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

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HIGHLIGHTS

- Isopods' survival was unaffected by the different soil moisture regimes.
- Consumption ratio showed higher sensitivity to soil moisture than survival or biomass change.
- Soil moisture influenced the toxicity of single pesticides and some pesticide mixtures.
- Interactions between soil moisture and pesticides were more important than between pesticides.

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ABSTRACT

A main source of uncertainty currently associated with environmental risk assessment of chemicals is the poor understanding of the influence of environmental factors on the toxicity of xenobiotics. Aiming to reduce this uncertainty, here we evaluate the joint-effects of two pesticides (chlorpyrifos and mancozeb) on the terrestrial isopod *Porcellionides pruinosus* under different soil moisture regimes. A full factorial design, including three treatments of each pesticide and an untreated control, were performed under different soil moisture regimes: 25%, 50%, and 75% WHC. Our results showed that soil moisture had no effects on isopods survival, at the levels assessed in this experiment, neither regarding single pesticides nor mixture treatments. Additivity was always the most parsimonious result when both pesticides were present. Oppositely, both feeding activity and biomass change showed a higher sensitivity to soil moisture, with isopods generally showing worse performance when exposed to pesticides and dry or moist conditions. Most of the significant differences between soil moisture regimes were found in single pesticide treatments, yet different responses to mixtures could still be distinguished depending on the soil moisture assessed. This study shows that while soil moisture has the potential to influence the effects of the pesticide mixture itself, such effects might become less important in a context of complex combinations of stressors, as the major contribution comes from its individual interaction with each pesticide. Finally, the implications of our results are discussed in light of the current state of environmental risk assessment procedures and some future perspectives are advanced.

1. Introduction

The cumulative evidence that different environmental conditions and chemical stressors can interact, influencing each other's effects on soil biota has been pushing ecotoxicologists to assess increasingly complex scenarios (van Gestel and van Diepen, 1997; Bednarska et al., 2009; Cardoso et al., 2014; Lima 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 realistic exposures to xenobiotics since they neglect the simultaneous occurrence of mul-

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tiple stressors, either natural or anthropogenic (Bednarska et al., 2013). Such procedures are mostly based on standard laboratory assays where organisms are exposed to a single compound, while all remaining conditions are 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 multiple stressors that might interact with organisms at the same time. These studies are particularly relevant for edaphic ecosystems in agricultural landscapes since these constitute amended systems that are continuously subject to several kinds of stress, including the contamination with a wide range of agrochemicals (pesticides, fertilizers) or severe tillage, while simultaneously experiencing severe abiotic conditions (Kibblewhite et al., 2008; Pretty, 2008; Santos et al., 2011b).

Soil is a heterogeneous compartment and, although normally acts as a buffer for some abiotic conditions, it is still featured by marked spatiotemporal variations in resources and conditions. Together with the limited mobility of most soil organisms, these conditions can make some of them highly vulnerable to adverse situations (Postma et al., 1989; Ettema and Wardle, 2002). Along with temperature, soil moisture is one of the most significant environmental factors shaping edaphic ecosystems (Singh and Gupta, 1977; Porporato et al., 2002; Iturbe and Porporato, 2004; Choi et al., 2006). Besides local precipitation history, also the properties of soil, the topography and the vegetation cover are factors strongly contributing to the soil moisture registered in a certain place and time (Mohanty and Skaggs, 2001; Weltzin et al., 2003). Even though there is still uncertainty regarding the ongoing climate changes, there are several lines of evidence pointing towards an intensification of the water cycle caused by an increasing atmospheric temperature ultimately, leading to changes in evaporation, evapotranspiration, and precipitation rates (Ragab and Prudhomme, 2002; Huntington, 2006; Rustad, 2008). As a consequence, soil communities will probably have to deal with different patterns of soil moisture, to which is added a higher unpredictability and frequency in the occurrence of extreme events. It, thus, becomes of paramount importance to evaluate how this environmental factor can affect pesticides' toxicity in agroecosystems.

Differences in soil moisture may lead to different pesticides' bioavailabilities by influencing their adsorption, volatilization and transformation/degradation rates (Arnold and Briggs, 1990). Moreover, such differences can also affect the fitness of edaphic organisms making them physiologically less tolerant to 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 constitute an additional source of stress to the organisms (Lima et al., 2011), which are known to play a vital role to soil functioning in agricultural landscapes (Altieri, 1999).

