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Joint effects of chlorpyrifos and mancozeb to the terrestrial isopod

***Porcellionides pruinosus*: a multiple biomarker approach**

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ABSTRACT

The exposure to pesticides by non-target soil biota has long been regarded as a serious downside of modern agriculture regimes and subject of heated debate. Of utmost relevance is the exposure to pesticide mixtures since their effects have shown not to necessarily reflect the individual toxicity of its components and even the simple addition of effects may lead to consequences not clearly anticipated. In this work, a multiple biomarker approach was employed to identify the mechanistic and time-effects underlying several single and mixture treatments of chlorpyrifos and

mancozeb in juveniles and adults of the terrestrial isopod *Porcellionides pruinosus*. The effects of both pesticides and mixture at recommended doses were mostly transitory under these controlled conditions and one-pulse exposure. While imbalances were identified on detoxification and oxidative stress-related enzymes, isopods generally showed the ability to recover until the end of the experiment. Juveniles showed, however, higher vulnerability than adults. The most notorious differences between life stages occurred in energy-related parameters where distinct performances and stress-handling behaviours were observed, suggesting higher metabolic costs in juveniles. Our results stress that understanding the time-dependence of the underlying mechanisms governing the joint-effects of pesticides can help assessing and anticipating mixtures' effects. Moreover, it is also emphasized the importance taking life stage-related differences in consideration when evaluating the environmental risks of pesticides and pesticide mixtures.

KEYWORDS

pesticide mixtures, soil invertebrates, terrestrial isopods, biomarkers, energy reserves, oxidative stress

INTRODUCTION

Nowadays, agriculture is a highly optimized process, that strongly relies on the application of multiple agrochemicals, including pesticides (Matson et al. 1998). These compounds are also known to pose serious problems to non-

target soil organisms that inhabit agroecosystems, so they must be thoroughly evaluated (Carvalho 2006). The traditional approach for assessing the environmental risks of using 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 et al. 1998; Aktar et al. 2009). Nevertheless, given the requirement of acting on a broad range of pests/pathogens, non-target organisms are often simultaneously exposed to several pesticides. Since the effects of pesticide mixtures were previously shown not to necessarily reflect the individual effects of its components (Lydy et al. 2004), a growing awareness has emerged regarding their interaction. Moreover, the mere addition of effects of co-occurring pesticides is often disregarded by these procedures, which may ultimately lead to underestimations of the environmental risk (Pape Lindstrom and Lydy 1997; Belden and Lydy 2006). Despite the attention received by mixture toxicity, the complexity and specific character of chemical compounds' interactions still constitutes an important constraint to ecotoxicologists and risk assessors. Particularly important seems to be the comprehension of mechanisms by which toxicity is induced during exposures to mixtures, how they can differ from single pesticides, and how they can be accurately predicted in a cost-effective way. 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 and Fossi 1994; Morgan et al. 1999), and widely used

to evaluate sub-lethal exposures to pesticides in an extensive number of organisms providing some insight on the mechanistic effects (Booth and O'Halloran 2001; Booth et al. 2003; Santos et al. 2010; Pereira et al. 2013). Of highest relevance is also the assessment of bioenergetic parameters such as the energy reserves content, energy consumption or the cellular energy allocation. The rates at which organisms assimilate, allocate or deplete energy constitute an accurate indication of their condition and therefore, play a central role in their tolerance to environmental stressors (De Coen and Janssen 1997).

Chlorpyrifos (CPF) and mancozeb (MCZ) are extensively used pesticides with different target pests/diseases and different modes of action, whose application is frequently simultaneous or with very short intervals (Cross and Berrie 1996). CPF is an organophosphate (OP) insecticide, used to control insect pests and has the inhibition of acetylcholinesterase as main mode of action (Fukuto 1990). 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 as a multi-site action compound (Gullino et al. 2010), that is frequently applied against a wide spectrum of fungal diseases (Cycon et al. 2010). MCZ breaks down quickly, when exposed to water, to release ethylene bisisothiocyanate sulfide, which is further converted into ethylene bisisothiocyanate. These metabolites are both active toxicants, thought to interfere with fungi enzymes containing sulphhydryl groups (Gullino et al. 2010).

The synantropic nature and wide distribution of several terrestrial isopod species, like *Porcellionides 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 a considerable attention among the research community, that is frequently using isopod species in soil ecotoxicology experiments (Sousa et al. 2000; Loureiro et al. 2002; Jänsch et al. 2005; Ferreira et al. 2010; Santos et al. 2010; Tourinho et al. 2013; Silva et al. 2014).

Aiming to contribute for further knowledge, here we evaluated the joint effects of CPF and MCZ to adults and juveniles of the terrestrial isopod *Porcellionides pruinosus*. In order to have an insight into the mechanism of toxicity prompted by this mixture on terrestrial isopods, a battery of biomarkers, energy reserves and energy allocation measurements were undertaken using both adults and juveniles.

MATERIAL AND METHODS

Test organism

The terrestrial isopod *P. pruinosus* was used as test-species. Isopods were collected in a horse manure heap and maintained in laboratory cultures at 22°C ($\pm 1^\circ\text{C}$), 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*). Life stages were selected according to their weight, with adults ranging from 15 to 25 mg and juveniles 5 to 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.

Chemical compounds and soil

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

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 CaCl₂), 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.

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) (a.i.- active ingredient). An additional group of organisms was also kept in clean soil adjusted to 60% WHC with distilled water and used as control. Mixture treatments were performed as shown in Figure 1SD, ranging

from 1CPF/1MCZ to 10CPF/10MCZ. This experimental design was employed for adults and for juveniles.

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. Soil moisture was then adjusted to 60% WHC by adding the necessary amount of distilled water. Soil was, then, transferred to rectangular plastic boxes (14.3 cm length x 9.3 cm width x 4.7 cm height) 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 throughout 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 ± 1 °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 T0. In every sampling time, isopods were individually weighted, freeze-dried in liquid nitrogen and stored at -80 °C until further analysis.

