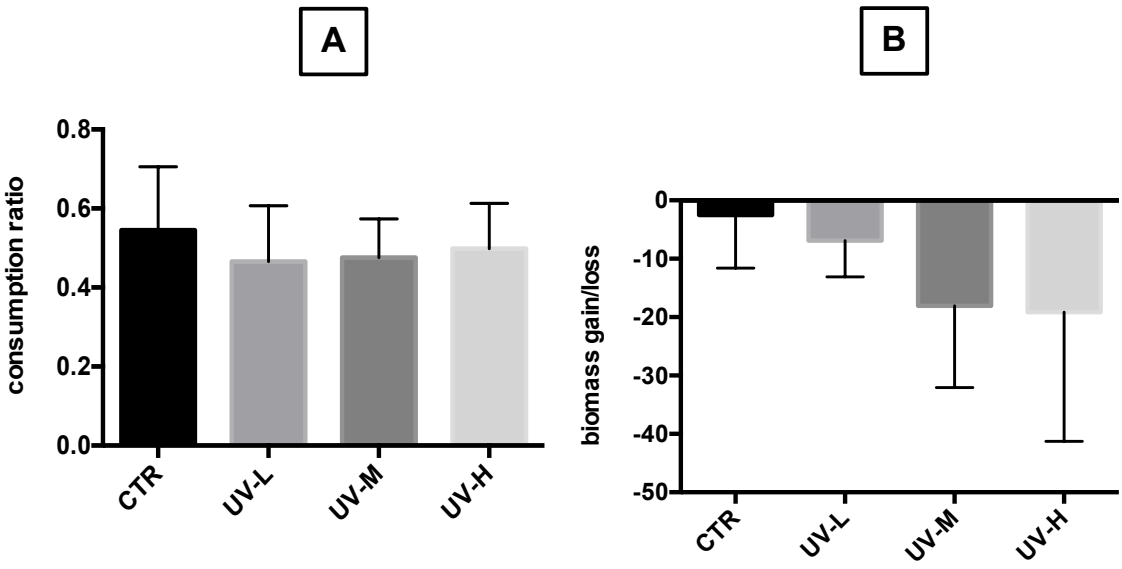

Figure 2.11 – Performance of feeding parameters of *Porcellionides pruinosus* after 14 days of exposure to different daily doses of UV radiation in LUFA 2.2 soil. Data are expressed as mean (± standard error). One-way ANOVA, $p<0.05$.

2.5. Discussion

A thorough knowledge on the effects of abiotic factors on the performance of soil organisms is a critical starting point to build an understanding of the potential implications of global environmental changes on edaphic ecosystems. This is particularly so for species, or groups, known to have an uneven importance on the structure and functioning of soil ecosystems, either by playing vital roles on important functional processes, constituting fundamental links within food webs or even by changing the habitat for other organisms (Stork & Eggleton 1992; Jouquet et al. 2006; Lavelle et al. 2006). In this study, we evaluated the influence of three abiotic factors on several traits of the terrestrial isopod *P. pruinosus*, a species that fulfil most of these criteria (Paoletti & Hassall 1999).

2.5.1. Temperature

Although the widespread colonization of terrestrial environments, isopods can still be considered physiologically poorly adapted to land life when compared to other groups like insects (Sutton et al. 1980). In this way, the weight of factors such as temperature and soil moisture on terrestrial isopods' performance was not surprising since they can be directly implicated on their water balance.

In spite of the higher mortality registered on both extremes of the temperature range, none of the treatments assessed in this experiment showed to be severe enough to affect isopods' survival in a significant way. The fact that the higher and lower LT_{50}'s modelled were out of the temperature range assessed, although being very close to both extremes, was therefore not entirely unexpected since the range did not include conditions that could not be felt within the natural habitat of this species. Hence, the main question would reside on how the isopods would deal with a constant regime of such high or low temperatures, having a limited ability to avoid it. This approach obviously places a considerable emphasis on their physiological hardiness to unfavourable temperatures but, contrary to the common approach of assessing the critical thermal limits, it also leaves room to the manifestation of behavioural strategies, either individual or collective, that aim to increase their thermal tolerance (Hassall et al. 2010; Broly et al. 2013a). As stated by Castañeda et al. (2004), the maintenance of thermal homeostasis and temperature-related performance in ectotherms is the "consequence of biochemical, physiological, morphological and/or behavioural traits and strategies". Aspects such as having soil as the exposure medium, testing animals in groups, or having a higher duration can, in our

opinion, contribute to have a more realistic insight into the effects of temperature on these organisms. Nonetheless, given the scarcity of studies with a similar approach, comparisons must necessarily include works with marked procedural differences.

To our knowledge, the only 14 days study assessing the effects of temperature in terrestrial isopods was performed by Römbke et al. (2011) and also reported no differences in *P. pruinosus* survival between 20 ^{o}C and 28 ^{o}C (Römbke et al. 2011). Furthermore, similar lethal temperatures were also reported by Edney (1964a) for other three isopod species (*Porcellio scaber*, *Porcellio laevis*, and *Armadillidium vulgare*), notwithstanding the differences between both studies. Contrary to ours, in this work isopods were individually assessed for their thermal tolerance during 30 minutes, and not in soil. *P. scaber* and *P. laevis* exhibited lethal temperatures of 38 ^{o}C and 38.5 ^{o}C, respectively, whereas in *A. vulgare* it was showed to be about 40 ^{o}C (Edney 1964a). It must be said, however, that this lower LT_{50} found in our study for *P. pruinosus* does not necessarily reflect a truly lower tolerance since it may instead be the consequence of the above mentioned differences between experimental set ups. This seems to be supported by the results of Römbke et al. (2011) in which *P. scaber* showed higher vulnerability to higher temperatures than *P. pruinosus*. Likewise, Quinlan and Hadley (1983) also showed the rate of water loss of *P. pruinosus* and *P. laevis* to be similar at 40 ^{o}C, but higher for *P. laevis* below this temperature and higher for *P. pruinosus* above. One can, therefore, suggest that for conditions known to be tolerable for these species (necessarily below the lethal temperature), *P. pruinosus* must be more resilient than *P. laevis*. By the opposite, *A. vulgare* is acknowledgedly the most xeric so having a higher lethal temperature confers no surprise (Carefoot 1993). Nonetheless, more than the species-specific difference in thermal tolerance, which is plausible in light of each one's habitat preferences, the most important thing to note is probably the short gap found between studies, even though the duration of the exposure was extensively different. This suggests that isopods can survive at conditions very close to their thermal limit, but once this limit is reached, their condition deteriorates very quickly.

Isopods' response to elevated temperatures is intimately associated to behaviours of water management, thus being highly dependent on the moisture of the environment. In fact, a clear distinction between direct and indirect effects of temperature (for instance, on body water content) is not straightforward for terrestrial isopods. Isopods are known to increase their evapotranspiration rates when exposed to high temperatures as a strategy to decrease the body temperature to normal levels (Edney 1951a; Edney 1951b). This

behavioural trait can, however, lead to severe dehydration/desiccation if the thermal stress is long enough (Edney 1951a; Edney 1951b). Furthermore, temperature and soil moisture are often highly correlated factors, which makes their interaction likely to occur. The physiological and behavioural mechanisms to cope with water loss will be further developed during the discussion of the soil moisture section.

The influence of high temperatures on the physiological functions of ectotherms can be felt at several levels: cellular, organism or whole-animal (Pörtner 2002; Stevens et al. 2010). First of all, by accelerating the biochemical reactions, higher temperatures are known to lead to higher metabolic rates in ectotherms, typically increasing nearly two-fold with every 10 ^{o}C (Abdel-Lateif et al. 1998; Donker et al. 1998; Lydy & Linck 2003). Such increase was already confirmed in isopod species, either by directly assessing the oxygen consumption (Hornung 1981; Salomon & Buchholz 2000; Klok et al. 2004; Stevens et al. 2010) or by mean of an increased heart rate (Edney 1964b), and is thought to be partly implicated on thermal stress. Pörtner et al. (2002) suggested an unified hypothesis for thermal limitation in ectotherms where a transition in mitochondrial functioning to an unsustainable anaerobic metabolism, prompted by oxygen insufficiency, is the underlying reason for the upper and lower limits in thermal tolerance. In a context of higher demands, such shortage in oxygen supply quickly leads to the failure of respiratory/ventilatory processes and eventually to death (Pörtner 2002). While recent works have questioned this hypothesis as a general rule for air breathing ectotherms, defending that it is unlikely in organisms such as insects with highly efficient tracheal systems, it thus seems to apply to terrestrial isopods in which the circulatory system plays a major role in oxygen delivery (Klok et al. 2004; Stevens et al. 2010).

At a cellular level, Ferreira et al. (unpublished data) found elevated temperatures to induce changes in several oxidative stress-related parameters. Namely, these authors reported significant and time-dependent inhibition of gluthathione *S*-transferases and catalase activity in *P. pruinosus* exposed to 30 ^{o}C.

Studies involving the assessment of terrestrial isopods' tolerance to cold stress are not so common and equally featured by considerable procedural differences. This must again be taken into consideration since it can lead to significantly different estimates, as shown by Stevens et al. (2010) who assessed the critical thermal limits in *P. scaber* using both visual determinations of knockdown temperature (i.e. "temperature at which animals

failed to respond to mild physical stimulation") and detection of the activity through thermo limit respirometry, reporting values as disparate as -7.3 ^{o}C and -1.0 ^{o}C, respectively. Tanaka and Udagawa (1993) assessed the cold hardiness in *P. scaber* and reported a seasonal variation in the lower LD_{50} (-1.37 ^{o}C in August and -4.58 ^{o}C in December), which is also substantially below the value derived through our experimental data. However, while the later authors calculated the LD_{50} after exposing the isopods during 24h, in our experiment the exposures lasted for 14 days. Two variables must therefore be considered. First, it seems clear that although being physiologically capable of bearing much lower temperatures, injury caused by cold may occur at temperatures well above the lower critical limit, as shown in our study. The second variable to have in mind is the acclimation temperature. Whereas the isopods in our experiment were long acclimated to 20 ^{o}C, in Tanaka and Udagawa (1993) isopods were field-collected at nearly zero temperature immediately before the experiments. The importance of acclimation on the tolerance to stress imposed by cold temperatures was also highlighted by Schuler et al. (2011) after evaluating the time required by *P. scaber* to recover from a chill inducing coma. In another study, Castañeda et al. (2004) found several populations of *P. scaber* acclimated to 21 ^{o}C to enter in a chill coma at nearly 3.5 ^{o}C which is similar to our results. Regarding the seasonal variations in tolerance, authors reported that they were concomitant with a seasonal accumulation of low molecular weight carbohydrates, that potentially conferred protection against chilling injury (Tanaka & Udagawa 1993).

The effects of temperature were also particularly noticeable on the sublethal endpoints assessed. The performance of *P. pruinosus* in every feeding parameter seemed to follow the classical right-shifted function that generally features ectotherms' response to temperature, consisting on a gradual increase of the performance along the temperature range, peaking at optimal conditions and starting thereafter to decline more abruptly (Castañeda et al. 2004; Huey & Berrigan 2001; Husain & Alikhan 1979). Since the peak observed for the maximum performance on the feeding parameters assessed was nearly 29-30 ^{o}C and our range only included one treatment above that value, one could not unequivocally confirm the abrupt decrease after the peak.

The first thing that stood out when analysing the feeding parameters for the different temperatures was the almost perfect match between the parameters assessed. This was partly surprising since isopods' assimilation of food was previously shown to be inversely related with food consumption (Paris 1963; Hassall & Rushton 1982). As stated by Hassall and Rushton (1982), "as the turn-over rate of the gut contents increases so

does the risk of failing to digest and absorb every component". These studies are however more focused on diet preferences and on how different quality food can influence isopods' feeding performance. Perhaps this sort of feeding strategies are more related with nutritionally poorer items, which is not the case of alder leaves as shown by Loureiro et al. (2006), Sousa et al. (1998) and Caseiro et al. (2000). In fact, Dudgeon et al. (1990) also suggested the ease of food assimilation to be an important factor on isopods' food selection and Loureiro et al. (2006) showed that regardless of the higher consumption found for alder, this food item was also the one showing the highest assimilation rate and efficiency. Such optimization in the feeding performance may nonetheless be partly related with higher energy demands. Interestingly, the response in our study was extremely similar to the patterns of oxygen consumption reported by Husain and Alikhan (1979) when exposing *P. scaber* to several temperatures, which suggests that more than an increased performance, it may instead be a requirement. This hypothesis is further reinforced when noticing that the temperature considered optima for the feeding performance was associated with nearly 20% mortality. In this way, 30 ^{o}C seems to be the threshold temperature at which the expected increase in energy consumption can be partly mitigated by an optimized feeding performance. Finally, to conclude this section of temperature effects on isopods' feeding parameters, it must be referred that consumptions, assimilations and egestions found in this experiment were generally slightly below the values reported by Loureiro et al. (2006) but were very similar to the values found in Silva et al. (2014).

Locomotion results provided another indication that temperature near 30 ^{o}C can hardly be considered optimum in the sense of a whole organismal fitness, despite showing an optimized feeding performance. Locomotor activity was extremely impaired after the 14 days of exposure to 30 ^{o}C and 35 ^{o}C. In fact, a similar right-shifted response was also registered for isopods' speed but the best performance was found for 20 ^{o}C – 25 ^{o}C with an abrupt decline for higher temperatures. To the best of our knowledge, no other study assessed the effects of such prolonged exposure to several temperatures on isopods' locomotor performance, nor any other soil invertebrate. Dailey et al. (2009) and Schuler et al. (2011) found a similarly shaped response but exhibiting maximum speeds at 34-35 ^{o}C. However, whereas both authors evaluated the effects of an instantaneous exposure to different temperatures, which seems to impute a particular focus on the behavioural component, in our experiment one intended to use locomotion as an indicator of organisms' fitness after prolonged exposure. This means that, although the immediate

exposure to 35 ^{o}C may result in incremental speeds, this probably does not occur for longer-term exposures where the fitness can be greatly affected. Interestingly, the shape of the response does not seem to vary but the peak seems to be moved across the range. In this way, effects such as the described here for locomotor performance or behaviour may seriously limit organisms' ability to avoid predation.

2.5.2. Soil moisture

Contrary to what is normally refered for terrestrial isopods, a considerable tolerance to dry environments was shown by *P. pruinosus* since almost no mortality was found when exposed to soil with a 20% WHC soil moisture content. It must be refered however, that the curve for isopods' tolerance to lower soil moisture environments appeared to decrease rather steeply after that value because when submitted to 10% WHC, 80% mortality was registered at the end of the experiment. As a cosmopolitan species, *P. pruinosus* seems to be capable of adapting to a wide range of conditions (Quinlan & Hadley 1983). The absence of a waxy waterproof barrier (similar to the hydrophobic lipid layer found in the cuticle of insects and arachnids) is offsetted with several other morphological, physiological and behavioural adaptations (Sutton et al. 1980; Broly et al. 2013a). The ultrastructural analysis of its integument revealed that it is surface-covered with sferical microstructures, responsible for its frosted appearence, that are thought to be involved on their tolerance to diseccation (Hadley & Hendricks 1985). Moreover, when submitted to drought events, they initially undergo a very high rate of water loss that is thereafter steadily decreased. This situation was described by Nair and Nair (1985) as a mechanism to further reduce the permeability since it leads to the shrinkage of their cuticle. These isopods are also provided of particularly effective mechanisms for actively absorbing atmospheric water vapour, which confers them some independence under environments with higher amounts of water available in this physical state than liquid sources (Wright & Machin 1993). Regarding the effects of overly humid environments, *P. pruinosus*, like most mesic/xeric isopods, is known to have a hydrophilic ventral cuticle and permeable pleopodal endopods that are responsible by problems to balance their water content (Sutton et al. 1980; Wright & Machin 1993). Since they are phisiologically unable to limit this water absorption, they usually rely on behavioral responses to avoid contact with too-wet surfaces (Wright & Machin 1993). In this way, the inability to escape caused by the limited mobility experienced during this experiment must have been the underlying reason for the significant increase in mortality found in isopods exposed to 80% WHC and particularly 90% WHC.

Despite no differences in survival were found within the range of 20% WHC to 70% WHC, isopods clearly showed to avoid the soil adjusted to 20% WHC when simultaneously provided with soil at 60% WHC. Interestingly, despite the significantly higher mortality found for 80% WHC, isopods' avoidance to this treatment did not seem as strong as for 20% WHC. This outcomes highlights the importance of isopods' behavioral patterns on dealing with dry conditions. It appears, thus, that avoiding dry environments is still a critical priority to this species, even though they are phisiologically capable to cope with such conditions. Warburg and Berkovitz (1978) evaluated the hygroreaction in *Armadillo officinalis* by exposing organisms to a low humidity range (0%-55% RH) and high humidity range (55%-96% RH) and observed positive hygroreactions for both situations, including with higher intensity of response in the latter. This is not in line with our results since *P. pruinosus* showed to avoid exceedingly moist environments as well. Besides the ecological differences between these species, it must also be noted, however, that in the above mentioned work, moisture was not adjusted through soil but instead by using different saturated solutions to control relative humidity inside petri dishes. In this way, although both studies address isopods' hygroreaction, the responses being measured vary considerably. Horowitz (1970) refered that isopods can stand exposures to elevated atmospheric humidities (saturated, 100% RH) as long as they are not in touch with the fluids. In soil, isopods can be in close contact with liquid water so they tend to avoid it when the amount is too high. Nevertheless, the reason *P. pruinosus* did not not show such a strong response for higher moistures as for lower ones is not clear because it seems to constitute an even higher physiological problem. Perhaps this stronger reaction to dry environments when compared to moist ones is grounded on evolutionary arguments since terrestrial isopods constitute a monophyletic group directly evolved from a common marine ancestral group, which probably had the limitation of water loss as one of the biggest priorities (Broly et al. 2013b). It would be interesting to continue developing this issue for instance by analysing how important can this factor be when other variables are present (i.e. other abiotic and biotic factors or even soil contaminants) in order to identify the possible trade-offs.

In this work we found no evidences supporting the influence of soil moisture on the consumption of *P. pruinosus*. In fact, isopods' consumption was rather similar between 20% WHC and 80% WHC. Only for 10% WHC consumption appeared to be reduced but that was not confirmed by statistical analysis as high lethality influenced results. This was

probably related with the direct effects of soil moisture on the health condition of the organisms, previously addressed for isopods' survival and avoidance or locomotor behaviours. Biomass variation also seemed to be independent of soil moisture. A worse performance was expected on this endpoint for organisms exposed to the most severe conditions since organisms could eventually allocate less energy to growth. Particularly on drier environments, isopods were expected to lose some weight because of the significant loss on body water content reported in other studies (Warburg 1965; Quinlan & Hadley 1983; Dailey et al. 2009).

2.5.3. Ultraviolet radiation

The present UV radiation experiment aimed at addressing several questions raised in previous works, where the effects of this stressor on *P. pruinosus* were evaluated (Morgado et al. 2013; Ferreira et al. unpublished data). First of all, while former studies always dealt with single UV exposures, just varying on its duration or recovery periods, in this experiment isopods were daily exposed to UV radiation since it became clear that a longer-term follow-up study with exposures on consecutive days would be necessary to enlighten about its effects under more realistic scenarios (Morgado et al. 2013). Furthermore, whereas those works focused on UV's infra-organismal effects, using a multiple biomarker approach, this intended to clarify the effects at higher organization levels. Although being very useful to have an insight into the pathways of damage of a stressor or to detect early signs of stress in organisms (Olsen et al. 2001; Ferreira et al. 2010), molecular-level aproaches such as biomarkers may provide limited information if not complemented with assessments at higher biological levels (Ferreira et al. 2015; Morgan et al. 1999). Hence, this work ultimately contributed to clarify the real meaning of the responses previously registered and to estimate more realistically the consequent effects expected on populations. In this sense, it seems now apparent that those sublethal responses can indeed have serious consequences in organisms' performance and eventually reduce survival. Both Morgado et al. (2013) and Ferreira et al. (unpublished data) suggested the effects of UV radiation on *P. pruinosus* to be related to at least the following three pathways: disruption of prooxidant/antioxidant balance in organisms, leading to changes in reactive oxygen species (ROS)-scavenging system; impairment in neurotransmission (impairment of acetylcholinesterase activity); and changes in energy-related parameters. However, isopods generally showed some ability to recover during the post-exposure period since significant UV-induced alterations were mostly found immediately after the exposure. Nevertheless, as shown in the present paper, isopods do

not seem to present the same plasticity when irradiated during consecutive days, even if the UV doses are considerably lower. No other studies are available for terrestrial isopods and literature is also scarce when considering terrestrial invertebrates. Cardoso et al. (2014) did not register any mortality in the collembolan *Folsomia candida* after a single UV exposure but found significant changes on the reproductive output. These authors showed UV to induce a shift on this species reproductive strategy that lead to an increased reproduction effort (Cardoso et al. 2014). Beresford et al. (2013) found a significant mortality when submited the same collembolan species to continuous UV radiation but contrary to the previous work, in this situation, the exposure took place in agar, which considering an eudaphic and umpigmented organism can be highly relevant. In fact, the environmental medium of exposure seems to be a chief factor influencing the susceptibility of organisms to UV radiation, even for surface dwellers like *P. pruinosus* (Morgado et al. 2013). In this regard, it must be pinpointed that *P. pruinosus* was not only irradiated in soil, which is *per se* a protective medium, but also had in alder leaves a shelter option. In another UV-supplementation study with daily exposures, Leinaas (2002) found different vulnerabilities to UV within an assemblage of Arctic collembolan species and also reported delayed effects of organisms' survival that were dose-related. Finally, Chuang et al. (2006) showed earthworms to be substantially more vulnerable to UV radiation than any of the aforementioned species, since short exposures to lower UV doses resulted in high mortalities.

Perhaps one of the most interesting findings of the previous experiments using molecular approaches was the effect of UV radiation on the acetylcholinesterase activity (Morgado et al. 2013; Ferreira et al. unpublished data). UV radiation had long been referred to induce oxidative stress to living organisms (Nichols & Katiyar 2010; Lesser et al. 2001; Sinha & Häder 2002) but effects on neurotransmission had rarely been addressed (Souza et al. 2010) and never in terrestrial organisms. Nevertheless, this outcome may have particular relevance on the results observed in this experiment for locomotor performance and feeding parameters. Acetylcholinesterase is an enzyme found in cholinergic synapses and neuromuscular junctions that is able to dissociate the neurotransmiter acetylcholine from cholinergic receptors, therefore regulating their activity (Soreq & Seidman 2001). Its inhibition may lead to an overstimulation of cholinergic receptors (Grisaru et al. 1999; Soreq & Seidman 2001) which, considering the wide distribution of this system, can entail severe symptoms of hyperexcitation, respiratory problems, loss of neuromotor faculties and eventually death (Roex et al. 2003; Aluigi et al. 2005). Several authors managed to identify associations between the inhibition of

acetylcholinesterase activity and weak performances on several traits in a wide range of organisms, including terrestrial isopods (Hart 1993; Pan & Dutta 1998; Engenheiro et al. 2005; García-de la Parra et al. 2006). Although a previous experiment showed acetylcholinesterase activity to recover quickly when the UV radiation exposures ceased (Morgado et al. 2013), that situation seems to be unlikely in the present experiment since exposures took place in a daily base. It must therefore be hypothesized that this enzyme played at least an important role on the poor performance observed on exposed isopods. Nevertheless, it was previously stressed by several authors that oxidative stress can also assume a preponderant role on most life-history traits, since it can act as a mediator of important life-history trade-offs (Monaghan et al. 2009). A higher investment on oxidative stress repairing systems such as enzymatic or non-enzymatic antioxidants often implies the dedication of resources that were supposed to be allocated to other traits, therefore decreasing overall organisms' performance (Monaghan et al. 2009). In the short-term, such trade-offs may well have impacts on the feeding activity, growth, or locomotor performance as found in our study.

2.5.4. General conclusions

In summary, our findings show that abiotic factors can indeed become an important factor influencing soil organisms. Temperature did not affect the survival but showed marked effects on sublethal endpoints. These parameters generally showed a right-shifted response featured by a gradual increase of performance until reaching a maximum value where an inflexion point occurs. Nevertheless, the maximum values were found to vary between parameters, which highlights the importance of having a broader perspective into the effects of a stressor on the organisms. Soil moisture showed to affect isopods survival, locomotor activity and avoidance behavior, but the effects on the feeding parameters were not so clear. Likewise, UV radiation also showed to affect survival and exert strong influence of sublethal endpoints such as locomotor behariour and feeding parameters. Considering the pervasive nature of these stressors and the extensive variability and heterogeneity at which soil communities are often exposed, our results suggest that taking abiotic factors into account is critical whenever soil organisms are being used as indicators of soil health. This can have serious implications, for instance, for the environmental risk assessment procedures associated to harmful practices for soil compartment since the use of optimum environmental conditions on these exposures may underestimate the real risk to soil communities. Furthermore, in a context of global

changes, with increasing unpredictability in environmental conditions, these issues assume an even higher relevance.

2.6. References

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CHAPTER 3: Environmental- and growth stage-related differences in the susceptibility of terrestrial isopods to UV radiation

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Environmental- and growth stage-related differences in the susceptibility of terrestrial isopods to UV radiation

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3.1. Abstract

Global environmental changes are nowadays one of the most important issues affecting terrestrial ecosystems. One of its most significant expressions is the increasing ultraviolet radiation (UVR) arising from the human-induced depletion in ozone layer. Therefore, to investigate the effects of UVR on the terrestrial isopod *Porcellionides pruinosus* a multiple biomarker approach was carried out. Two experiments were performed in order to analyze the importance of the exposure environment and the growth stage on the UV-induced damages. First, adult individuals were exposed to UVR in three exposure environments (soil, soil with leaves, and plaster). Thereafter, three growth stages using soil as the exposure condition were tested. Integrated biomarker responses (IBR) suggested that UV effects were higher in plaster, and mostly related to acetylcholinesterase and glutathione-*S*-transferases activities, lipid peroxidation rates, and total energy available. The effects in soil and soil with leaves were not so clear. In the growth stages' experiment, juveniles and pre-adults were found to be more affected than adults, with the greatest differences between irradiated and non-irradiated isopods occurring in energy-related parameters. Our findings suggest that soil surface-living macrofauna may be prone to deleterious effects caused by UVR, highlighting the importance of taking the media of exposure and growth stage in account.

Keywords: ultraviolet radiation, terrestrial isopods, biomarkers, energy reserves, integrated biomarker response, growth stage

3.2. Introduction

Over the last decades, a growing awareness has emerged concerning the effects of ultraviolet radiation (UVR) in terrestrial ecosystems. The main factor contributing to this concern is the human-induced depletion of stratospheric ozone layer, that is leading to a higher amount of UVR reaching Earth's surface (Mintzer 1992). Notwithstanding the recent efforts to deal with the problem at a global scale, it is unlikely that radiation levels can return to pre-1980 values in the next decades (Liu et al. 2004; Weatherhead & Andersen 2006). These projections highlight the importance of understanding how this increment in UVR will affect terrestrial biota.

A considerable amount of work was already published concerning the effects of UVR in terrestrial organisms. Nevertheless, much of this work has been focused on plant species (Rozema et al. 1997; Jansen et al. 1998) or vertebrates, mostly in a human health perspective (Hightower 1995; Scharffetter-Kochanek et al. 2000; Ichihashi et al. 2003). Little attention has been paid to soil invertebrates since they are often assumed to be morphologically well protected and/or able to escape from high intensity radiation (Paul & Gwynn-Jones 2003; Caldwell et al. 2007). However, when analyzing the situation in a long-term perspective, organisms may be unable to cope with the cumulative effects predicted and their defense mechanisms can be overwhelmed (Kemp 1994). Indeed, several examples of UV-induced injury were already reported in soil biota and a multiplicity of physiological pathways were shown to be affected (Misra et al. 2005; Chuang et al. 2006; Ye et al. 2008; Sato et al. 2010). These effects are thought to be mostly related with the generation of reactive oxygen species (ROS), responsible for oxidative damage in biomolecules (Lesser et al. 2001; Sinha & Häder 2002; Nichols & Katiyar 2010). When irreversibly damaged, these organisms' cells may undergo apoptosis (Sato et al. 2010). Otherwise, damages can be fixed through cells' repairing mechanisms (e.g. glutathione related enzymes) (Renzing et al. 1996), which will also lead to higher energy consumption, that in other conditions would be allocated to other traits, like growth, or reproduction, possibly impairing their ecological function (Maltby 1999). In the end, such sub-lethal effects can still decrease organisms' performance and might therefore

represent strong impairments at the population level, being highly ecologically relevant (Lesser et al. 2001).

Biomarkers have been successfully used to evaluate the effects of sub-lethal levels of a wide range of stressors in an extensive number of different organisms (e.g. Vieira et al. 2008; Domingues et al. 2010; Santos et al. 2010a; Colacevich et al. 2011; Novais et al. 2011). Hence, they are widely acknowledged as a good indication of early signs of stress (Olsen et al. 2001; Ferreira et al. 2010), becoming particularly useful with stressors expected to have long-term cumulative effects, which is the case of UVR (Kemp 1994). Likewise, the measurement of the energy budget is also a valuable tool to have an insight into organisms' condition because it influences all life-history traits (De Coen & Janssen 1997). Some attempts have been done recently to develop indices that can integrate the overall results of biomarkers. One of them is the Integrated Biomarker Response (IBR) designed by Beliaeff and Burgeot (2002). Originally conceived to optimize the use of biomarkers in field studies, it is also expected to be very useful in laboratory tests. After the transformation of biomarkers' results in a general index value, they may be computed as the area of a star plot, providing an overview of the variations found within the battery of biomarkers under study (Beliaeff & Burgeot 2002).

In this work we evaluated the effects of UV radiation in *Porcellionides pruinosus*, a widely distributed terrestrial isopod that is considered a key species in edaphic ecosystems because of its involvement on decomposition and nutrient recycling processes (Loureiro et al. 2005). Moreover, it is frequently used in ecotoxicological tests, being described as a good test-species (Loureiro et al. 2002).

When assessing the effects of a stressor, one must have into account that organisms' sensitivity may be influenced by several factors, such as their surrounding environment and the growth stage. In order to analyze the relative importance of these factors, we divided our work in two experiments. First we exposed adult individuals of *P. pruinosus* to high doses of UVR in three simulated environments (soil, soil with leaves, and plaster). In the second experiment, we exposed individuals of *P. pruinosus* in three different growth stages (juveniles, pre-adults and adults) to high doses of UV radiation, using soil as an ecological relevant exposure condition.

In order to evaluate if there were differences in the susceptibility of this species to UVR that could be related to the environment surroundings or the growth stage, a battery of biomarkers and measurements of energy reserves was undertaken and plotted in an IBR index.

3.3. Material and methods

3.3.1. Test organisms and soil

The terrestrial isopod *Porcellionides pruinosus* was used as test-species. Animals were collected in a horse manure heap and kept in laboratory cultures at 20 ^{o}C (±1 ^{o}C), 16h:8h (light:dark) photoperiod, with soil adjusted to a moisture content of 60% and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Juveniles, pre-adults and adults were considered based on their weight range as 5-10 mg, 10-15 mg and 15-25 mg, respectively. Nevertheless, isopods whose weight was too close to these limits were avoided. Moulting animals or those showing any visible problem (e.g. lack of an antenna, problems in locomotion) were also not used in this study. No sex differentiation was done, but pregnant females were not used.

All tests performed in soil used the certified loamy sand soil LUFA 2.2 (LUFA Speyer). The properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), water holding capacity = 41.8 ± 3.0 (g/100g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

3.3.2. UV irradiation

Exposure to UVR took place in a room with controlled temperature and light conditions (20 ± 1 ^{o}C and 16h : 8h, light:dark). UV irradiance was supplied by a UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 313 nm and 365 nm corresponding to UV-B and UV-A respective peaks) that was placed 30 cm above the boxes containing the isopods. Isopods were simultaneously exposed to UV-A and UV-B radiation. In order to filter UV-C wavelengths, the UV lamp was covered with a clear cellulose acetate film (0.003 mm, Grafix plastics, USA). This cellulose sheet had been previously irradiated during 12h to allow the stabilization of radiation intensity passing through it. Isopods were exposed to a single irradiation event with 8h. The intensity across the radiation spectrum was measured with a spectro-radiometer connected to a monochromator and analyzed with BenWin+ software (Bentham Instruments, Reading, UK). UV-A and UV-B average peak intensities in the simulated environments' experiment were 74.46 mW/m^2 nm and 141.14 mW/m^2, respectively, and 44.61 mW/m^2 and 99.21 mW/m^2 nm in the growth stages' experiment. Since the effectiveness of damages to biological tissues varies with the wavelength, intensity values were corrected by using the weighting factors of the CIE reference action spectrum for erythema in human skin

(McKinlay & Diffey 1987). Total biologically effective doses of UVR (UVD_{eff}) used in the simulated environments' and growth stages' experiments were 18.08 kJ/m^2 and 10.3 kJ/m^2, respectively. They were calculated as follows (1), using the biologically effective UV irradiance (I_{eff}) between 280 and 400 nm and integrated it into time (2).

$$UVD_{eff}\ (\mathrm{J.\,cm^{-2}}) = \frac{I_{eff}\ (\mathrm{mW.\,cm^{-2}})\ \mathrm{x}\ time\ of\ exposure\ (\mathrm{s})}{1000} \quad (1)$$

$$\left[UVD_{eff\,(\mathrm{J.cm^{-2}})}\right]_{0h}^{8h} = \frac{UVD_{eff\,0h} - UVD_{eff\,8h}}{2} + UVD_{eff\,8h} \quad (2)$$

3.3.3. Influence of exposure environment

Isopods were selected from cultures and randomly divided into rectangular plastic boxes (14,3 cm x 9,3 cm x 4,7 cm) with three different substrates (soil, soil with leaves, and plaster), and then exposed to UV radiation. Five replicates were used for each treatment, each one consisting in a box containing twenty isopods. Boxes with the bottom covered with plaster were water saturated overnight prior to the experiment in order to provide isopods an adequate moisture level. Likewise, soil moisture was also adjusted to 60% WHC. Additional water would be added during the course of the experiment whenever necessary. A 35-40% coverage was obtained by including one alder leave on each box of the soil with leaves treatment. After the UV exposure, animals were kept for recovery in soil (60% WHC), and placed inside a climatic chamber at 20 ^{o}C (±1 ^{o}C), 16h : 8h (light:dark) photoperiod and supplied with alder leaves. An additional set of 70 unexposed organisms was kept in soil during all the experiment and used as a control. Four isopods per treatment were collected in every sampling time: immediately after the UV exposure (henceforth, T_{Exp}), and after a recovery time of 48h, 96h, and 7 days. In all situations, they were individually weighted, killed in liquid nitrogen to minimize handling-induced effects on the biomarker response and stored at -80 ^{o}C until further analysis.