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 different soil moisture regimes. These pesticides were chosen as they are extensively used in several Mediterranean crops, like orchards and vineyards, and their application is frequently simultaneous. *Porcellionides pruinosus* was used as a model organism as this is a synantropic and widely distributed terrestrial isopod, frequently used in soil ecotoxicology experiments (Loureiro et al., 2002; Santos et al., 2010; Morgado et al., 2013; Tourinho et al., 2013; Silva et al., 2014; Ferreira et al., 2015). As a decomposer, it is involved in critical processes, such as the turn-over of soil organic matter, nutrient recycling, and also in promoting the degradation of contaminants (Loureiro et al., 2005). All the processes improve plant uptake and performance and are critical to increase the stability of agroecosystems (Wolters and Ekschmitt, 1997). Moreover, they also contribute to biological control of weeds through seed consumption (Saska, 2008).

Albeit the undeniably successful colonization of terrestrial habitats, the best when considering the Crustacea subphylum, 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 depend on effective behavioural patterns such as aggregation and avoidance of unsuitable habitats (Warburg, 1968; Broly et al., 2013). Isopods' tolerance to desiccation has been 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 and 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 pivotal ecological role 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 factors, especially at different moisture levels.

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

2. Material and methods

2.1. 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 °C (±1 °C), 16/8 h (light/dark) photoperiod, with commercial garden soil adjusted to a moisture content of 40–60% 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 moulting isopods and gravid females were discarded in order to avoid poten-

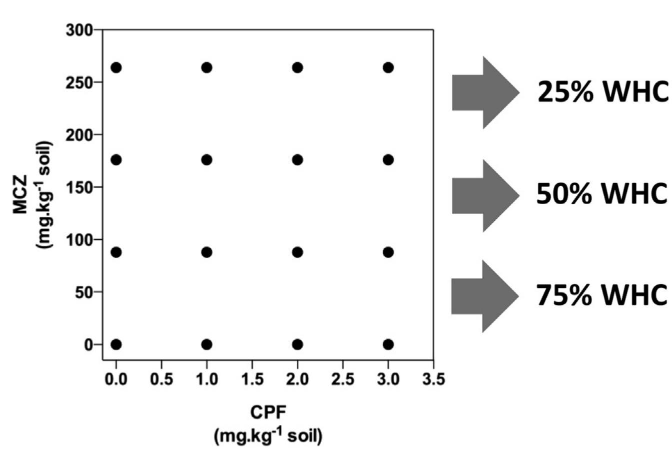

Fig. 1. Experimental design scheme. Full factorial design with three concentrations of each pesticide (plus control), performed for three soil moistures: 25%, 50%, and 75% WHC.

tial differences in tolerance arising from physiological, metabolic or behavioural alterations occurring in these stages.

2.2. 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 standard 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/100 g), organic C = 1.77 ± 0.2 (%), total nitrogen = 0.17 ± 0.02 (%), texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand (loamy soil).

2.3. Experimental design

The selection of treatments for 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 experiment including an untreated control plus three nominal concentrations of each pesticide was performed in three different soil moisture conditions: 25%, 50%, and 75% of the soil water holding capacity (WHC) (Fig. 1). These soil moisture treatments were selected based on a previous work where the performance of *P. pruinosus* was assessed throughout a range of soil moisture treatments (Morgado et al., 2015). The rationale behind this choice was to use treatments whose effects on mortality were not significantly different from each other, so they could possibly interact with the pesticides without masking their effects. Fifty per cent of soil WHC was considered the control treatment because it is normally used in ecotoxicology experiments with *P. pruinosus* (Loureiro et al., 2009). Pesticide concentrations were selected so the highest concentration for each compound was equivalent to 1TU, derived from preliminary tests. CPF concentrations included 1 mg kg^{-1} soil, 2 mg kg^{-1} soil, and 3 mg kg^{-1} soil, henceforth referred to as CPF1, CPF2, and CPF3, respectively. MCZ concentrations included 88 mg kg^{-1} soil, 176 mg kg^{-1} soil, and 264 mg kg^{-1} soil, henceforth referred to as MCZ1, MCZ2, and MCZ3, respectively. Apart from the soil moisture, all the remaining conditions were kept constant: temperature was always 20 °C (±1 °C) and the photoperiod set for 16:8 h (light:dark).