Measured parameters

Protocols for analysis of biomarkers and energy parameters followed the methods described by Ferreira et al (2015) and are thoroughly detailed in

supplementary material. In order to measure lipid peroxidation (LPO), glutathione *S*-transferase (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. For determination of energy-related parameters one organism per replicate was used. Five replicates per treatment were always used.

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). GST activity was determined using the method described by Habig et al (1974). CAT activity was determined using the method described by Clairborne (1985) and adapted to microplate by Ferreira et al (2010). AChE activity was determined according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). For all biomarkers, 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.

Energy-related parameters were assessed as described by de Coen and Janssen (1997) and included: energy reserves content (lipids, carbohydrates, proteins), total energy available (Ea), consumed energy (Ec) and cellular energy allocation (CEA). Ea was calculated by summing the energetic equivalents of each energy reserve fraction (mJ/mg org). Ec was calculated as oxygen consumption rate in the electron transport system (mJ/mg org/h). CEA was calculated as follows:

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

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.

Statistical analysis

A multivariate analysis of variance (MANOVA) with Pillai's trace statistics was performed, using log-transformed ($\log(x)+1$) biomarkers responses and energy-related parameters as dependent variables, and "life stage", "sampling time", "CPF treatment" and "MCZ treatment" as independent variables. Both main effects and interactions were assessed. Whenever significant differences were found in factorial MANOVA, univariate statistical methods were applied to test individual dependent variables. Two-way analysis of variance (ANOVA) with "CPF treatment" and "MCZ treatment" as independent variables, were used to assess differences in individual biomarkers. When significant differences were detected, multiple comparisons were performed using Tukey HSD post-hoc test. Both factorial MANOVA and ANOVAs were conducted in R version 3.3.0 (R Core Team, 2016, <https://www.R-project.org>). After converting data into percentage of control, a Student's t -test or Mann-Whitney rank-sum test (nonparametric equivalent) were performed in Sigmaplot statistic pack (SigmaPlot 12.0 statistic pack; Systat Software, Inc., San Jose, CA, USA) to compare the responses of different life stages. Normality and homoscedasticity assumptions were visually inspected and tested using Shapiro–Wilk test and Levene's equal variance test, respectively.

Significant differences were assumed if probability values were equal or higher than 95% ($\alpha = 0.05$).

RESULTS

The factorial MANOVA revealed highly significant main effects and interactions for all independent variables considered in the present study (statistical details summarized on table 1SD). Hence, the univariate main effects and interactions for each biomarker/energy-related parameter were examined (two-way ANOVA, details summarized in table 2SD).

Neurotoxicity

The responses of AChE activity in adults and juveniles throughout the experiment are shown in Figure 1. A significantly higher activity of this enzyme in adult isopods was found after 48h 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, a significant increase of AChE was observed at 48h, for 5CPF/1MCZ and 10CPF/10MCZ when compared to control. After 96h a significantly lower activity was found for every treatment except 1CPF. At day 7, significant increase in AChE activity was observed for 1CPF/5MCZ and 5CPF/1MCZ treatments when compared to control.

Detoxification

Regarding GST activity (Figure 2), a dose-related increase was found in adults after 48h with significant differences to control in all treatments except 1CPF. 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. GST activity in juveniles followed a similar pattern to adults in the first sampling time, except for the most severe mixture treatments where values were similar to control. Contrary to adults, however, 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.

Oxidative stress

No significant differences were registered for CAT activity in adults at 48h but significant decreases were found at 96h for treatments 1CPF/5MCZ, 5CPF/1MCZ and 5CPF/5MCZ and after 7 days for 10CPF/10MCZ (Figure 3). 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, even though a decrease was found in mixture treatments at day 7, reaching 15-30% of control.

We found no differences within the LPO rates measured during this experiment, either for adults or juveniles (Figure 2SD).

Energy-related parameters

The only significant difference observed in adults' Ea at 48h was an increase in 5CPF/5MCZ (Figure 4). 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 (see supplementary material) we found the largest contribution for the total energy available to be provided by lipids (Figure 3SD). 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. No significant differences were found between control and pesticide-exposed juveniles except at 96 h for the 5MCZ treatment.

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 4SD). Adults' carbohydrates content 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.

As for the previous energetic components, proteins also seemed to increase in adult isopods, particularly after 48h and 7 days (Figure 5SD). 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. There was no clear response to pesticides by juveniles 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 Ec (Figure 5), 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, a statistically significant decrease in Ec was found for several mixture treatments after 48h. After 96h, these differences had already disappeared but a 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 6), 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.

Comparison of life-stage responses

The significant differences registered when comparing the responses of adults and juveniles to the several single and mixture treatments are summarized in table 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. There was not a clear pattern for life stage-related differences in AChE activity, which seemed to be mainly found 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 life stages' 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 compared to juveniles. Few differences were detected on the response of CAT and LPO between life stages.

DISCUSSION

In recent years, mixture toxicity of pesticides has received an increased attention. However, only a minor fraction has focused on lower organizational

levels. By providing an insight into the mechanisms of toxicity, biomarkers can provide a forewarning of 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 and 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 overall, the effects of CPF and MCZ field dose exposures to adult isopods were mostly transitory, as shown by detoxification and oxidative stress-related enzymes that resulted in a new homeostasis status within the course of the experiment. However, it was noteworthy that some effects on juveniles were be more prominent and/or lasted longer than in adults. This could be seen in GST activity and several energy-related parameters, becoming particularly clear when comparing the responses of both life-stages as percentage of the respective control. This is in agreement with findings reported by several authors after evaluating life stage-related differences in terrestrial isopods' vulnerability to several kinds of stress (Ribeiro et al. 2001; Stanek et al. 2006; Morgado et al. 2013). These doses may pose problems to juveniles, which might have 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 usually 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 as fortnightly repeated applications during prolonged periods (Adam Wightwick and Menzies 2010). CPF can also be applied with similar periodicity, notwithstanding its higher persistence. These situations may prevent organisms from completely recovering of previous exposures and thus increase the vulnerability to further events. In addition, exposures were tested under controlled conditions, which are optimum for isopods, with no predation risk or other abiotic influences that may impair their physiology or survival.