3.3.4. Influence of growth stage

Isopods of three growth stages (juveniles, pre.adults and adults) were collected from cultures and placed inside circular plastic boxes (Ø 8 cm x 4,5 cm high) with soil adjusted to 60% WHC. Twenty boxes were prepared for each growth stage, each one containing 5 isopods. Ten out of these boxes prepared for each growth stage were then

submitted to 8 hours of UVR whereas the remaining were not exposed and kept as control in a chamber at 20 ºC (±1 ºC), 16h:8h (light:dark) photoperiod. After the UV-exposure, five out of the ten exposed boxes for each growth stage were sampled, along with another five controls, and the remaining were kept for recovery in the control conditions. Food was then supplied in all boxes. After seven days of recovery, the rest of the boxes (five exposed and five controls) were also sampled. In every sampling time, isopods were collected, individually weighted, killed with liquid nitrogen, and stored at -80 ºC until further analysis.

3.3.5. Biomarkers analysis

Biomarkers were analysed using the protocol described by Ferreira et al. (2010). For the lipid peroxidation (LPO), glutathione-*S*-transferases (GST) and catalase (CAT), a pool of two full-body organisms was used in each replicate. For testing acetylcholinesterase (AChE) activity an organism's head was used. A total of five replicates were obtained as final measurement of each biomarker. Organisms were sonicated (Kika Labortechnik U2005 Control^{TM}), for approximately 5 sec, using 100% amplitude, with one pulse with 1 mL of potassium phosphate buffer 0.1M, (pH 7.4) and 500 µL of potassium phosphate buffer 0.1M (pH 7.2) respectively for the pool of organisms and the head. After sonication, 150 µL of the homogenate was separated from the pool of two full-body organisms, 2.5 µL of butylated hydroxytoluene (BHT) 4% in methanol were added, and it was used as sample for LPO determination. The remaining homogenate was centrifuged at 10,000 rpm (4 ^{o}C) for 20min to obtain the post-mitochondrial supernatant (PMS). The isopod head was centrifuged at 3500 rpm (4 ºC) during 3 min to extract the enzyme to the supernatant and used as sample.

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate (Ferreira et al. 2010) by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue and BHT 4% in methanol, 500 µL trichloro acetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v), and 400 µL Tris-HCl 60 mM with diethyle-netriamine penta acetic acid (DTPA) 0.1 mM. Samples were then incubated at 100 ^{o}C in a water bath for 1h, and finally centrifuged for 5 min at 11,500 rpm (25 ^{o}C). They were kept away from light and immediately read at 535 nm. LPO is expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10^{-3} M^{-1} cm^{-1}.

Glutathione-*S*-transferases (GST) activity was determined based on the method described by Habig et al. (1974). After sonication and centrifugation, 100 µL of the PMS was mixed with 200 µL of a reaction solution. The reaction solution was a mixture of 4.95 mL K-phosphate buffer 0.1 M (pH 6.5) with 900 µL L-glutathione reduced (GSH) 10 mM, and 150 µL 1-chloro-2.4-dinitrobenzene (CDNB) 10 mM and it was measured at 340 nm. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to 1 nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6×10^{-3} M^{-1} cm^{-1}.

Catalase (CAT) activity was determined based on the method described by Claiborne (1985) and previously adapted to microplates by Ferreira et al. (2010). We mixed 15 µL of PMS with 150 µL H_2O_2 0.030 M, and 135 µL K-phosphate 0.05 M (pH 7.0) and measured the decomposition of the substrate (H_2O_2) at 240 nm. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to 1 mmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The AChE activity determination was performed according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996) as follows. In a 96 well microplate 250 mL of the reaction solution was added to 50 µL of the sample and the absorbance was read at 414 nm, after 10, 15, and 20 min. The reaction solution had 1 mL of 5.50-dithiobis-2-nitrobenzoicacid (DTNB) 10 mM solution, 1.280 mL of 0.075 M acetylthiocholine iodide solution and 28.920 mL of 0.1 M phosphate buffer. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to 1 nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36×10^{-3} M^{-1} cm^{-1}.

3.3.6. Energy reserves, available energy, energy consumption and CEA

For the energy reserves (lipids, carbohydrates and proteins), total energy available, consumed energy and cellular energy allocation (CEA) determination, one organism per replicate was sonicated (Kika Labortechnik U2005 Control^{TM}), for approximately 5 s, using 100% amplitude, with one pulse, with 1 mL of ultra-pure water. This homogenate was then divided into three microtubes, each one containing a total of 300 µL. One part was used to determine the proteins and carbohydrates fraction, another one to determine the lipids fraction and the final one to determine the energy consumption (electron transport activity – ETS).

To determine total proteins and carbohydrates content, 100 µL of 15% trichloroacetic acid (TCA) were added to the 300 µL fraction and incubated at -20 ^{o}C for 10 min. A centrifugation was then performed (3500 rpm, 10 min, 4 ^{o}C), and the supernatant was separated to be used as the carbohydrate fraction. The remaining pellet was resuspended in 625 µL sodium hydroxide (NaOH), incubated at 60 ^{o}C for 30 min, and, after being neutralised with 375 µL hydrocloric acid (HCl), it was finally used as the protein fraction. Total protein content was then determined using the Bradford's reagent, and by measuring the absorbance at 590 nm using bovine serum albumin as standard. Five replicates were used in each processing methodology. Total carbohydrate content was determined by adding 50 µL of 5% phenol and 200 µL sulphuric acid (H_2SO_4) to 50 µL of sample in a multiwell microplate, incubated for 30 min at 20 ^{o}C and then the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as J/mg org (expressed as fresh weight).

Total lipid quantification was determined by adding 500 µL chloroform (spectrofotometric grade) to the 300 µL fraction. After vortexed, 500 µL methanol (spectrofotometric grade) and 250 µL ultra-pure water were added, and centrifuged (3500 rpm, 5 min, 4 ^{o}C). The botom phase which contained the lipid extraction was used for lipid measurement. Then, 500 µL H_2SO_4 were added to 100 µL of lipid extract and it was heated for 15 min (200 ^{o}C). After cooling down, 1.5 mL of ultra-pure water were added and the total lipid content determined by measuring the absorbance at 375 nm using tripalmitin as a standard. The lipids content is expressed as J/mg org (expressed as fresh weight).

The final 300 µL fraction was used to determine the energy consumption (electron transport activity – ETS). Initially, 150 µL of a buffer of 0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100 were added to this fraction. Extract was then centrifuged at 3500 rpm during 10 min (4 ºC), and the supernantant was removed and used as sample. In a microplate, 150 µL buffered substrate solution (0.13M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH) were added to 50 µL of sample. The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490 nm for 3 min. The amount of formazan formed was calculated using a molar extinction coefficient of 15,900 M^{-1} cm^{-1}.

The different energy reserve fractions (Ea): protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17,500 mJ/mg carbohydrate,

24,000 mJ/mg protein and 39,500 mJ/mg lipid. The cellular respiration rate (Ec) was determined, using the ETS data, based on the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system. The quantity of oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 $kJ.mol^{-1}$ O_2 (Gnaiger 1983). The Ea value was calculated by integrating the change in the different energy reserve fractions over the exposure period. Similarly, the Ec value was obtained by integrating the change in energy consumption over the exposure period. The total net energy budget was then calculated as follows, where *t* is the time of the exposure from the measured sample; E_{at} is the energy available at time *t*; E_{a0} is the energy available at time 0h; E_{ct} is the energy consumption at time *t* and E_{c0} is the energy available at time 0h.

$$CEA\ (mJ/org) = \frac{\left[\left(E_{at} - E_{a0}\right)*t\right] - \left[\left(E_{ct} - E_{c0}\right)*t\right]}{2} \quad (3)$$

3.3.7. Integrated biomarkers response (IBR)

IBR calculations followed the procedure described by Beliaeff and Burgeot (2002) and were either applied by combining all data or separating biomarkers from energy-related parameters. Briefly, it started with the computation of the general means for each biomarker (*m*) and corresponding standard deviations (*s*), followed by the mean values for the several treatments inside of each biomarker (*X*). Standardization of *X* was then carried on to obtain *Y*, where

$$Y = \frac{X - m}{s} \quad (4)$$

At next, values of *Z* were computed as *Z* = -*Y*, if the negative biological effect was an inhibition, or *Z* = *Y*, if it was related with an increment. Minimum values were calculated for all treatments, transformed into their absolute number and summed to *Z* to get the scores (*S*). These scores were displayed as a star plot, with S_i and S_{i+1} being two consecutive clockwise score values (radius coordinates), and *n* the number *of* radii (parameters) used. The area (*A*) of each radius was then calculated as:

$$A_i = \frac{S_i}{2} \sin\beta\ (S_i \cos\beta + \ S_{i+1} \sin\beta) \quad (5)$$

where,

$$\beta = \arctan \frac{S_{i+1} \sin \alpha}{S_i - S_{i+1} \cos \alpha} \quad (6)$$

With $\alpha = 2\pi / n$ radius and $S_{n+1} = S_1$. Finally, IBR value, corresponding to the total area of the plot, was calculated by

$$IBR = \sum_{i=1}^{n} A_i \quad (7)$$

Regarding the biological effect considered for each biomarker, AChE, lipids, carbohydrates, proteins, Ea and CEA were all initially assumed to decrease under situations of phototoxicity. Ec can either increase or decrease depending on the intensity of the stressor, and with organisms' strategy as well. Theoretically, when submitted to some deleterious factor, organisms are obliged to expend extra energy in dealing with it. However, some organisms might simultaneously decrease the energy devoted to other physiological processes in such a way that the overall energy consumption would be less than if they were not under stress. For simplicity and coherence, IBR values were always calculated using the first rationale ($Z = -Y$). Likewise, the activity of GST and CAT following UV-exposure can also be inducted to prevent, or cope with the formation of lipid peroxides or inactivated by ROS-mediated denaturation (Iizawa et al. 1994). In this way, their kinetics must be followed through time to consider their biological effect. Finally, although an increase in LPO rates is known to have deleterious effects to the organisms' health condition, it seemed evident that the decrease that followed the exposure had been triggered by the UVR. It was therefore decided to consider it an effect and the formula $Z = -Y$ was used instead of $Z = Y$.

3.3.8. Statistical analysis

On the simulated environments experiment, one-way analysis of variance (ANOVA) was used to test differences between treatments, among each sampling time. When significant differences were detected, a Dunnet's *post-hoc* test was applied to compare each treatment against the control. On the growth stages' experiment, a non-paired *t*-test was applied to determine if there were differences in means between the UV-

exposed animals and the control ones. These comparisons were only made among the same growth stage and sampling time. After converting data into percentage to control, a *t*-test was also used to compare results of different sampling times within the same growth stage. Likewise, using data in the same format, a one-way analysis of variance (ANOVA) was applied, followed by a Tukey's *post-hoc* test, in order to compare the overall response of all growth stages. For all comparisons, significant differences were assumed if probability values were equal or higher than 95% ($p \leq 0.05$). Normality and equal variance tests were performed before these statistical tests, and if data failed on showing a normal distribution, an appropriate transformation was applied. When transformation was not possible, an unparametric Krushkal-Wallis ANOVA on ranks was used. All statistical procedures were performed using SigmaPlot 11.0 statistic pack (Systat Software, Inc., San Jose, CA, USA).

3.4. Results

No mortality was observed for adult individuals, either in the exposure environment experiment or in the growth stages' experiment. As regards to the juveniles and pre-adults, no mortality was found during the exposure. At day 7, two juveniles and two pre-adults had died in the control and one of each had also died among the UV-exposed ones.

3.4.1. Influence of the exposure environment

Average IBR indices showed to be 1.3-1.6 times higher in UV-exposed animals than in the control (Table 3.1a). If only considering the first sampling time (T_{Exp}) these differences were substantially more pronounced (i.e. almost 4 times higher in plaster and almost 3 times in soil with leaves), showing from there on a progressive improvement. In order to better identify the processes responsible for the UVR effects we also calculated separate IBR indices to biomarkers and energy-related parameters (Table 3.1b and 3.1c, respectively). When including only biomarkers, differences between exposed and control isopods were, not only more evident, but also longer lasting during the course of the experiment (Table 3.1b). On the other hand, when only looking to the energy-related

parameters, there was not possible to identify any negative effect that could be related to the UVR exposure (Table 3.1c).

At T_{Exp}, LPO was the parameter contributing the most to the differences between exposed and non-exposed treatments, along with GST, CAT, and AChE, although these last three less notoriously (Figure 3.1). At 48h of recovery, GST was the main responsible for the higher IBR values for biomarkers in exposed animals. However a higher IBR value for energy-related parameter was found in control what is influencing the overall IBR result for this sampling time (Figure 3.1). After 96h, similar IBR scores were found in all treatments with exception to soil with leaves which presented the higher IBR values, mainly caused by an increased energy consumption (Figure 3.1). At day 7, the lower IBR for energy-related parameters in UV-exposed individuals became even more noticeable while in biomarkers they generally continued to show higher values when compared to control.

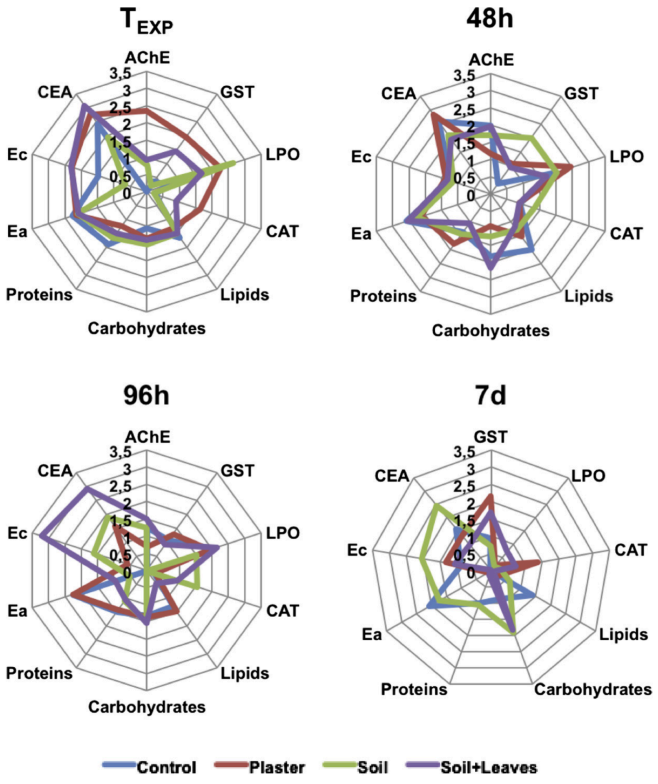

Figure 3.1 – Integrated biomarker response (IBR) star plots showing the variation in biomarkers and energy-related parameters on adult individuals of *Porcellionides pruinosus* exposed to increased ultraviolet radiation in 3 simulated environments: plaster (red lines), soil + leaves (purple lines) and soil (green lines). Blue lines show results from control.

Table 3.1 – Integrated biomarker response (IBR) mean values (± standard error) measured in *Porcellionides pruinosus* in relation to sampling time and exposure environment. (a) including all biomarkers and energy reserves used in this work; (b) including only biomarkers of exposure (AChE, GST, CAT, LPO); (c) including only energy-related parameters (Lipids, Proteins, Carbohydrates, Ea, Ec and CEA).

	Control	**Soil**	**Soil + leaves**	**Plaster**
(a)				
T_{Exp}	5.72	6.50	14.04	21.97
48 h	12.18	14.18	10.32	10.51
96 h	4.78	5.81	12.83	6.28
7 d	3.32	7.64	2.31	2.60
IBR	6.50 ± 1.96	8.53 ± 1.92	9.88 ± 1.92	10.34 ± 4.20
(b)				
T_{Exp}	0.2	0.69	3.05	8.25
48 h	2.51	6.16	3.33	3.57
96 h	2.09	2.14	3.38	2.04
7 d	0	0.12	1.06	1.28
IBR	1.2 ± 0.64	2.28 ± 1.36	2.705 ± 0.55	3.79 ± 1.56
(c)				
T_{Exp}	10.81	8.04	13.82	11.61
48 h	11.99	8.04	8.59	9.98
96 h	3.90	3.44	9.59	6.01
7 d	10.81	8.46	0.84	1.30
IBR	9.38 ± 1.85	7.00 ± 1.19	8.21 ± 2.71	7.23 ± 2.30

Figure 3.2 shows the effects of UV irradiation on each individual parameter. Significant differences in the measured biomarkers were almost exclusively found in isopods collected immediately after the exposure. The AChE activity showed a significant decrease in isopods exposed in plaster after the exposure (One-way ANOVA, $F_{3,13}$=12.876, p<0.001), showing a recovery right after that in all exposure scenarios. Regarding the GST activity, a significant increase was also detected in plaster at T_{Exp} (One-way ANOVA, $F_{3,16}$=4.021, p=0.026) and a significant inhibition was found in soil after 48h of recovery (One-way ANOVA, $F_{3,16}$=3.893, p=0.029). Values for CAT activity showed an overall similar trend to GST, although no significant differences were found. A significant decrease in LPO rate was registered at T_{Exp} in all treatments involving UV irradiation (One-way ANOVA, $F_{3,16}$=12.417, p<0.001), but no significant results were observed thereafter.

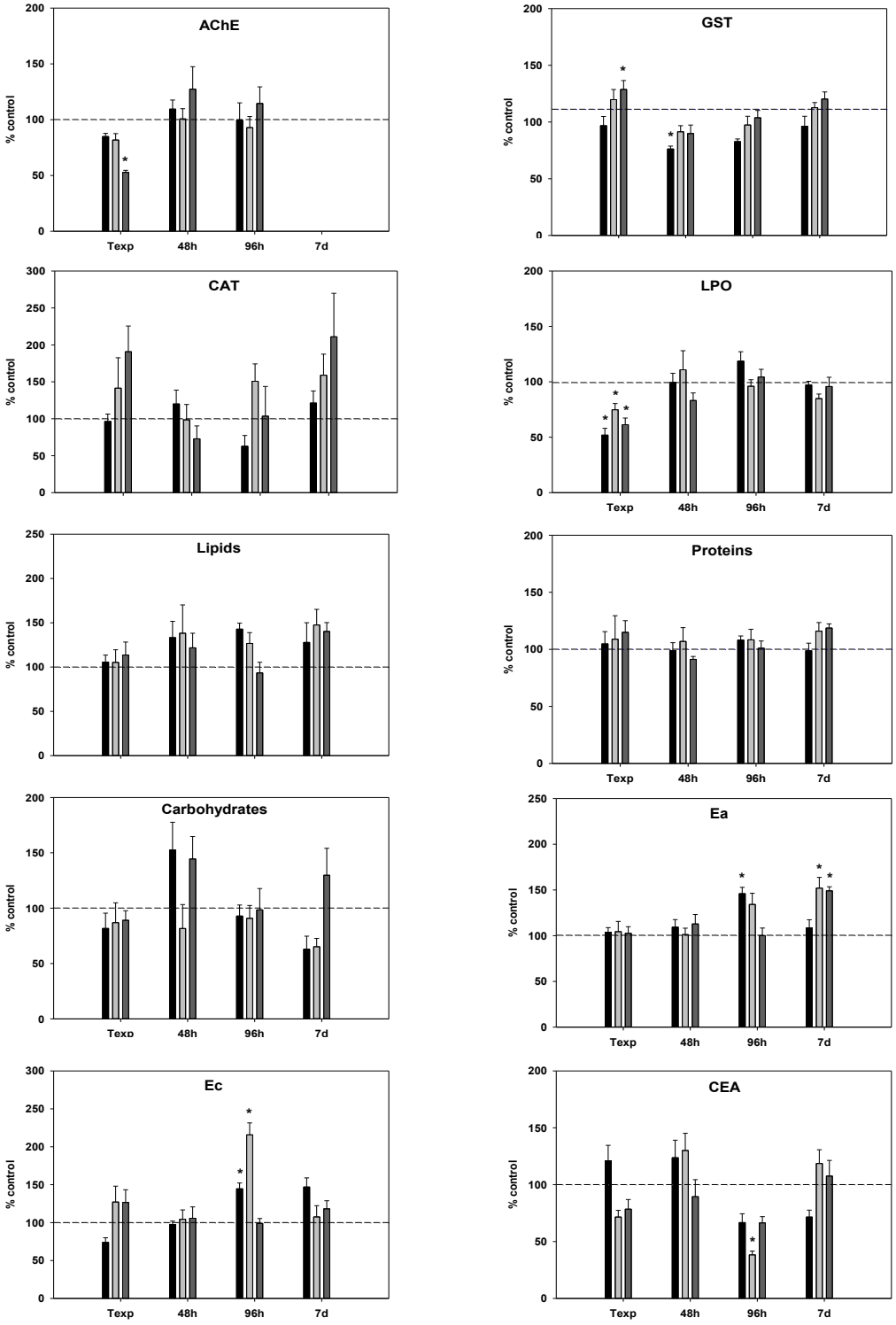

Figure 3.2 – Temporal variation in biomarkers and energy-related parameters on adult individuals of *Porcellionides pruinosus* exposed to ultraviolet radiation in 3 simulated environments: soil (black bars), soil + leaves (grey bars), and plaster (dark grey bars). All results are shown in percentage to control. Asterisks indicate significant differences to control (*t*-test, p<0,05).

Concerning energy reserves and energy budget, significant differences were only found in the last two sampling times (96h and 7d). After 96h, isopods exposed in soil showed a significant increase in total energy available (Ea) when compared against control (Krushkal-Wallis, H=9.963, df=3, p=0.019), and the same situation occurred at day 7 in soil with leaves and plaster treatments (One-way ANOVA, $F_{3,12}$=10.552, p=0.001). A similar recovery-time-dependent increase was apparently found in lipids, but no significance was observed. Carbohydrates seemed to decrease immediately after the exposure in irradiated animals, nevertheless, no consistent patterns were observed from there on. Regarding the energy consumption (Ec), significant increases were registered in soil and soil with leaves after 96h (One-way ANOVA, $F_{3,16}$=22.907, p<0.001). Finally, a significant decrease in cellular energy allocation (CEA) was also found at 96h in soil with leaves (One-way ANOVA, $F_{3,16}$=5.558, p=0.008).

3.4.2. Influence of growth stage

The IBR showed that the overall difference between control and UV-exposed animals varied across the different growth stages, with the UVR exposure showing more than 3 times higher adverse effects on juveniles and pre-adults and no noticeable effects on adults (Table 3.2).

At T_{Exp}, biomarkers responding to each growth stage were different. In LPO, for instance, effects were only visible in juveniles, whereas almost no responses were shown in pre-adults and adults (Figure 3.3). Nevertheless a response in all growth stages was observed for CAT and for carbohydrates content. These effects in carbohydrates, along with those found in the amount of lipids, were also responsible for the differences found in total available energy and CEA index of juveniles and pre-adults.

After 7 days of recovery, the observed scenario was somehow different from the after-exposure sampling with all groups showing a clear recovery in carbohydrates' (particularly prominent in juveniles and pre-adults) and lipids' content, and with juveniles and pre-adults joining adults in the decrease of energy consumption (Ec). This resulted in a recovery of these classes' CEA.

Table 3.2 - Integrated biomarker response (IBR) as mean values (± standard error) measured in UV-exposed individuals of *Porcellionides pruinosus* in relation to sampling time and growth stage. "T_{Exp}" refers to organisms sampled immediately after the exposure whereas "7d" refers to those sampled after seven days of recovery.

	Juveniles		**Pre-adults**		**Adults**	
	Control	UV	Control	UV	Control	UV
T_{Exp}	0.46	2.55	1.20	3.86	1.92	0.50
7d	0.96	2.26	0.44	1.31	0.80	0.24
IBR	0.71 ± 0.25	2.41 ± 0.15	0.82 ± 0.38	2.59 ± 1.28	1.36 ± 0.56	0.37 ± 0.13

Results for each parameter are shown on Figure 3.4. When compared with control, significant differences in AChE activity were only found in adults sampled 7 days after the UV exposure where an inhibition was observed (*t*-test, $t_{(8)}$=4.103, *p*=0.003). No significant differences were found in GST and CAT activities and LPO rates decreased in exposed juveniles, almost reaching significance at day 7 (*t*-test, $t_{(8)}$=4.103, *p*=0.051). However, a significant increase was found in exposed adults' LPO from T_{Exp} to day 7 (Mann-Whitney, *p*=0.016).

Energy reserves also seemed to be differently affected in these growth stages. When compared against control, only carbohydrates showed a decrease in pre-adults at T_{Exp} (*t*-test, $t_{(7)}$=3.964, *p*=0.006). Nevertheless, when analyzing the evolution of results in exposed individuals, a significant increase was found in lipids for pre-adults (*t*-test, $t_{(8)}$= -2.869, *p*= 0.021), and in all growth stages for carbohydrates (juveniles: *t*-test, $t_{(8)}$= -4.674, *p*= 0.002; pre-adults: *t*-test, $t_{(8)}$= -3.026, *p*= 0.016; adults: Mann-Whitney, *p*= 0.016), and significant decreases were found in energy consumption for juveniles (*t*-test, $t_{(8)}$= -3.070, *p*= 0.015) and pre-adults (*t*-test, $t_{(8)}$=3.014, *p*= 0.017).

Comparing the responses induced by UVR in the different growth stages, significant differences were registered between pre-adults and adults for AChE activity at day 7 ($F_{2,10}$ = 8.915, *p*=0.006) and between all the growth stages for LPO, also at day 7 ($F_{2,11}$ = 28.575, *p*<0.001). Regarding the energy-related parameters, significant differences were observed in lipids between adults and the remaining growth stages at T_{Exp} ($F_{2,12}$ = 7.618, *p*=0.007) and also in energy consumption ($F_{2,12}$ = 5.726, *p*=0.018). Furthermore, adults also showed differences to pre-adults in total energy available ($F_{2,12}$ = 4.948, *p*=0.027) and to juveniles in CEA index ($F_{2,12}$ = 8.862, *p*=0.004). After 7-days of recovery significant differences were only found in the CEA between adults and juveniles ($F_{2,11}$ = 6.672, *p*=0.013).

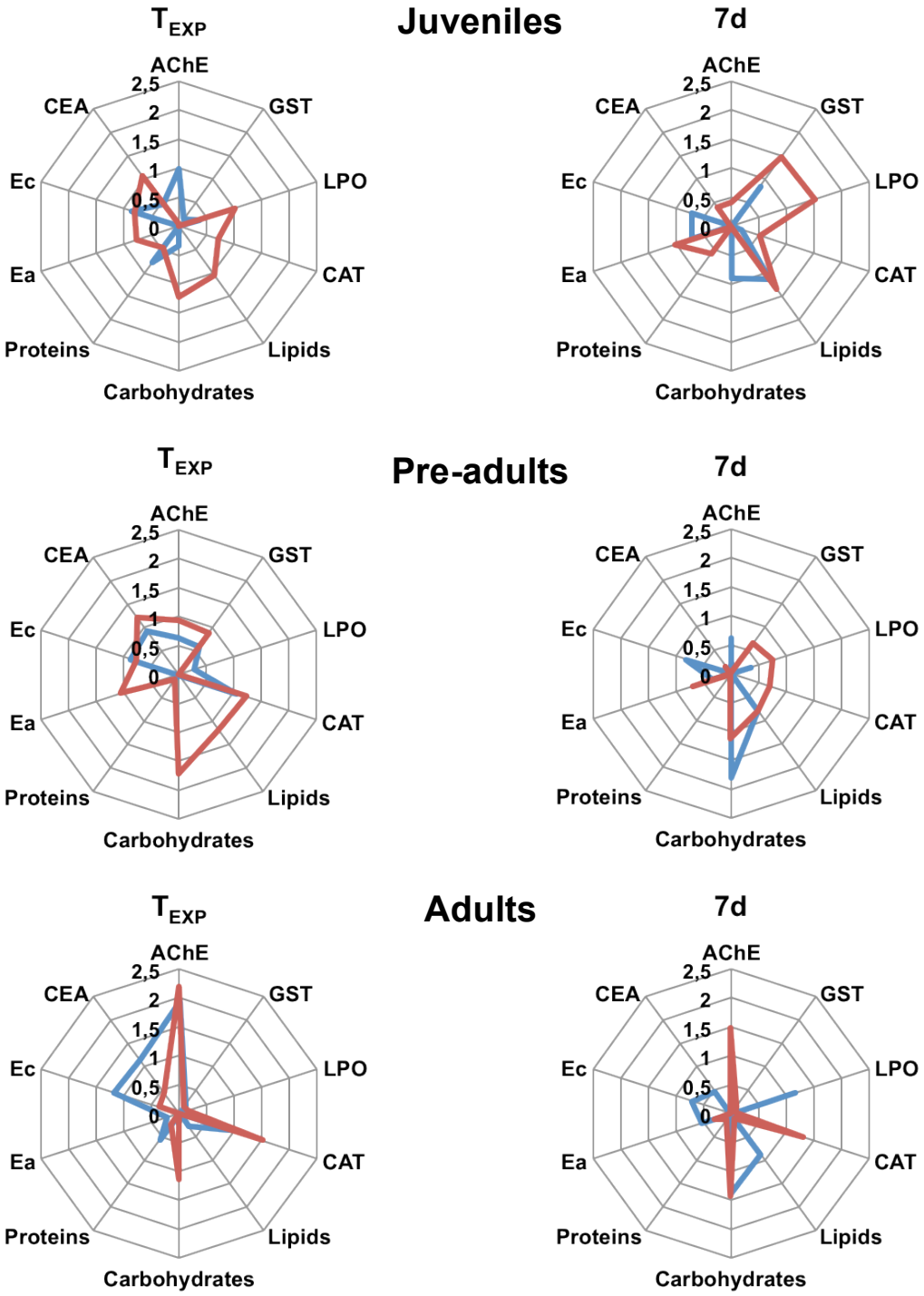

Figure 3.3 – Temporal variation of biomarkers and energy-related parameters on juveniles, pre-adults and adult individuals of the terrestrial isopod *Porcellionides pruinosus* exposed to ultraviolet radiation in soil. Black bars show results found immediately after the exposure (T_{Exp}) and grey bars show results found after 7 days of recovery. All results are shown in percentage to control. Lines over bars indicate significant differences between T_{Exp} and 7d (*t*-test, $p<0.05$). Asterisks indicate significant differences to control (*t*-test, $p<0.05$).

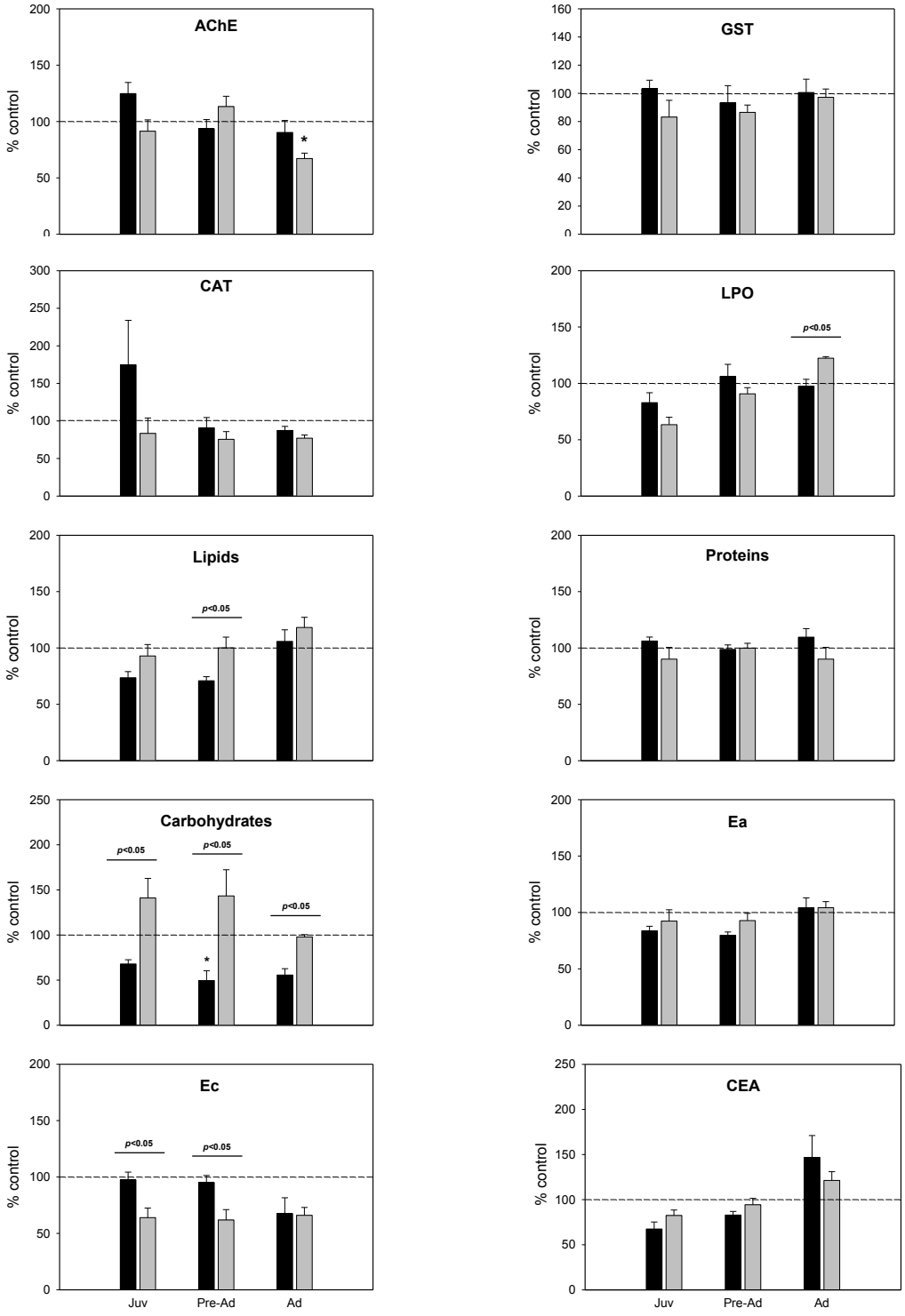

Figure 3.4 – Temporal variation of biomarkers and energy-related parameters on juveniles, pre-adults and adult individuals of the terrestrial isopod *Porcellionides pruinosus* exposed to ultraviolet radiation in soil. Black bars show results found immediately after the exposure (T_{Exp}) and grey bars show results found after 7 days of recovery. All results are shown in percentage to control. Lines over bars indicate significant differences between T_{Exp} and 7d (*t*-test, $p<0.05$). Asterisks indicate significant differences to control (*t*-test, $p<0.05$).

3.5. Discussion

In this study we investigated the effects of UVR to *P. pruinosus* when exposed in different simulated environments (plaster, soil and soil with leaves) or in different growth stages (juveniles, pre-adults and adults). The option for a multiple biomarker approach was taken in order to have an insight into the possible pathways of damage. Hence, the effects observed in biomarkers with a completely different nature are a clear demonstration of the broad spectrum of processes involved and, on the other hand as some biomarkers are process related (e.g. GST and CAT) this can also integrate enzymatic reactions straightly related. Moreover, the integration of all these parameters on a single index allowed us to identify different patterns of susceptibility arising from the exposure to UVR in different environments or by different growth stages.