2.4. 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 would not 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 (International Organization for Standardization, 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 treatment with one individual per replicate. All the boxes were supplied with three previously weighted disks

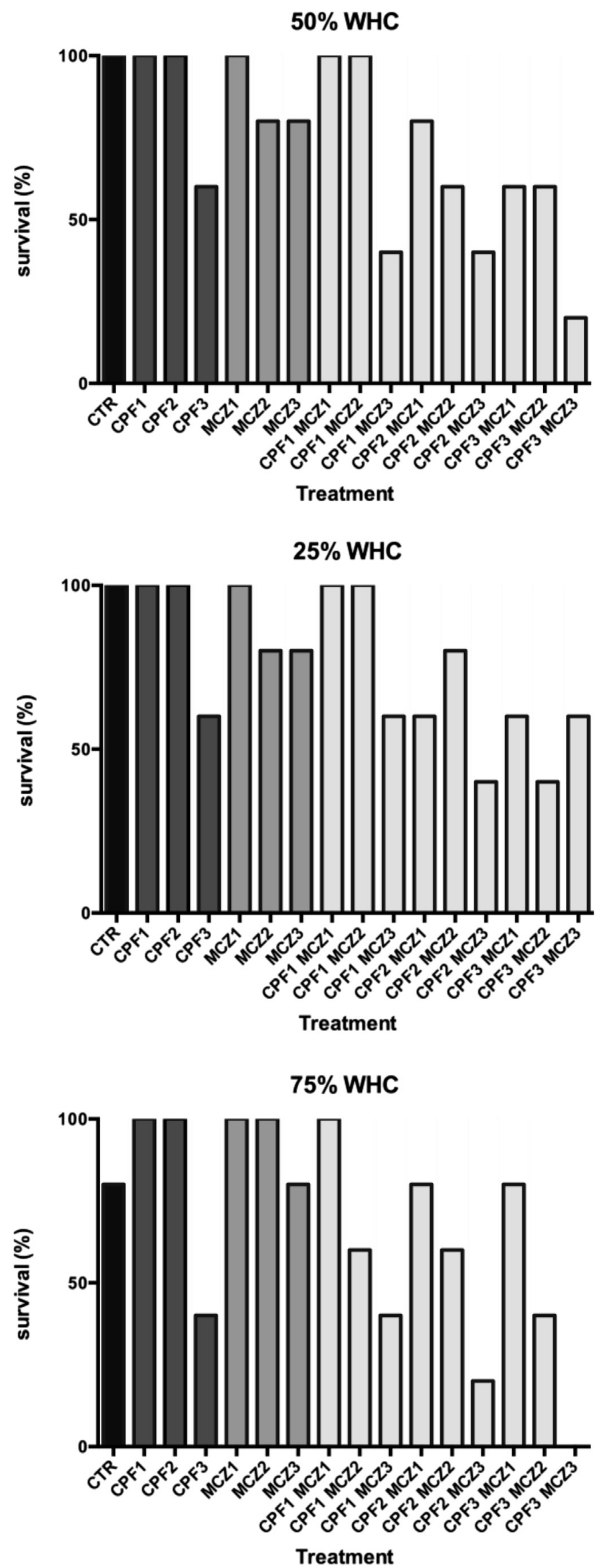

Fig. 2. Survival of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% WHC; 25% WHC; 75% WHC.

Table 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 chlorpyrifos and mancozeb, under three different moisture regimes: 25%, 50%, and 75% WHC. IA is the reference model of independent action; S/A is synergism/antagonism, DR is “dose-ratio” and DL is “dose-level” deviations 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% WHC					50% WHC					75% 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	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

****p* 0.001; ***p* 0.01; **p* 0.05; “–” non significant.

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 ratio and their biomass change (Loureiro et al., 2006).

$$\text{Consumption Ratio} = (W_{\text{Li}} - W_{\text{Lf}})/W_{\text{isop}}$$

$$\text{Biomass Change} = \left[\left(W_{\text{isop}} - W_{\text{isop f}}\right)/W_{\text{isop}}\right] \times 100$$

where, dw – dry weight; W_{Li} – initial leaf weight (mg dw); W_{Lf} – final leaf weight (mg dw); W_{isop} – initial isopod weight (mg); $W_{\text{isop f}}$ – final isopod weight (mg).