Neurotoxicity

There were no evidences of CPF-induced inhibition in AChE activity, for the treatments used in this experiment. Strong CPF-induced inhibitions were already shown in a wide range of soil organisms but the effective doses seem to vary considerably. 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 hiliaris* after field dose exposure to CPF, but they were restricted to the first 24h. Similar early effects would be skipped in the present experiment because the first sampling time was performed at 48h. Nevertheless, as reported by the same authors, such short-lasting transient effects may not lead to serious fitness costs to the organisms in the long-term (Booth et al. 2003). On the other hand Collange et al (2010) only found AChE inhibition in

the earthworm *Lumbricus terrestris* at much higher concentrations (≥ 12 mg CPF / kg soil).

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

Two additional outcomes must also be pinpointed for AChE. First, the significantly lower activity observed in juveniles at 96h may have been partly influenced by a higher control value, when comparing to the remaining sampling times and to additional values reported for juveniles of *P. pruinosis* (Morgado et al. 2013). The second is related to the significant increases observed in AChE activity. Since these were restricted to MCZ-containing treatments, they may be related to the metallic content of this compound (complex of Mn^{2+} and Zn^{2+} chelated with ethylenethiourea (Brody et al. 2013)), as summarised in Barillet et al (2007). Another hypothesis is that these exposures triggered the production of the monomeric AChE-R that shares similar hydrolytic activity but remains soluble within the synaptic cleft (Soreq 2001). Santos et al (2010) also observed a similar increase in AChE activity of *P. pruinosis* 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.

Detoxification

The major differences 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. Complete homeostasis was also not achieved before day 7 as shown by the significant inhibition at 96h in mixtures. Overall, GST results seemed to highlight the complexity of dose and time-dependencies in the kinetics of detoxification enzymes. Such interplay can help explaining the responses observed for juveniles exposed to mixture treatments. Hence, whereas adults seemed to be effective on dealing with the contamination stress, juveniles did not show such 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 (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. Nebbia et al (1993) showed the action of zineb (another Zn²⁺-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. Pesticide biotransformation by organisms can be an important process for promoting interaction between pesticides (Spurgeon et al. 2010). A potential effect of MCZ on the mixed function oxidases may have affected the transformation of CPF to CPF oxon, therefore attenuating CPF effects on the mixture (particularly in AChE). Assessing the kinetics of both pesticides and their metabolites could enlighten about their actual exposure pattern, therefore contributing to explain in detail the time-course of responses observed.

Oxidative stress

Despite no direct evidences of oxidative damage were registered on LPO, results suggest that the ROS-scavenging enzymatic activity was actually being required. GST and CAT were the only enzymes with such properties assessed in this experiment but several other molecules (enzymatic or non-enzymatic) must also have contributed to the maintenance of this balance. Albeit none of these pesticides has oxidative stress as the primary mode of action, both were previously referred to constitute pro-oxidant agents (Jager et al. 2007; Tsang and Trombetta 2007). We found almost no differences in CAT activity 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 life stage-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

CPF while the opposite pattern seemed to occur for MCZ. The effects observed for CPF on CAT activity seemed to be in line with those reported by Ferreira et al (2015) for the sampling times after exposure to dimethoate, another OPs pesticide. It was not clear if complete homeostasis was achieved during this experiment because both life stages seemed to present a dose-dependent decrease in CAT activity at day 7, particularly pronounced in mixture treatments.

Energy-related parameters

The most relevant aspects arising from the analysis of the energy-related parameters are possibly those associated to the comparison of life stage-related responses. We found few evidences of abnormal depletion on any of the energy reserves assessed, neither in adults nor juveniles, which was expected 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 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. When considering the whole energy available the most relevant outcome was the dose-dependent increase at day 7. A similar situation was observed by Morgado et al (2013) leading to the hypothesis that it could have been due to behavioural changes on feeding and/or metabolism. Such increase was concurrent with reductions of Ec, which, however, were the opposite in the present experiment. In this way, although not ruling out this

hypothesis, additional factors may as well be involved (e.g. . differences in 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 an hypothesis by Ferreira et al (2015) for dimethoate and further confirmed using transcriptomic and metabolomics approaches (Loureiro and Soares 2014 Dec 16). Transcriptomic analysis showed dimethoate-induced impairments to moult by affecting, for instance, the regulation of genes coding for chitinase (chitin-degrading enzymes) or haemocyanin (respiratory pigment also involved in moult processes) transcription (Loureiro and Soares 2014 Dec 16). Similarly, a metabolomic approach showed once more, metabolites associated with moult (i.e. the aminoacids lysine, valine, leucine, isoleucine, (Loureiro and Soares 2014 Dec 16)). Considering that isopods, and crustaceans in general, accumulate reserves during the pre-ecdysis period (Sánchez-Paz et al. 2006; Carter and Mente 2014), such increase in energy available might not imply greater fitness. Instead, it may indicate a disruption in moult cycles that, by assessing total energy content, the analytical method was unable to distinguish. Further research is needed to infer about this hypothesis (i.e. prolonged studies to relate moult cycles and energy reserves). As stated previously, no significant differences were found in adults' Ec, neither with treatment nor time. By the opposite, juveniles exposed to mixtures showed decreased Ec after 48h and significantly higher Ec at day 7. This is different from Morgado et al (2013) but probably reflects the different source, nature and duration of the stress event. Regarding CEA, the most important results were also associated with

mixture treatments. Contrary to adults, CEA in juveniles exposed to mixtures seemed to be impaired throughout the study period becoming mostly negative at day 7. While this biomarker provides an indication of differentiated effects on bioenergetics parameters, complementary assessments focused on determining the aerobic scope, would help clarify the consequences of such different responses for isopods' fitness (Sokolova 2013).

Conclusions

Briefly, this study showed that whereas the recommended doses of each pesticide elicited effects that were mostly transient, slightly higher single pesticide concentrations and mixture treatments impaired some physiological processes in these organisms, particularly in juveniles. We found these pesticides to affect detoxification and antioxidant systems, and identified differences in energy-related parameters, suggesting life-stages to respond differently to contamination stress and to have different metabolic costs associated. Our results stress that understanding the time-dependence of the underlying mechanisms governing the joint-effects of pesticides can help assessing and anticipating mixtures' effects. Moreover, it is also emphasized the importance of taking life stage-related differences in consideration when evaluating the environmental risks of pesticides and pesticide mixtures. Future approaches using multiple pulse exposures that mimic field application strategies could help enlighten about the real safety of these pesticide mixtures.