An overview in findings regarding the influence of exposure environment showed that isopods exposed in plaster were the most affected by UVR, followed by those exposed in soil with leaves and soil, respectively. This suggests that environmental media can partly mitigate the effects of UVR in *P. pruinosus*. Isopods' ability to avoid several types of adverse conditions was already described in literature (Loureiro et al. 2005; Hassall & Tuck 2007; Santos et al. 2010b). Thus, *P. pruinosus* can probably take advantage of the heterogeneity provided by soil as a rather complex compartment, by seeking for refuge in less exposed sites, either on the surface or in the very first centimeters of soil column. However, more important than identifying the most severe treatments, since they obviously provide different shelter possibilities, it is the identification of the underlying mechanisms behind each stress situation that needs to be addressed. In this way, IBR values also showed that biomarkers (mainly those related to oxidative stress like GST, CAT or LPO) were the parameters that consistently presented higher differences to control throughout the study period in all exposure scenarios. By the opposite, when only including the energy-related parameters, the average IBR values on exposed organisms were always lower than control. In relation to the exposure performed with soil with leaves, lower or similar effect levels would be expected when compared to soil given this species' negative phototaxis and the aforementioned ability for seeking shelter. However, this assumption was not confirmed and, by the opposite, higher IBR scores were generally found in this treatment indicating a higher stress to these organisms. One hypothesis is that the addition of leaves did not totally prevent isopods to be exposed to UVR, but instead resulted in a higher exposure. For instance, alder leaves

may have influenced their sheltering behavior by also providing a food source, thus making them more prone to be exposed while feeding.

In the other experiment, younger animals generally showed less resistance to UV than adults. Terrestrial isopods' normal survivorship is known to have a very high death rate of new born individuals and a lower mortality in adults (Paris 1963; Al-Dabbagh & Block 1981). Therefore, this size-dependent susceptibility was also anticipated when exposed to UVR. Unlike the simulated environments' experiment, the greatest differences in this assay were not found in oxidative stress biomarkers, in which responses had the same intensity, but in energy-related parameters where two patterns could be found. Adults showed to be more resilient to the depletion of energy reserves and seemed to have an effective response to this stress event. On the contrary, a considerable parallelism could be found in the physiological response of juveniles and pre-adults, whose reserves were extensively affected. In fact, the overall comparison of the three growth stages' response to UVR confirmed these relationships by showing adults to be significantly different from the remaining groups. Since pre-adults present intermediate features between both the other groups, their position so close to juveniles is somewhat surprising and seems to emphasize the costs of development that are common to both classes. This is therefore an important point to have in mind when selecting the most adequate growth- or age-classes to use in similar studies. It should also be stressed that the young isopods generally referred as mancas were not used in this experiment. Contrary to those, the isopods considered to be juveniles in these experiments had already a well-developed exoskeleton pigmentation. Nonetheless, adult terrestrial isopods still generally have a thicker and darker exoskeleton when compared to younger ones, so they can be expected to have lower sensitivity to UV radiation. On the contrary, smaller isopods could theoretically take better advantage of soil for shelter purposes but it did not seem enough to counteract their higher intrinsic sensitivity.

After identifying the overall patterns, one must have a more detailed insight into the effects of UVR on each individual parameter. UVR is best-known for being an effective prooxidant agent, strongly inducing the production of ROS (Lesser et al. 2001; Sinha & Häder 2002; Nichols & Katiyar 2010). This results from the incomplete reduction of oxygen and might react with key macromolecules, such as lipids, causing cellular damage (D'Autréaux & Toledano 2007). In this way, one would expect that UV irradiated isopods would show signs of lipid peroxidation but, surprisingly, a decrease on their LPO rates was the general rule immediately after exposure. The rational for this is not clear, and to our knowledge, there is no other similar situation reported on literature. However, the lack

of similar studies using terrestrial isopods, nor terrestrial arthropods, constitutes an additional constraint and entails a careful analysis of these results. As previously stated, most of the research done with UVR refers to vertebrates, using both *in situ* tests or cultured cells, and an increase in lipid peroxidation seems generally well established after acute UV irradiation (Jurkiewicz & Buettnerf 1996; Yuen & Halliday 1997; Flamarique et al. 2000). Regarding invertebrates, however, few studies have been carried out, and results do not seem to be so conclusive. For instance, Gouveia et al. (2005) exposed both intact or eyestalkless individuals of the estuarine crab *Chasmagnathus granulate* and found no significant differences for LPO, nor any increasing trend. Using another crab species, Vargas et al. (2010) exposed individuals acclimatized to 3 different photoperiods and, contrary to those kept at constant light or dark, no differences were found in eyestalks of crabs acclimatized to natural light regime when compared against control. Photoperiod is indeed, a factor that one must have in mind because, besides influencing cells' susceptibility to oxidative stress (Vargas et al. 2010), it may also affect the recovery (Sato et al. 2010; Ribeiro et al. 2011). Nevertheless most of the UV-exposed organisms in our study showed significantly lower LPO rates at T_{Exp} than control ones, suggesting that it might be an UVR effect. In the growth stages' experiment, this was not so noticeable, except for juveniles, but perhaps this difference can be attributed to the different UV doses used and only the most sensitive organisms were affected. Thus, it is possible that, with higher UV doses, lipids may undergo some biochemical mechanisms that leads to the removal and/or change of their structure, making it difficult with this method, to visualize differences (Davies 2000). For instance, the removal and replacing of peroxidized lipids from the membrane is known to help preventing further propagation reactions (Davies 2000). Another possibility is that UVR can trigger some defense mechanisms, such as the production of antioxidants, whose action masked this method results. If this was the case, the stress induced by the UVR was not sufficient to induce oxidative stress, but it still induced the regular functioning of protections towards ROS production. Iizawa et al. (1994) reported the existence of a complex relationship between lipid peroxides and induction of ROS scavenging enzymes. This actually seems consistent with the activity of both CAT and GST in the simulated environments' experiment. CAT is an enzyme that removes or degrades the hydrogen peroxide (H_2O_2) (Halliwell 1974), whereas GST is a multifunctional enzyme that, despite not being directly involved in ROS scavenging, can play an important role against oxidative stress by acting as a thioltransferase-like redox regulator (Carbone et al. 2003; Terada 2005). In the growth stage experiment, this could not be clearly seen, nevertheless, it seems evident that oxidative stress was, actually,

taking place, at least in the most exposed or those considered to be the most sensitive. Some authors have defended that the best way to assess organisms' susceptibility to oxidative stress is by evaluating the total antioxidant capacity, instead of only measuring a limited number of antioxidants (Amado et al. 2009). Organisms are provided with a complex antioxidant protection system that relies on both enzymatic and non-enzymatic compounds acting synergistically against the several free radicals (Kono & Fridovich 1982; Rikans & Hornbrook 1997). This system is normally in equilibrium with the endogenous production of ROS, but when submitted to some prooxidant agent, an imbalance may occur (Sies 1997). In this work, we opted for a holistic approach to the effects of UV in *P. pruinosus*, rather than exclusively an oxidative-stress research. In this way, we selected some of the most usual biomarkers representing each process. In a further work, it would be interesting to compare the variations of total antioxidant capacity with those obtained in our study for LPO, GST and CAT.

Neurotransmission also seemed to be affected in UV-irradiated isopods by significantly inhibiting AChE activity, being the most severe damages observed in the simulation of the unprotected exposure environment (plaster). Nevertheless, this treatment was also where the highest reactivation took place at 48h, but it may not necessarily entail a true organismal recovery. Abnormal accumulations of AChE were already reported after situations of its severe inhibition and they were mainly attributed to a feedback mechanism consisting of c-Fos mediated transcriptional responses (Kaufer et al. 1999b). Maybe the hyperexcitation of the cholinergic receptors (that probably occurred at T_{Exp}) could have induced a cascade of reactions responsible for the simultaneous downregulation of Acetylthiocholine (Ach) production and enhancement in AChE expression (Grisaru et al. 1999; Kaufer et al. 1999a; Kaufer et al. 1999b). Although hyperexcitation can be immediately reduced, these processes can result in an overexpression of AChE and were already associated to delayed problems in neuromotor faculties (Grisaru et al. 1999). Another possibility is that the reactivation observed at 48h in the first experiment would just represent a "transient recovery" as reported by Souza et al. (2010) after the UV-irradiation of two lacustrine copepods. Unfortunately, a problem occurred with AChE samples of day 7 in the exposure environments experiment, and they couldn't be used. This is, a highly relevant issue given the central role of this enzyme (Soreq & Seidman 2001) so the time-lapsed until full recovery, when it is possible, must be worth of concern. This question needs to be addressed in a further study. Of interest was also the fact that AChE activity was apparently more affected in adults than in younger animals. The opposite is generally established regarding stressors that target the

AChE activity, mainly pesticides. Stanek et al. (2006), for instance, reported that after acute exposure of *Porcellio scaber* to diazinon, the lowest observed effect concentration inhibiting AChE activity was lower for juveniles than adults. However, it has also been reported that results can be quite variable, even among compounds belonging to the same chemical family, like organophosphorus pesticides (OP). Moser (1999) found no differences between young and adult rats when exposed to methamidophos. On the other hand, Pope and Liu (1997) reported a "remarkably faster" recovery of AChE activity in OP-exposed young rats' brain when compared to adults. This reference to differences related with the stressors' nature seems to be particularly important for this study. To our knowledge, there is no other work with age-related differences in AChE activity after exposure to UVR, and contrary to OPs whose AChE inhibition is the main target, the way that UVR affects this enzyme *in vivo* is not so clearly understood. It may be a consequence of the direct incidence of UVR that causes its denaturation as reported by Bishop et al. (1980), an indirect effect of the long exposure to UVR by inducing some kind of psychological stress to the organisms (Grisaru et al. 1999; Meshorer & Soreq 2006), or more probably a combination of causes.

Some interesting results were also found when analyzing energy-related parameters. In overall, they highlight the lower susceptibility of adults' energy reserves when compared to juveniles or pre-adults. Regarding the simulated environments' experiment, results were not so clear, but some common trends could be found. First of all, and without surprise, carbohydrates were always the first energy reserve to be depleted after exposure, confirming their importance as a first-order response to stress events and the first type of energy reserve to be consumed. Second, proteins seemed not to be greatly affected after the exposure. Finally, a hormetic-like, and recovery-time-dependent, response seemed to occur in lipids content of UV-exposed adult isopods. In fact, in irradiated adults, lipids exceeded control values on both experiments, and regardless of the treatment. This situation was not observed in younger isopods in whose exposure seemed to have negatively affected lipids. Some similar results were previously reported by Ribeiro et al. (2011) regarding carbohydrates and proteins but the hormetic-like response in lipids was not found in that study. Several hypotheses can be advanced to explain such response. The first one is that isopods changed their feeding behavior to face this stressor, and this could have occurred by increasing the intake of food, altering processes like assimilation or egestion, or even by increasing coprophagy (Hassall & Rushton 1982). Another option, but not necessarily alternative, lies on the decrease in energy consumption. Such plasticity in the expression of metabolism would allow isopods

to compensate the amount of energy expended in dealing with a stressor (Brown et al. 2004) and it was already identified in other soil invertebrates (Testerink 1983). Only adults seemed to have had the ability to use this strategy immediately after the exposure, whereas juveniles and pre-adults only had their rates decreased at day 7. For these reasons, cellular energy allocation in exposed isopods only seemed to be higher than control in adults. Saying that, one cannot obviously state, however, that irradiation had a positive effect on adults' energy budget but that adults can balance their budget under this kind of exposure. In a mechanistic perspective, metabolic rate is responsible for controlling ecological processes at all ecological levels (Brown et al. 2004). By lowering its values to cope with a certain event, organisms might be impairing other fitness-enhancing processes like feeding, growth or reproduction and consequently affecting the biologically-regulated flux of energy at population or ecosystem levels (Testerink 1983).

In this work, we only assessed the effects of UVR at infra-organismal levels so an interesting next step would be to complement our parameters with the evaluation of some of the above-mentioned processes. For instance, severe impairments were already found by Ribeiro et al. (2011) on the feeding rate, reproduction and body length of *Daphnia magna* exposed to UV. Linkage to higher-level parameters assume particular significance in organisms that, like isopods, can exert a strong influence on ecosystems' processes with their regular activity. In addition, one would suggest the assessment of a chronic exposure with repeated pulses, on consecutive days or in a periodic basis. In this work we opted for an acute exposure with a high dose of UVR so that we could clearly identify the various insults related to this source of stress. However, long-term follow-up studies with regular exposure to UVR have already proved their importance in mice particularly when considering the evolution of antioxidant defense mechanisms (Iizawa et al. 1994). Finally, the likelihood and nature of its interaction with other ubiquitous stressors is another field that needs to be studied in soil organisms.

Despite further work is needed to answer some questions raised in the meantime, our findings put in evidence that soil biota, and terrestrial isopods in particular, can also be affected by the increasing UVR. Moreover, they strongly suggest the influence of the exposure conditions and growth stage as mediators of organisms' susceptibility to this stressor. Whereas differences between simulated environments' were mainly related with biomarkers of exposure (AChE, GST and LPO), in the growth stages' assessment, energy-related parameters were the most differentiating factors. Hence, one would recommend the inclusion of these variables in similar studies, in order to improve the strength of risk assessment predictions and also to identify some other mechanistic

detoxification processes that may not be the most common pathways, but are known to exist since energy depletion is observed.

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CHAPTER 4: Joint toxicity of chlorpyrifos and mancozeb to *Porcellionides pruinosus*: a multiple biomarker approach

Joint toxicity of chlorpyrifos and mancozeb to *Porcellionides pruinosus*: a multiple biomarker approach

4.1. Abstract

The increasing concerns with the safeguarding of crop productivity led pesticides to become a critical tool in modern intensive agriculture regimes worldwide. However, pesticides are also known to entail deleterious effects to non-target organisms and these are often simultaneously exposed to multiple compounds. Since mixtures' effects have been shown not to necessarily reflect the toxicity of its components and even the simple addition of effects may lead to consequences not clearly anticipated, a thorough understanding of the underlying mechanisms of toxicity during exposures to multiple compounds becomes critical to predict possible effects associated to the use of these compounds in agriculture fields. Aiming to comply with this goal, in this work we evaluated the age-related susceptibility differences on the terrestrial isopod *Porcellionides pruinosus*, when exposed to binary mixtures of chlorpyrifos and mancozeb. In order to have an insight into the several pathways of toxicity prompted by this mixture, a multiple biomarker approach was employed in juveniles and adult organisms, as well as the measurement of energy reserves and the assessment of cellular energy allocation. Results showed impairments on detoxification mechanisms and oxidative stress enzymes, along with shifts in behaviour observed by the increase/decrease of energy consumption rates and energy reserves content. It was also possible to observe distinctive behaviours of stress handling by adults and juveniles.

Keywords: pesticide mixtures, terrestrial isopods, biomarkers, cellular energy allocation, oxidative stress, neurotransmission

4.2. Introduction

Agriculture is nowadays a highly optimized process, that strongly relies on the application of multiple agrochemicals to reduce losses and increase yield production (Matson et al. 1997; Carvalho 2006). Pesticides, in particular, constitute a rapid, effective and economical mean of controlling crop pests and pathogens and have been largely responsible for the steady increase in productivity and cost-effectiveness experienced by this activity (Matson 1998; Aktar et al. 2009). Nevertheless, although being admittedly important, these compounds are also known to pose serious problems to non-target organisms that inhabit agroecosystems, so they must be thoroughly evaluated (Carvalho 2006; Santos et al. 2010b; Santos et al. 2011). The traditional approach for assessing the environmental risks associated to the use of pesticides consists mainly on standard laboratory assays, where model species are exposed to a range of concentrations of a single test compound, allowing the estimation of acceptable threshold values that entail no risk to soil ecosystems (Matson 1998; Arapis et al. 2006; Aktar et al. 2009). Nevertheless, given the requirement of acting on different kind of pests and pathogens, non-target organisms can be often simultaneously exposed to several pesticides (Lydy et al. 2004). Since the effects of pesticide mixtures were previously shown not to necessarily reflect the individual toxicity of its components (Lydy et al. 2004), a growing awareness has emerged regarding the interactions between pesticides. Moreover, the mere addition of effects of co-occurring pesticides is often disregarded by these standard procedures, which may in the worst case scenario lead to underestimations of the environmental risk (Pape-Lindstrom & Lydy 1997; Belden & Lydy 2006). Despite the higher attention lately received by mixture toxicity research, soon became clear that the complexity and specific character of these interactions would constitute an important constraint to ecotoxicologists and risk assessors. Particularly important seems to be the comprehension of the mechanisms by which toxicity is induced during exposures to mixtures, how they can differ from the single pesticides, and how they can be accurately predicted in a cost-effective way. In a context of continuous development of new agrochemicals, only knowing these mechanisms will enable an accurate generalisation regarding the behaviour of one chemical within several chemical mixtures.

Aiming to contribute for further knowledge, in this work, the joint toxicity of two pesticides, chlorpyrifos (CPF) and mancozeb (MCZ), to the terrestrial isopod *Porcellionides pruinosus* was evaluated. In particular, we aimed at analysing the age-

related differences on the susceptibility of this species to the joint effects of these pesticides using a multiple biomarker approach.

Biomarkers were described by van Gestel and van Brummelen (1996) as “any biological response to a xenobiotic at the below- individual level, measured inside an organism or in its products”. They have long been suggested to provide a good indication of early signs of exposure to xenobiotics (Depledge & Fossi 1994; van Dam et al. 1998; Morgan et al. 1999), and widely used to identify and evaluate the effects of sub-lethal exposures to pesticides in an extensive number of different organisms (Booth & O'Halloran 2001; Booth et al. 2003; Santos et al. 2010a; Pereira et al. 2013). Of utmost relevance is also the assessment of energy-related parameters such as the energy reserves content, energy consumption or the cellular energy allocation. The rates at which organisms assimilate or allocate energy constitute an accurate indication of their condition under any circumstances (De Coen & Janssen 1997). Furthermore, since energy is the common ground linking all organisms within an ecosystem, energy-related parameters can also assume primordial relevance at multiple organizational levels (Brown et al. 2004). In this way, biomarkers and energy-related parameters may be important tools in future integrated approaches of assessing the health of the environment since they can provide the predictive capability required when dealing with substances of long-term cumulative effects (Moore et al. 2004).

CPF is an organophosphate (OP) insecticide, used to control outbreaks of Coleoptera, Diptera, Homoptera and Lepidoptera, both in soil or foliage, having as main mode of action the inhibition of acetylcholinesterase (Fukuto 1990; Schreck et al. 2008). This inhibition leads to the synaptic over-accumulation of acetylcholine, a major neurotransmitter in the nervous systems of vertebrates and invertebrates, and consequently to an overstimulation of cholinergic receptors, ultimately resulting on the disruption of nervous system function (Mileson et al. 1998). MCZ is a dithiocarbamate fungicide, classified by the Fungicide Resistance Action Committee as a multi-site action compound (Gullino et al. 2010), that is frequently applied against a wide spectrum of fungal diseases (Cycoń et al. 2010). Actually, MCZ must be considered as a pro-fungicide since it is not fungicidal itself. It breaks down quickly, when exposed to water, to release ethylene bisisothiocyanate sulfide (EBIS), which is further converted into ethylene bisisothiocyanate (EBI). These metabolites are both active toxicants, thought to interfere with fungi enzymes containing sulphydryl groups (Gullino et al. 2010). Moreover, it is also known to release Mn^{2+} and Zn^{2+} that are chelated within its molecular structure (Atamaniuk et al. 2013; Houeto et al. 1995; Hwang et al. 2003). Some authors suggested

MCZ to have neurodegenerative effects, mainly at dopaminergic and glutamatergic receptors (Negga et al. 2012; Brody et al. 2013). Moreover, MCZ is also thought to inhibit the activity of cytochrome P450, thereby limiting the ability of organisms to detoxify (Lewerenz & Plass 1984; Szépvölgyi et al. 1989). Both these pesticides are extensively used in several crops, like horticulture, vineyards and orchards, and their application frequently happens to be simultaneous (Cross & Berrie 1996).

The synantropic nature and wide distribution of several terrestrial isopod species, like *P. pruinosus*, make them particularly prone to be exposed to human-induced stressors, like chemical contaminants. Alongside with its ecological importance (Loureiro et al. 2002), this factor has contributed for considerable attention among the research community, that is frequently using this species in soil ecotoxicology experiments (Sousa et al. 2000; Loureiro et al. 2002; Jänsch et al. 2005; Ferreira et al. 2010; Santos et al. 2010b; Morgado et al. 2013; Tourinho et al. 2013; Silva et al. 2014).

In order to have an insight into the several pathways of toxicity prompted by this mixture on terrestrial isopods, a battery of biomarkers, energy reserves and energy allocation measurements were undertaken using both adult individuals and juveniles.

4.3. Material and methods

4.3.1. Test organism

The terrestrial isopod *Porcellionides pruinosus* was used as test-species. Isopods were collected in a horse manure heap and maintained in laboratory cultures at 22 ^{o}C (±1 ^{o}C), 16:8 h (light:dark) photoperiod, garden soil at 40%-60% of its water holding capacity (WHC) and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Different growth stages were selected according to their weight, with the adults considered to have between 15-25 mg and juveniles between 5-10 mg. Nevertheless, isopods whose weight was too close to these limits were avoided. No gender differentiation was considered, but moulting isopods and pregnant females were not used in this experiment.

4.3.2. Chemical compounds and soil

Two commercial formulations were used as soil contaminants in this experiment: one formulation whose main active principle was the OP insecticide chlorpyrifos (CICLONE® 48 EC with 480 g/L of chlorpyrifos). The second commercial formulation was mainly composed by the dithiocarbamate fungicide mancozeb (MANCOZEBE SAPEC® with 80% of mancozeb).

The certified loamy sand soil LUFA 2.2 (Speyer, Germany) was used as test soil. The main properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), WHC = 41.8 ± 3.0 (g/100g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

4.3.3. Experimental design

Chemical treatments were selected according to the application rate recommended by the manufacturer, ranging from the field dose (FD) to 10 times the FD, for each commercial formulation. For CPF, the nominal concentrations included 8.72 µg a.i./kg soil (FD), 43.64 µg a.i./kg soil (5FD), and 87.28 µg a.i./kg soil (10FD). For MCZ, nominal concentrations included 15.91 mg a.i./kg soil (FD), 79.55 mg a.i./kg soil (5FD), and 159.1 mg a.i./kg soil (10FD). An additional set of organisms was also kept in clean soil adjusted to 60% of WHC with distilled water and was used as control. Mixture treatments were performed as shown in Figure 4.1, ranging from 1CPF/1MCZ to 10CPF/10MCZ. This experimental design was employed twice, for adults and for juveniles.

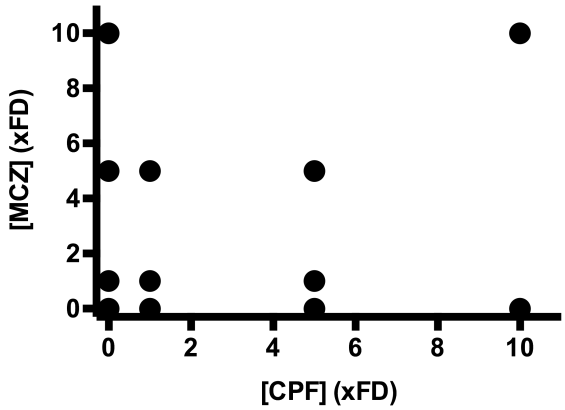

Figure 4.1 – Experimental design applied to evaluate toxicity of mixtures of chlorpyrifos (CPF) and mancozeb (MCZ) to the terrestrial isopod *Porcellionides pruinosus* in LUFA 2.2 soil. The axes units correspond to the concentrations of the pesticides expressed as number of field doses (FD) applied for each pesticide.

4.3.4. Experimental set up

The incorporation of pesticides into the soil was made as aqueous solutions. For each treatment, the whole batch of soil was spiked together and thoroughly mixed in order to homogeneously distribute the pesticides. Soil moisture was then adjusted to 60% of the WHC by adding the necessary amount of ultra-pure water. Soil was, then, transferred to rectangular plastic boxes (14.3 length cm x 9.3 width cm x 4.7 height cm) in portions of 100 g. Five replicates were used for each treatment, each one containing 10 isopods.

Isopods were selected from the laboratory cultures and randomly distributed in the test boxes. All the boxes were supplied with a similar amount of alder leaves, closed with perforated lids and kept for 7 days in a temperature-controlled room at 20 °C and 16:8 h (light:dark) photoperiod. Soil moisture was readjusted every two days by adding the necessary amount of distilled water. Three isopods per replicate were collected in every sampling time: 48h, 96h, and 7 days after the beginning of the exposure. An additional set of isopods was also sampled before the exposure, hereafter considered as the sampling time zero (T0). In every sampling time, isopods were individually weighted, freeze-dried in liquid nitrogen and stored at -80 °C until further analysis.

4.3.5. Biomarker analysis

Biomarker analysis followed the protocol described by Ferreira et al. (2010) with few adjustments. In order to measure lipid peroxidation (LPO), glutathione-*S*-transferases (GST) and catalase (CAT), a pool of two isopods' bodies (without the heads) was used per replicate. A pool of the two corresponding heads was further used for testing acetylcholinesterase (AChE) activity. Five replicates per treatment were used for each biomarker. After this separation, samples were sonicated (Kika Labortechnik U2005 Control™), for approximately 5 s, using one pulse and 100% amplitude, in 1 mL of potassium phosphate buffer 0.1 M (pH 7.4) and 1 mL of potassium phosphate buffer 0.1 M (pH 7.2), respectively for the pool of bodies (PB) and the pool of heads (PH). After sonication, 2.5 µL of butylated hydroxytoluene (BHT) 4% in methanol was added to the 150 µL of PB homogenate, and it was used as sample for LPO determinations. The remaining PB homogenate was centrifuged at 10,000 rpm (4 °C) for 20 min to obtain the post-mitochondrial supernatant (PMS). The PH homogenate was centrifuged at 3,500 rpm (4 °C) during 3 min to extract the enzyme to the supernatant and used as sample.

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate by Ferreira et al. (2010) by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue and BHT 4% in methanol, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v), and 400 µL Tris–HCl 60 mM with diethyle-netriamine penta acetic acid (DTPA) 0.1 mM. Samples were then incubated at 100 °C in a water bath for 1 h, and finally centrifuged for 5 min at 11,500 rpm (25 ^{o}C). They were kept in the dark and immediately read at 535 nm. LPO was expressed as nmol TBARS hydrolysed per minute per mg of wet weight, and was calculated using a molar extinction coefficient of 1.56 x 10^{-3} M^{-1} cm^{-1}.

Glutathione-*S*-transferases (GST) activity was determined based on the method described by Habig et al. (1974). After sonication and centrifugation, 100 µL of the PMS was mixed with 200 µL of a reaction solution. The reaction solution was composed by a mixture of 4.95 mL K- phosphate buffer 0.1 M (pH 6.5) with 900 µL L-glutathione reduced (GSH) 10 mM, and 150 µL 1-chloro-2.4-dinitrobenzene (CDNB) 10 mM and it was measured at 340 nm. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to 1 nmol of substrate hydrolysed per minute, and was calculated using a molar extinction coefficient of 9.6 x 10^{-3} M^{-1} cm^{-1}.

Catalase (CAT) activity was determined based on the method described by Clairborne (1985) and previously adapted to microplate by Ferreira et al. (2010). For this 15 µL of PMS was mixed with 150 µL H_2O_2 0.030 M, and 135 µL K-phosphate 0.05 M (pH 7.0) and measured the decomposition of the substrate (H_2O_2) at 240 nm. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to 1 mmol of substrate hydrolysed per minute, and was calculated using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The AChE activity determination was performed according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). In a 96 well microplate 250 µL of the reaction solution was added to 50 µL of the sample and the absorbance was read at 414 nm, after 10, 15, and 20 min. The reaction solution had 1 mL of 5.50-dithiobis-2-nitrobenzoicacid (DTNB) 10 mM solution, 1.280 mL of 0.075 M acetylthiocholine iodide solution and 28.920 mL of 0.1 M phosphate buffer. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to 1 nmol of substrate hydrolysed per minute, and was calculated using a molar extinction coefficient of 1.36 x 10^{-3} M^{-1} cm^{-1}.

For all biomarker, protein concentration was determined according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

4.3.6. Energy reserves, available energy, energy consumption and CEA

For the energy reserves (lipids, carbohydrates and proteins), total energy available (Ea), consumed energy (Ec) and cellular energy allocation (CEA) determination, one organism per replicate was sonicated (Kika Labortechnik U2005 Control^{TM}), for approximately 5 s, using 100% amplitude, with one pulse, with 1 mL of ultra-pure water. This homogenate was then divided into three microtubes, each one containing a total of 300 µL. One part was used to determine the proteins and carbohydrates fraction, another one to determine the lipids fraction and the final one to determine the energy consumption (electron transport activity – ETS).

To determine total proteins and carbohydrates content, 100 µL of 15% trichloroacetic acid (TCA) were added to the 300 µL fraction and incubated at –20 °C for 10 min. A centrifugation was then performed (3,500 rpm, 10 min, 4 °C), and the supernatant was separated to be used as the carbohydrate fraction. The remaining pellet was resuspended in 625 µL sodium hydroxide (NaOH), incubated at 60 °C for 30 min, and, after being neutralised with 375 µL hydrochloric acid (HCl), it was finally used as the protein fraction. Total protein content was then determined using the Bradford's reagent, and by measuring the absorbance at 590 nm using bovine serum albumin as standard. Five replicates were used in each processing methodology. Total carbohydrate content was determined by adding 50 µL of 5% phenol and 200 µL sulphuric acid (H_2SO_4) to 50 µL of sample in a multiwell microplate, incubated for 30 min at room temperature and then the absorbance was measured at 492 nm using glucose as standard. The protein and carbohydrate contents were expressed as J/mg org (expressed as fresh weight).

Total lipid quantification was determined by adding 500 µL chloroform (spectrophotometric grade) to the 300 µL fraction. After vortexed, 500 µL methanol (spectrophotometric grade) and 250 µL ultra-pure water were added, and centrifuged (3,500 rpm, 5 min, 4 °C). The bottom phase, which contained the lipid extraction, was used for lipid measurement. Then, 500 µL H_2SO_4 were added to 100 µL of lipid extract and it was heated for 15 min (200 °C). After cooling down, 1.5 mL of ultra-pure water was added and the total lipid content determined by measuring the absorbance at 375 nm

using glycerol tripalmitin as a standard. The lipids content was expressed as J/ mg org (expressed as fresh weight).

The final 300 µL fraction was used to determine the energy consumption (electron transport activity – ETS). Initially, 150 µL of a buffer of 0.3 M Tris–HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100 were added to this fraction. The extract was then centrifuged at 3,500 rpm during 10 min (4 °C), and the supernatant was removed and used as sample. In a microplate, 150 µL buffered substrate solution (0.13 M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH) were added to 50 µL of sample. The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490 nm for 3 min. The amount of formazan formed was calculated using a molar extinction coefficient of 15,900 M^{-1} cm^{-1}.

The different energy reserve fractions: protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17,500 mJ/mg carbohydrate, 24,000 mJ/ mg protein and 39,500 mJ/mg lipid and summed up to obtain the available energy (Ea). The energy consumption (Ec) was determined, using the ETS data, based on the theoretical stoichiometrical relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system. The quantity of oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O_2 (Gnaiger 1983). The Ea value was calculated by integrating the change in the different energy reserve fractions over the exposure period. Similarly, the Ec value was obtained by integrating the change in energy consumption over the exposure period. The total net energy budget was then calculated as follows,

$$CEA\ (mJ/org) = \frac{\left[\left(E_{at} - E_{a0}\right) * t\right] - \left[\left(E_{ct} - E_{c0}\right) * t\right]}{2} \quad (1)$$

where *t* is the time of the exposure from the measured sample; E_{at} is the energy available at time *t*; E_{a0} is the energy available at time 0 h; E_{ct} is the energy consumption at time *t* and E_{c0} is the energy available at time 0h.

4.3.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to test differences between treatments, among each sampling time. When significant differences were detected, a Holms-Šidak *post-hoc* test was applied to compare each treatment against the control. After converting data into percentage of control, a *t*-test was also used to compare results of different growth stages. For all comparisons, significant differences were assumed if probability values were equal or higher than 95% ($\alpha = 0.05$). Normality and equal variance tests were checked prior to ANOVA analysis and if data failed on showing a normal distribution or homoscedasticity, an appropriate non-parametric test was used. All statistical procedures were performed using Sigmaplot statistic pack (SigmaPlot 12.0 statistic pack; Systat Software, Inc., San Jose, CA, USA) or GraphPad Prism 6 statistical pack (GraphPad Software, La Jolla, CA, USA).

4.4. Results

The details of the following statistical analysis were summarized on Table 4.1SD. The evolution of AChE activity in adults and juveniles throughout the study period can be depicted in Figure 4.2. A significantly higher activity of this enzyme in adult isopods was found after 48h of exposure for treatments 1MCZ, 5MCZ, 1CPF/1MCZ, 1CPF/5MCZ and 5CPF/1MCZ when compared with control. No differences were found at 96h whereas after 7 days AChE activity was again higher than control for 1MCZ and 5MCZ. For juveniles the situation was different. After 48h of exposure a significant increase of AChE was observed for 5CPF/1MCZ and 10CPF/10MCZ when compared to control. After 96h significant inhibitions were found for every treatment except 1CPF where it showed a significant increase when compared to control. At day 7 significant increase in AChE activity was also observed for 1CPF/5MCZ and 5CPF/1MCZ treatments when compared to control.