2.5. Statistical analysis

A Generalized Linear Model (GLM) with binomial distribution and logit link function was used to estimate the effects of “soil moisture”, “CPF concentration”, “MCZ concentration” and their interaction on isopods’ survival. GLMs were fitted using the brglm function in brglm library using the R software package (version 3.1.3, 2015). A backward stepwise procedure was used for model simplification by starting to fit the full model (i.e. including all possible terms of interaction) and afterwards removed the non-significant terms. Several GLMs were fitted to our survival dataset and the most parsimonious was selected by comparing the respective Akaike Information Criteria (AIC). For a comprehensive description of GLM application to survival data in mixture toxicity experiments see Iwasaki and Brinkman (2015). Two-way ANOVAs were performed in order to test for differences in consumption ratio and biomass change 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 (IA) 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 the theory underlying this 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/predictor) 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 ($\alpha = 0.05$). In order to analyse data from continuous variables (e.g. consumption ratio, biomass change), the probability of nonresponse to the toxicants can be calculated according to the following equation:

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

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). Binary and ternary IA predictions were performed for both consumption ratio and biomass change. Binary combinations only included the pesticides as predictors and were performed independently for each soil moisture regime, using the corresponding control and single pesticide treatments to calculate IA estimations. Ternary combinations were performed by also using soil moisture as a third predictor. To do so, all data were included in a single analysis and 50% WHC was considered to be the control condition for soil moisture, based on which predictions were estimated. Biomass change data was converted to positive values and log-transformed as described by Wicklin (2011).

3. Results

As shown in Fig. 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 the GLM analysis where the predictor “soil moisture” failed to show significant influence on survival. The final model (i.e. the one with the lowest AIC value) was only composed by three terms including the concentration of CPF (cpf, z-value = −3.640, $p < 0.001$), the concentration of MCZ (mcz, z-value = −3.549, $p < 0.001$) and the interaction between these pesticides (cpf*mcz, z-value = 1.707, $p < 0.001$ – see Table 1SD). Additional details of the GLM analysis are provided as Supplementary data (see file “Generalized Linear Model”). 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 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.