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DATA AVAILABILITY

Data, associated metadata, and calculation tools are available from the corresponding authors (ruimorgado@ua.pt and sloureiro@ua.pt)

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FIGURE CAPTIONS

Figure 1 – 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Figure 2 – 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Figure 3 – 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Figure 4 – 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Figure 5 – Consumed energy 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Figure 6 – 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 (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

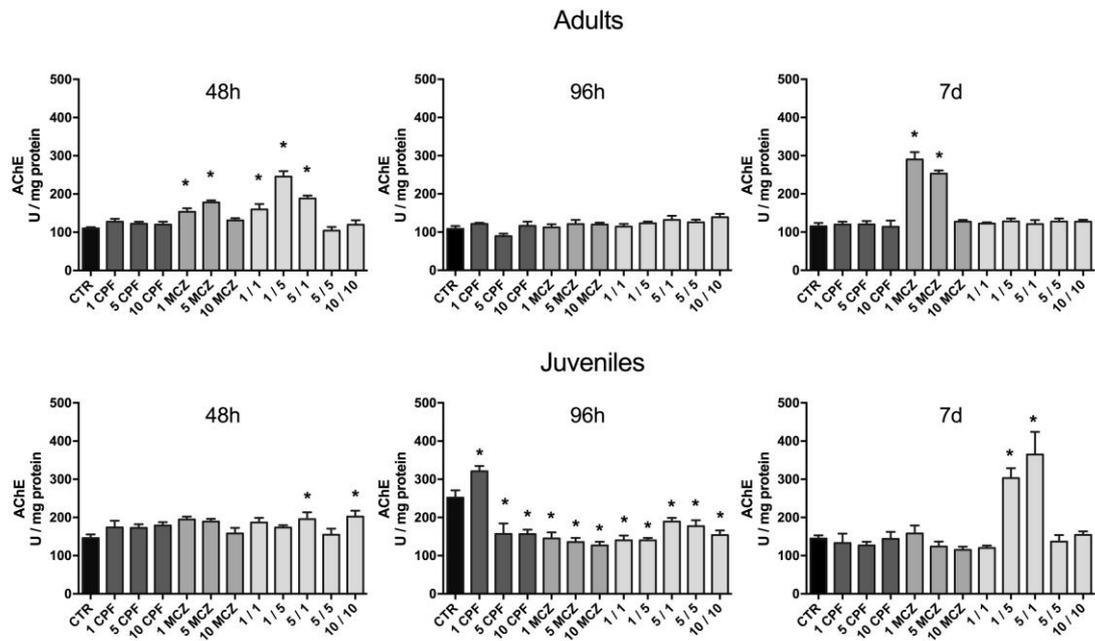


Figure 1 – Mean AChE activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

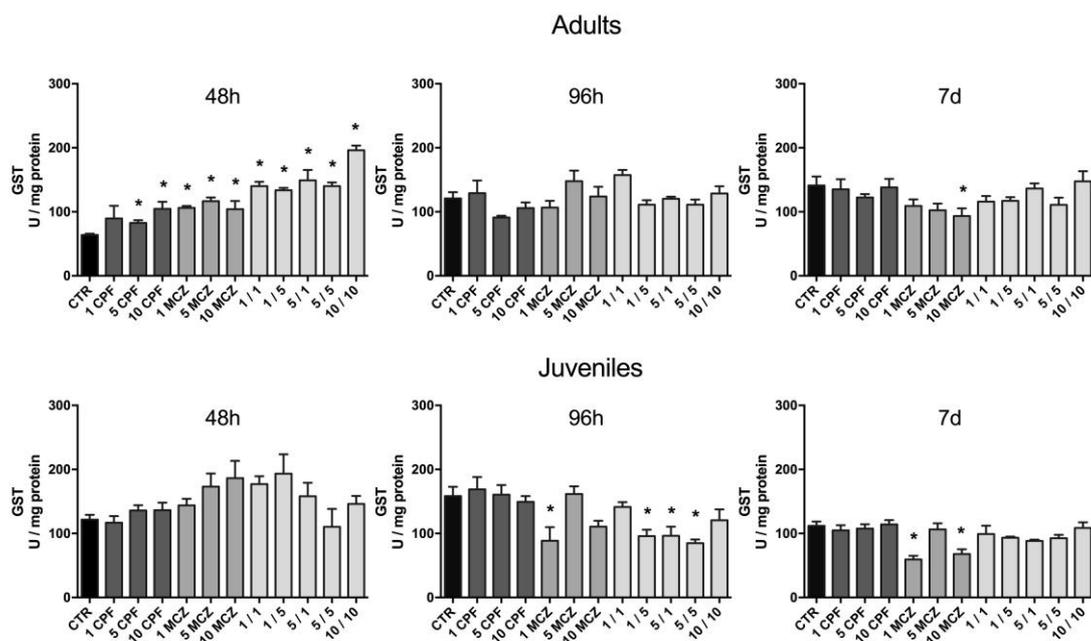


Figure 2 – Mean GST activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

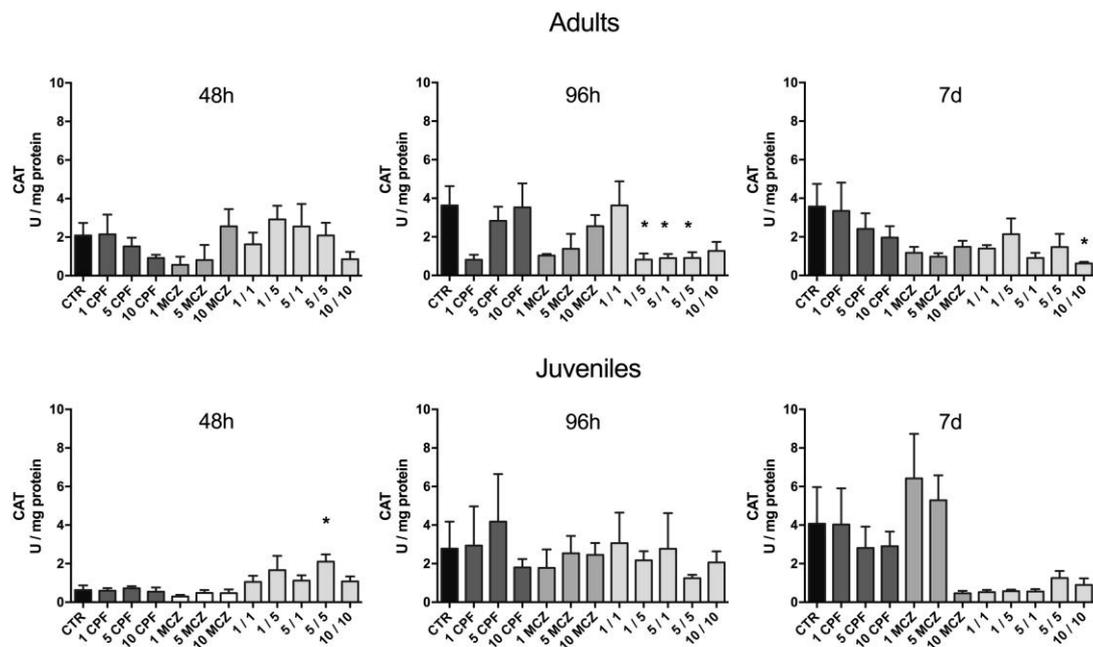


Figure 3 – Mean CAT activity and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

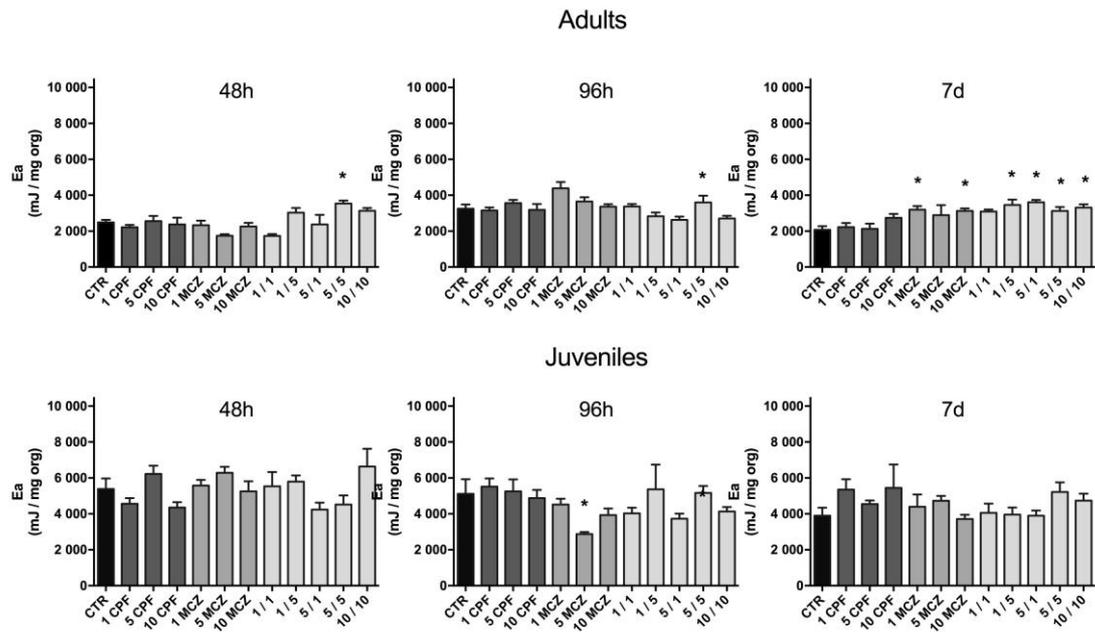


Figure 4 – Total energy available and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

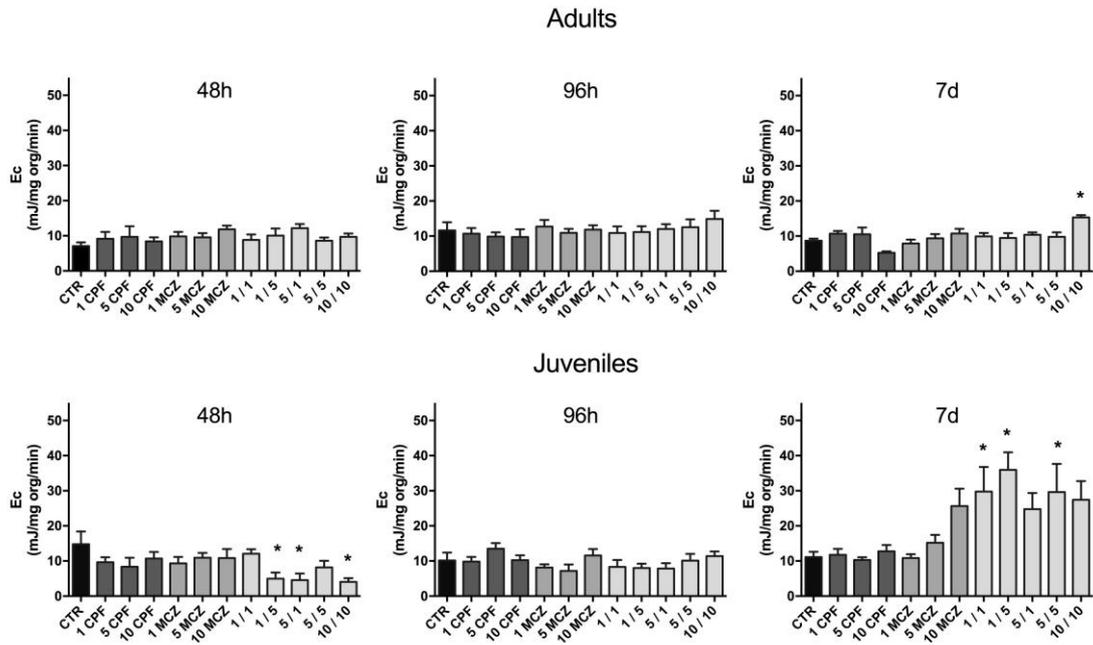


Figure 5 – Consumed energy and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