Regarding GST activity (Figure 4.3), a dose-related increase was found in adults after 48h with significant differences to control in almost every treatment, except for 1 CPF. After 96h such increasing pattern disappeared and no significant differences to control were found. At day 7, the only significant result was the decrease registered for the 10MCZ exposure. In juveniles, after 48h, GST activity apparently followed a similar pattern to adults in the first sampling time, except for the most severe mixture treatments

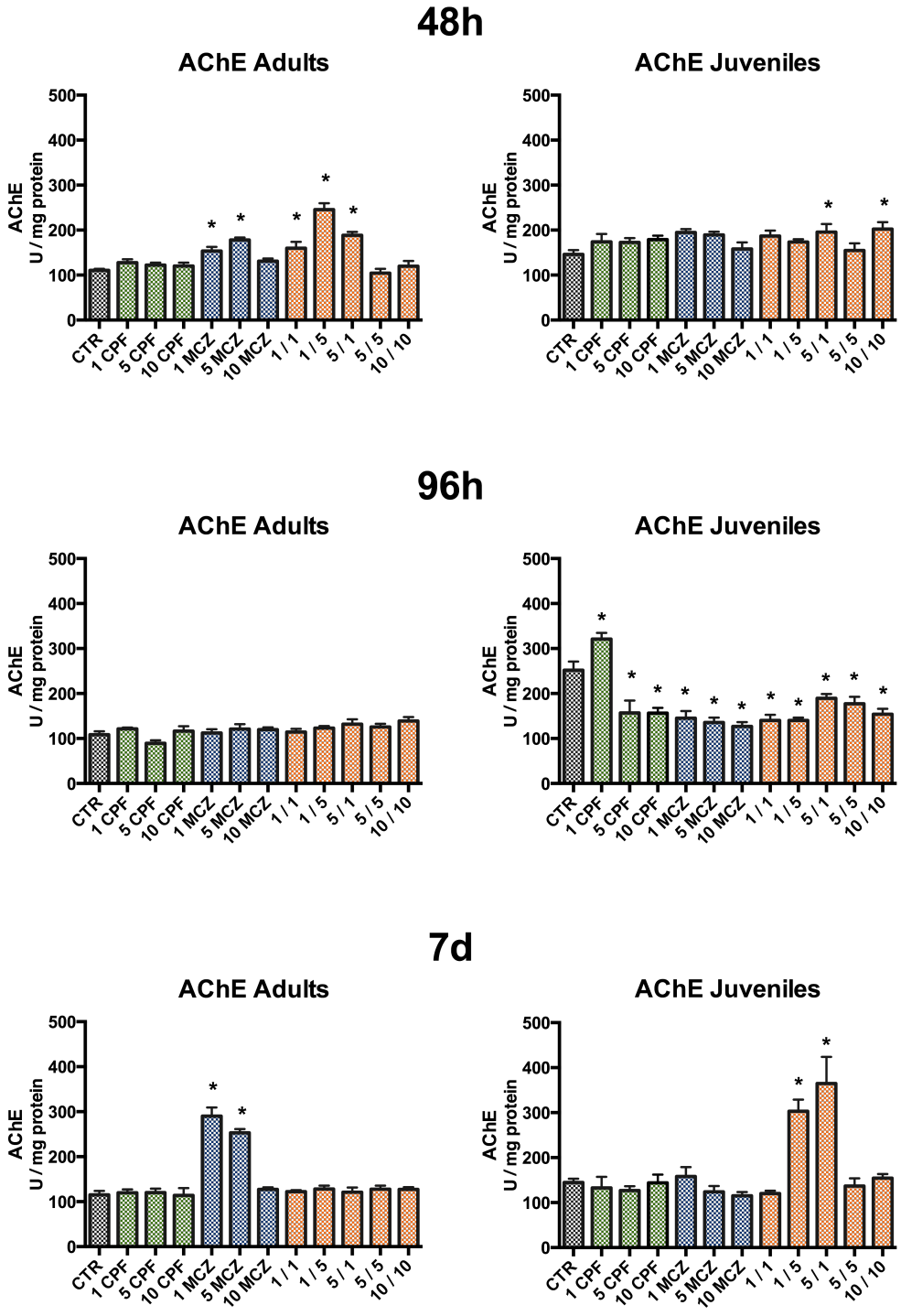

Figure 4.2 – Mean AChE activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α=0.05).

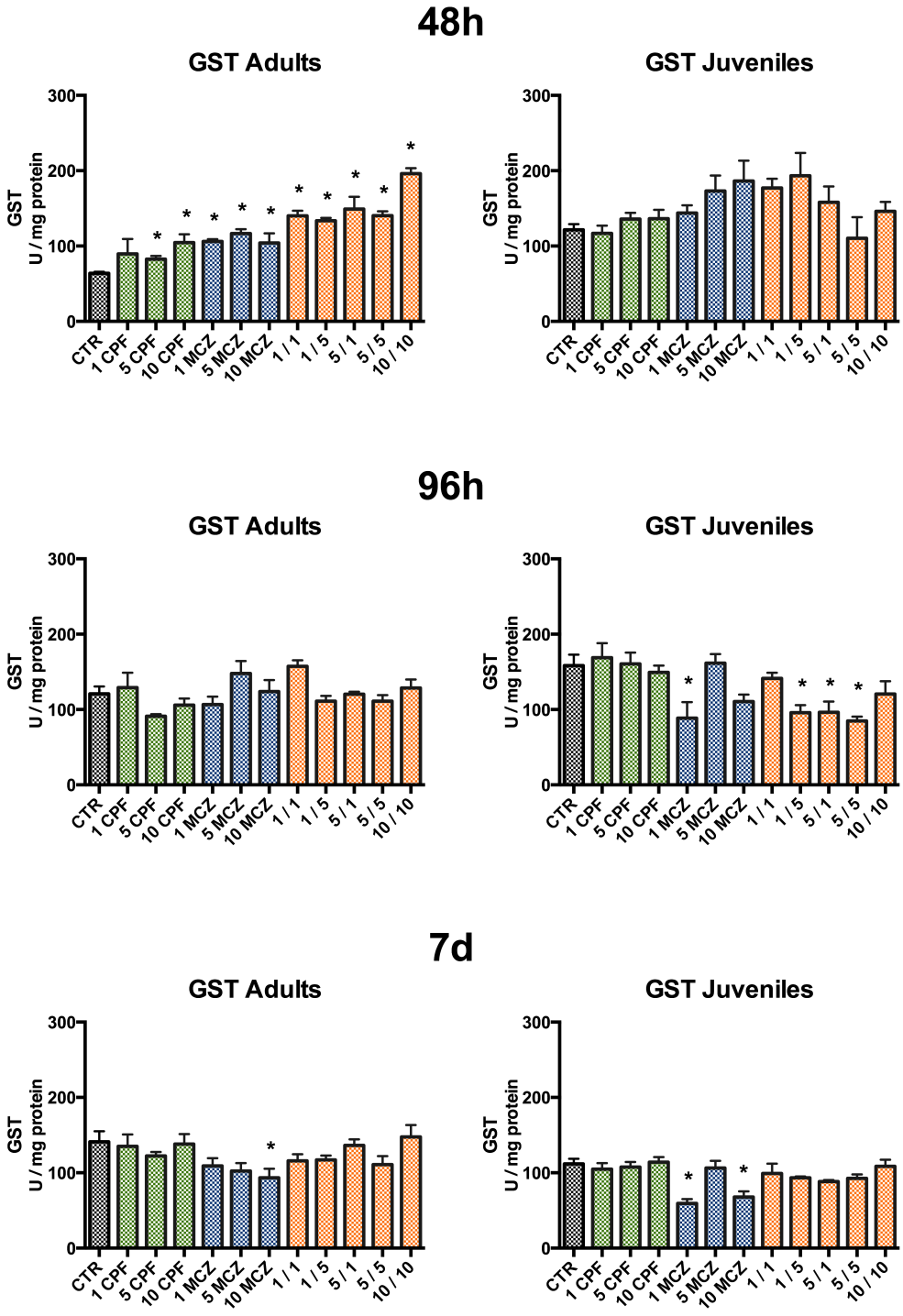

Figure 4.3 – Mean GST activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

where a decreasing trend was observed. However, contrary to adults, no significant differences were found for any treatment in this sampling time. After 96h the decreasing trend previously restricted to the most severe treatments was registered for almost all the contaminated treatments, with significant differences to control for 1MCZ, 1CPF/5MCZ, 5CPF/1MCZ, and 5CPF/5MCZ. At day 7, significant differences to control only consisted on the inhibitions observed for 1MCZ and 10MCZ exposures.

No significant differences were registered for CAT activity in adults at 48h but significant decreases were found at 96h for treatments 1CPF, 1CPF/5MCZ, 5CPF/1MCZ, 5CPF/5MCZ and 10CPF/10MCZ and after 7 days for 10CPF/10MCZ (Figure 4.4). In fact, although at day 7 only 10CPF/10MCZ showed to be different from the control, CAT activity appeared to decrease in a dose-related manner. In juveniles, CAT seemed to have increased after 48h in a dose-dependent way, but significant differences to control were restricted to 5CPF/5MCZ. No differences were found on the remaining sampling times, though the remarkable decreases found in mixture treatments at day 7 where CAT activity only reached 15-30% of control.

We found no differences within the LPO rates measured during this experiment, either for adults or juveniles (Figure 4.5).

Regarding the energy reserves, the only significant difference at 48h observed in adults' total available energy (Ea) was an increase for the 5CPF/5MCZ treatment (Figure 4.6). After 96h Ea was significantly higher than control in adult isopods exposed to 1MCZ. At day 7, a dose-related increase in total energy available could be found in adults with significant results for 1MCZ, 10MCZ, 1CPF/5MCZ, 5CPF/1MCZ, 5CPF/5MCZ and 10CPF/10MCZ. No significant differences to control were found in juveniles for any of the three sampling times. However, juveniles' total energy available seemed to be lower than in the control for most of the mixture treatments after 96h of exposure.

When analysing each one of the energetic components we could find that the largest contribution for the total energy available was provided by lipids (Figure 4.7). In fact the pattern of this energetic component was found to be quite similar to the one found for Ea. No significant differences to control were found in the first 48h; after 96h isopods exposed to 1MCZ showed significantly more lipid contents than those kept in control. Furthermore, after 7 days of exposure, adults exposed to 1MCZ, 1CPF/5MCZ, 5CPF/1MCZ and 10CPF/10MCZ showed a similar dose-related increase in lipids statistically higher than the control. This lipid preponderance was also visible in juveniles where lipid patterns were again similar to the Ea patterns. No significant differences were found between control and pesticide-exposed juveniles except at 96 h for 5MCZ.

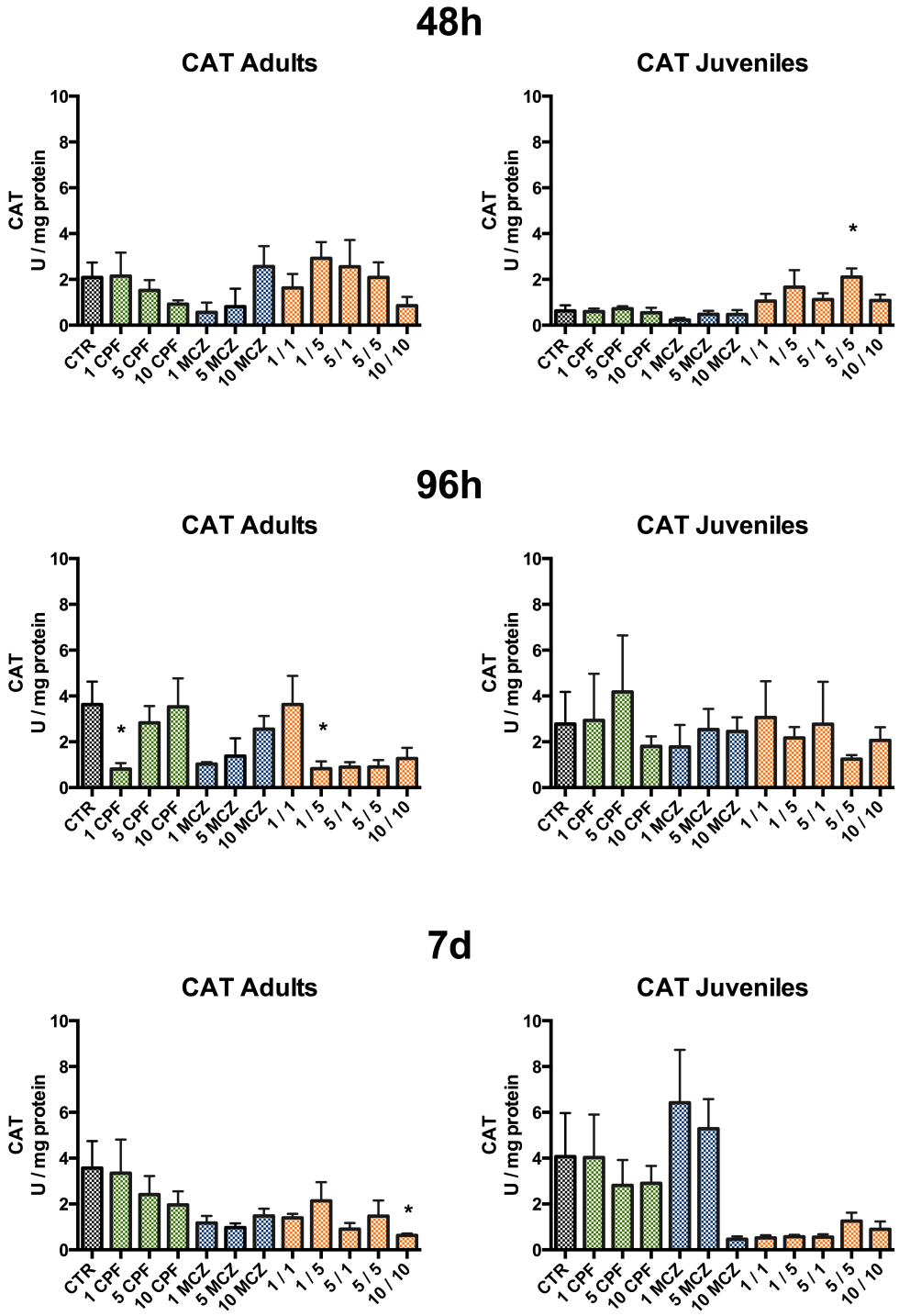

Figure 4.4 – Mean CAT activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

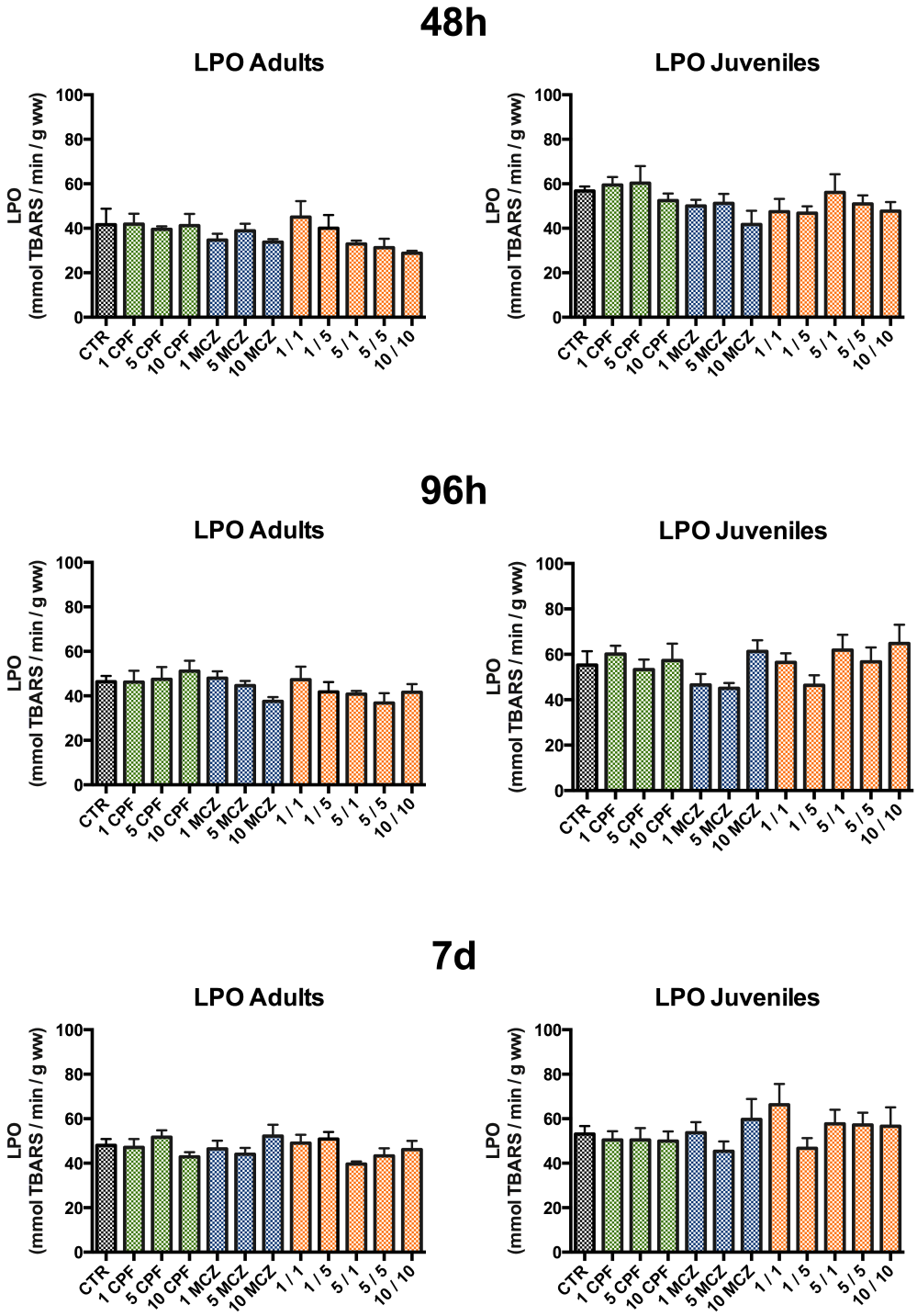

Figure 4.5 – LPO rates and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

Regarding carbohydrates, significant differences were only found for adult isopods exposed to 1MCZ at 48h and for isopods exposed to 5CPF/1MCZ and 5CPF/5MCZ for 7 days (Figure 4.8). Content in carbohydrates from adult isopods was generally higher than control in the mixture treatments, particularly at 48h and after 7 days. Regarding juveniles, no significant differences to control were observed except for isopods that had been exposed to 1CPF/1MCZ for 96h. However, although none of the treatments showed to be different from control, after 48h exposed juveniles seemed to have lower carbohydrates content than control ones. Moreover, although some exceptions were found, this lower content in carbohydrates seemed to follow a decreasing dose-related pattern.

As for the previous energetic components, proteins also seemed to increase in adult isopods, particularly after 48h and 7 days (Figure 4.9). However, significant increases to control were only found for adult isopods exposed to 5CPF/5MCZ for 48h and those exposed to 10MCZ, 5CPF/1MCZ and 5CPF/5MCZ for 7 days. Apparently there was no clear response by juveniles upon pesticides' exposures regarding the protein content. Significant differences to control in juveniles consisted on the lower protein content observed when exposed to 5CPF after 48h. There were no significant differences to control, or any visible pattern of effects at 96h and day 7 in juveniles.

Regarding the Ec (Figure 4.10), the only significant difference to control registered in adults was found at day 7 in individuals exposed to 10CPF/10/MCZ where an increase was observed. In juveniles, there seemed to be a decrease in Ec of isopods exposed to mixture treatments for 48h, with significant differences for 5CPF/1MCZ and 10CPF/10MCZ. After 96h, these differences had already disappeared but an even more prominent increase in Ec seemed to occur after 7 days in mixture treatments, with significant differences to control at 1CPF/1MCZ, 1CPF/5MCZ and 5CPF/5MCZ.

Regarding CEA (Figure 4.11), no differences were found in juveniles throughout the study period whereas in adults differences were mainly registered at day 7, with a higher allocation of energy in 1MCZ, 10MCZ, 1CPF/1MCZ, 1CPF/5MCZ, 5CPF/1MCZ, 5CPF/5MCZ and 10CPF/10MCZ.

The significant differences registered when comparing the responses of adults and juveniles to the several single and mixture treatments are summarized on Table 4.1. AChE and GST activities, along with the energy reserves, Ec and CEA were the parameters at which adults' and juveniles' responses differed the most. Age-related differences in AChE activity seemed to be mostly associated to the higher values registered in adults exposed

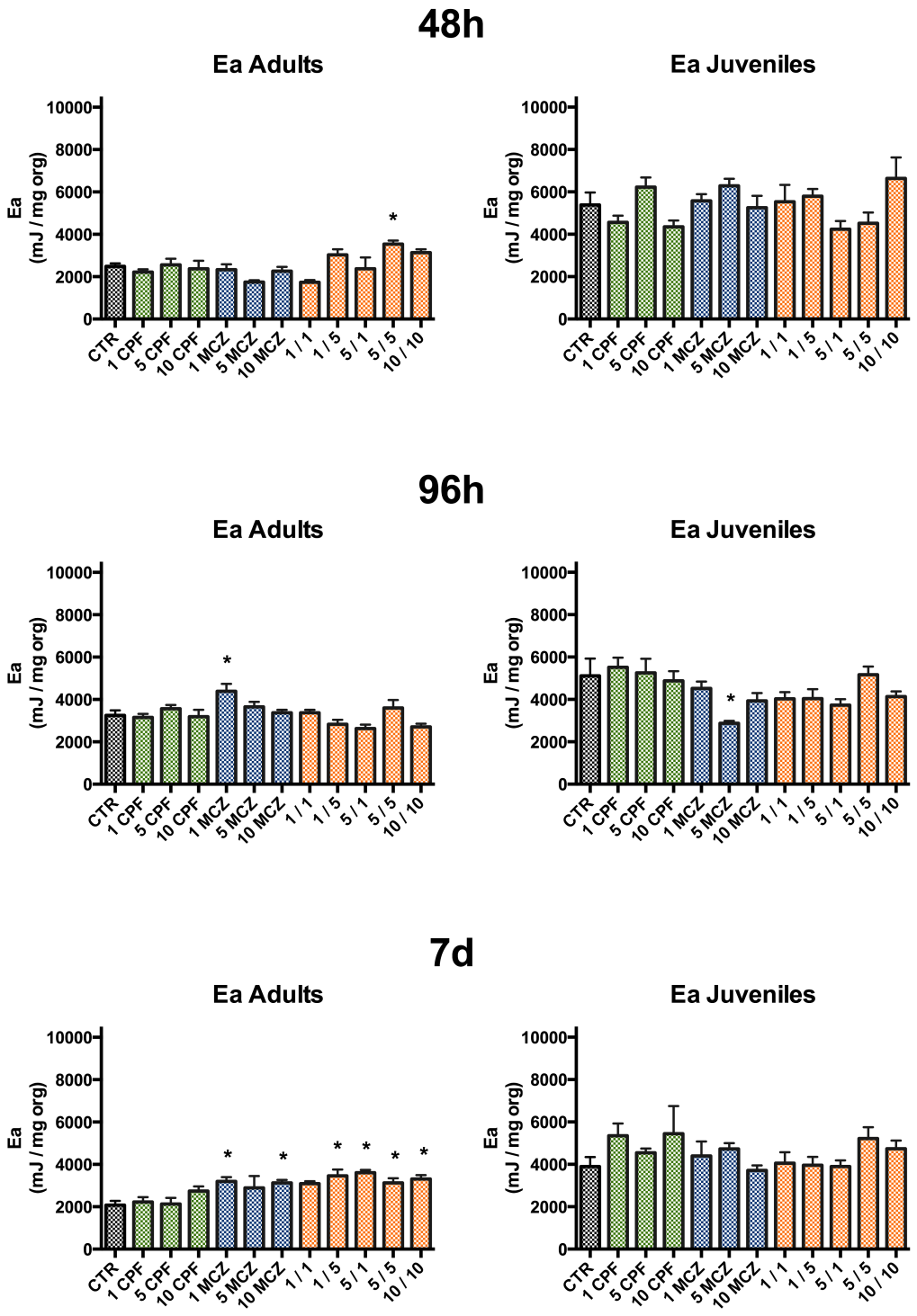

Figure 4.6 – Total energy available and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

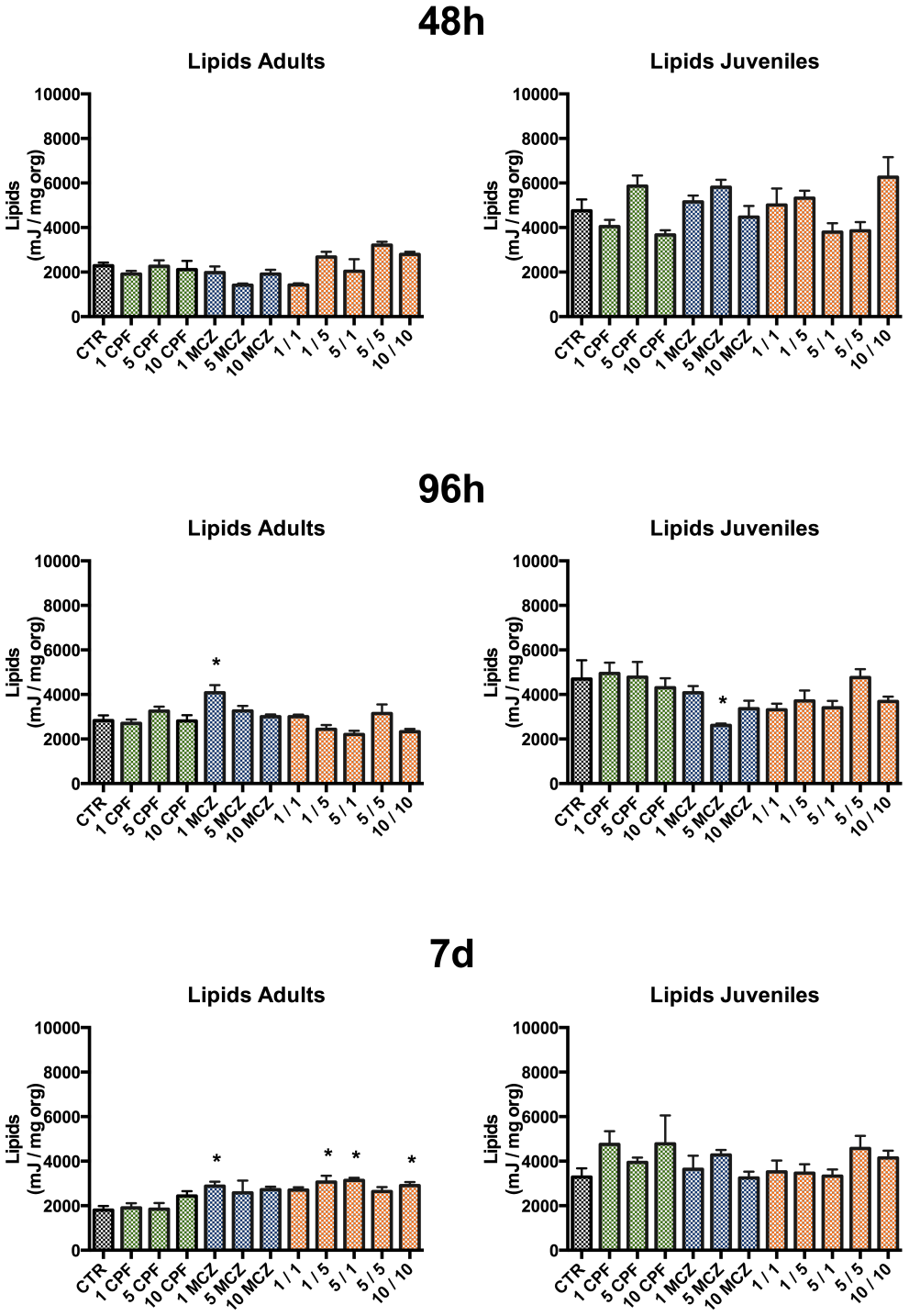

Figure 4.7 – Lipids content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

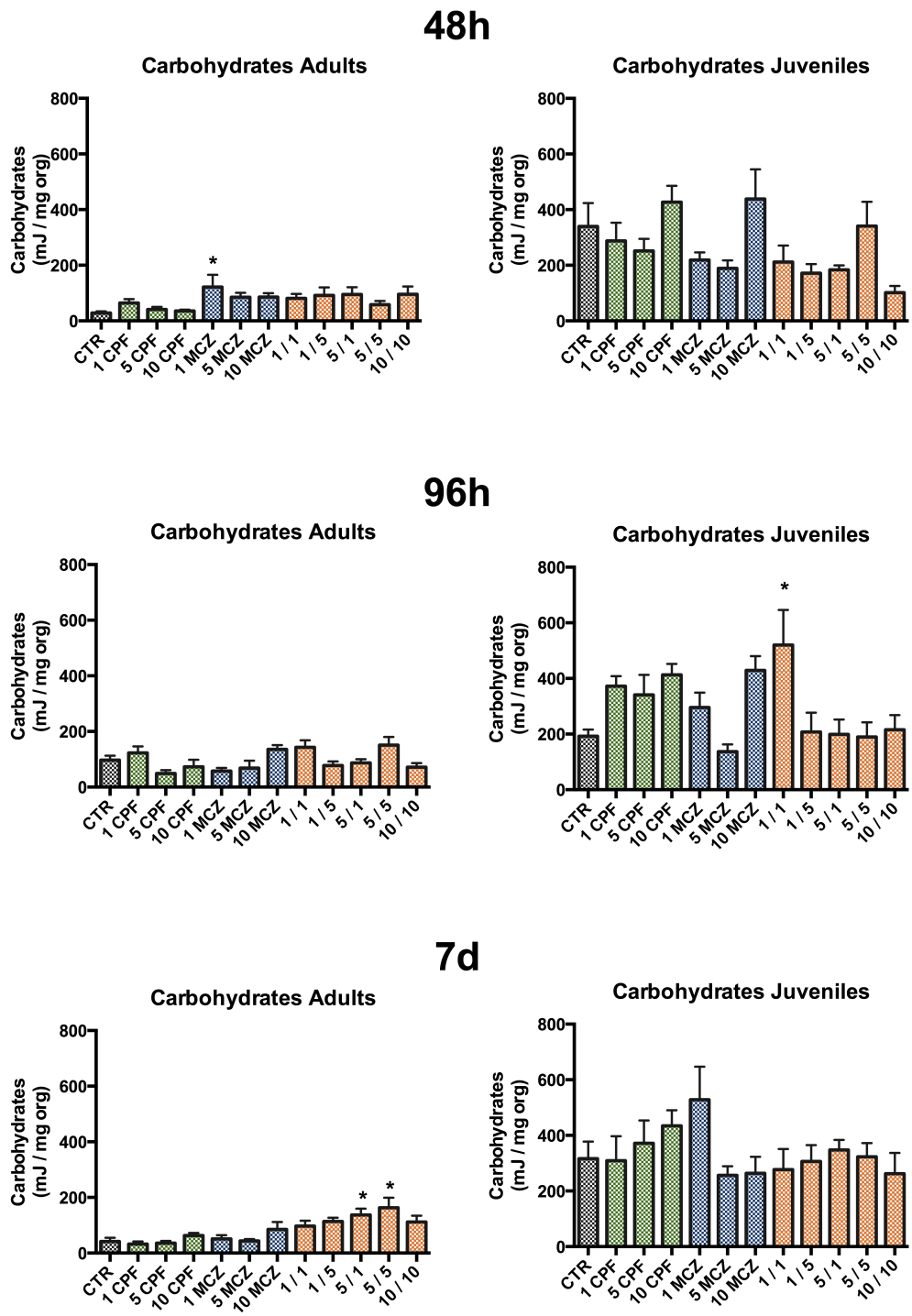

Figure 4.8 – Carbohydrates content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

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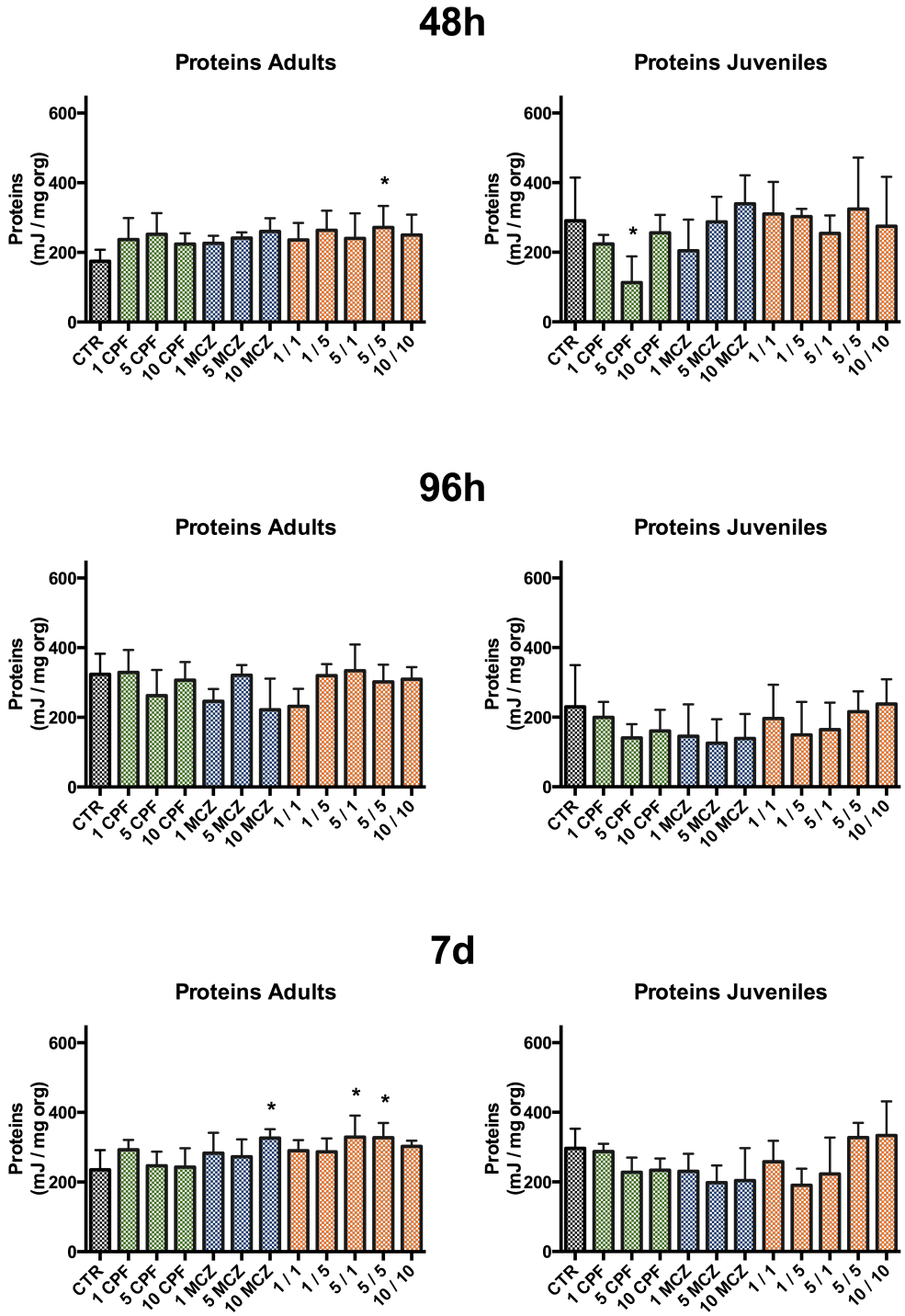

Figure 4.9 – Proteins content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