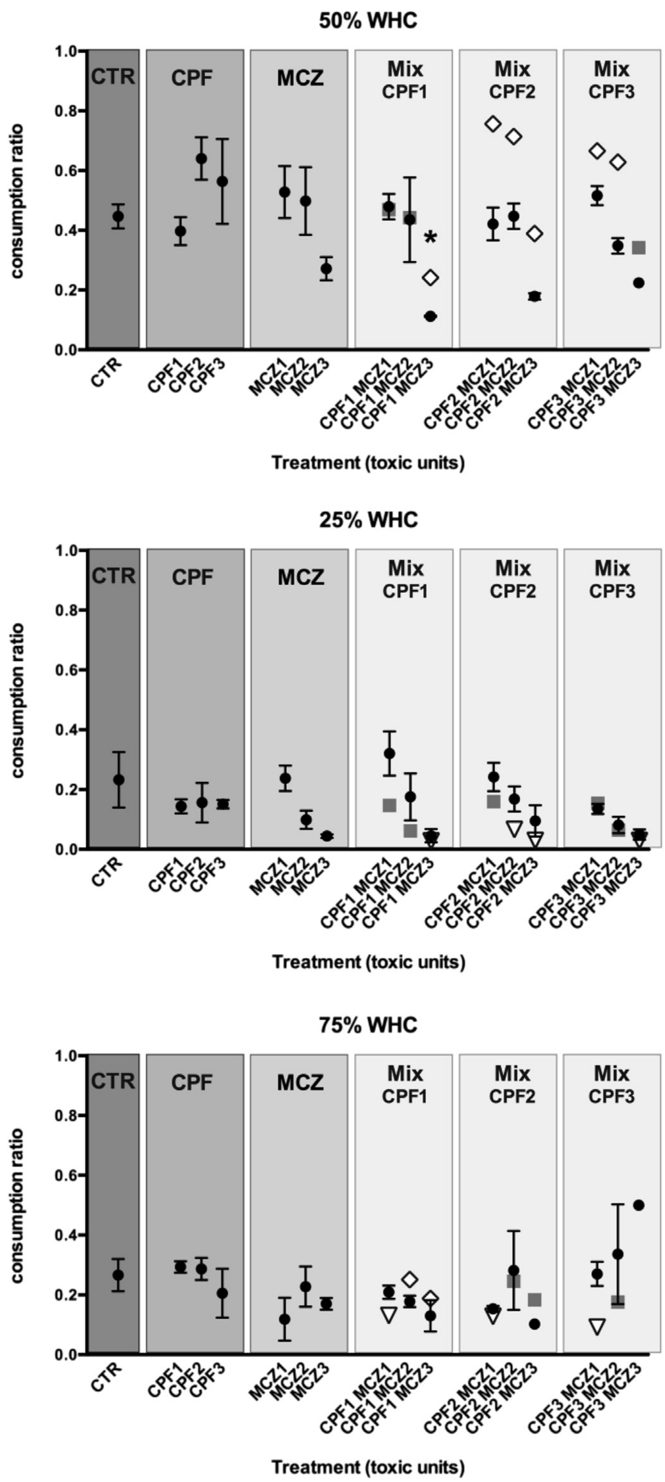

Fig. 3. Observed (circles; ± standard error) and predicted consumption ratios of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: 50% WHC, 25% WHC, and 75% WHC. Grey squares (■) represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), open inverted triangles (▽) represent prediction values that were significantly higher than observed results (i.e. antagonism), and open diamonds (◇) 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, $\alpha = 0.05$).

Contrary to survival, isopods' consumption ratios were not only influenced by the "chemical treatment" (two-way ANOVA, $F_{14,124} = 2.02$, $p < 0.01$), but also by "soil moisture" (two-way ANOVA, $F_{2,124} = 2.02$, $p < 0.001$). Their interaction was however not significant (two-way ANOVA, $F_{28,124} = 1.304$, $p > 0.05$). When comparing controls, isopods kept at 25% WHC consumed significantly less than those kept at 50% WHC indicating that soil moisture can alone influence this parameter (Fig. 3; Table 2). No significant differences were, however, registered between the remaining control treatments. Overall, isopods submitted to 25% WHC and to 75% WHC showed lower consumptions than at 50% WHC, even though significant differences were mostly associated to single-pesticide treatments and low-concentration mixtures (Table 2). No differences were registered between the same chemical treatments at 25% WHC and 75% WHC. Within-group comparisons to control only showed statistical differences for the treatment CPF1/MCZ3 at 50% WHC (Dunnett's test, $p < 0.05$).

Different patterns were detected when comparing the observed consumption ratio to IA-predicted values (Fig. 3; Table 1SD). While at 50% WHC isopods generally consumed significantly less than would be expected by the effects on single pesticide treatments, at 25% WHC mixtures' effects on isopods consumption were predominantly antagonistic and at 75% WHC mixed effects were registered (Fig. 3 and Table 1SD). However, when including all three factors to model the IA predictions (i.e. treating all data as ternary combinations instead of assessing mixtures' effects as a binary combination within each moisture regime), only synergistic relationships were found in isopods' consumption, regardless of the regime assessed (Table 2SD).

Observed and IA-predicted biomass change is shown in Fig. 4. After the 14 days period, biomass change was predominantly negative and significantly affected by both "soil moisture" (two-way ANOVA, $F_{2,125} = 19.41$, $p < 0.001$), and "chemical treatment" (two-way ANOVA, $F_{14,125} = 1.316$, $p < 0.001$), but not by their interaction (two-way ANOVA, $F_{28,125} = 1.316$, $p > 0.05$). No significant differences were found between control groups kept at different soil moistures (Table 2). Significant differences were again mostly found in single-pesticide treatments and highlight the poorer performance registered at 75% WHC, where isopods showed stronger biomass losses than those kept at 50% WHC. Contrary to consumption ratios, almost no differences were found between 50% WHC and 25% WHC (Table 2). Multiple comparisons to control within the same soil moisture conditions showed that isopods exposed to MCZ3 (Dunnett's test, $p < 0.001$), CPF1/MCZ1 (Dunnett's test, $p < 0.05$), CPF2/MCZ2 (Dunnett's test, $p < 0.05$), and CPF2/MCZ3 (Dunnett's test, $p < 0.05$) lost significantly more weight when kept at 50% WHC. The same happened with the treatments CPF2 (Dunnett's test, $p < 0.05$) and MCZ3 (Dunnett's test, $p < 0.05$) at 75% WHC (Fig. 4).