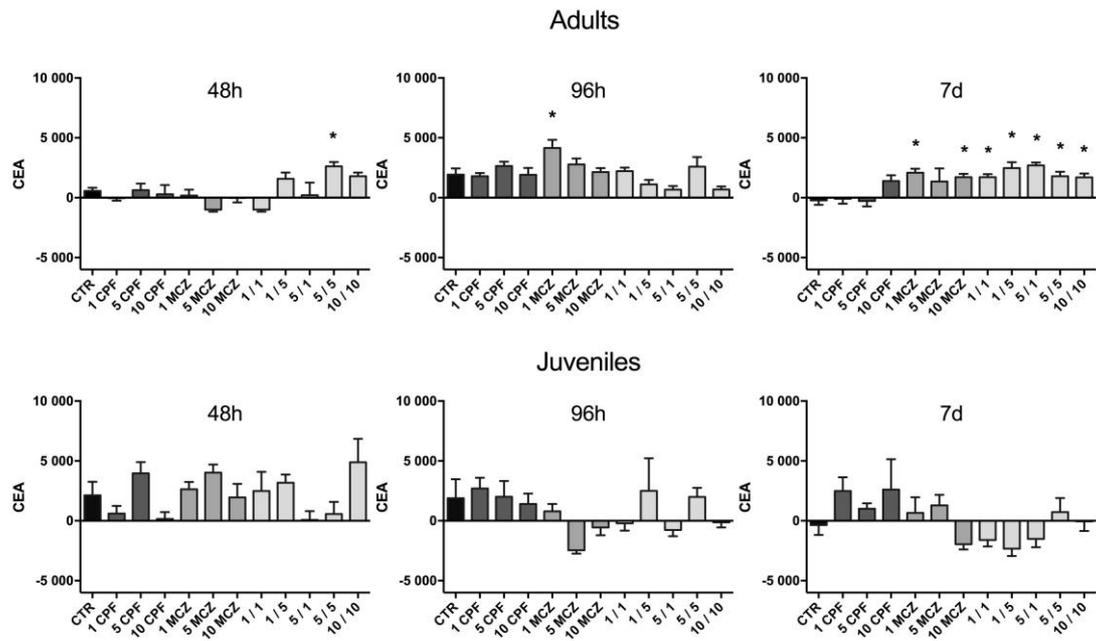


Figure 6 – Cellular energy allocation and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

Table 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 to 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* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 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/10MCZ		↘***		↗*			↘**		↘***		
96h	1CPF	↗*									
	5CPF		↗*					↗*		↗*	
	10CPF	↘**						↗***	↘*		
	1MCZ	↘**				↘**	↘**	↗**	↘**		↘*
	5MCZ	↘***		↘*		↘**	↘**		↘*		↘***
	10MCZ	↘***	↘*	↗*			↘**	↗*			↘**
	1CPF/1MCZ	↘***	↘**			↘**	↘***				↘**
	1CPF/5MCZ	↘***	↘**		↗**						
	5CPF/1MCZ	↘**	↘**								
	5CPF/5MCZ	↘***	↘***			↘*					
10CPF/10MCZ	↘***										
7d	1CPF										
	5CPF										
	10CPF									↗*	
	1MCZ	↘***	↘*		↗*				↘*		
	5MCZ	↘***			↗*				↘**		
	10MCZ	↘**			↘*	↘***	↘**		↘**		↘***
	1CPF/1MCZ	↘**			↘**	↘**	↘*	↘*	↘**		↘**
	1CPF/5MCZ	↗**				↘**	↘*	↘***	↘***	↗**	↘***
	5CPF/1MCZ	↗**	↘*			↘***	↘***	↘**	↘*		↘***
	5CPF/5MCZ							↘*	↘*		
10CPF/10MCZ					↘**	↘*	↘*			↘*	

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

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

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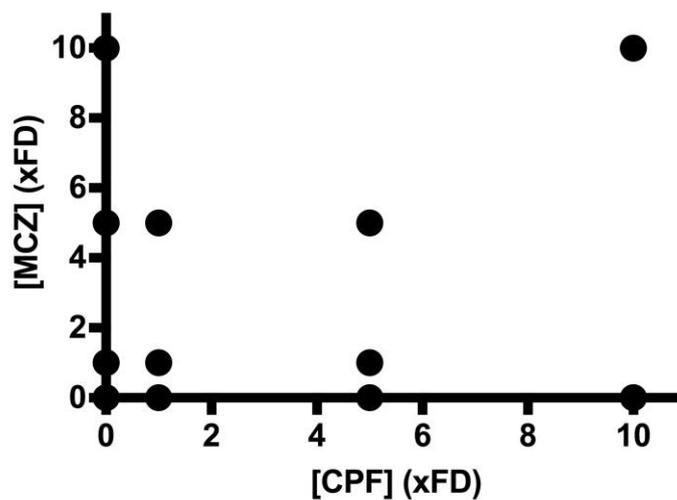


Figure 1SD – Experimental design applied to evaluate toxicity of mixtures of chlorpyrifos (CPF) and mancozeb (MCZ) to the terrestrial isopod *Porcellionides pruinosus* in LUFA 2.2 soil. The axes units correspond to the concentrations of the pesticides expressed as number of field doses (FD) applied for each pesticide.