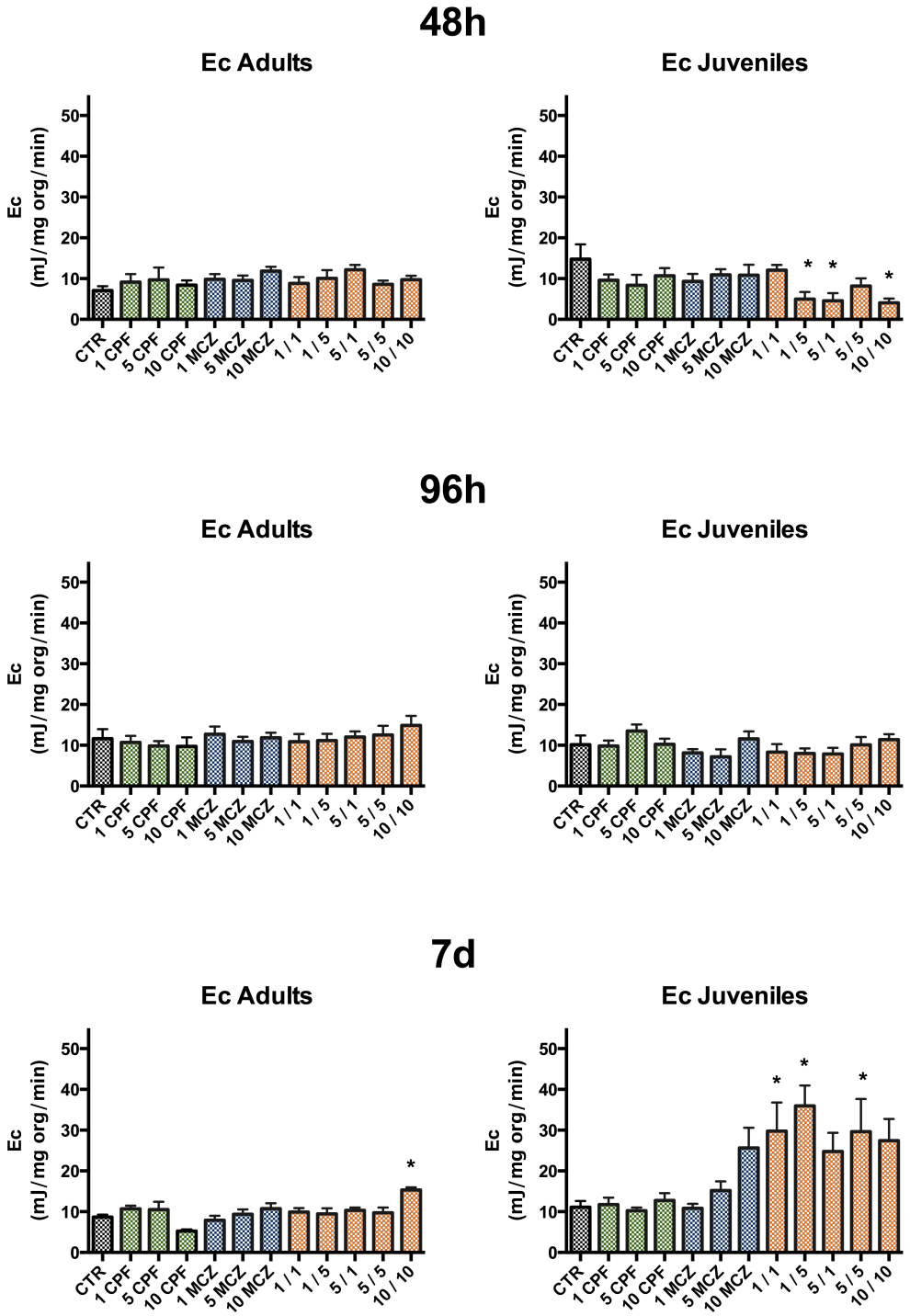

Figure 4.10 – Consumed energy and corresponding standard error in adults and juveniles of *Porcellionides* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

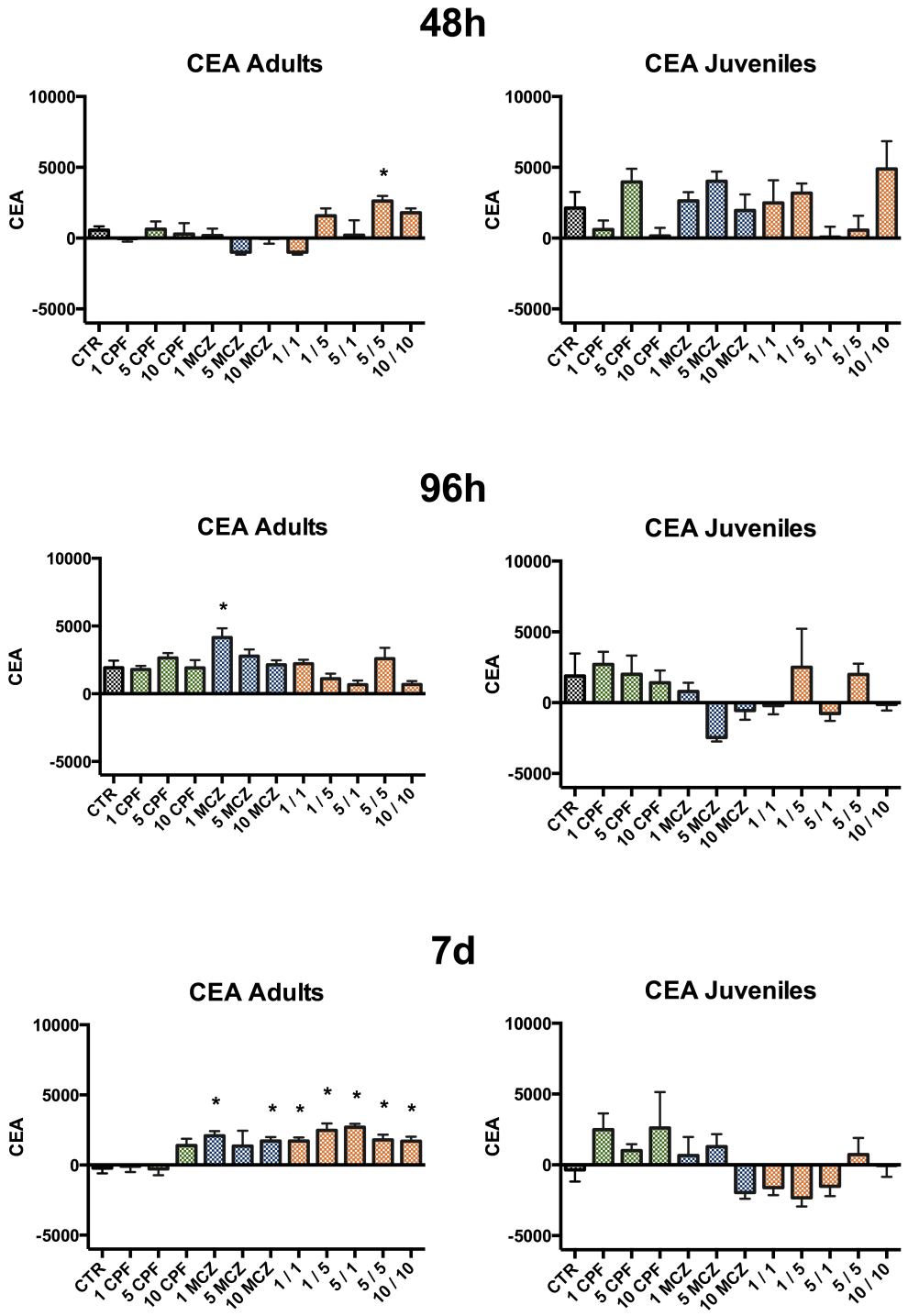

Figure 4.11 – Cellular energy allocation and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (One-way ANOVA, Holms-Šidak, α =0.05).

Table 4.1 – Significant differences found when comparing the responses measured in biomarkers and energy-related parameters of juveniles and adult individuals of *Porcellionides pruinosus* after exposure to single and combined treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 during 48h, 96h and 7 days. In order to standardize the responses for both age-classes, all values were converted to percentages of the respective control and compared using a Student's *t*-test. ↗ denotes a significant increase in juveniles when compared to adults whereas ↘ denotes a significant decrease in juveniles when compared to adults. Asterisks provide details regarding the magnitude of those differences: * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$.

		AChE	GST	LPO	CAT	Ea	Lipid	Carb	Prot	Ec	CEA
48h	1CPF							↘*	↘**		
	5CPF								↘***		
	10CPF		↘*							↘*	
	1MCZ		↘**					↘*	↘**	↘**	
	5MCZ	↘**				↗***	↗***	↘**		↘*	↗***
	10MCZ							*		↘*	
	1CPF/1MCZ		↘***				↗*	↘**			↗**
	1CPF/5MCZ	↘***	↘**					↘*	↘*	↘**	
	5CPF/1MCZ	↘*	↘**					↘*	↘*	↘***	
	5CPF/5MCZ		↘**		↗**	↘***	↘***			↘**	↘***
	10CPF/10MC		↘***		↗*			↘**		↘***	
96h	1CPF	↗*									
	5CPF		↗*					↗*		↗*	
	10CPF	↘**						↗***	↘*		
	1MCZ	↘**				↘**	↘**	↗**	↘**		↘*
	5MCZ	↘***		↘*		↘**	↘**		↘*		↘***
	10MCZ	↘***	↘*	↗*			↘**	↗*			↘**
	1CPF/1MCZ	↘***	↘**			↘**	↘***				↘**
	1CPF/5MCZ	↘***	↘**		↗**						
	5CPF/1MCZ	↘**	↘**								
	5CPF/5MCZ	↘***	↘***			↘*					
	10CPF/10MC	↘***									
7d	1CPF										
	5CPF										
	10CPF									↗*	
	1MCZ	↘***	↘*		↗*				↘*		
	5MCZ	↘***			↗*				↘**		
	10MCZ	↘**			↘*	↘***	↘**		↘**		↘***
	1CPF/1MCZ	↘**			↘**	↘**	↘*	↘*	↘**		↘**
	1CPF/5MCZ	↗**				↘**	↘*	↘***	↘***	↗**	↘***
	5CPF/1MCZ	↗**	↘*			↘***	↘***	↘**	↘*		↘***
	5CPF/5MCZ								↘*		
	10CPF/10MC					↘**	↘*	↘*			↘*

to several MCZ treatments and to the markedly lower activity observed in almost all pesticide-exposed juveniles at 96h. Regarding GST activity, differences in response generally denoted a comparatively higher increase found in adults at 48h and an activity decrease only observed in juveniles at 96h. Differences regarding age-classes' responses to pesticides measured in the energy reserves content were generally associated to the lower energy accumulation or even the decrease found in juveniles when compared to

adults, particularly in the most severe treatments. One of the few exceptions to this pattern was the higher accumulation of carbohydrates in juveniles exposed to single CPF treatments for 96h. In Ec, most differences were registered at 48h where a decrease in consumption seemed to occur in juveniles but not in adults. Differences in CEA generally highlighted a higher allocation of energy in pesticide-exposed adults when exposed to juveniles. Few differences were detected on the response of CAT and LPO between age-classes.

4.5. Discussion

Although the considerable attention received by mixture toxicity in recent years, a sizable proportion of these studies have dealt with effects at individual/population levels whereas only a minor fraction focused on lower organizational levels, such as molecular biomarkers. However, by providing an insight into the mechanisms of toxicity, biomarkers can provide a forewarning on the interaction between chemicals, highlighting for instance, modes of action that were not evident by the individual action of each component (Walker 1998). This is particularly important for mixtures that include chemicals with specific and reactive modes of action, such as pesticides, since they can modulate the toxicokinetics and/or toxicodynamics of one another (Escher & Hermens 2002). Moreover, an earlier perception of mixture's effects is important to understand the effects of chronic, low-concentration exposures, which seems to be more appropriate to current ecotoxicology challenges (Eggen et al. 2004). In this work a multiple biomarker study was performed to evaluate the single- and joint-effects of low concentrations of CPF and MCZ to the terrestrial isopod *P. pruinosus*.

In overall, two general aspects of our results need to be highlighted. First of all, it was important to note that few effects were observed in adult isopods when the toxicants were applied at the recommended doses, even for endpoints that actually constitute the main target of such compounds such as AChE. Furthermore, the few field dose effects detected on these organisms seemed to be mostly transitory, as shown by detoxification and oxidative stress-related enzymes that resulted in new homeostasis status. Another important result, though not entirely unexpected, was that in some parameters the effects on juveniles seemed to be more prominent and/or last longer than in adults. This could be seen in GST activity and several energy-related parameters, and became particularly

clear when comparing the responses of both age-classes as percentage of the respective control. This is in agreement with findings reported by several authors after evaluating age-related differences in terrestrial isopods' vulnerability to several kinds of stress, from pesticides (Ribeiro et al. 2001; Stanek et al. 2006) to ultraviolet radiation (Morgado et al. 2013). In this regard, despite being apparently safe for adults, these doses may pose more problems to juveniles, which might lead to consequences at higher organizational levels if the costs were too high in the medium/long-term (i.e. impaired recruitment/population growth/reproduction). When evaluating the effects of pesticides or mixtures, besides their application doses, one must also consider the strategy of application normally followed in the field. This is particularly relevant for MCZ since, although having a short half-life in soil (one to seven days depending on soil and conditions), it is used in fortnightly repeated application during prolonged periods (Wightwick et al. 2010). Similarly, although presenting a higher persistence, CPF can in some circumstances be applied with similar periodicity. These situations may prevent organisms from completely recovering of previous exposures and thus increase the vulnerability to further events.

As previously mentioned, the absence of inhibition in AChE activity is a noteworthy observation of this experiment, particularly when one of the compounds applied to soil was an OP insecticide. Strong CPF-induced inhibitions were already shown in a wide range of soil organisms but the effective doses seem to vary considerably. In a microcosm experiment, Reinecke and Reinecke (2007) showed AChE activity in the earthworm *Aporrectodea caliginosa* to be affected after two weeks of exposure, even by treatments intended to mimic background concentrations registered in orchards. Booth et al. (2003) also found effects on the wolf spider *Lycosa hilaris* after field dose exposure to CPF, but they were restricted to the first 24h. Perhaps the fact that we only had our first sampling time at 48h led us to skip earlier inhibitory effects. Nevertheless, as reported by the same authors, such short-lasting transient effects may not, in fact, represent serious fitness costs to the organisms in the long-term (Booth et al. 2003). Collange et al. (2010), on the other hand, only found inhibition in the earthworm *Lumbricus terrestris* at much higher concentrations.

On the other hand, literature shows that dithiocarbamates such as MCZ have minimal inhibitory effects on AChE activity (Espigares et al. 1998). Although also having neurotoxicity effects as well, MCZ is thought to affect primarily other neurotransmission sub-types such as the GABAergic or the dopaminergic systems (Negga et al. 2012; Brody et al. 2013).

Two additional outcomes must also be pinpointed for AChE. The first is relative to the significantly lower activity observed in juveniles at 96h because rather than an inhibitory response in pesticide-exposed treatments, results seemed to have been influenced by a higher control value. In fact, AChE activity in control seemed to be twice as many the values registered for the remaining sampling times, which in turn are similar to values reported for juveniles of this species in other studies (Morgado et al. 2013). In this way, few conclusions could be drawn for this enzyme in juveniles at 96h. The second is related to the significant increases observed in AChE activity that, interestingly, were only associated with MCZ exposures. Perhaps these exposures triggered the production of less common variants of AChE. AChE-S is normally the prevalent isoform known to regulate acetylcholine signalling that is found in the synaptic cleft linked to the membrane of neurons (Soreq & Seidman 2001). Nevertheless, when under stress or anti-cholinesterase agents, stress-induced alternative splicing in ACHE genes may lead to an overproduction of AChE-R, a monomeric variant that shares similar hydrolytic activity but remains soluble within the synaptic cleft (Soreq & Seidman 2001). Although the exact role of this variant on dealing with stress is not clear yet, correlative studies point towards a higher vulnerability of organisms that are unable to codify this monomeric isoform (see Grisaru et al. 1999 for a review). Another possible explanation for the fact that such increases were exclusively associated to treatments containing MCZ must be related to the metallic content of this compound. MCZ is a complex of manganese (Mn^{2+}) and zinc (Zn^{2+}) chelated with a major metabolite of this pesticide, ethylenethiourea (Brody et al. 2013). Similar increases in AChE activity were already described after exposure to metals and some different explanations were advanced, briefly summarised in Barillet et al. (2007). For instance, Abou-Donia et al. (2002) referred that this situation might reflect an increased axonal repair and synaptic modelling. On the other hand, Berman and Leonard (1990) showed AChE to be highly responsive to changes in the medium, such as in ionic strength, and suggested that peripheral anion sites can play a significant role on this catalytic activity increase. Regarding pesticide mixtures, Santos et al. (2010a) also observed a similar increase in AChE activity of *P. pruinosus* when exposed to mixture of molluscicidal baits and suggested that it could be related to intra-specific responses of the central nervous system of this isopod.

The major differences in vulnerability between adults and juveniles were related to the detoxification enzyme GST. In fact, a dose-dependent increase of GST activity was found in adult isopods after 48h of exposure. However, after 96h these organisms seemed to have already achieved a homeostasis status that was maintained until day 7.

On the contrary, the significant GST increase was not found in juveniles, despite the apparently similar dose-dependent increase observed in all the treatments except the most severe ones. Furthermore, complete homeostasis was not achieved before day 7 as shown by the significant inhibition at 96h in mixtures. Hence, whereas adults seemed to be effective on dealing with the contamination stress, juveniles did not show such a prompt response, which led to longer-lasting effects. The fact that juveniles had more problems in effectively using this detoxification mechanism when exposed to mixtures achieves an extra relevance if considering that other protective systems can also be simultaneously affected. GST is a group of multi-functional enzymes involved on phase II of xenobiotics' biotransformation (Lagadic et al. 1994; Xu et al. 2005). However, MCZ was previously reported to inactivate also the phase I's cytochrome P450 enzymes (Lewerenz & Plass 1984; Borin et al. 1985; Szépvölgyi et al. 1989; Siddiqui et al. 1991), which in combination with GST inhibition is likely to constitute a serious impairment on overall detoxification capacity. In fact, Nebbia et al. (1993) showed the action of zineb (another Zn^{2+}-containing dithiocarbamate fungicide) to affect mixed function oxidases, even before acting on the glutathione-related enzymes, which highlights the generalized impairments on multiple detoxification mechanisms.

Despite no direct evidences of oxidative damage were registered on LPO, CAT responses seemed to suggest that the ROS-scavenging activity of this enzyme was actually being required. In fact, albeit none of these pesticides has oxidative stress as the primary mode of action, both of them were previously referred to constitute strong pro-oxidant agents (Jager et al. 2007; Tsang & Trombetta 2007). In this regard, we found almost no differences between juveniles and adults since both followed the typical time- and dose-dependent "bell-shaped" or "inverted bell-shaped" curves that normally feature oxidative stress enzymes (Iizawa et al. 1994; Walker 1998). The few age-related differences registered must be therefore related to the pace of activation/inactivation processes rather than actual differences in overall responses. Our results also suggest that CPF and MCZ differed on the type of curve since an inhibition followed by an increase was registered for the former while the opposite pattern seemed to occur for the later. The effects observed for chlorpyrifos seemed to be in line with those reported by Ferreira et al. (2015) who found a similarly shaped curve when *P. pruinosus* were exposed to dimethoate. This seems to suggest the existence of a common response for OP insecticides on these organisms. It was not clear if complete homeostasis was achieved during this experiment because both age-classes seemed to present a dose-

dependent decrease in CAT activity at day 7, particularly pronounced in mixture treatments.

The most relevant aspects arising from the analysis of the energy-related parameters are possibly those associated to the comparison of age-related responses. In fact, in this experiment we found few evidences of abnormal depletion on any of the energy reserves assessed, neither in adults nor juveniles. This result is not entirely unexpected given the short duration of the experiment. Stanek et al. (2006) also found no depletion in *Porcellio scaber* after two weeks of exposure to diazinon via contaminated food and claimed that such duration was insufficient to provoke effects. Whilst the 21 days study of Ribeiro et al. (2001) with *Porcellio dilatatus* exposed to parathion showed significant decreases, Ferreira et al. (2015) did not find consistent effects on reserves after exposing *P. pruinosus* to dimethoate during 28 days. Duration seems, therefore, not to be the only relevant variable on this subject. When considering the whole energy available the most relevant outcome is the dose-dependent increase observed at day 7. A similar situation had been previously observed by Morgado et al. (2013) leading the authors to hypothesize that it might have been the result of behavioural changes, either on feeding, on metabolism, or even both. In that study, however, such increase was concurrent with reductions of energy consumption, which is exactly the opposite of the present experiment. In this way, although not discarding the former hypothesis, one must also consider the possibility that additional factors are involved as well. For instance, it can also be related with the moult cycle. Drobne and Štrus (1996) reported chemical contamination with Zn^{2+} to disturb the moult cycle in *P. scaber*, leading isopods to enter ecdysis during the first week of exposure. This seems not to be restricted to metals since it was also highlighted as a possibility by Ferreira et al. (2015) for dimethoate. Considering that isopods, and crustaceans in general, may accumulate reserves during the pre-ecdysis period (Carter & Mente 2014) and part of the accumulated reserves will need to be incorporated into the new carapace resulting from moult, such increase in energy available might not imply a greater fitness but instead be an artefact of the analytical method. Further research is needed to infer about this hypothesis. As stated previously, adults did not show to vary significantly the energy consumption, neither with treatment nor with time. By the opposite, juveniles exposed to mixtures showed a different response since they showed to decrease energy consumption in the first sampling time but present significantly higher energy consumption at day 7. This seems to follow a different pattern from the exposure to UV radiation in Morgado et al. (2013), but probably reflects the different source, nature and duration of the stress event. Regarding cellular energy

allocation, the most relevant results seemed to be associated with mixture treatments as well. Contrary to adults that generally showed positive or near-zero values, CEA in juveniles exposed to mixtures seemed to be impaired throughout the study period becoming mostly negative at day 7.

Briefly, this study highlighted the age-related differences in susceptibility of *P. pruinosus* to single and combined treatments of CPF and MCZ. Whereas the recommended doses of each pesticide appeared not to pose considerable problems to these organisms, higher concentrations and mixture treatments seemed to impair several physiologic processes, particularly in juveniles. By assessing a set of biomarkers and energy-related parameters, we found these pesticides to affect both isopods' detoxification and antioxidant systems, as shown by the significant increase/inhibition of GST and CAT activity. Moreover, we also identified differences in energy related parameters, which suggest different age-classes to respond differently to contamination stress and to have different metabolic costs.

4.6. References

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Table 4.1SD – Statistical details for biomarkers and energy-related parameters of *Porcellionides pruinosus* between individuals in control and exposed to several single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in Lufa 2.2 soil. Data refers to *F* and respective *p* values from one-way ANOVA, a=0.05.

	AChE	GST	LPO	CAT	Ea	Lipid	Carb	Prot	Ec	CEA
Adults										
48h	F=20.44;*p*<0.001	F=13.09;*p*<0.001			F=4.259;*p*<0.001	F=4.372;*p*<0.001				F=4.495;*p*<0.001
96h	F=2.473;*p*=0.015	F=2.683;*p*=0.009		F=2.88;*p*=0.006	F=4.075;*p*<0.001	F=4.929;*p*<0.001	F=3.122;*p*=0.003			F=4.259;*p*<0.001
7d	F=35.03;*p*<0.001	F=2.248;*p*=0.027			F=4.065;*p*<0.001	F=3.051;*p*=0.001	F=5.371;*p*<0.001	F=2.411;*p*=0.018	F=4.436;*p*<0.001	F=4.586;*p*<0.001
Juvenile										
48h	F=2.017;*p*=0.048	F=2.308;*p*=0.023		F=3.109;*p*=0.003	F=2.424;*p*=0.018	F=3.463;*p*=0.001	F=2.916;*p*=0.005	F=2.187;*p*=0.032	F=2.356;*p*=0.021	F=2.475;*p*<0.001
96h	F=15.12;*p*<0.001	F=5.42;*p*<0.001			F=3.129;*p*=0.003	F=3.463;*p*=0.008				
7d	F=11.94;*p*<0.001	F=5.364;*p*<0.001		F=3.236;*p*=0.002			F=3.983;*p*=0.01	F=2.635;*p*=0.01	F=4.491;*p*<0.001	F=2.322;*p*=0.022

AChE – acetylcholinesterase; GST – gluthathione *S*-reductase; LPO – lipid peroxidation; CAT – catalase; Ea – energy available; Lipid – lipids content; Carb – carbohydrates content; Prot – proteins content; Ec – energy consumption; CEA – cellular energy allocation.

CHAPTER 5: Temperature induces different pesticide mixture effects on the terrestrial isopod *Porcellionides pruinosus*

Temperature induces different pesticide mixture effects on the terrestrial isopod *Porcellionides pruinosus*

5.1. Abstract

The recognition that climate changes can act jointly with several other stressors, influencing their individual effects, is raising concerns among ecotoxicologists about the effectiveness of current risk assessment procedures in predicting the real effects of an exposure to xenobiotics. This issue assumes particularly importance for agroecosystems since these are highly prone to be exposed to multiple pesticides while simultaneously experiencing severe environmental conditions. In this work, we evaluated the individual and joint effects of the pesticides chlorpyrifos and mancozeb to the terrestrial isopod *Porcellionides pruinosus*, when submited to different temperature regimes. Interestingly, isopods' survival to these pesticides was found to be oppositely affected by temperature, either in single or mixture treatments. Whereas chlorpyrifos' acute toxicity increased under higher temperatures, the toxicity of mancozeb was more prominent at lower temperatures. However, although the weight of each compound on the toxicity of the mixture showed to vary with temperature, this influence was always felt in a non-interactive way since no deviations were found to the reference model of independent action in any of the temperature scenarios. Regarding the assessment of feeding parameters, a significantly lower consumption was found in isopods kept at colder temperatures when compared with the remaining regimes. Furthermore, isopods generally showed a dose-dependent decrease in consumption, either in individual pesticides or mixture treatments. On the other hand, a higher variance was found in the biomass gain/loss results. Regarding mixture treatments, additivity was also the pattern that better explained the combined action of these pesticides on the feeding parameters, although some antagonistic situations were also observed. These results highlight the importance of including the joint effects of multiple stressors in the risk assessment procedures, since they are all partly contributing for the effects observed and their addition may, in some situations, become unbearable to soil communities. In this way, toxicity effects like those described here are

likely to entail severe implications on several functional ecosystems' processes at different levels of organization.

Keywords: Multiple stressors; climate changes; chlorpyrifos; mancozeb; independent action model

5.2. Introduction

Environmental contamination and climate changes are two of the most important factors affecting soil ecosystems in agricultural fields. Agriculture is nowadays featured by the use of a wide range of pesticides whose application might be coincident. Although such mixtures may constitute, *per se*, a serious problem to soil biota (Loureiro et al. 2009; Santos et al. 2010; Santos et al. 2011a; Santos et al. 2011b), in the present scenario of climate changes they assume an even higher relevance (Chen & McCarl 2001; Kattwinkel et al. 2011). This is particularly important in the Mediterranean region since it probably includes some of Europe's most adversely affected agroecosystems by climate changes (Olesen & Bindi 2002; Giorgi & Lionello 2008). Decreased agricultural yields, increasingly frequent heat waves, droughts and violent winter storms, soil erosion, and ecosystems degradation are some of the most common expectations for this area (Miraglia et al. 2009).

Furthermore, the projected rises in temperature might disrupt the existing balance of these biological systems by desynchronizing populations' cycles, thereby changing their communities' stable state. The consequences of such imbalance will likely include, for instance, a higher probability of issues related with crop pests and diseases that will certainly demand for additional efforts in order to optimize production levels (Wolters et al. 2000; Chen & McCarl 2001; Miraglia et al. 2009). This suggests that, notwithstanding all the concern regarding the widespread use of pesticides, these compounds will certainly hold a major importance in Mediterranean agriculture and emphasize the significance of evaluating the effects to soil biota, upon their application under different climatic scenarios.

Traditional risk assessment procedures have usually been conducted in a conceptually simplistic design, consisting on the exposure of test organisms to a range of concentrations of a single compound, under near-optimal conditions (Sjursen & Holmstrup 2004). None of these circumstances is, however, common in agricultural fields, since most

crops require the application of more than just one type of pesticide over the growing cycle (Santos et al. 2011b) and organisms surely have to cope with considerable fluctuations on biotic and abiotic conditions during their lifespan (van Gestel & van Diepen 1997). In this way, new approaches are needed that keep the pragmatism and simplicity of single tests while simultaneously accounting on the complexity of multiple stressors' interactions.

Pesticides' toxicity is particularly influenced by temperature. It can act directly on the bioavailability of the pesticide by changing its adsorption, desorption, volatilization and/or degradation rates (Arnold & Briggs 1990), or indirectly by influencing the uptake, metabolism, and detoxification processes at the organism level (Noyes et al. 2009). These interactions are often rather complex varying with the properties of the chemical, the organisms, and the magnitude of stressors involved (Holmstrup et al. 2010). As an example, Ferreira et al. (2015) reported a higher dimethoate-induced mortality in *Porcellionides pruinosus* at 25 ^{o}C when compared to those exposed at 20 ^{o}C, but Martikainen and Rantalainen (1999) observed long lasting toxicity effects of dimethoate in *Folsomia candida* exposed under low temperatures. Among the different approaches employed to evaluate the joint effects of environmental conditions and toxicants, the application of the Independent Action model (IA) constituted a significant improvement since it provided a sound theoretical framework to detect deviations to the simple addition of effects of stressors with dissimilary modes of action (Long et al. 2009; Ferreira et al. 2010). This model assumes that the probability of effect of each stressor is fully independent from one another (Martin et al. 2009; Santos et al. 2011b), so if any deviation is found to the model projection, it would mean that stressors are actually interacting, either in a synergistic or antagonistic way (Jonker et al. 2005).

Regardless of the growing attention that these issues have received lately, several gaps of knowledge must still be fulfilled. For instance, studies involving more than binary combinations of natural and chemical stressors are still scarce, despite being, definitely, the most usual circumstance in nature (Laskowski et al. 2010). However, the interaction between toxicants may lead to different outcomes when occurring under different environmental conditions, as shown by Bednarska et al. (2009), by exposing carabid beetles to chlorpyrifos and nickel.

This study aimed at evaluating the joint effects of two widely used pesticides, the acetylcholinesterase-inhibiting insecticide chlorpyrifos (CPF) and the broad-spectrum contact fungicide mancozeb (MCZ), in the terrestrial isopod *Porcellionides pruinosus*, under four temperature regimes that mimic different but real contiditions for

temperate/mediterranean climates. Both pesticides are commonly applied in many crops, like orchards and vineyards, and their application frequently takes place together or within short time intervals.

Terrestrial isopods constitute one of the most conspicuous elements of soil macrodecomposer guild. Their deep involvement on nutrient and organic matter recycling processes makes them a key group in most edaphic ecosystems and earned them an important role as model species for assessing the effects of soil contamination in ecotoxicology experiments (Drobne 1997; Loureiro et al. 2002). *Porcellionides pruinosus* is a synanthropic species that is generally regarded as the most widely distributed terrestrial isopod (Lefebvre & Marcadé 2005). Since a great deal of its ecological role is mostly related with its feeding activity, the main rationale behind this work was to assess whether chlorpyrifos, mancozeb, and temperature could act synergistically affecting isopods' feeding habits (or ultimately their survival), therefore impairing the delivery of their services to edaphic ecosystems.

5.3. Material and methods

5.3.1. Test organism

The terrestrial isopod *Porcellionides pruinosus* was used as test-species. Animals were collected in a horse manure heap and kept in laboratory cultures at 20 ^{o}C (±1 ^{o}C), 16:8 (light:dark) photoperiod, with soil adjusted to a moisture content of 60% of its water holding capacity (WHC) and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Only adults were selected to this experiment (15-25 mg wet weight). No sex differentiation was considered, but pregnant females were not used for the trials. Likewise, moulting animals or those showing any visible problem were not used in the experimental set up.

5.3.2. Chemical compounds and soil

The commercial formulations of both pesticides were used to perform these experiments: the organophosphorus insecticide chlorpyrifos (CICLONE® 48 EC with 480 g/L of chlorpyrifos) and the dithiocarbamate fungicide mancozeb (MANCOZEBE SAPEC® with 80% of mancozeb).

The certified loamy sand soil LUFA 2.2 (Speyer, Germany) was used. The main properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), water holding capacity = 41.8 ± 3.0 (g/100g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

5.3.3. Experimental set up

Preliminary tests were undertaken to assess the toxicity of both pesticides on *P. pruinosus*. Based on these results, a full factorial design experiment with three nominal concentrations of each pesticide (1 $mg.kg^{-1}$ soil, 2 $mg.kg^{-1}$ soil and 3 $mg.kg^{-1}$ soil of CPF and 88 $mg.kg^{-1}$ soil, 176 $mg.kg^{-1}$ soil and 264 $mg.kg^{-1}$ soil of MCZ) and an untreated control was conceived. Using the Toxic Units (TU) concept (1 TU=LC_{50}), these treatments were selected so that they were equivalent to 0.33TU, 0.66TU, and 1TU of each pesticide, found in the preliminary tests. Thus, when arranged in a full factorial design, chemical treatments ranged from 0.33 TU, in situations where the minimum concentration of a single pesticide was used, to 2 TU, when combining the maximum concentration of both pesticides. This experimental design was repeated using four different temperature regimes (Figure 5.1): a) constant 20 ^{o}C; b) mild daily cycle (15 ^{o}C and 25 ^{o}C); c) hot daily cycle (25 ^{o}C to 35 ^{o}C); d) cold daily cycle (5 ^{o}C to 15 ^{o}C). Constant 20 ^{o}C is the most commonly used temperature in similar ecotoxicological assays with isopods and other soil species. The remaining cycles were selected aiming to resemble the temperature conditions felt in Portugal during winter, spring and summer days, respectively. Photoperiod was always set for 16:8 hours (light:dark).

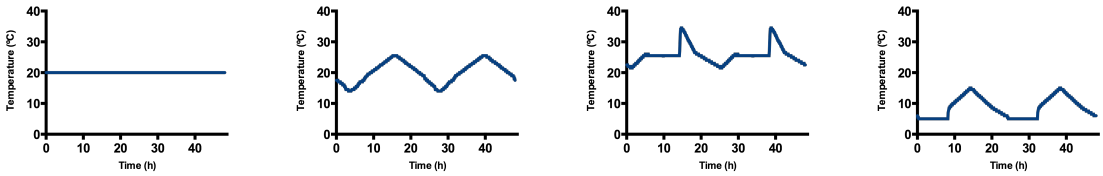

Figure 5.1 – Temperature cycles used for the exposures during the experiment: a) constant 20 ^{o}C; b) Mild cycle (daily cycle between 15 ^{o}C and 25 ^{o}C); c) Hot cycle (daily cycle between 25 ^{o}C and 35 ^{o}C); d) Cold cycle (daily cycle between 5 ^{o}C and 15 ^{o}C).

Pesticides were incorporated into soil as aqueous solutions. The whole batch of soil for each treatment was spiked together and thoroughly mixed to ensure an homogeneous distribution of contaminants. Soil moisture was subsequently adjusted to 60% of soil's WHC by adding distilled water. At next, portions of 18g were taken from these batches into circular plastic boxes (Ø 6.5 cm). Soil pH was measured in distilled water, both in the beginning and the end of the experiment, and followed the ISO standard procedure 10390 (ISO 2005).

When starting the experiment, isopods were collected from culture boxes and placed into boxes with bottom covered by water saturated plaster. Then, each one's body weight was registered (±0.1 mg), and they were randomly divided individually and placed into the test boxes. A total of 320 isopods was used in this experiment, which consisted on 5 replicates on each treatment, with one individual per replicate. Every isopod was supplied with 4 previously weighed disks of alder leaves (±30 mg dw). All the boxes were closed with perforated lids to allow gas changes, and they were kept for 14 days inside a climatic chamber, set for the required temperature regime. Soil moisture was controled every second day (daily during the hot cycle) and readjusted by adding the necessary amount of distilled water. At the end of the experiment, isopods and leaves were reweighted to determine both the isopods' consumption rate and their biomass gain/loss, calculated according to Loureiro et al. (2006):

$$\text{Consumption rate} = (W_{Li} - W_{Lf}) / W_{isop} \quad (1)$$

$$\text{Biomass gain/loss} = [(W_{isop} - W_{isop\,f}) / W_{isop}] \times 100 \quad (2)$$

where, dw - dry weight; $W_{L\,i}$ - initial leaf weight (mg dw); $W_{L\,f}$ - final leaf weight (mg dw); W_{isop} - initial isopod weight (mg); $W_{isop\,f}$ - final isopod weight (mg).