When kept at 50% WHC, isopods lost significantly more weight than predicted by the IA model in CPF1/MCZ2 and CPF2/MCZ2 (Fig. 4; Table 3SD). Regarding those kept at 25% WHC, antagonism was found in treatments CPF1/MCZ1, CPF2/MCZ2, and CPF2/MCZ3 (Fig. 4; Table 3SD). Finally, antagonistic relationships were found for almost every mixture treatments in isopods kept at 75% WHC (Table 3SD). As for isopods' consumption, the inclusion of soil moisture as a predictor in IA model estimations for biomass change (i.e. ternary combinations) showed significant synergistic deviations in all mixture treatments, irrespective of the regime assessed (Table 4SD).

4. Discussion

Our results showed that soil moisture can, indeed, influence the toxicity of these commercial formulations on *P. pruinosus*, but only

Table 2
Dunnett's post-hoc test results to compare the consumption ratio and the biomass change registered in isopods exposed to the same chemical treatments, but under different soil moisture regimes.****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns-non significant.

		CTR	CPF1	CPF2	CPF3	MCZ1	MCZ2	MCZ3	CPF1 MCZ1	CPF1 MCZ2	CPF1 MCZ3	CPF2 MCZ1	CPF2 MCZ2	CPF2 MCZ3	CPF3 MCZ1	CPF3 MCZ2	CPF3 MCZ3
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 change	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

for the feeding parameters since it showed no effects on isopods' survival.

To our knowledge, there are no similar studies with terrestrial isopods that assess the combined effects of unfavourable soil moisture levels and pesticides. 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, which contributes for an extensive case-specificity. Sørensen and Holmstrup (2005) found that neither dimethoate nor cypermethrin reduced the drought tolerance in the collembolan *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 some of them having a strongly lipophilic character, pesticides have generally well defined modes of action and therefore are toxic to organisms 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. Contrarily, Everts et al. (1991) reported an interaction between low humidity and deltamethrin for spiders, suggesting the mutual capability of these stressors to disturb arthropods' water balance as the underlying reason for this synergic relationship. Given the limitations of terrestrial isopods' water regulation (Sutton et al., 1980), if similar pesticide-induced discharges had occurred, they could imply elevated costs to their body water content. 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 other soil dwelling organism, the collembolan *Folsomia candida* to the same pesticide and drought stress conditions and, contrary to the former authors, an antagonistic interaction was reported for survival.

Regarding the toxicity of pesticides under very high soil moistures, fewer studies are available in literature, and they have generally reported no effects on the survival to the pesticides, which is consistent with the present work. Puurtinen and Martikainen (1997) found no differences in the survival of enchytraeids to dimethoate and benomyl at 70% WHC (when compared to 55% WHC), and Lima et al. (2011) reported a similar situation when exposing *E. andrei* to carbaryl at 100% WHC (compared to 60% WHC). Nevertheless, a different result was obtained by Cardoso (2012) in *F. candida*, where the same moisture treatments showed to interact with carbaryl toxicity changing it in a "dose-ratio" dependent manner. Among these species, *P. pruinosus* is the most susceptible to exceedingly humid environments and it could not stand the moisture levels assessed by Lima et al. (2011) or Cardoso (2012) (see Morgado et al., 2015).