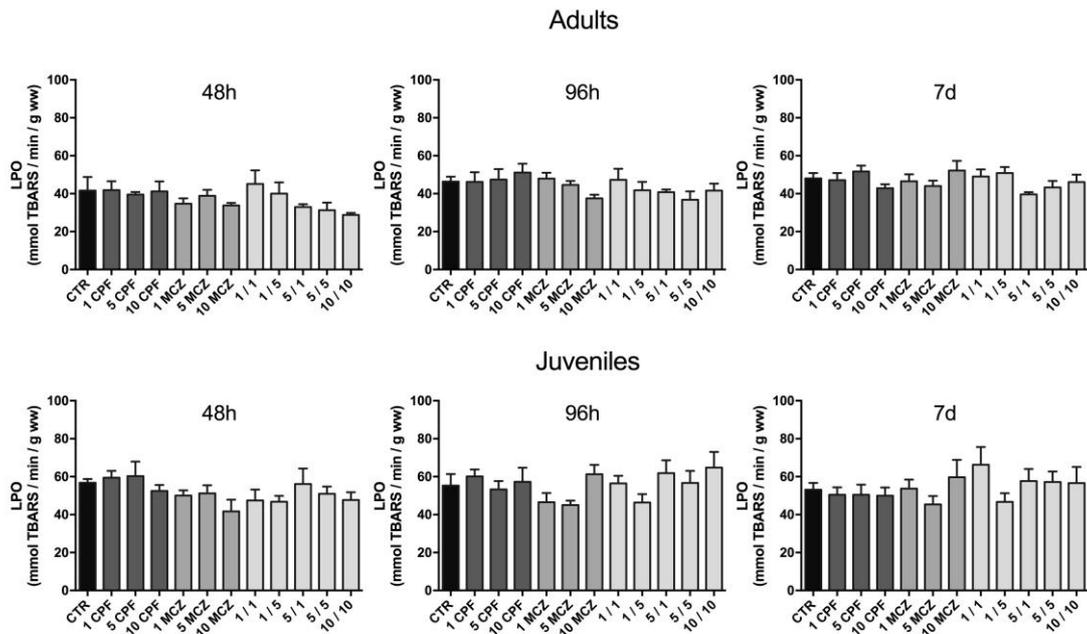


Figure 2SD – LPO rates and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

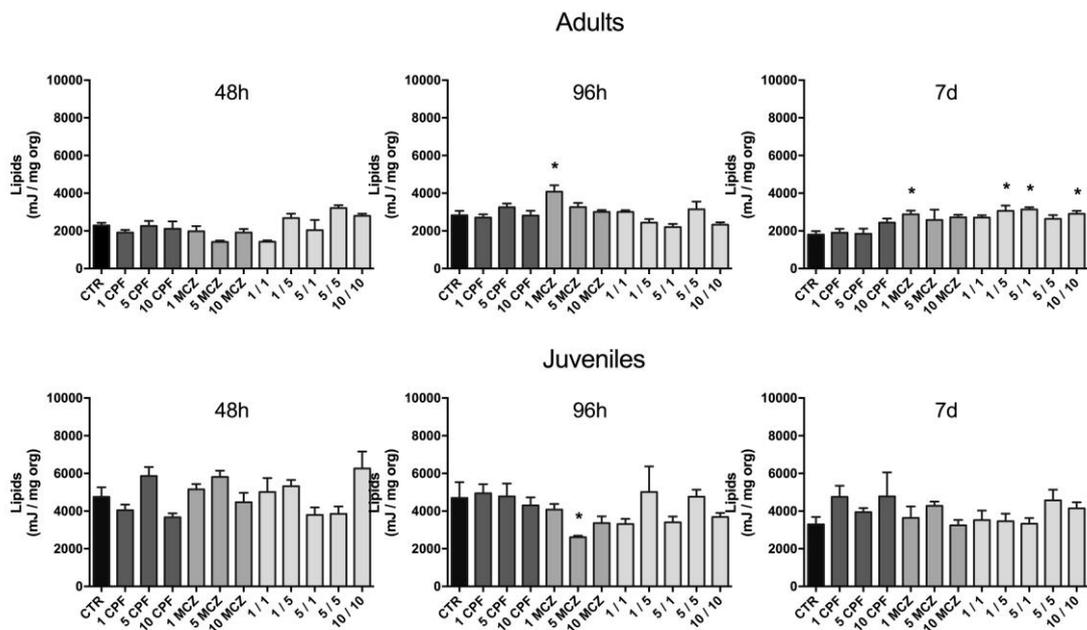


Figure 3SD – Lipids content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

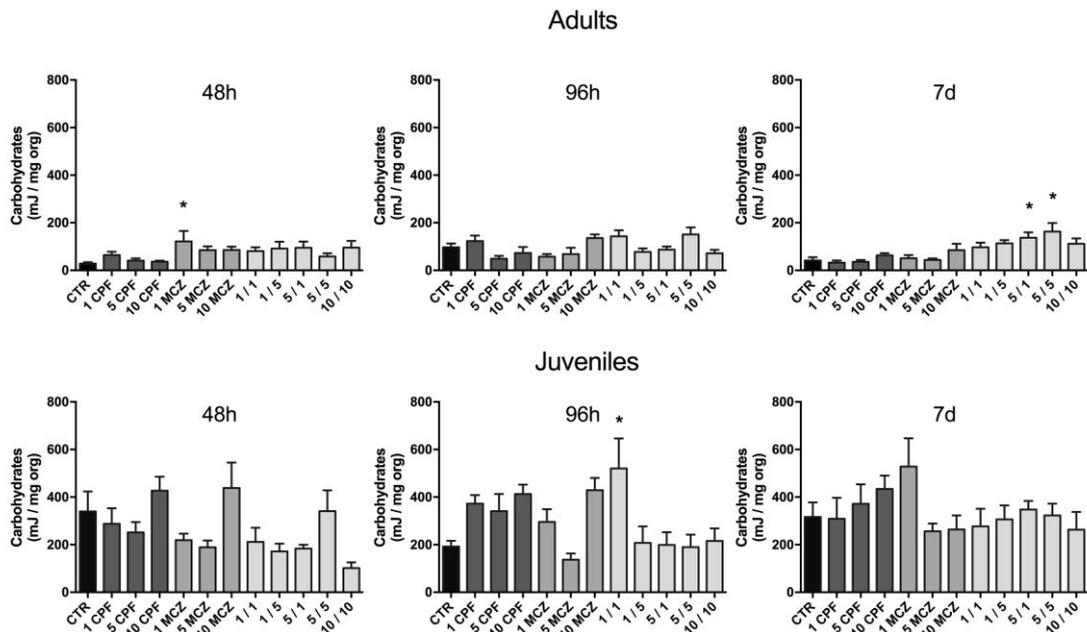


Figure 4SD – Carbohydrates content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