5.3.4. Statistical analysis

Isopods' survival for the single compounds was analyzed by calculating the concentration after which 50% of the exposed animals were found dead (LC50), using the probit regression scheme (Priprobit 1.63). A three-way analysis of variance (ANOVA) was performed to compare isopods' survival between treatments using "temperature", "CPF

concentration" and "MCZ concentration" as variables. In the mixture exposures, the effects of pesticides' interactions on isopods' survival were assessed for each temperature regime using the MixTox tool conceived by Jonker et al. (2005). This model allows the comparison of the observed data with the expected toxicity effects of the mixture calculated from a reference model (Jonker et al. 2005). In this work the independent action model (IA) was selected as a reference model because all stressors had different modes of action (Jonker et al. 2005). Since this reference model assumes that effects of each component of a mixture are statistically independent from one another, it must be extended with deviation functions to describe synergistic/antagonistic (S/A), dose-level (DL), and dose-ratio dependency (DR). To do so, two extra quantitative parameters (*a* and *b*) were added forming a nested framework. Data was fitted to the reference model and all its deviation functions, using the method of maximum likelihood; the best fit was chosen through likelihood testing. If significant deviations to the reference model were detected, a direct deduction of the effects could be done from the parameter values, to infer about the nature of these deviations, as shown in Jonker et al. (2005). A one-way ANOVA was used to test for differences in consumption ratio and biomass gain/loss, either between temperature regimes for comparing the same chemical treatments or within temperatures for comparing different chemical treatments (α=0.05). *Post-hoc* tests were performed whenever multiple comparisons were required: the Dunnetts' test for comparisons to control within temperatures and Holms-Šidak's test for all pairwise comparison between temperature regimes. A non-parametric Kruskal-Wallis analysis of variance by ranks followed by Dunn's *post hoc* test were performed whenever data showed not to follow a Gaussian distribution. It must be stated, though, that some treatments had to be excluded from the analysis because of the high mortality registered. The MixTox was also impossible to use on feeding parameters, since results failed on showing clear dose-response relationships in single treatments, as required by this framework. Nevertheless the mixture toxicity could still be predicted through the IA model by directly/mathematically comparing the observed data to the predicted toxicity (based on the individual effect of each stressor) as shown in Martin et al. (2009) and Santos et al. (2011a). Although having the drawback of preventing the accurate description of the deviation patterns, this analytical procedure still enabled the identification of their nature after calculation of the confidence interval (α=0.05). In order to analyze data from continuous variables (e.g. consumption ratio, biomass gain/loss), the probability of nonresponse to the toxicants can be calculated according to the following equation:

$$\text{mixture toxicity}\ (q_1, \ldots, q_n) = \max \prod_{i=1}^{n} q_1\ (c_1) \qquad (3)$$

where $q_i(c_i)$ is the probability of nonresponse at concentration c of toxicant i and max is the maximum value observed (assumed to be the control). Biomass variation data was converted to positive values and log-transformed as described by Wicklin (SAS 2011)

5.4. Results

As shown in Figure 5.2, no mortality was found in any of the control treatments, but a significantly higher mortality occurred in the hot and cold cycles when compared to 20 °C and the mild cycle (Three-way ANOVA, $F_{5,63}$= 31.67, p<0.001). Nevertheless, temperature seemed to have influenced both pesticides in different ways since the individual toxicity of CPF was more prominent at higher temperatures whereas MCZ was more toxic at lower temperatures (Figure 5.2). A similar situation was also observed for the mixture treatments since the greatest mortalities found after the hot cycle occurred whenever high concentrations of CPF were present, whereas in the cold cycle, treatments with higher concentrations of MCZ showed to be more lethal (Figure 5.2). However, when searching for interactions between the pesticides, the MixTox tool always indicated the reference model of IA as the most parsimonious outcome since none of the three deviation patterns that complete the nested framework showed to improve the fit to our experimental data (see Table 5.1). Thus, if one consider exclusively the joint-toxicity of these pesticides as binary mixture, their effects can be assigned as non-interacting or additive, irrespective of the temperature regime.

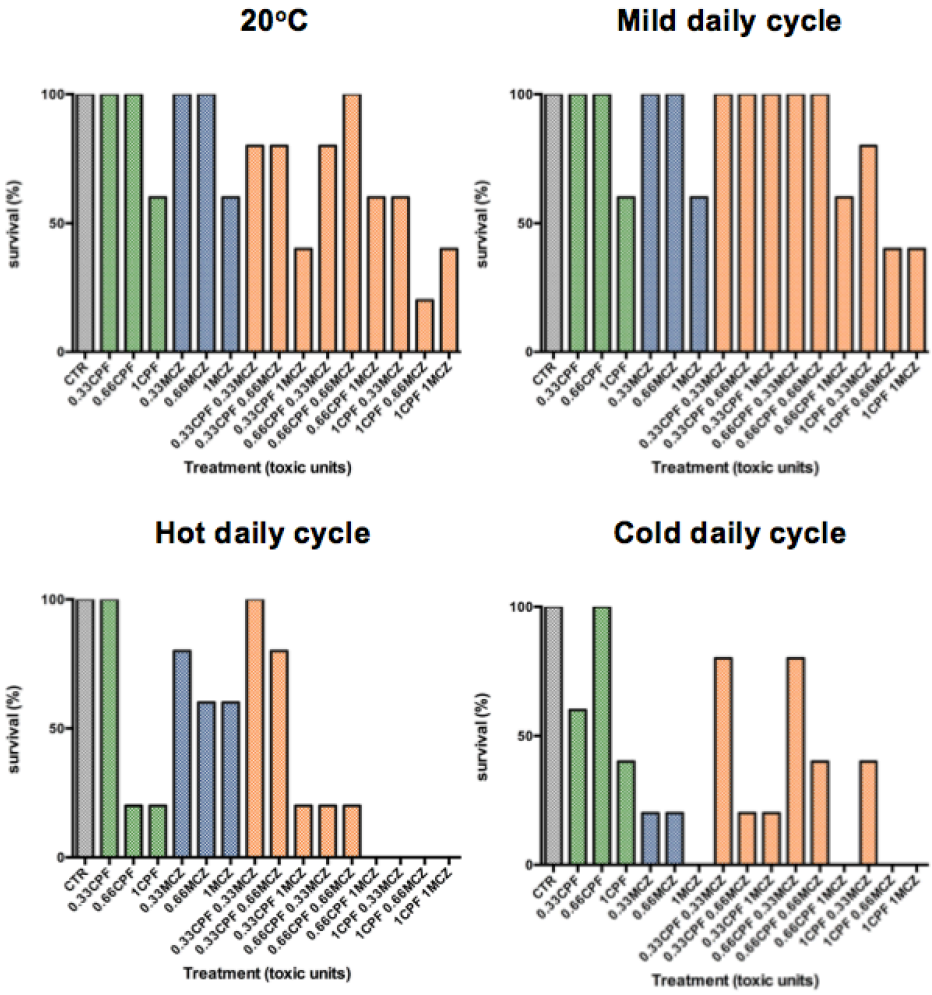

Figure 5.2 – Total survival of *Porcellionides pruinosus* (as percentage) after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four different temperature regimes: constant 20 °C, mild cycle (daily cycle between 15 °C and 25 °C), cold cycle (daily cycle between 5 °C and 15 °C) and hot cycle (daily cycle between 25 °C and 35 °C).

Table 5.1 – Parameter estimates and tests of fit of the reference model of independent action using the MixTox framework applied to the survival of *Porcellionides pruinosus* after exposed to single and mixture treatments of chlorpyrifos and mancozeb for 14 days in different temperature regimes. IA is independent action; S/A is synergism/antagonism, DR is "dose ratio" and DR is "dose level deviation from the reference; r^2 is the coefficient of determination, $p(\chi^2)$ indicates the outcome of the likelihood ratio test and SS are the objective functions; *a* and *b* are parameters of the deviation functions.

	20 °C					**Mild Cycle**					**Hot Cycle**					**Cold cycle**				
	r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*
IA	0.76	***	34.58	-	-	0.80	***	32.35	-	-	0.85	***	60.61	-	-	0.79	***	66.72	-	-
S/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

In some cases, the high mortality found in the more extreme treatments restrained the assessment of feeding parameters, since in some of them, there were no enough isopods still alive at day 14. In this way, as regards to the hot and cold cycles, the results reported are mostly related to treatments with the lowest TUs.

Within this, some interesting outcomes were observed. First of all, significant differences were found in consumption ratio between controls (One-way ANOVA, $F_{3,19}$=3.680, p=0.031) suggesting that temperature alone can influence this endpoint. While isopods' consumption at 20 ^{o}C was similar to after the ones under the mild and hot cycles, those kept for 14 days at colder temperatures were found to consume significantly less (Holm-Sidak, p=0.034). Regarding the other treatments, significant differences were only found between the cold cycle and: (i) all the remaining regimes at 0.33CPF (One-way ANOVA, $F_{3,17}$=16.638, p=<0.001), (ii) the hot cycle at 0.33MCZ (One-way ANOVA, $F_{2,13}$=7.332, p=0.009), (iii) 20 ^{o}C and the hot cycle in 0.33CPF/0.33MCZ (Kruskal-Wallis, H=11.484, df=3, p=0.009); (iv) 20 ^{o}C and the mild cycle at 1 CPF/0.33MCZ (One-way ANOVA, $F_{2,8}$=21.237, p=0.002). Consumption also seemed to decrease with the increase of pesticides' concentration (or with the increase of treatments' TUs), showing a dose-response relationship. However, significant differences for the respective control were only found at 20 ^{o}C for isopods exposed to 0.66MCZ (Kruskal-Wallis, H=31.350, df=15, p=0.008). Consumption patterns of the hot cycle resembled the 20 ^{o}C results until the treatment 0.33CPF/0.66MCZ, from where survival started to be too low to allow an accurate measuring of feeding parameters (Figure 5.3).

Comparisons between predicted and observed consumption ratios for mixture treatments showed that isopods generally consumed significantly more than what would be expected in light of the IA model, mainly at 20 ^{o}C. The only registered situations of a significantly less consumption when compared to the IA-predicted values were found in the mild cycle, in treatments 0.33CPF/0.66MCZ and 1CPF/1MCZ, and in the hot cycle for treatments 0.33CPF/0.33MCZ and 0.33CPF/0.33MCZ (Figure 5.3 and Table 5.1SD). Likewise, when including temperature effects in the IA model predictions, several significant deviations to the reference model were found but they all consisted of antagonistic interactions (Table 5.2SD).

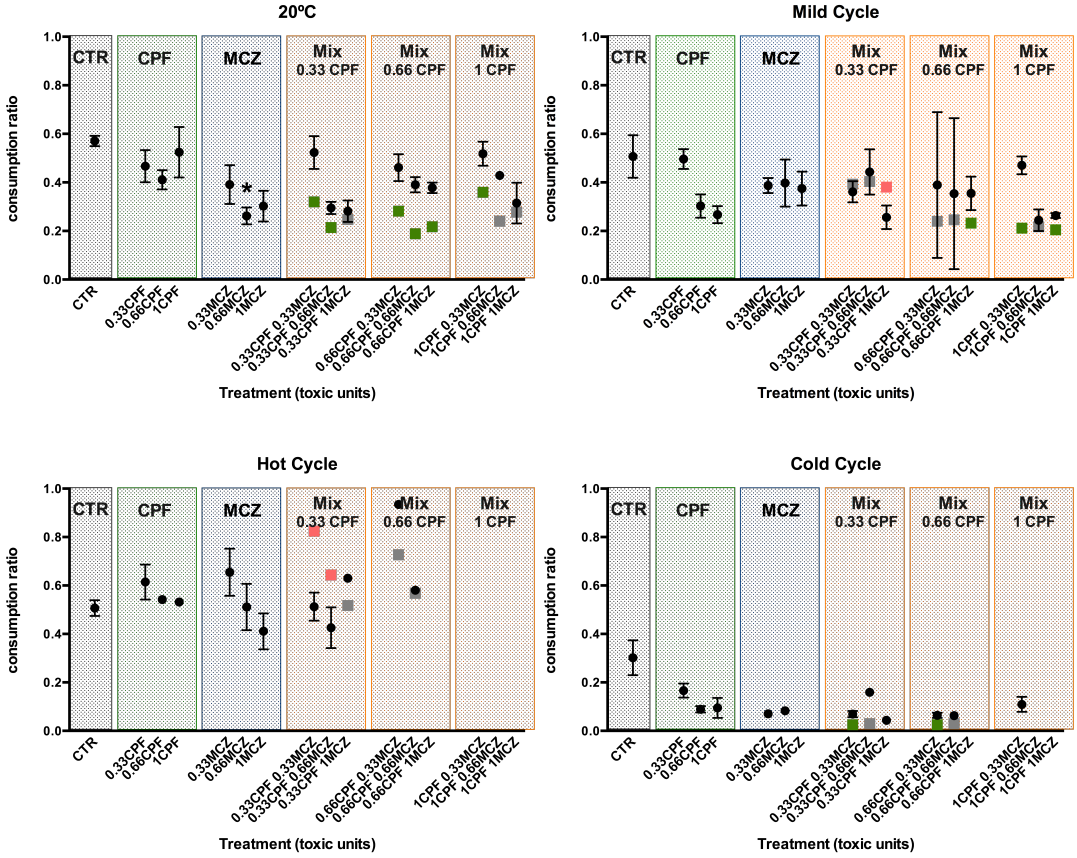

Figure 5.3 – Observed (circles; ± standard error) and predicted (triangles) consumption ratios of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four different temperature regimes: constant 20 °C, mild cycle (daily cycle between 15 °C and 25 °C), cold cycle (daily cycle between 5 °C and 15 °C), and hot cycle (daily cycle between 25 °C and 35 °C). Grey squares represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), green squares represent prediction values that were significantly higher than observed results (i.e. antagonism), and red squares represent prediction values that were significantly lower than observed results (i.e. synergism). Treatments indicated by asterisks are significantly different from control (one-way ANOVA followed by Dunnett's *post hoc* test, α=0.05).

Significant differences between control groups were also found in biomass gain/loss (Kruskal-Wallis, H=10.451, df=3, p=0.015), suggesting that similarly to consumption ratio, this endpoint can also be influenced by temperature alone. Whereas no differences were registered between 20 °C and the mild- and hot cycle's controls, a significant decrease in isopods' biomass was detected when comparing control groups in 20 °C and mild cycle to those kept under the cold cycle (Holms-Šidak, p<0.05). No differences were observed between hot and cold cycles. As regards to the other

treatments, significant differences were found between the cold and mild cycles at 0.33CPF (One-way ANOVA, $F_{3,17}$=4.52, p=0.020) and 0.66CPF (One-way ANOVA, $F_{2,14}$=7.575, p=0.007). Most significant deviations found to the IA model predictions were again antagonistic, though the occurrence of some synergistic deviations in every temperature regime (Figure 5.4 and Table 5.3SD). This pattern was not altered with the inclusion of temperature as a third stressor on the model (Table 5.4SD).

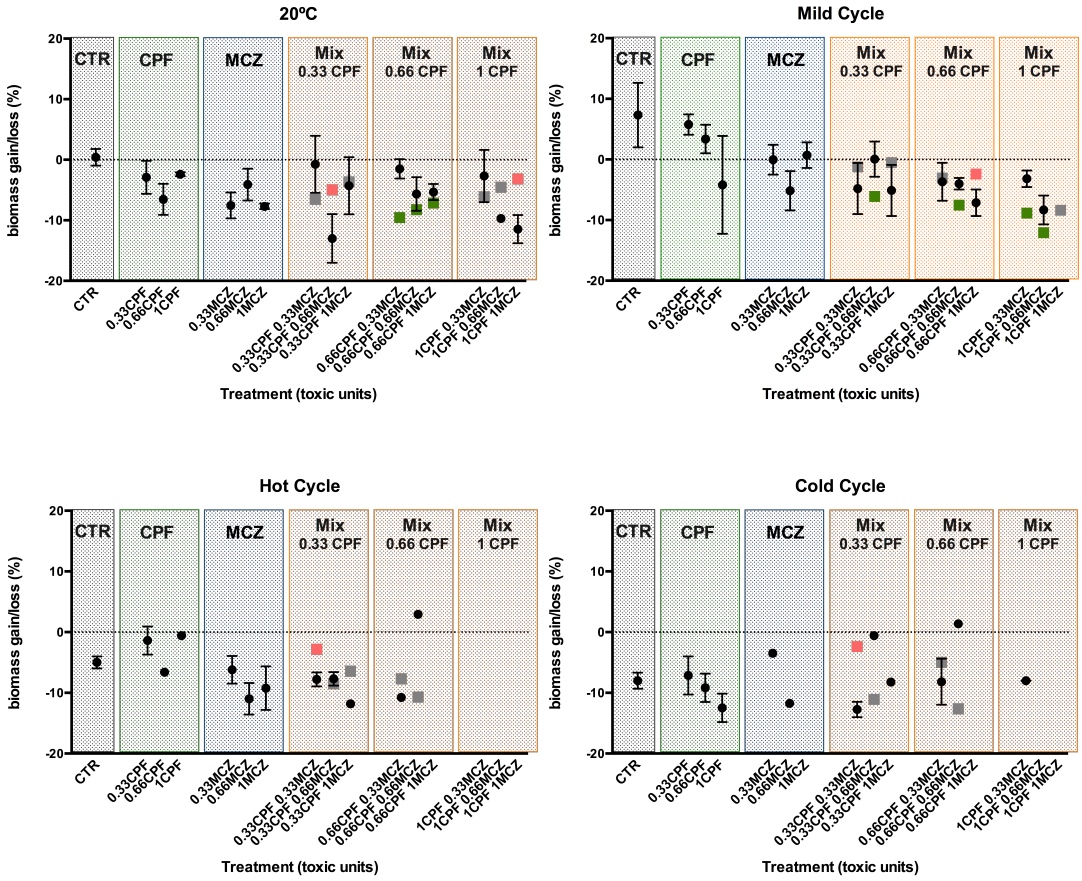

Figure 5.4 – Observed (circles; ± standard error) and predicted (triangles) biomass gain/loss of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four different temperature regimes: constant 20 °C, mild cycle (daily cycle between 15 °C and 25 °C), cold cycle (daily cycle between 5 °C and 15 °C), and hot cycle (daily cycle between 25 °C and 35 °C). Grey squares represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), green squares represent prediction values that were significantly lower than observed results (i.e. antagonism), and red squares represent prediction values that were significantly higher than observed results (i.e. synergism).

5.5. Discussion

More than the individual effects of global warming or environmental pollution, nowadays the interaction between multiple factors is raising the biggest concerns among soil ecologists. Albeit the considerable amount of work published on this area in the last few years (see Holmstrup et al. 2010 for a review), the influence of environmental conditions on the behavior and toxicity of chemical mixtures remains still poorly understood (Laskowski et al. 2010). Our results suggest that the toxicity of CPF and MCZ can, indeed, be strongly influenced by temperature, either when individually or jointly applied. Moreover, having a hierarchical and complementary design that included both lethal and sub-lethal parameters, allowed us to have a broader perspective into the several levels of toxicity, while avoiding the complexity that these multiple stressors' assessments generally require.

None of the temperature regimes *per se* showed to affect isopods' survival, as they resemble natural conditions on different seasons. In fact, the use of temperature daily fluctuating cycles seems to be a more accurate approach relating to real scenarios, as temperatures are never constant over a day period. Even if organisms were exposed to extreme temperatures in several consecutive days, these conditions were possibly ameliorated during at least some part of the day/night. By using the continuous 20 ^{o}C, we did not intend to find differences when comparing to the mild cycle, but mostly to provide a situation that could be compared to in similar mixture studies. However, if continuous regimes of extreme temperatures had been used instead of daily cycles, it is possible that results might had been fairly different.

Regarding survival results, several findings must be highlighted. First of all, and without surprise, CPF was acutely toxic at substantially lower doses than MCZ. Like all the organophosphorus insecticides, CPF is known to induce severe, and often irreversible, inhibitions of acetylcholinesterase (Fukuto 1990). Acetylcholinesterase is a synaptic enzyme mostly known for its role on the hydrolysis of the neurotransmitting agent acetylcholine, although additional roles have also been identified (Grisaru et al. 1999; Soreq & Seidman 2001). Its inhibition causes an overaccumulation of this neurotransmitter, leading to a hyperexcitation of cholinergic receptors, with organisms showing signs of hyperactivity, problems with neuromotor faculties, and eventually death (Roex et al. 2003). Although more effects are described in literature (Crumpton et al. 2000; Jager et al. 2007; Mansour & Mossa 2009), the extent of acute CPF toxicity on

living organisms is generally closely related to the degree of acetylcholinesterase inhibition caused by the contamination event (Jager et al. 2007; Kavitha & Rao 2008).

Contrary to CPF, MCZ was not conceived to directly target soil invertebrates so its acute effects to these non-target organisms are still far less clear. Dithiocarbamates like MCZ are known to act on several target organs, with several different effects (Liesivuori & Savolainen 1994). They were shown to inhibit the P450-containing mixed function oxidase system (Lewerenz & Plass 1984; Szépvölgyi et al. 1989), induce oxidative stress (Tsang & Trombetta 2007), and have neurodegenerative effects on several classes of receptor systems, mainly at glutamatergic and dopaminergic synapses (Negga et al. 2012; Brody et al. 2013). Since MCZ is often assumed to have low acute toxicity to soil invertebrates, survival has been reported as a less sensitive endpoint to evaluate its individual effects on non-target soil organisms. Better toxicity estimates were generally described with sub-lethal parameters, like avoidance behavior (Reinecke et al. 2002), reproduction (De Silva et al. 2010), or even molecular markers (Guven et al. 1999).

Even though temperature did not affect survival by itself, it was clearly able to influence the toxicity of both pesticides by increasing mortality at both highest and lowest temperature regimes. When compared to the mild cycle, both pesticides showed to induce a temperature-dependent increase in isopods' mortality. Interestingly, however, the extent of this influence was felt in opposite ways for CPF and MCZ, which demonstrates the case-specificity of these temperature-pesticide relationships. Whereas CPF toxicity was raised under higher temperatures, the toxicity of MCZ was markedly more prominent at lower temperatures. As previously stated, there are several ways that the toxicity of a pesticide can be influenced by temperature, so the definitive outcome will ultimately be the result of a complex weighting of many factors, including the properties of the compound and the physiologic status of the organism (Noyes et al. 2009). In fact, these differences in temperature-dependency might, to some extent, be related with the lower persistence and higher thermal lability of MCZ. As usual for all dithiocarbamate fungicides, MCZ is a compound with a very short half-life in soil (1-5 days), whose strategy of use is always preventive and based on fortnightly repeated applications, during periods of rapid foliage growth (Wightwick et al. 2010). In this way, a faster MCZ decay is likely to occur under higher temperatures, reducing the time of the exposure, whilst lower temperatures may extend its fate and, consequently the extent of its effects. CPF, on the other hand, is considered to be moderately persistent in soil so, despite being also expected to degrade faster at higher temperatures, it may not be enough to balance the expected increase in toxicity caused by its biotransformation. The biotransformation rates and the resulting

products must, indeed, have been the most important factor determining these temperature-related differences found in the toxicity of both pesticides. CPF, as a parental compound, is a poor anticholinesterase compound since it is not very reactive (Fukuto 1990). However, after undergoing into processes of metabolic activation mediated by the mixed-function oxidases, it leads to highly reactive metabolites, such as the oxon-analog, with a much higher anticholinesterase potency (Fukuto 1990). This study is, therefore, in line with previous works that reported a higher biotransformation rate at higher temperatures, with consequent increase in toxicity of organophosphourus insecticides (Lydy et al. 1999; Harwood et al. 2009).On the contrary, none of the main MCZ metabolites showed previously to have a higher short-term toxicity than the parental compound (Guven et al. 1999; Easton et al. 2001), despite having a much longer persistence (Wightwick et al. 2010). In this way, contrary to CPF, the major enhancing factor to the toxicity of MCZ would not be the breakdown processes but instead with the longer maintenance of the parental compound, and this is more likely to occur under low temperatures. This does not mean, however, that an increase in temperature cannot imply an increase in MCZ toxicity as well, as seen by the higher-than-predicted mortality found in single MCZ treatments during the hot cycle. Nevertheless, it suggests that conditions that maximize the preservation of this pesticide have a preponderant effect on its toxicity.

Although more pronounced to CPF, the increase in mortality found in both pesticides with higher temperatures (when compared to 20 ^{o}C or the mild cycle) can probably be explained by the action of this abiotic factor on the organisms. Considering the Q10 concept, the metabolism of ectotherms usually increases nearly twofold every 10 ^{o}C (Abdel-Lateif et al. 1998; Donker et al. 1998; Lydy & Linck 2003), potentially affecting an array of different physiological and behavioral parameters. Edney (1964) observed a remarkable temperature-dependent increase in the heart rate of *Porcellio laevis*. Similarly, Hornung (1981) and Salomon and Buchholz (2000) showed that temperature enhanced oxygen consumption in several isopod species, although the later authors have suggested that this is most probably due to a behavioral response rather than a consequence of direct metabolic adaptation processes. Pörtner (2001), in other hand, stated that, particularly in ectotherms, these increases in heart and ventilation rates are generally required to compensate the rises in oxygen demands at warmer temperatures. Either of physiologic or behavioral nature, these factors are certainly capable of influencing both organisms' condition and pesticides' kinetics on their body. Since metabolism is closely linked to energetics, a higher metabolic rate is expected to affect the partition of energy, consequently decreasing the energy available to some other processes, including in

dealing with any other possible stressors (Baas et al. 2010). Similarly, behavioral changes like temperature-driven increases on the activity patterns were already identified in terrestrial isopods (Warburg 1968; Dailey et al. 2009) and, according to Bayley and Baatrup (1996), are likely to enhance the uptake of pesticides. Nevertheless, in some situations these increments in metabolism can also help organisms getting rid of the toxicant by enhancing detoxification and elimination phases (Martikainen & Rantalainen 1999). These later authors also stated that this higher elimination performance could be due to increased molting or excretion rates. In this study, egestion was not evaluated but assuming that higher consumption rates may lead to higher egestions, it would be expected that some differences could also have happened here. However, no indication of this toxicity-ameliorating mechanism was observed or it just was not noticeable in this experiments, probably by being masked by the higher uptake rates.

Although influencing the acute toxicity and behavior of each component, temperature failed to show changes on the mixture itself. No deviations were found to the reference model projections meaning that there was no interaction between these pesticides, or none of them influenced the acute toxicity of the other one. In fact, we could observe that mixtures containing the same ratios could lead to different acute toxicities when under different temperatures. However, these differences could not be considered has deviations to the additivity behavior since the same had also happened on the individual treatments. Instead, we can only state that the weight of each compound to the toxicity of the mixture can be variable with temperature, but always in a non-interacting way.

This outcome should not, however, withdraw the significance of assessing this particular mixture, nor to be considered of low toxicological concern because, as shown above, the simple addition of their acute toxicities may entail severe consequences to soil communities when under the influence of extreme temperature conditions. For instance, insecticides are commonly applied in the summer, which is exactly the period when they can affect soil ecosystems the most. To assess whether the additional application of MCZ does not raise the ecological risk to unacceptable levels is critical since, according to our data, different levels of concern are expected depending on the season or conditions. Furthermore, in a context of elevated unpredictability, short episodes of unusually high or low temperatures in combination with chemical contamination may constitute an even stronger constraint to these ecosystems than the long-term steady warming (Parmesan et al. 2000).

Literature can show several examples of interacting chemical mixtures, either synergistic or antagonistic, so one should quarrel whether mixtures with a different behavior would be differently affected by temperature conditions. In one of the few ecotoxicological studies accounting on three-way stressors' interactions, Bednarska et al. (2009) found complex relationships between nickel, CPF and temperature and concluded that multiple stressors can lead to outcomes that are not possible to predict when studying each stressor separately. Additional research will be needed in order to clearly identify the features of the most concerning mixtures and to understand their relationships. In this study, toxicants were selected for their relevance in agricultural fields and, in our opinion, this must always be one of the chief parameters to be considered. To our knowledge, there is only one other study evaluating the impact of this mixture on invertebrate non-target organisms, despite being ubiquitously used. In a field study, Cross and Berrie (1996) evaluated this mixture's effects to the predatory mite *Typhlodromus pyri* and concluded that mixture-treated areas ended up having significantly less organisms per leave than either CPF or MCZ alone. This seems to be in accordance with our findings. Nevertheless these authors were not clear as regards to the occurrence, or not, of truly synergistic interactions between compounds. In addition they employed a longer study with repeated applications of both individual and mixture treatments. In this way, given the lack of information and the frequency of its application, more research seems to be advisable.

Regarding the feeding parameters, the first observation that immediately stood out from the present study was the difference found in consumption patterns between temperature regimes. Isopods kept at colder temperatures consumed significantly less when compared with the other regimes, not only in the control group but also in most of the additional treatments. This must be due to the temperature-induced decrease on metabolism, and might also have important implications on the uptake of the pesticides. Generally, either individual pesticides or mixture treatments seemed to have decreased isopods' consumption rates in exposed individuals. This may have resulted from an avoidance behavior and/or, more likely to be the consequence of a gradual deterioration in isopods' health condition so they simply could not undergo the normal functional and metabolic processes associated with food consumption and allocation.

Despite the reductions in consumption generally registered for the mixture treatments, most of the several significant deviations found to the IA reference model still showed an antagonistic nature. This indicates that mixture effects on this parameter were not as strong as would be expected, allowing isopods to keep some of their life traits

relatively unaltered. However, it should be pointed out that although the reduction was less steep than expected, it often coincided with notorious losses in isopods' biomass, suggesting that less energy was still being allocated to growth. Consumption and growth patterns were very similar under the 20 ^{o}C and mild cycle regimes. Fewer conclusions could be drawn for some of the mixture treatments in the hot and cold cycles since there were often no isopods still alive at the end of the experiment. When we conceived this experiment, it was our intention to employ a nested hierarchical design in order to maximize the information with the minimum complexity and extensiveness. This means that endpoints are not supposed to have the same relative importance, but instead to be organized in tiers. Whenever treatments showed a severe impairment on survival, feeding parameters could not be assessed and sub-lethal effects do not assume much relevance.

In this experiment, the results on the biomass parameter showed to be quite variable. This is however an important parameter since it influences several isopods' life-traits like feeding, locomotor activity, fecundity or reproduction, which closed relates to the eficiency of their ecological function as macrodecomposers.

Given their ecological importance, terrestrial isopods constitute a key group in soil ecosystems and, along with other elements of the decomposer macrofauna guild, they are often considered to be an ecosystem engineer (Loureiro et al. 2005). In this way, toxicity effects like those described in this study are expected to entail strong implications on ecosystems' processes at different levels of organization (Loureiro et al. 2005).

5.6. References

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Table 5.1SD - Binary combination of chlorpyrifos and mancozeb in isopods' consumption rates. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted mortality of *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four temperature regimes: constant 20 °C, a mild daily cycle (between 15 °C and 25 °C), a hot daily cycle (25 °C-35 °C), and a cold daily cycle (5 °C-15 °C). Predicted values were calculated by measuring the joint probability of effects on isopods' consumption found for single exposures to chlorpyrifos and mancozeb in separate for each soil moisture. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 20 °C was assumed to be the control for temperature.

	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
20 °C					
	0.33	0.33	0.523 (0.402-0.644)	0.320	Antagonism
	0.33	0,66	0.295 (0.269-0.320)	0.208	Antagonism
	0.33	1	0.282 (0.239-0.324)	0.246	ns
	0,66	0.33	0.461 (0.373-0.549)	0.282	Antagonism
	0,66	0,66	0.390 (0.349-0.432)	0.183	Antagonism
	0,66	1	0.378 (0.350-0.406)	0.216	Antagonism
	1	0.33	0.518 (0.430-0.606)	0.359	Antagonism
	1	0,66	0.429 (--- - ---)	0.233	-----
	1	1	0.120 (0.063-0.336)	0.276	ns
Mild cycle					
	0.33	0.33	0.370 (0.307-0.433)	0.393	ns
	0.33	0,66	0.453 (0.288-0.619)	0.404	ns
	0.33	1	0.262 (0.213-0.312)	0.381	synergism
	0,66	0.33	0.399 (-0.07-0.868)	0.240	ns
	0,66	0,66	0.362 (-0.079-0.803)	0.246	ns
	0,66	1	0.363 (0.266-0.461)	0.232	Antagonism
	1	0.33	0.481 (0.413-0.55)	0.212	Antagonism
	1	0,66	0.250 (0.207-0.294)	0.217	ns
	1	1	0.270 (0.259-0.280)	0.205	Antagonism
Hot cycle					
	0.33	0.33	0.525 (0.406-0.644)	0.824	Synergism
	0.33	0,66	0.437 (0.292-0.580)	0.644	Synergism
	0.33	1	0.646 (--- - ---)	0.518	-----
	0,66	0.33	0.958 (--- - ---)	0.727	-----
	0,66	0,66	0.596 (--- - ---)	0.568	-----

	0,66	1	-----	-----	-----
	1	0.33	-----	-----	-----
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----
Cold cycle					
	0.33	0.33	0.069 (0.063-0.075)	0.025	Antagonism
	0.33	0,66	0.159 (--- - ---)	0.03	-----
	0.33	1	0.044 (--- - ---)	-----	-----
	0,66	0.33	0.064 (0.06-0.069)	0.021	Antagonism
	0,66	0,66	0.063 (--- - ---)	0.024	-----
	0,66	1	-----	-----	-----
	1	0.33	0.109 (0.087-0.131)	-----	-----
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----

Table 5.2SD – Ternary combination of temperature, chlorpyrifos and mancozeb in isopods' consumption rates. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted biomass variation by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four temperature regimes: constant 20 °C, a mild daily cycle (between 15 °C and 25 °C), a hot daily cycle (25 °C-35 °C), and a cold daily cycle (5 °C-15 °C). Predicted values were calculated by measuring the joint probability of effects on isopods' biomass variation found for single exposures to different temperature regimes, concentrations of chlorpyrifos and concentrations of mancozeb. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 20 °C was assumed to be the control for temperature.