Two points must be emphasized before concluding this survival section. The first is that in the present work, as in a previous where the effects of temperature in the same mixture were assessed (Morgado, 2014), no deviations were found to the reference model of IA using MixTox. This shows a consistency of non-interacting effects between these pesticides on the survival of *P. pruinosus*, irrespective of the environmental conditions. However, it was interesting to note that while no interaction was found by the MixTox tool, the most parsimonious GLM model actually included the interaction term for CPF and MCZ. This emphasizes the need of considering different approaches to model the combined effects of multiple stressors, particularly if the factors involved have a different nature. The application of GLM models to mixture toxicity data consisting of binary biological responses was recently proposed Iwasaki and Brinkman (2015) and may constitute an important complement to toxicological approaches in the future.

Contrary to survival, it seems clear by analysing the feeding parameters that isopods exposed to overly dry or moist conditions showed a fairly worse feeding and growth performance when simultaneously exposed to pesticides. The effects on the feeding parameters were more evident for MCZ than for CPF, as shown by the well-defined dose-dependent decrease in consumption ratios and increase in biomass loss. This does not mean that MCZ was more toxic than CPF, since the concentrations used were of different order of magnitude. However, it suggests that the difference between lethal and sublethal toxicity must be smaller for CPF than for MCZ (i.e. for the endpoints here considered). Similarly, MCZ showed a higher impact on the definition of mixtures' effects since the joint effects of the pesticides were generally more dependent of the MCZ concentration. If one looks, for instance, at each group of three mixture treatments in Fig. 3 (grouped according to the CPF concentration and with increasing concentrations of MCZ), a steep decrease in isopods' consumption ratio could generally be found whenever MCZ concentration was increased within the mixture. Increasing CPF concentrations, on the contrary, did not show equally relevant effects, which was unexpected since the mechanisms potentially leading to changes in organisms' feeding activity, and consequently growth, are more obvious for CPF. In fact, when it comes to assessing pesticide-induced changes in behaviour, a particular attention has been devoted to acetylcholinesterase-inhibiting compounds because of its widespread neuromuscular effects. 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. Likewise, Blažič et al. (2005) showed the pesticide-induced inhibition of feeding in *P. scaber* to be concomitant to acetylcholinesterase inhibition. Our results, however, suggest that additional mechanisms may have an even stronger effect on organisms' feeding activities since dithiocarbamates were claimed to have low potential for acetylcholinesterase inhibition (Espigares et al., 1998). MCZ effects must instead be related to a

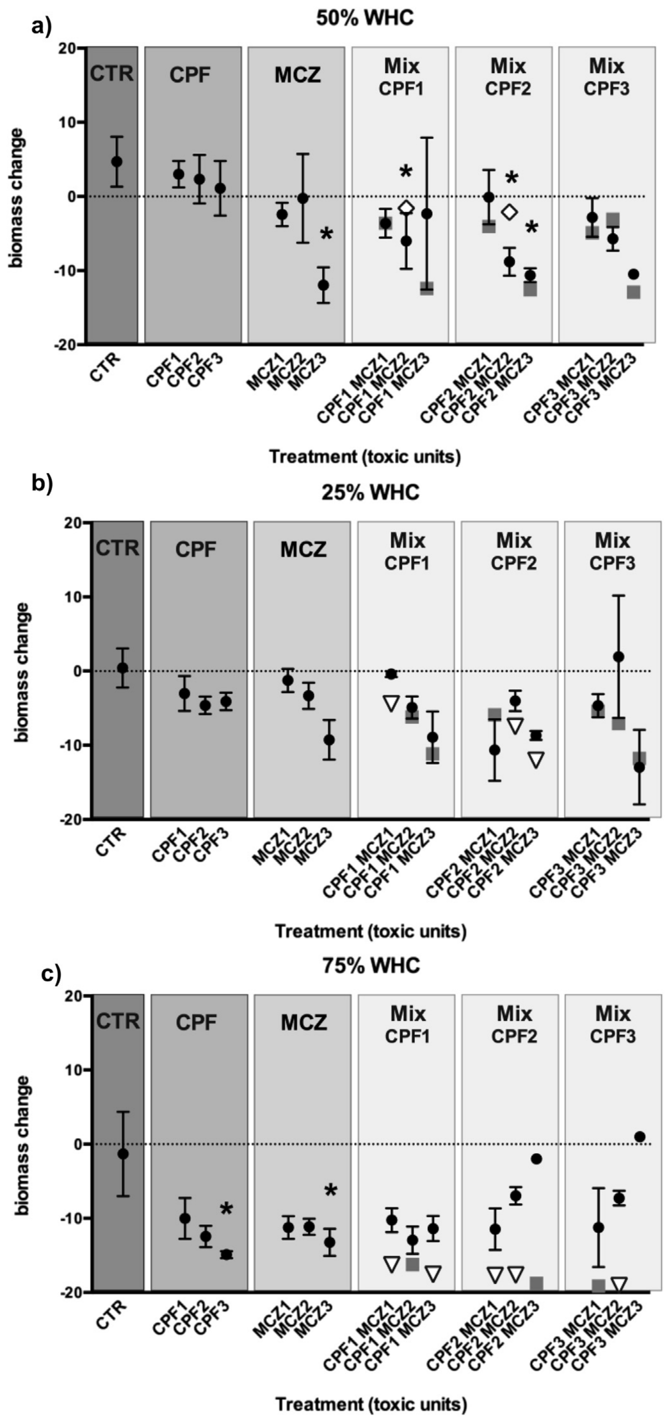

Fig. 4. Observed (circles; ± standard error) and predicted biomass change of *Porcellionides pruinosus* after exposure to single and mixture treatments of chlopyrifos (CPF) and mancozeb (MCZ), under three different soil moisture regimes: a) 50% WHC, b) 25% WHC, and c) 75% WHC. Grey squares (■) represent values predicted by the independent action model (IA) that were not significantly different from the observed results (i.e. were inside the confidence intervals), open inverted triangles (▽) represent prediction values that were significantly lower than observed results (i.e. antagonism), and open diamonds (◇) 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, $\alpha = 0.05$).

general impairment in organisms condition since this compound is still known to induce several non-specific responses such as oxidative stress (Tsang and Trombetta, 2007) and impairments on phases I and II of organisms' detoxification systems (Lewerenz and Plass, 1984; Szépvölgyi et al., 1989; Siddiqui et al., 1991; Nebbia et al., 1993). Besides, indirect effects related to the fungicidal effects of MCZ may also have been involved. 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 and Singh, 2003). These communities are known to dominate the first stages of decomposition processes and by rendering the leaf material more attractive they can stimulate detritivores' consumption, thus becoming highly relevant in short-term exposures like ours (Zimmer et al., 2003; Gessner et al., 2010).

Perhaps more important is the fact that different moistures led to different joint-effects of the pesticides on isopods' consumption and biomass change. 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, the significant synergistic deviations to the IA model registered when considering the ternary combinations of factors clearly emphasized the risks comprised by such approach. It was not always clear whether such effects were actually related to changes in the behaviour of the mixture or just to the interaction of each pesticide with soil moisture. Since most of the significant differences between soil moisture regimes were found in single pesticide treatments, it is likely that the major contribution was related to the influence of soil moisture on each pesticide. Yet, several significant deviations to the IA predictions were registered for the binary combinations of CPF and MCZ (within each soil moisture regime), and different patterns of mixture behaviour could be distinguished. This indicates that soil moisture indeed has the potential to influence the mixture itself but this effect might be of minor importance when compared to its own interaction with each chemical, particularly in a context of complex combinations of stressors. This is an important finding insofar as significant enhancements in accuracy could be achieved in the environmental mixture toxicity field, only by knowing the individual effects of the environmental factors on each component of a mixture. After knowing such effects, the overall toxicity could be estimated using reference models, such as the IA or other, with potential gains in accuracy. This would 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 (as proposed by the funnel hypothesis, see Warne and Hawker, 1995), one may speculate that such approach could also apply to more complex mixtures. Non-additive combinations of chemicals weaken when included in multicomponent mixtures, likely leading to negligible effects (Warne and Hawker, 1995; Belden et al., 2007). As such, if the interaction between chemicals is low, the additional effects of environmental factors on each component of the mixture is likely to become the main source of uncertainty, being thus the main driver for potential deviations to the predicted toxicity. More studies are however needed to confirm this hypothesis since the effects of natural environmental factors on pesticide mixtures, or mixtures of xenobiotics in general, are still largely unknown (Bednarska et al., 2009; Laskowski et al., 2010). 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.

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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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.10.034.

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