Temperature	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
20 °C	0.33	0.33	0.523 (0.402-0.644)	0.32	Antagonism
	0.33	0,66	0.259 (0.269-0.32)	0.214	Antagonism
	0.33	1	0.282 (0.239-0.324)	0.247	ns
	0,66	0.33	0.461 (0.373-0.549)	0.282	Antagonism
	0,66	0,66	0.3989 (0.349-0.432)	0.189	Antagonism
	0,66	1	0.378 (0.35-0.406)	0.218	Antagonism
	1	0.33	0.518 (0.43-0.606)	0.359	Antagonism
	1	0,66	0.429 (--- - ---)	0.241	-----
	1	1	0.315 (0.224-0.405)	0.278	ns
Mild cycle	0.33	0.33	0.361 (0.307-0.415)	0.284	Antagonism
	0.33	0,66	0.443 (0.301-0.585)	0.19	Antagonism
	0.33	1	0.256 (0.214-0.299)	0.219	ns
	0,66	0.33	0.39 (-0.012-0.791)	0.25	ns
	0,66	0,66	0.354 (-0.024-0.732)	0.167	ns
	0,66	1	0.355 (0.271-0.438)	0.193	Antagonism
	1	0.33	0.470 (0.412-0.529)	0.319	Antagonism
	1	0,66	0.244 (0.207-0.282)	0.214	ns
	1	1	0.264 (0.254-0.273)	0.246	Antagonism
Hot cycle	0.33	0.33	0.513 (0.411-0.614)	0.284	Antagonism
	0.33	0,66	0.426 (0.303-0.549)	0.19	Antagonism
	0.33	1	0.63 (--- - ---)	0.22	-----
	0,66	0.33	0.935 (--- - ---)	0.25	-----
	0,66	0,66	0.581 (--- - ---)	0.167	-----
	0,66	1	-----	0.193	-----
	1	0.33	-----	0.319	-----

	1	0,66	-----	0.214	-----
	1	1	-----	0.246	-----
Cold cycle	0.33	0.33	0.069 (0.066-0.072)	0.193	ns
	0.33	0,66	0.159 (--- - ---)	0.319	-----
	0.33	1	0.044 (--- - ---)	0.214	-----
	0,66	0.33	0.064 (0.062-0.067)	0.246	ns
	0,66	0,66	0.063 (--- - ---)	0.169	-----
	0,66	1	-----	-----	-----
	1	0.33	0.19 (0.098-0.121)	0.113	ns
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----

Table 5.3SD - Binary combination of chlorpyrifos and mancozeb in isopods' biomass variation. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted mortality of *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four temperature regimes: constant 20 °C, a mild daily cycle (between 15 °C and 25 °C), a hot daily cycle (25 °C-35 °C), and a cold daily cycle (5 °C-15 °C). Predicted values were calculated by measuring the joint probability of effects on isopods' consumption found for single exposures to chlorpyrifos and mancozeb in separate for each soil moisture. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 20 °C was assumed to be the control for temperature.

	CPF (TU)	MCZ (TU)	Observed Biomass gain/loss (CI)	IA predicted biomass gain/loss	Output
20 °C					
	0.33	0.33	1.33 (1.137-1.529)	1.133	ns
	0.33	0,66	0.832 (0.463-1.2)	1.212	Synergism
	0.33	1	1,282 (1.09-1.474)	1.443	ns
	0,66	0.33	1.349 (1.294-1.405)	1.061	Antagonism
	0,66	0,66	1.245 (1.136-1.354)	1.136	Antagonism
	0,66	1	1.269 (1,214-1.323)	1.072	Antagonism
	1	0.33	1.308 (1.127-1.489)	1.156	ns
	1	0,66	1.155 (--- - ---)	1.238	-----
	1	1	1.091 (0.965-1.217)	1.168	ns
Mild cycle					
	0.33	0.33	1.203 (0.951-1.455)	1.377	ns
	0.33	0,66	1.368 (1.271-1.465)	1.252	Antagonism
	0.33	1	1.230 (1.061-1.399)	1.397	ns
	0,66	0.33	1.287 (1.171-1.403)	1.340	ns
	0,66	0,66	1.299 (1.261-1,337)	1.218	Antagonism
	0,66	1	1.219 (1.124-1.315)	1.359	Synergism
	1	0.33	1.315 (1.262-1.369)	1.082	Antagonism
	1	0,66	1.19 (1.084-1.296)	0.984	Antagonism
	1	1	1.053 (0.208-1.899)	1.098	ns
Hot cycle					
	0.33	0.33	1.202 (1.144-1.260)	1.303	Synergism
	0.33	0,66	1.206 (1.154-1.259)	1.153	ns
	0.33	1	1.083 (--- - ---)	1.194	-----
	0,66	0.33	1.119 (--- - ---)	1.204	-----
	0,66	0,66	1.426 (--- - ---)	1.107	-----

	0,66	1	-----	-----	-----
	1	0.33	-----	-----	-----
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----
Cold cycle					
	0.33	0.33	1.224 (1.159-1.289)	1.289	Synergism
	0.33	0,66	1.095 (--- - ---)	1.1	-----
	0.33	1	1.154 (--- - ---)	-----	-----
	0,66	0.33	1.209 (1.122-1.297)	1.248	ns
	0,66	0,66	1.227 (1.079-1.375)	1.036	Antagonism
	0,66	1	-----	-----	-----
	1	0.33	1.221 (1.164-1.277)	1.149	ns
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----

Table 5.4SD – Ternary combination of temperature, chlorpyrifos and mancozeb in isopods' biomass variation. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted biomass variation by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under four temperature regimes: constant 20°C, a mild daily cycle (between 15 °C and 25 °C), a hot daily cycle (25 °C-35 °C), and a cold daily cycle (5 °C-15 °C). Predicted values were calculated by measuring the joint probability of effects on isopods' biomass variation found for single exposures to different temperature regimes, concentrations of chlorpyrifos and concentrations of mancozeb. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 20 °C was assumed to be the control for temperature.

Temperature	CPF (TU)	MCZ (TU)	Observed biomass gain/loss (CI)	IA predicted biomass gain/loss	Output
20°C	0.33	0.33	1.33 (1.137-1.529)	1.133	ns
	0.33	0,66	0.832 (0.463-1.2)	1.212	Synergism
	0.33	1	1,282 (1.09-1.474)	1.443	ns
	0,66	0.33	1.349 (1.294-1.405)	1.061	Antagonism
	0,66	0,66	1.245 (1.136-1.354)	1.136	Antagonism
	0,66	1	1.269 (1,214-1.323)	1.072	Antagonism
	1	0.33	1.308 (1.127-1.489)	1.156	ns
	1	0,66	1.155 (--- - ---)	1.238	-----
	1	1	1.091 (0.965-1.217)	1.168	ns
Mild cycle	0.33	0.33	1.203 (0.936-1.469)	1.197	ns
	0.33	0,66	1.368 (1.266-1.471)	1.281	ns
	0.33	1	1.230 (1.052-1.409)	1.209	ns
	0,66	0.33	1.287 (1.164-1.41)	1.122	Antagonism
	0,66	0,66	1.299 (1.259-1.339)	1.201	Antagonism
	0,66	1	1.219 (1.118-1.32)	1.133	ns
	1	0.33	1.315 (1.259-1.372)	1.222	ns
	1	0,66	1.190 (1.078-1.302)	1.308	Synergism
	1	1	1.053 (0.16-1.947)	1.234	ns
Hot cycle	0.33	0.33	1.206 (1.152-1.259)	1.044	Antagonism
	0.33	0,66	1.21 (1.161-1.258)	1.118	Antagonism
	0.33	1	1.086 (--- - ---)	1.055	-----
	0,66	0.33	1.122 (--- - ---)	0.979	-----
	0,66	0,66	1.430 (--- - ---)	1.047	-----
	0,66	1	-----	-----	-----
	1	0.33	-----	-----	-----

	1	0,66	-----	-----	-----
	1	1	-----	-----	-----
Cold cycle	0.33	0.33	1.042 (0.96-1.124)	0.987	
	0.33	0,66	1.370 (--- - ---)	1.066	-----
	0.33	1	1.2 (--- - ---)	1.141	-----
	0,66	0.33	1.153 (0.953-1.354)	1.077	
	0,66	0,66	1.431 (1.377-1.485)	0.98	
	0,66	1	-----	-----	-----
	1	0.33	1.204 (1.203-1.205)	1.049	Antaginism
	1	0,66	-----	-----	-----
	1	1	-----	-----	-----

CHAPTER 6: Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to *Porcellionides pruinosus*

Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*

6.1. Abstract

One of the main sources of uncertainty currently associated with environmental risk assessement procedures is the poor understanding of the influence that environmental factors can exert on the toxicity of xenobiotics. Aiming at contributing for this topic, the joint-effects of two pesticides (chlorpyrifos and mancozeb) to the terrestrial isopod *Porcellionides pruinosus* were evaluated under different soil moisture conditions. A full factorial design, including three treatments of each pesticide and an untreated control, were performed and repeated under three different soil moisture regimes: 25% WHC, 50% WHC, and 75% WHC.

Soil moisture showed no effects on isopods' survival nor did show any influence on isopods resilience to single and mixture treatments of these pesticides. Additivity was always the most parsimonious result when both pesticides were present.

By the opposite, the feeding parameters showed to be rather more sensitive to the treatments used, with isopods clearly showing a worse performance when exposed to exceedingly dry or too-moist conditions. The most significant differences between soil moisture regimes were found for the single pesticide treatments. Nevertheless, soil moisture still showed to influence the effects of the binary mixture, both in the consumption ratio and biomass gain/loss. A lower than expected consumption ratio and a higher than expected decrease in biomass were found for most of the mixture treatments at 50% WHC. On the contrary, for 25% and 75% WHC, consumption was found to be higher than perdicted by the IA model and biomass variation showed to be less negative. Given the key role of terrestrial isopods in several soil ecosystems processes, this situation is likely to constitute a serious imbalance at different levels of organization.

Kewwords: Multiple stressors; climate changes; pesticides; mixtures; terrestrial isopods; independent action model

6.2. Introduction

The increasing evidences that different natural and chemical stressors can interact influencing each other's toxicity has been pushing ecotoxicologists to assess increasingly complex scenarios (Jones 1975; van Gestel & van Diepen 1997; Chen et al. 2004; Heugens et al. 2006; Bednarska et al. 2009; Lima et al. 2011; Cardoso et al. 2014; Ferreira et al. 2015). This situation has been prompted by the growing awareness that studies currently supporting environmental risk assessments may not be representative of real exposures to xenobiotics since they neglect the simultaneous occurrence of multiple stressors (Bednarska et al. 2013). Such procedures are mostly based on standard laboratory bioassays where organisms are exposed to a single compound, with all the remaining conditions kept at near-optimal conditions (Holmstrup et al. 2010; Laskowski et al. 2010). Since these conditions are seldom met in nature, a new approach is required in order to provide risk assessments, with an appropriate perspective into the joint effects of simultaneously acting stressors, in a pragmatic and cost-effective way.

Such studies seem to be particularly relevant for edaphic ecosystems from agricultural landscapes since these constitute amended ecosystems that are continuously subject to several kinds of stress, including the exposure to a wide range of pesticides, while simultanously experiencing severe abiotic conditions (Hope 2005; Pretty 2008; Santos et al. 2011b).

Soil is a heterogeneous matrix, with marked spatial and temporal variations in resources and conditions which, together with the limited mobility of its organisms, make some of these particularly vulnerable to adverse situations (Postma et al. 1989; Ettema & Wardle 2002). Along with temperature, soil moisture is one of the most significant environmental factors shaping edaphic ecosystems (Singh & Gupta 1977; Porporato et al. 2002; Iturbe & Porporato 2004; Choi et al. 2006). Besides local precipitation history, the properties of soil, the topography and the vegetation coverage can also contribute strongly to the soil moisture registered in a certain place and time (Mohanty & Skaggs 2001; Weltzin et al. 2003). Although the extensive uncertainty still assigned to the ongoing climate changes, several lines of evidence suggest that the expected increase in

atmospheric temperature will probably lead to an intensification of the water cycle, mainly due to changes in evaporation, evapotranspiration, and precipitation rates (Ragab & Prudhomme 2002; Huntington 2006; Rustad 2008). In this way, soil communities will probably have to deal with different patterns of soil moisture, to which is added a higher unpredicatbility in the occurrence of other events, like the co-ocorrence of pesticides. Therefore, it becomes of paramount importance to evaluate how can this natural stressor affect pesticides' toxicity.

Differences in soil moisture may lead to different pesticides' bioavailabilities by influencing their adsorption, volatilization and transformation/degradation rates (Arnold & Briggs 1990). Moreover, such differences can also affect the fitness of organisms making them physiogically less tolerant to slightly unfavourable conditions (Everts et al. 1991). In this way, the stress imposed by unfavourable soil moisture conditions may, in some situations, interact with pesticides' toxicity or just constitute an additional source of stress to the organisms (Lima et al. 2011).

In this work, we evaluated the effects of two pesticides to the terrestrial isopod *Porcellionides pruinosus*, the insecticide chlorpyrifos (CPF) and the fungicide mancozeb (MCZ), under three soil moisture regimes. Both these pesticides are extensivelly used in several Mediterranean crops, like orchards and vineyards, and their application is frequently simultaneous.

Porcellionides pruinosus is a synanthropic and widely distributed terrestrial isopod that has been frequently used in multiple soil ecotoxicology experiments (Santos et al. 2010; Ferreira et al. 2015). As a decomposer, it is involved in critical soil processes, such as the organic matter turn-over, nutrient recycling, and also in promoting the degradation of soil contaminants (Loureiro et al. 2005). Albeit the undeniable successful colonization of terrestrial habitats, the best when considering the Crustacea subphyllum, terrestrial isopods still compare poorly to other arthropods, like insects, regarding the water-balance capabilities (Edney 1954; Sutton et al. 1980). In order to maintain a correct balance of their body fluids, they are known to depend on effective behavioral patterns such as aggregation and avoidance of unsuitable habitats (Warburg 1968; Broly et al. 2013a;). Isopods' tolerance to dessication has been extensivelly investigated and several degrees of susceptibility to dry conditions were already identified among this group (Warburg 1968). Although being generally considered to be a mesic isopod, *P. pruinosus* is a cosmopolitan species that is also present in more xeric habitats, indicating some tolerance to water loss (Quinlan & Hadley 1983). Furthermore, by being unable to avoid water absorption through the cuticle, they also become prone to water overload in too-moist

environments, if they are unable to escape (Horowitz 1970; Sutton et al. 1980). These particular features, along with their ecological importance and the likelihood of being exposed to pesticides, makes of *P. pruinosus* a good surrogate species to assess the joint effects of different pesticides and natural stressors.

The aim of this study was therefore to investigate if soil moisture could influence the toxicity of two pesticides to *Porcellionides pruinosus*, either individually or in mixtures, by measuring survival, consumption ratio and biomass gain/loss. The independent action model (IA) was used in order to assess the possible occurrence of any significant interaction between the stressors.

6.3. Material and methods

6.3.1. Test organism

In this experiment, the terrestrial isopod *Porcellionides pruinosus* was used as test-species. These organisms were collected in a horse manure heap and kept in laboratory cultures at 22 ^{o}C (±1 ^{o}C), 16:8 (light:dark) photoperiod, with soil adjusted to a moisture content of 60% of its water holding capacity (WHC) and fed *ad libitum* with alder leaves (*Alnus glutinosa*). Only adult isopods were used in this experiment (15-25 mg wet weight). No gender differentiation was made, but pregnant females were discarded. Similarly, moulting isopods were not used in this experimental set up.

6.3.2. Chemical compounds and soil

Two pesticides were used to perform this experiment, both as commercial formulations: the organophosphorus insecticide chlorpyrifos (CICLONE® 48 EC with 480 g/L of chlorpyrifos) and the dithiocarbamate fungicide mancozeb (MANCOZEBE SAPEC® with 80% of mancozeb).

The certified loamy sand soil LUFA 2.2 (Speyer, Germany) was used as test soil. The main properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), water holding capacity = 41.8 ± 3.0 (g/100g), organic C = 1.77 ± 0.2 (%), nitrogen = 0.17 ± 0.02, texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

6.3.3. Experimental design

The selection of treatments to use in this experiment was based on preliminary tests where the effects of soil moisture and the toxicity of both pesticides were assessed individually. Then, a full factorial design including an untreated control plus three nominal concentrations of each pesticide (ranging from 1 $mg.kg^{-1}$ soil to 3 $mg.kg^{-1}$ soil of CPF and 88 $mg.kg^{-1}$ soil and to 264 $mg.kg^{-1}$ soil of MCZ) was repeated in three different soil moisture conditions: 25%, 50%, and 75% of the soil water holding capacity (WHC) (Figure 6.1). Chemical treatments were selected so that, in a toxic unit perspective (1 TU = LC_{50}) they were equivalent to 0.33 TU, 0.66 TU, and 1 TU of each pesticide. Hence, in this experiment treatments ranged from 0.33 TU when the minimum concentration of a single pesticide was applied, to 2 TU, when combining the maximum concentration of both pesticides. Apart from the soil moisture, all the remaining conditions were kept constant: temperature was always 20 ^{o}C (±1 ^{o}C) and the photoperiod set for 16:8 hours (light:dark).

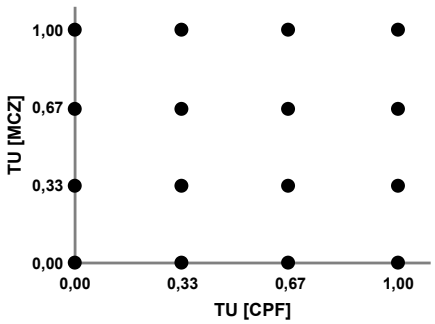

Figure 6.1 – Experimental design scheme. Pesticide treatments are presented as toxic units (TU) being 1TU assigned to the LC_{50}

6.3.4. Experimental set up

Different soil spiking procedures were used to incorporate each pesticide into the soil. Whereas CPF was incorporated in the form of aqueous solutions, MCZ was directly included in soil as a powder and thoroughly mixed with distilled water in order to ensure its homogeneous distribution. This difference was due to the extremely low water solubility of MCZ, that wouldn't enable the dissolution of this compound in the water necessary to adjust soil to the lowest soil moisture treatment (25% WHC). Hence, we decided to keep this procedure when spiking MCZ in the remaining soil moisture treatments, as well. The whole batch of soil for each treatment was spiked together and transferred to small

circular plastic boxes (Ø 6.5 cm) used for the exposure. Soil pH was measured after suspending a soil sample in distilled water, following the ISO standard protocol 10390 (ISO 2005), in the beginning and at the end of the experiment.

Isopods were collected from cultures, weighted and individually placed inside the test boxes. A total of 240 isopods were used in this experiment, 5 per chemical treatment with one individual per replicate. All the boxes were supplied with three previously weighted disks of alder leaves. The boxes were also weighted, in order to further readjust soil moisture during the course of the experiment, closed with perforated lids, and kept for 14 days inside a temperature-controlled room. Soil moisture was readjusted every second day adding the necessary amount of distilled water. At the end of the experiment, isopods' fresh weight and the dry weight of leaves were re-determined in order to calculate the isopods' consumption rate and their biomass variation (Loureiro et al. 2006).

$$\text{Consumption Rate} = (W_{Li} - W_{Lf}) / W_{isop} \quad (1)$$

$$\text{Biomass gain/loss} = [(W_{isop} - W_{isop\,f}) / W_{isop}] \times 100 \quad (2)$$

where, dw - dry weight; $W_{L\,i}$ - initial leaf weight (mg dw); $W_{L\,f}$ - final leaf weight (mg dw); W_{isop} - initial isopod weight (mg); $W_{isop\,f}$ - final isopod weight (mg).

6.3.5. Statistical analysis

The Probit regression (Priprobit 1.63) was used to calculate the concentration after which 50% of the exposed isopods were found dead (LC_{50}) in the individual treatments. Two-way ANOVAs were performed in order to test for differences in survival, consumption rate and biomass gain/loss, that could be related to the factors "soil moisture" and "chemical treatment". When significant differences were detected, a Dunnett's *post-hoc* test was used to compare each treatment against the respective control, and a Tukey's test was used to compare the same treatments in different soil moisture regimes. These statistical procedures were performed using the GraphPad Prism 6 statistical pack (GraphPad Software, La Jolla, CA, USA). In order to analyse the mixture toxicity in survival, data was fitted to the reference model of Independent Action using the MixTox framework conceived by Jonker et al. (2005). This framework allows the comparison of observed toxicity results with the expected mixture effects, calculated from the reference

model (Jonker et al. 2005). It also helps to identify and infer about the nature of any possible deviations by extending the reference model with deviation functions to describe synergistic/antagonistic (S/A), dose-level (DL), and dose-ratio dependency (DR). A more detailed insight into these framework should refer to Jonker et al. (2005). Regarding the feeding parameters, however, this framework could not be used because results failed on showing a clear dose-response relationship in the single pesticide treatments. Nevertheless predictions for the mixture toxicity could still be done through the IA model by mathematically comparing the observed results to the predicted effects (based on the individual effect of each stressor) as shown in Martin et al. (2009) and Santos et al. (2011a). The nature and statistical significance of the deviations to additivity were evaluated after calculation of the confidence intervals (α=0.05). In order to analyse data from continuous variables (e.g. consumption ratio, biomass gain/loss), the probability of nonresponse to the toxicants can be calculated according to the following equation:

$$\text{mixture toxicity}\,(q_1, \ldots, q_n) = \max \prod_{i=1}^{n} q_1\,(c_1) \qquad (3)$$

where $q_i(c_i)$ is the probability of nonresponse at concentration c of toxicant i and max is the maximum value observed (assumed to be the control). Biomass variation data was converted to positive values and log-transformed as described by Wicklin (SAS 2011).

6.4. Results

As shown in Figure 6.2, isopods' survival generally followed the same pattern, independently of soil moisture, with just a slightly higher mortality at 75% WHC. This was confirmed by a two-way ANOVA, where the factor "soil moisture" failed on showing a significant influence on survival (two-way ANOVA, $F_{2,192}$=1.117, *p*=0.3294), as did factor "chemical treatment" (two-way ANOVA, $F_{15,192}$=5,623, *p*=<0.0001). In fact, for isopods' survival, the only significant differences found in this experiment between soil moisture regimes was the higher mortality registered for treatments 1CPF/1MCZ at 75% of soils' WHC when compared to 25% WHC (Tukey's test, *p*=0.0492). Significant differences to control within soil moistures were only found for treatments 1CPF/1MCZ at 50%

(Dunnett's test, p=0.0212) and 75% of soil WHC (Dunnett's test, p=0.0212). The Two-way ANOVA also showed no significant interaction between "soil moisture" and "chemical treatment" (two-way ANOVA, $F_{30,192}$=0.5628, p=0.9683). When looking for interactions between the pesticides, MixTox framework always indicated the reference model of IA as the most parsimonious outcome since none of the additional deviation parameters showed to provide a better fitting to our survival data (Table 6.1). In this way, as far as isopods' survival is concerned, the joint-effects of CPF and MCZ could always be considered as non-interacting, or additive, regardless of the soil moisture assessed.

Contrary to survival, isopods' consumption rates were not only influenced by the "chemical treatment" (two-way ANOVA, $F_{14,124}$=2.02, p<0.0023), but also by "soil moisture" (two-way ANOVA, $F_{2,124}$=2.02, p<0.0001), whereas the interaction between the two stressors was again not significant (two-way ANOVA, $F_{28,124}$=1.304, p=0.1635). Indeed, isopods kept in control conditions at 25% WHC showed to consume significantly less than those kept in the same control conditions but at 50% WHC indicating that soil moisture can alone influence this parameter (Figure 6.3; Table 6.2). No significant differences were, however, registered between the remaining controls. Significant differences between soil moistures were mostly associated to the single-pesticide treatments and to mixtures of low toxic units at 25% and 75% WHC that showed lower consumptions than the corresponding treatments at 50% WHC (Table 6.2). Few differences were found for mixtures with higher toxic units and none when comparing consumption between treatments at 25% WHC to those at 75% WHC. Within-group comparisons to control only showed statistical differences for the treatment 0.33CPF/1MCZ at 50% WHC (Dunnett's test, p=0.0395).

Two different patterns were detected when comparing the observed consumption ratio to IA-predicted values (Figure 6.3). When exposed to 50% WHC, isopods generally consumed significantly less than would be expected after the single treatments (Figure 6.3 and Table 6.1SD). By the opposite, consumption values registered at 25% and 75% WHC were predominantly antagonistic (i.e. isopods consumed significantly more than predicted using the IA model) or non-significant (Figure 6.3 and Table 6.1SD). Regarding situations of synergism, only one situation was found in each of them, 1CPF/0.33MCZ at 25% WHC and 0.33CPF/0.66MCZ at 75% WHC.

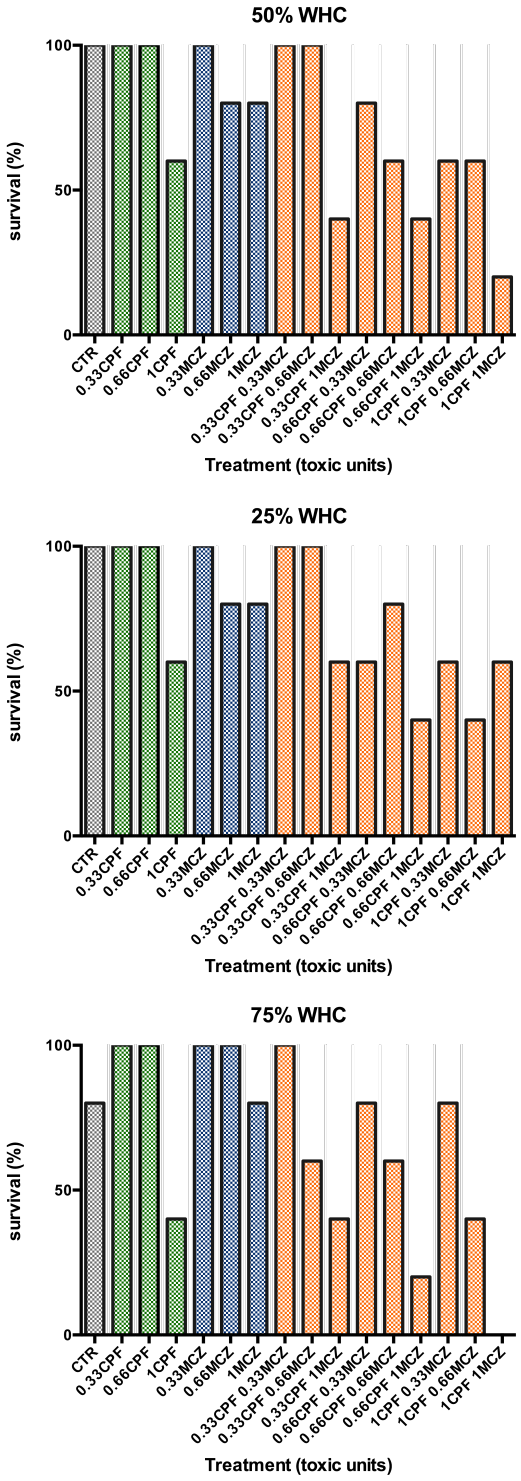

Figure 6.2 – Survival of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: a) 50% of soil WHC; b) 25% of soil WHC; c) 75% of soil WHC.

Table 6.1 – Parameter estimates and tests of fit of the Independent Action model using the MixTox framework applied to the survival of *Porcellionides pruinosus* after 14 days of exposure to single and mixture treatments of chlopyrifos and mancozeb, under three different moisture regimes: 25% of soil WHC, 50% of soil WHC, and 75% of soil WHC. IA is independent action; S/A is synergism/antagonism, DR is "dose ratio" and DR is "dose level deviation from the reference; r^2 is the coefficient of determination, $p(\chi^2)$ indicates the outcome of the likelihood ratio test and SS are the objective functions; *a* and *b* are parameters of the deviation functions.

	25% soil WHC					**50% soil WHC**					**75% soil WHC**				
	r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*	r^2	$p(\chi^2)$	SS	*a*	*b*
IA	0.65	***	25.59	-	-	0.71	***	31.71	-	-	0.63	***	30.44	-	-
S/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

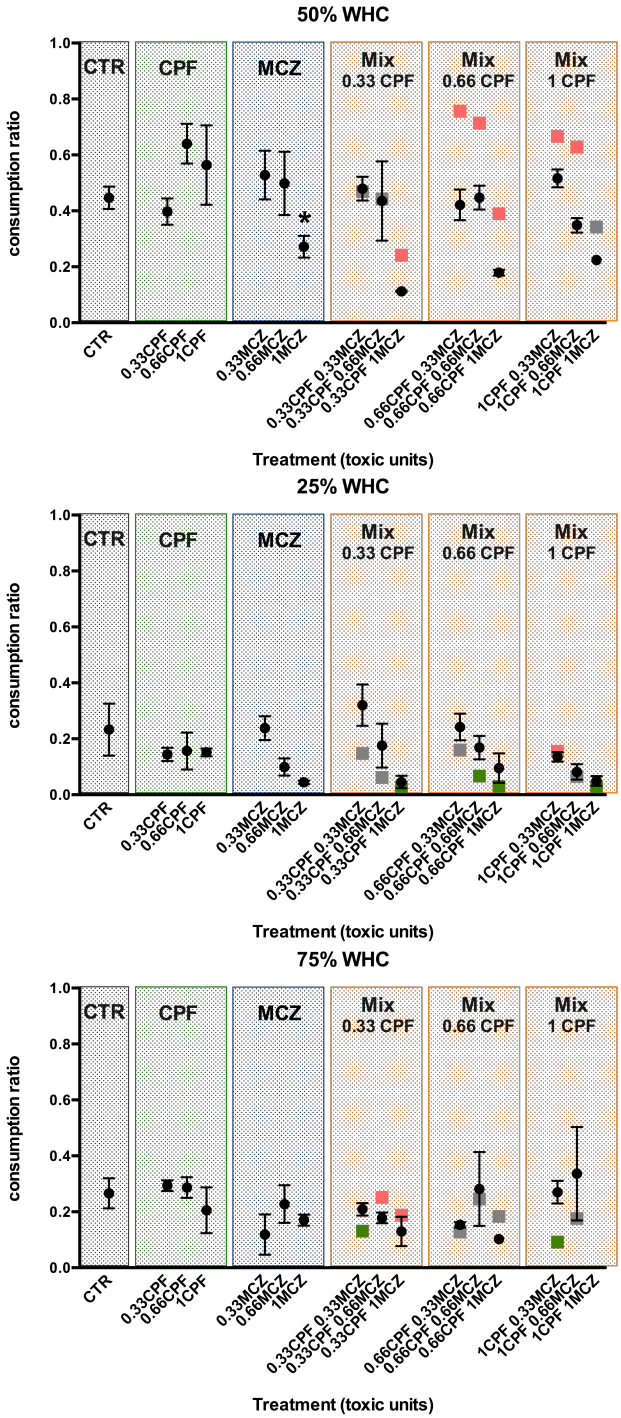

Figure 6.3 – Observed (circles; ± standard error) and predicted (triangles) consumption ratios of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: a) 50% of soil WHC, b) 25% of soil WHC, and c) 75% of soil WHC. Grey squares represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), green squares represent prediction values that were significantly higher than observed results (i.e. antagonism), and red squares represent prediction values that were significantly lower than observed results (i.e. synergism). Treatments indicated by asterisks are significantly different from control (two-way ANOVA followed by Dunnett's *post hoc* test, α=0.05).

Observed and IA-predicted biomass variation is shown in Figure 6.4. After the 14 days period, biomass variation was predominantly negative and significantly affected by both "soil moisture" (two-way ANOVA, $F_{2,125}$=19.41, p<0.0001), and "chemical treatment" (two-way ANOVA, $F_{14,125}$=1.316, p=0.0005), but not by their interaction (two-way ANOVA, $F_{28,125}$=1.316, p=0.1554). Multiple comparisons showed no significant differences between the control groups kept at different soil moistures, but identified several significant differences for the remaining treatments (Table 6.2). This was mainly observed for the single pesticide treatments between 50% WHC and 75% WHC (Table 6.2). The only differences found between 50% and 25% WHC occurred for 0.66CPF/0.33MCZ while between 25% and 75% was registered for 0.33CPF/0.33MCZ (Table 6.2). Multiple comparisons to control within the same soil moisture showed that isopods exposed to 1MCZ (Dunnett's test, p=0.0.0003), 0.33CPF/0.33MCZ (Dunnett's test, p=0.0352), 0.66CPF/0.66MCZ (Dunnett's test, p=0.0157), and 0.66CPF/1MCZ (Dunnett's test, p=0.0172) lost significantly more weight when kept at 50% WHC. The same happened with the treatments 0.66CPF (Dunnett's test, p=0.0408) and 1MCZ (Dunnett's test, p=0.0360) at 75% WHC (Figure 6.4).

When kept at 50% WHC, isopods seemed to lose significantly more weight than predicted by the IA model in some mixture treatments: 0.33CPF/0.66MCZ, 0.66CPF/0.66MCZ, and 1CPF/0.66MCZ (Table 6.3SD). Biomass variation in isopods exposed to 0.66CPF/1CPF was not so negative as would be expected, and no significant differences were found in the remaing treatments (Table 6.3SD). Regarding those kept at 25% WHC, synergism was found for 0.66CPF/0.33MCZ and antagonism was found in treatments 0.33CPF/0.33MCZ, 0.66CPF/0.66MCZ, and 0.66CPF/1MCZ (Table 6.3SD). No significant deviations to the IA model were found for the remaining mixture treatments in this soil moisture. Finally, antagonistic relationships were found for every mixture treatments in isopods kept at 75% WHC (Table 6.3SD).

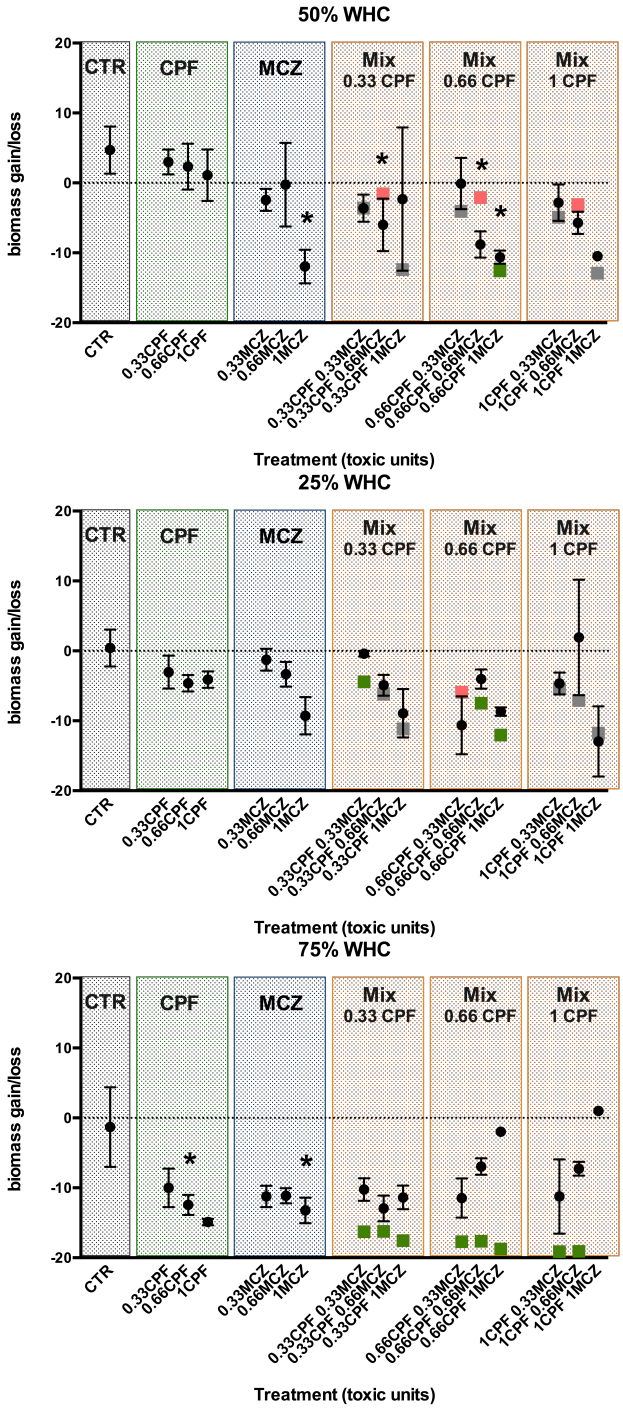

Figure 6.4 – Observed (circles; ± standard error) and predicted (triangles) biomass gain/loss of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: a) 50% of soil WHC, b) 25% of soil WHC, and c) 75% of soil WHC. Grey squares represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), green squares represent prediction values that were significantly lower than observed results (i.e. antagonism), and red squares represent prediction values that were significantly higher than observed results (i.e. synergism). Treatments indicated by asterisks are significantly different from control (two-way ANOVA followed by Dunnett's *post hoc* test, α=0.05).

Table 6.2 - Dunnett's *post-hoc* test results to compare the consumption ratio and the biomass gain/loss registered in isopods exposed to the same chemical treatments, but under different soil moisture regimes. ****-*p*<0,001; ***-*p*<0,001; **-*p*<0,01; *-*p*<0,05; ns- non significant

		CTR	0.33 CPF	0,66 CPF	1CPF	0.33 MCZ	0.66 MCZ	1MCZ	0.33 CPF 0.33 MCZ	0.33 CPF 0.66 MCZ	0.33 CPF 1 MCZ	0.66 CPF O.33 MCZ	0.66 CPF 0.66 MCZ	0.66 CPF 1 MCZ	1CPF 0.33 MCZ	1CPF 0.66 MCZ	1CPF 1MCZ
Consumption ratio	50% vs 25%	*	*	***	***	**	***	ns	ns	**	ns	ns	*	ns	**	ns	ns
	25% vs 75%	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	50% vs 75%	ns	ns	***	*	***	**	ns	**	*	ns	*	ns	ns	ns	ns	ns
Biomass gain/loss	50% vs 25%	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
	25% vs 75%	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
	50% vs 75%	ns	**	***	**	*	*	ns	ns	ns	ns	*	ns	ns	ns	ns	ns

6.5. Discussion

The results of this experiment showed that soil moisture can, indeed, influence the toxicity of these commercial formulations on *P. pruinosus*, but only for the feeding parameters since it showed no effects on isopods' survival. Considering the limitations normally assigned to terrestrial isopods' water balance (Warburg 1968; Sutton et al. 1980), one had hypothesized that such suboptimal soil moisture conditions could markedly decrease their resilience to single and mixture treatments. However, regardless of the worse performance showed by desiccated organisms or those under exceedingly moist environments, none of such regimes was severe enough to increase the pesticide-related mortality in the 14 days period.

As a cosmopolitan species, *P. pruinosus* is capable of adapting to a wide range of conditions (Quinlan & Hadley 1983). Despite lacking of an effective waterproof barrier on the epicuticle, similar to the hydrophobic lipid layer in insects (Sutton et al. 1980), this species still owns several morphological, physiological, and behavioral adaptations to cope with drought situations (Hadley & Hendricks 1985; Broly et al. 2013b). Likewise, the avoidance of too-moist environments is also the primary strategy of mesic/xeric isopods, as *P. pruinosus*, since they are known to have a hydrophilic ventral cuticle and permeable pleopodal endopods that pose them serious problems to balance its water content (Horowitz 1970; Sutton et al. 1980).

Hitherto, there are no similar studies with terrestrial isopods that assess the combined effects of unfavourable soil moisture and pesticides, nor xenobiotics in general. Furthermore, contrasting results can be found in literature for assessments with different soil organisms, indicating that the intrinsic vulnerability of the species and the properties of the compound can play critical roles in these interactions contributing for the extensive case-specificity. Sørensen and Holmstrup (2005) found that neither dimethoate nor cypermethrin reduced the drought tolerance in *Folsomia fimetaria*, which is in line with our survival results. In order to infer about the hypothesis advanced by Sjursen and Holmstrup (2004) that the lipophilicity of a toxicant could be partly responsible for the reduction of drought tolerance, Sørensen and Holmstrup (2005) assessed the effects of several compounds belonging to different classes and found that this effect was mostly observed for chemicals with non-specific modes of action (narcosis). Despite having, some of them, a strongly lipophilic character, pesticides have well defined modes of action which makes them generally toxic at very low doses. Puurtinen and Martikainen (1997) also found no

decrease on the survival of an *Enchytraeid* species to dimethoate that could be attributed to differences in soil moisture. By the opposite, Everts et al. (1991) reported an interaction between low humidity and deltamethrin and suggested that the mutual capability of these stressors to disturb arthropods' water balance must be the reason for this synergic relationship. Given the aforementioned singularities of terrestrial isopods' water regulation, if similar pesticide–induced discharges were registered for these organisms, they could imply elevated costs to their body water content and cause an imbalance on their behavioural mechanisms. Lima et al. (2011) also found synergism between the toxicity of carbaryl and drought stress in the earthworm *Eisenia andrei*, and explained it as being the result of dehydration that consequently leads to higher toxicant concentrations within the body. Interestingly, however, Cardoso (2012) exposed the collembola *Folsomia candida* to the same pesticide and drought stress conditions and, contrary to the former authors, an antagonistic interaction was reported for survival.

There are few studies in literature that evaluated the toxicity of pesticides under very high soil moistures and most of them reported these conditions as not having any effect on the survival to pesticides. Puurtinen and Martikainen (1997) found no differences in the survival of enchytraeids to dimethoate and benomyl at 70% WHC. A similar situation was observed by Lima et al. (2011) when exposed *E. andrei* to carbaryl at 100% soil WHC. Nevertheless, a different result was obtained by Cardoso (2012) in *F. candida*, where the same moisture treatments showed to interact with carbaryl toxicity, presenting a "dose-ratio" deviation to the reference model of IA. This deviation consisted of a synergistic pattern when the moisture stress was the dominant factor and antagonism when the toxicity of carbaryl was dominant. Among these species, *P. pruinosus* is certainly the most susceptible to exceedingly humid environments and it could not stand the moisture levels assessed by Lima et al. (2011) or Cardoso (2012). In this way, we opted for using moisture treatments that had previously shown to be sublethal, so they could possibly interact with the pesticides without masking their effects. However, no significant interactions were found.

Regarding the behavior of the mixture on isopods survival, no deviations were found to the reference model of IA, meaning that, as far as this endpoint is concerned, these two commercial formulations do not seem to influence each others' toxicity, irrespective of the soil moisture assessed. In a previous experiment using the same pesticides, temperature was also found not to change the mixture's behavior since the reference model was likewise the most parsimonious (Chapter 5). Nevertheless, contrary to soil moisture, temperature showed to influence the toxicity

of each pesticide individually, so albeit they were not interacting, the final outcome was actually different depending on the temperature assessed (Chapter 5). In this way, there seems to be a consistency of non-interacting effects between these two pesticides on the survival of *P. pruinosus*, irrespective of the environmental conditions.

Notwithstanding, other ecologically relevant responses to such conditions were still identified in this experiment. Contrary to survival, it seems clear by analysing the feeding parameters that isopods exposed to exceedingly dry or moist conditions showed a fairly worse feeding and growth performance, including in their respective controls. The lower consumptions, for instance, must have resulted of a worse health condition that end up impairing the intake and assimilation of food and/or constitute a consequence of a reduced appeal of the food. Zimmer et al. (2003) suggested previously that the colonization of leaves by microbiota can stimulate isopods consumption and constitute an additional and higher-quality food item. Since microbial biomass is highly dependent on soil water content (Barros et al. 1995), it is possible that this was the underlying reason for the lower consumption values at lower soil moistures. It is, however, unlikely that this was also the reason behind the difference in feeding activity observed between 50% WHC and 75% WHC because Barros et al. (1995) also indicated the water content at field capacity as the soil moisture *optima* for microbial activity. This suggests that, at least under excessive soil moistures, the impaiment of the isopods' health status must have been the prevailing factor for the overall decrease in feeding activity and not the effects on the food quality.

Regarding the effects of pesticides in isopods' feeding activity, MCZ generally seemed to cause higher effects than CPF, as shown by the well defined dose-dependent decrease in consumption rates. Similarly, MCZ also seemed to have a higher weight on the definition of mixtures' effects since consumption patterns observed for these treatments generally followed the single MCZ treatments. In fact, if one look at each group of three mixture treatments (grouped according to the CPF concentration), a steep decrease in isopods' consumption rate could be found whenever the MCZ concentration increases within the mixture. On the contrary, increasing concentrations of CPF did not seem to have particulaly higher effects on isopods consumption when the concentration of MCZ was low. Perhaps this was indirectly related with the fungicidal effects of MCZ. Despite not having been contaminated themselves, it is possible that pesticide transference from soil to leaves may have limited the proliferation of their own microbiome, particularly in case of MCZ since CPF was previously shown to increment fungal communities (Pandey &

Singh 2004). These communities are known to dominate the first stages of decomposition processes and by rendering the leaf material more atractive they can stimulate detritivores' consumption, thus becoming highly relevant in short-term exposures like ours (Zimmer et al. 2003; Gessner et al. 2010;).

Direct effects on isopods must however also be considered since they can be responsible for differences in consumption rates as well. Although not affecting survival, pesticides and pesticide mixtures must still have led to the impoverishment of isopods' health condition, consequently affecting their regular activity patterns, including feeding. For instance, Bayley and Baatrup (1996) reported dimethoate to induce hyperactivity in *Porcellio scaber* and suggested that such pattern might potentially disrupt this species' feeding activity. Similar results were also reported for other isopod species (Engenheiro et al. 2005; F. F. Sørensen et al. 1997) collembolans (Sørensen et al. 1995), carabids (Jensen et al. 1997) and other species (Lundebye et al. 1997; Roast et al. 2000; Rao et al. 2005;). Blažič et al. (2005) showed the imidacloprid-induced feeding inhibition in *P. scaber* to be concomitant to suborganismal manifestations of stress, namely on acetylcholinesterase (AChE) and glutathione *S*-tranferases. Similar findings were reported by Xuereb et al. (2009) after exposing the crustacean *Gammarus fossarus* to CPF and methomyl which led these authors to suggest AChE activity to be well correlated with this species' feeding rates. The use of AChE-inhibiting compounds is actually a common feature of the above mentioned works. In fact, when it comes to assessing pesticide-induced changes in behaviour, a particular attention has been devoted to AChE activity because of its widespread neuromuscular effects. Nevertheless, this work suggests that additional factors may have an even stronger effect on organisms' feeding activities. The mechanisms by which MCZ can directly affect organisms' feeding rates are not so straightforward and probably are not related with AChE inhibitions since dithiocarbamates were claimed to have low inhibitory potential (Espigares et al. 1998). MCZ effects must instead be related to a general impairment in organisms condition since this compound is still known to induce several non-specific responses such as oxidative stress (Tsang & Trombetta 2007) and impairments on phases I and II of organisms' detoxification systems (Siddiqui et al. 1991; Szépvölgyi et al. 1989; Lewerenz & Plass 1984; Nebbia et al. 1993).

If, on the contrary, there were any active mechanisms by the isopods aiming to decrease consumption so the burden of pesticides was also decreased, it must have been relevant enough for them to assume a clear trade-off with biomass loss, with all the implications that may potentially arise on the different life traits. As Jager et al. (2013) pointed out, this strategy is common in organisms dealing with

xenobiotics and, even if it leads to increased survival, it still implies a decrease on organisms' fitness, since long-term effects are expected to occur. In fact, positive variations on isopods' biomass were only found for control and for the single CPF treatments at 50% WHC. All the remaining showed a negative variation when compared to the initial biomass. Regarding these single CPF treatments, the positive biomass values are probably related with the remarkable consumption ratios found for most of the single pesticide treatments at 50% WHC, being even higher than control. The fact that such higher consumptions have not been followed by equally higher positive variations in biomass, seems to indicate that they may consist on a compensatory behavior triggered by the increased energetic demands of detoxification (Ribeiro et al. 2001). The reason why this situation is not noticeable on the other moisture conditions is not clear.

The comparison of the observed feeding results with the IA predicted values, showed several significant differences, both for consumption ratio and for biomass variation, suggesting that soil moisture can, indeed, influence the interaction of these two pesticides. Furthermore, different patterns could be found depending of the moisture assessed. Whereas a synergistic action was found in consumption ratios for almost every mixture treatments at 50% WHC, at 25% and 75% WHC pesticides either did not show any interaction or antagonistic situations were found. A similar situation was found for the biomass with synergism at 50% WHC and antagonism in most of the mixture treatments at 25% and 75% WHC.

An important rationale behind this work was to try to evaluate the possible consequences of only using near-optimal moisture conditions, when performing mixture toxicity assays with pesticides. In this way, despite no effects were registered on isopods survival, organisms simultaneously exposed to exceedingly dry or moist conditions and pesticides clearly showed a worse performance on consumption and marked decreases in biomass variation, suggesting that delayed effects may occur. To our knowledge, no other work was performed that aimed at assessing the influence of different soil moistures on the toxicity of pesticide mixtures. Nevertheless, given the multiplicity of responses already found in literature for the joint action of soil moisture and one single pesticide, a similar case-specificity is likely to be the general rule. It is thus of paramount importance to continue deepening this subject towards a better understanding of the real consequences of non-including the environmental factors on the risk assessment procedures.

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Table 6.1SD – Binary combination of chlorpyrifos and mancozeb in isopods' consumption. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted consumption ratios by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% of soil WHC, 25% of soil WHC, and 75% of soil WHC. Predicted values were calculated by measuring the joint probability of effects on isopods' consumption found for single exposures to chlorpyrifos and mancozeb in separate for each soil moisture. Results assigned as "synergism" or "antagonism" mean that predicted consumption ratio was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 50% soil WHC was assumed to be the control for soil moisture.

	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
50% WHC					
	0.33	0.33	0.48 (0.39-0.57)	0.47	ns
	0.33	0,66	0.44 (0.17-0.71)	0.44	ns
	0.33	1	0.11 (0.11-0.11)	0.26	Synergism
	0,66	0.33	0.42 (0.32-0.52)	0.76	Synergism
	0,66	0,66	0.45 (0.36-0.53)	0.71	Synergism
	0,66	1	0.18 (0.17-0.19)	0.42	Synergism
	1	0.33	0.52 (0.44-0.59)	0.75	Synergism
	1	0,66	0.35 (0.31-0.39)	0.71	Synergism
	1	1	0.22 (--- - ---)	0.41	-----
25% WHC					
	0.33	0.33	0.32 (0.12-0.52)	0.15	ns
	0.33	0,66	0.18 (0.06-0.29)	0.06	ns
	0.33	1	0.05 (0.04-0.05)	0.03	Antagonism
	0,66	0.33	0.24 (0.15-0.34)	0.16	ns
	0,66	0,66	0.17 (0.11-0.23)	0.07	Antagonism
	0,66	1	0.10 (0.05-0.14)	0.03	Antagonism
	1	0.33	0.14 (0.16-0.16)	0.16	Synergism
	1	0,66	0.08 (0.06-0.10)	0.06	ns
	1	1	0.05 (0.04-0.06)	0.03	Antagonism
75% WHC					
	0.33	0.33	0.21 (0.17-0.24)	0.13	Antagonism
	0.33	0,66	0.18 (0.15-0.20)	0.25	Synergism
	0.33	1	0.13 (0.08-0.18)	0.19	Synergism
	0,66	0.33	0.15 (0.14-0.16)	0.13	ns
	0,66	0,66	0.28 (0.01-0.56)	0.25	ns

0,66	1	0.10 (--- - ---)	0.18	-----
1	0.33	0.27 (0.19-0.35)	0.09	ns
1	0,66	0.34 (-0.08-0.75)	0.18	Antagonism
1	1	-----	-----	-----

Table 6.2SD – Ternary combination of soil moisture, chlorpyrifos and mancozeb in isopods' consumption. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted consumption ratios by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% of soil WHC, 25% of soil WHC, and 75% of soil WHC. Predicted values were calculated by measuring the joint probability of effects on isopods' consumption found for single exposures to different soil moistures, concentrations of chlorpyrifos and concentrations of mancozeb. Results assigned as "synergism" or "antagonism" mean that predicted consumption ratio was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 50% soil WHC was assumed to be the control for soil moisture.

Soil moisture	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
50% WHC	0.33	0.33	0.48 (0.39-0.57)	0.47	ns
	0.33	0,66	0.44 (0.17-0.71)	0.44	ns
	0.33	1	0.11 (0.11-0.11)	0.26	Synergism
	0,66	0.33	0.42 (0.32-0.52)	0.76	Synergism
	0,66	0,66	0.45 (0.36-0.53)	0.71	Synergism
	0,66	1	0.18 (0.17-0.19)	0.42	Synergism
	1	0.33	0.52 (0.44-0.59)	0.75	Synergism
	1	0,66	0.35 (0.31-0.39)	0.71	Synergism
	1	1	0.22 (--- - ---)	0.41	-----
25% WHC	0.33	0.33	0.32 (0.22-0.42)	0.25	ns
	0.33	0,66	0.18 (0.12-0.24)	0.23	ns
	0.33	1	0.05 (0.04-0.05)	0.14	Synergism
	0,66	0.33	0.24 (0.19-0.29)	0.39	Synergism
	0,66	0,66	0.17 (0.14-0-20)	0.37	Synergism
	0,66	1	0.10 (0.07-0.12)	0.22	Synergism
	1	0.33	0.14 (0.13-0.15)	0.39	Synergism
	1	0,66	0.08 (0.07-0.09)	0.37	Synergism
	1	1	0.05 (0.05-0.05)	0.37	Synergism
75% WHC	0.33	0.33	0.21 (0.19-0.23)	0.28	Synergism
	0.33	0,66	0.18 (0.16-0.19)	0.26	Synergism
	0.33	1	0.13 (0.10-0.16)	0.15	ns
	0,66	0.33	0.15 (0.15-0.16)	0.45	Synergism
	0,66	0,66	0.35 (0.14-0.56)	0.43	ns
	0,66	1	0.07 (--- - ---)	0.25	-----
	1	0.33	0.23 (0.18-0.28)	0.45	Synergism

	1	0,66	0.34 (--- - ---)	0.42	-----
	1	1	-----	-----	-----

Table 6.3SD – Binary combination of chlorpyrifos and mancozeb in isopods' biomass variation. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted biomass variation by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% of soil WHC, 25% of soil WHC, and 75% of soil WHC. Predicted values were calculated by measuring the joint probability of effects on isopods' consumption found for single exposures to chlorpyrifos and mancozeb in separate for each soil moisture. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 50% soil WHC was assumed to be the control for soil moisture.

	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
50% WHC					
	0.33	0.33	1.32 (1.24-1.40)	1.44	Synergism
	0.33	0,66	1.24 (1.08-1.41)	1.42	Synergism
	0.33	1	1.31 (0.91-1.71)	1.17	ns
	0,66	0.33	1.38 (1.25-1.52)	1.42	ns
	0,66	0,66	1.20 (1.12-1.29)	1.40	Synergism
	0,66	1	1.16 (1.11-1.21)	1.15	ns
	1	0.33	1.34 (1.24-1.44)	1.40	ns
	1	0,66	1.28 (1.21-1.44)	1.38	Synergism
	1	1	1.16 (--- - ---)	1.14	-----
25% WHC					
	0.33	0.33	1.39 (1.38-1.40)	1.31	Antagonism
	0.33	0,66	1.30 (1.23-1.36)	1.27	ns
	0.33	1	1.18 (1.00-1.36)	1.12	ns
	0,66	0.33	1.11 (0.86-1.36)	1.28	ns
	0,66	0,66	1.32 (1.27-1.37)	1.25	Antagonism
	0,66	1	1.21 (1.19-1.24)	1.10	Antagonism
	1	0.33	1.31 (1.24-1.37)	1.30	ns
	1	0,66	1.41 (1.14-1.68)	1.26	Antagonism
	1	1	1.00 (0.74-1.27)	1.11	Antagonism
75% WHC					
	0.33	0.33	1.16 (1.09-1.23)	0.96	Antagonism
	0.33	0,66	1.07 (0.97-1.18)	0.97	ns
	0.33	1	1.13 (1.04-1.22)	0.90	Antagonism
	0,66	0.33	1.10 (0.96-1.25)	0.91	Antagonism
	0,66	0,66	1.28 (1.22-1.34)	0.92	Antagonism

0,66	1	1.10 (--- - ---)	0.86	-----
1	0.33	0.82 (0.23-1.40)	0.85	ns
1	0,66	1.25 (1.20-1.29)	0.85	Antagonism
1	1	-----	-----	-----

Table 6.4SD – Ternary combination of soil moisture, chlorpyrifos and mancozeb in isopods' biomass variation. Comparison between observed (± confidence intervals, α=0.05) and Independent Action model-predicted biomass variation by *Porcellionides pruinosus* after exposure to mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% of soil WHC, 25% of soil WHC, and 75% of soil WHC. Predicted values were calculated by measuring the joint probability of effects on isopods' biomass variation found for single exposures to different soil moistures, concentrations of chlorpyrifos and concentrations of mancozeb. Results assigned as "synergism" or "antagonism" mean that predicted biomass variation was above the upper limit or below the lower limit, respectively, of the confidence intervals computed for observed data. "ns" means no significant differences between observed and prediced values. "-----" means the comparison was not possible to perform. 50% soil WHC was assumed to be the control for soil moisture.

Soil moisture	CPF (TU)	MCZ (TU)	Observed CR (CI)	IA predicted CR	Output
50% WHC	0.33	0.33	1.32 (1.24-1.40)	1.44	Synergism
	0.33	0,66	1.24 (1.08-1.41)	1.42	Synergism
	0.33	1	1.31 (0.91-1.71)	1.17	ns
	0,66	0.33	1.38 (1.25-1.52)	1.42	ns
	0,66	0,66	1.20 (1.12-1.29)	1.40	Synergism
	0,66	1	1.16 (1.11-1.21)	1.15	ns
	1	0.33	1.34 (1.24-1.44)	1.40	ns
	1	0,66	1.28 (1.21-1.35)	1.38	Synergism
	1	1	1.16 (--- - ---)	1.14	-----
25% WHC	0.33	0.33	1.39 (1.38-1.41)	1.48	Synergism
	0.33	0,66	1.30 (1.23-1.37)	1.46	Synergism
	0.33	1	1.18 (1.00-1.37)	1.20	ns
	0,66	0.33	1.11 (0.85-1.36)	1.46	Synergism
	0,66	0,66	1.32 (1.26-1.37)	1.44	Synergism
	0,66	1	1.21 (1.18-1.24)	1.19	ns
	1	0.33	1.31 (1.24-1.37)	1.45	Synergism
	1	0,66	1.41 (1.13-1.69)	1.42	ns
	1	1	1.00 (0.72-1.28)	1.17	ns
75% WHC	0.33	0.33	1.16 (1.09-1.23)	1.43	Synergism
	0.33	0,66	1.07 (0.97-1.17)	1.40	Synergism
	0.33	1	1.13 (1.04-1.22)	1.16	ns
	0,66	0.33	1.10 (0.96-1.25)	1.41	Synergism
	0,66	0,66	1.25 (1.21-1.30)	1.38	Synergism
	0,66	1	1.36 (--- - ---)	1,14	-----
	1	0.33	0.89 (0.43-1.34)	1.39	Synergism

	1	0,66	1.25 (--- - ---)	1.37	-----
	1	1	-----	-----	-----

CHAPTER 7: General discussion and conclusions

General discussion and conclusions

As environmental values evolve and modern societies grow increasingly risk averse, a higher pressure is exerted by public opinion toward a stringent regulation of toxic substances, in particular pesticides. Nevertheless, in spite of the remarkable effort invested on the development and improvement of environmental risk assessments (ERA), a considerable uncertainty still persist in these procedures. This fact is actually acknowledged by risk managers, and accommodated by using arbitrary safety factors without a solid scientific background, which however does not seem to serve either environmental conservation or industry stakeholders (Chapman et al., 1998; Hunka et al., 2014).

One of the main shortcomings already identified in these frameworks is the disregard of environmental stressors as factors liable to influence organisms' susceptibility to pesticides, or pesticide mixtures (Bednarska et al., 2013). Despite the numerous scientific works suggesting the relevance of these stressors in ecotoxicology studies (see Sih et al., 2004; Relyea and Hoverman, 2006; Holmstrup et al., 2010), such knowledge is still to be incorporated in ERA frameworks. This incorporation appears thus as a priority for future improvement of these frameworks since it can deliver significant net benefits to all concerned parties.

This work intended to contribute for such improvement by analysing: i) the individual effects of several abiotic stressors to the performance of soil organisms; ii) the joint-effects of pesticide mixtures; and iii) the joint-effects of environmental stressors on the toxicity of pesticide mixtures. The terrestrial isopod *Porcellionides pruinosus* was selected as model species because of its relevance on soil ecosystems, a compartment to which less attention has been directed.

The individual effects of three abiotic factors on the performance of *P. pruinosus* were evaluated in Chapter 2 using multiple endpoints: survival, locomotor activity, feeding parameters, and avoidance behaviour. The results showed that abiotic factors might indeed affect this species at relevant environmental conditions

therefore suggesting the importance of their consideration in ecotoxicological assays and further on risk assessment. At the range assessed, temperature did not affect survival but showed marked effects on sublethal endpoints. The feeding parameters and locomotor activity showed a right-shifted response featured by a gradual increase in performance until the maximum is reached and an abrupt decline thereafter. On the contrary, soil moisture was found to significantly affect isopods' survival but the effects on the feeding parameters were not clear. Isopods exhibited a clear preference for intermediate soil moisture values tending to avoid overly dry or wet conditions. UV radiation showed to affect survival, body weight and locomotor performance.

The near-absence of studies assessing the effects of UV radiation in terrestrial invertebrates led to a more deep assessment of these effects by using a multiple biomarker approach (Chapter 3). In this way, the effects observed in biomarkers with completely different physiological functions were a clear demonstration of the broad spectrum of processes involved: oxidative stress, neurotransmission and responses in energetic parameters. The environmental medium of exposure showed to partly mitigate the effects of UV radiation in *P. pruinosus*, as shown by the significantly higher damages registered in plaster (offers no shelter). Nonetheless, UV effects were also detected in environments with higher shelter possibilities as shown by the higher IBR scores registered for animals exposed in soil or soil with litter when compared with unexposed ones. Juveniles and pre-adults were found to be more affected than adults, with the greatest differences between irradiated and non-irradiated isopods occurring in energy-related parameters.

A similar multiple biomarker approach was employed to assess the underlying mechanisms of toxicity induced by single and mixture treatments of the insecticide chlorpyrifos (CPF) and the fungicide mancozeb (MCZ) (Chapter 4). Results also suggested the occurrence of age-related differences in susceptibility to pesticides, with adults showing to be more resilient than juveniles. This was particularly noticeable on the different responses registered for energy-related parameters, which suggested age-classes to respond differently to contamination stress and to have different metabolic costs associated. Whereas the recommended application doses did not seem to pose considerable problems to these organisms, higher concentrations and mixture treatments seemed to impair several physiological processes, particularly in juveniles. These pesticides were found to induce changes

in isopods' detoxification and antioxidant systems, as shown by the significant increase/inhibition of GST and CAT activities.

Chapters 5 and 6 dealt with the influence of environmental stressors on the toxicity of pesticide mixtures. In Chapter 5, a full-factorial design experiment, including three treatments of each pesticide plus an unexposed control, was performed under four temperature regimes: constant 20 ^{o}C, mild daily cycle, hot daily cycle and cold daily cycle. Isopods' survival was found to be oppositely affected by temperature, either in single or mixture treatments: whereas CPF toxicity was raised under higher temperatures, the toxicity of MCZ was more prominent at lower temperatures. Notwithstanding, although the weight of each compound to the mixtures' toxicity showed to vary with temperature, this influence was always felt in a non-interactive since no deviations to the reference model of independent action (IA) were registered. Isopods' consumption rates showed to be decreased when exposed to colder temperatures. Furthermore, isopods generally showed a dose-dependent decrease in consumption, either in single pesticide treatments or mixtures. Biomass gain/loss, results were less clear. Additivity was also the most common pattern in feeding parameters.

A similar full factorial experiment was repeated under different soil moistures: 25%, 50% and 75% of soil WHC (Chapter 6). Soil moisture showed minor influence on isopods' resilience to single and mixture treatments of CPF and MCZ. Like in Chapter 5, additivity of effects was the best pattern to describe survival data. However, while temperature showed to influence differently the individual toxicity of each pesticide, making the overall toxicity in mixtures actually different, soil moisture did not show that ability. A different situation seemed to occur on feeding parameters with isopods clearly showing a worse performance when exposed to exceedingly dry or moist conditions. Additivity was again the most frequent result but several significant deviations were still found to IA predictions, mostly of synergistic nature at 50% WHC and antagonistic at 25% and 75% WHC.

In brief, findings reported in this thesis demonstrated why the negligence of natural stressors, or multiple stressors in general, by ERA is not a good solution. Even if no interaction occurs, the magnitude of responses observed in Chapters 2 and 3 is a clear indication that natural stressors should not be ignored since they can constitute an extra source of stress and decrease considerably the burden of xenobiotics handled by organisms. In fact, another important highlight of this thesis

was the relevance of additivity on risk assessments. As previously stressed by several authors, ecotoxicology studies involving multiple stressors responses are normally biased towards the seek of synergistic interactions while situations of additivity or antagonism between components are frequently seen as having few ecological importance (Silva et al., 2002; Holmstrup et al., 2010). While a special attention must be devoted to situations of synergism since these potentially constitute the most dangerous threat to the precautionary principle evoked by ERA, it is equally true that antagonistic interactions may indicate over-protective situations with similarly relevant socio-economic implications. Likewise, the simple addition of effects may entail consequences hard to predict if only the single effects of each stressor were assessed (Silva et al. 2002). As shown in this work (Chapters 5 and 6), significant enhancements in accuracy could be achieved in ERA just by considering the additive effects of multiple stressors on these predictions.

Given the impossibility of assessing every single pesticide under all exposure scenarios, a critical step would have to be the prioritization of the most relevant conditions to be assessed for a particular compound. Such information could be acquired from the integration of application patterns and properties of the compound in order to limit the extensiveness of such efforts. This approach would also be useful to identify the likely co-occurring pesticides.

Further complications may arise when assessing the effects of environmental stressors on pesticide mixtures, as shown by studying isopods' survival in Chapter 5. While no evidences of interaction were found between these pesticides at any of the conditions assessed, different survival patterns were found under different temperature conditions. If in one hand this introduces some reservations about the effects of mixtures previously referred as non-interactive, on the other it suggests that by knowing the individual effects of natural stressors on each component, the overall toxicity can be reasonably estimated using common reference models such as IA model. This seems to create new insights into a more realistic evaluation of the toxicity of pesticide mixtures and should probably be taken into account in risk assessments. Considering that the interaction between chemicals in a mixture is generally reduced as the number of constituents increase (Warne and Hawker, 1995), one may speculate that such method could also apply to more complex mixtures. More studies are however needed to confirm this hypothesis since the effects of natural stressors on pesticide mixtures, or mixtures of xenobiotics in general, are still largely unknown (Bednarska et al. 2009; Laskowski et al. 2010).

Another important conclusion of this work is that solid inferences about the effects of any stressors can only be drawn if using multiple endpoints otherwise it is not possible to have an encompassing perspective of the risks. This is particularly important when assessing the joint effects of multiple stressors since these may act on different processes and might therefore be underestimated if a single approach is considered. The problem is that this normally ends up reducing the cost-effectiveness, which is a decisive feature in ERA. In this sense, when the experiments of Chapters 5 and 6 were conceived, it was intended to maximize the information gathered while keeping complexity and extensiveness at minimum. In this way, endpoints were organized in tiers to allow the assessment of sublethal effects whenever acute toxicity was not registered. Besides constituting a good indication of isopods' health condition, the option for feeding parameters as sublethal endpoints was also related with their involvement on critical processes occurring in soil like decomposition of organic matter and nutrient recycling (Drobne 1997; Loureiro et al. 2006).

It must be noted that, although having progressed significantly in the last few years, further research is needed so an acceptable understanding of the joint effects of multiple natural and chemical stressors can be reached. In spite of academics' criticism regarding the slow incorporation of scientific findings in ERA frameworks, it is also true that no consensual methodology was yet achieved due to the lack of accuracy, simplicity, and/or pragmatism. There are several gaps of knowledge still to fill, in particular for labile and reactive compounds like pesticides. For instance, no conclusions could be drawn in this thesis about a possible time-dependence on multiple stressors responses. A small indication was possible for the mixture itself (Chapter 4) but it did not involve natural stressors that in some situations might become decisive factors for the overall effects (Chapter 5). Moreover, one must also refer that this follow-up study performed for mixtures of CPF and MCZ only lasted for 7 days. While this seems appropriate to identify short-term hazard pathways induced with the application of these pesticides, it may however neglect the occurrence of delayed effects only arising at later stages of the exposure. Recent studies have suggested that new homeostasis statuses are not easily reached after exposure to pesticides and a strong dependence on the environmental conditions was also highlighted (Ferreira et al. 2015). In addition, for future consideration it is also suggested the assessment of the cumulative effects of repeated pesticide application, or pulse exposures. When evaluating the effects of pesticides, or

pesticide mixtures, besides their application rates, it is important to consider also the strategy of application normally followed. MCZ, for instance, has a very short half-life in soil (one to seven days) but its application is normally reinforced fortnightly during some critical periods of the crop cycle (Wightwick et al. 2010). The same happens for CPF, even though it is considered to be moderately persistent (PPDB, 2006). Using our 14-days studies as an example, this would mean that a similar application of both pesticides would be likely at day 15. This suggests that in agroecosystems, organisms may not have enough time to recover from previous exposures and might be continuously under stress. A promising approach to assess the effects of pulse exposures to pesticides is the use of toxicokinetic-toxicodynamic modelling (i.e. GUTS; see Jager et al. 2011; Nyman et al. 2012).

Regarding the ways of assessing the effects of abiotic stressors, a future approach must also include stochastic variations since these can introduce a different type of stress in the organisms. Furthermore, in a context of global changes, including climate changes, the unpredictability caused by increasingly frequent episodic events may assume an even higher importance than changes in average conditions (Jentsch et al. 2007).

Finally, it seems important that future approaches consider the development of higher tier tests such as microcosms, mesocosms or even field studies so that structural and functional endpoints can also be included in these assessments (Menezes-Oliveira et al., 2013; Menezes-Oliveira et al. 2014). Moreover, the concept of ecosystems services should also be adapted since it seems to be a promising approach to ERA in the future.

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