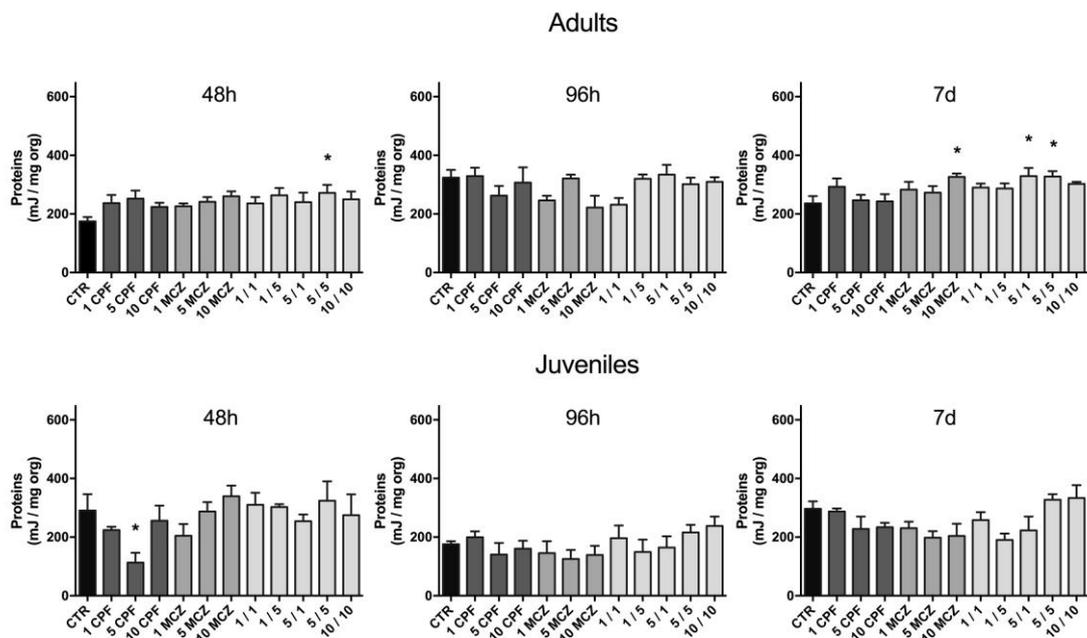


Figure 5SD – Proteins content and corresponding standard error in adults and juveniles of *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ) in LUFA 2.2 soil. Asterisks refer to significant differences from its correspondent time control (Two-way ANOVA, Tukey HSD, $\alpha=0.05$).

1 Table 1SD – Details of the factorial multivariate analysis of variance (MANOVA) for biomarkers and energy-related parameters
 2 measured in *Porcellionides pruinosus* exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ). Life
 3 stage, sampling time, CPF and MCZ were used as independent variables and the entire set of parameters assessed was used as
 4 dependent variables. Significant differences are shown in bold ($\alpha = 0.05$).
 5

	Pillai's Trace	<i>F</i>	<i>df</i>	Error <i>df</i>	<i>p</i>
Life stage	0.88426	200.925	10	263	<i>p</i> < 0.001
Time	0.51881	9.247	20	528	<i>p</i> < 0.001
CPF	0.16656	1.558	30	795	<i>p</i> = 0.030
MCZ	0.16656	3.781	30	795	<i>p</i> < 0.001
Life stage*Time	0.62511	12.003	20	528	<i>p</i> < 0.001
Life stage*CPF	0.23638	2.267	30	795	<i>p</i> < 0.001
Life stage*MCZ	0.37380	3.772	30	795	<i>p</i> < 0.001
Time*CPF	0.38344	1.830	60	1608	<i>p</i> < 0.001
Time*MCZ	0.75834	3.877	60	1608	<i>p</i> < 0.001
CPF*MCZ	0.64354	3.944	50	1335	<i>p</i> < 0.001
Life stage*Time*CPF	0.43718	2.106	60	60	<i>p</i> < 0.001
Life stage*Time*MCZ	0.47526	2.305	60	60	<i>p</i> < 0.001
Life stage*CPF*MCZ	0.47933	2.831	50	50	<i>p</i> < 0.001
Time*CPF*MCZ	0.74157	2.179	100	100	<i>p</i> < 0.001
Life stage*Time*CPF*MCZ	0.75299	2.215	100	100	<i>p</i> < 0.001

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8 Table 2SD – Details of the two-way ANOVAs for biomarkers and energy-related parameters measured in *Porcellionides pruinosus*
 9 exposed to single and mixture treatments of chlorpyrifos (CPF) and mancozeb (MCZ). CPF and MCZ were the independent
 10 variables and the parameters assessed were individually analyzed as dependent variables. Significant differences are shown in
 11 bold ($\alpha = 0.05$).

		AChE	GST	CAT	LPO	Ea	Lipid	Carb	Prot	Ec	CEA
Adults											
48h	CPF	F=14.14; p<0.001	F=10.169; p<0.001	F=1.724; p=0.177	F=2.046; p=0.120	F=3.007; p=0.040	F=2.385; p=0.081	F=0.559; p=0.644	F=0.993; p=0.404	F=0.148; p=0.931	F=2.881; p=0.045
	MCZ	F=17.46; p<0.001	F=33.535; p<0.001	F=0.805; p=0.498	F=2.497; p=0.071	F=3.686; p=0.018	F=3.428; p=0.024	F=6.343; p=0.001	F=2.795; p=0.050	F=1.701; p=0.179	F=3.715; p=0.018
	CPF*MCZ	F=16.57; p<0.001	F=0.457; p=0.806	F=1.248; p=0.304	F=0.718; p=0.613	F=4.533; p=0.002	F=4.142; p=0.003	F=1.09; p=0.378	F=1.056; p=0.396	F=0.781; p=0.568	F=4.682; p=0.001
96h	CPF	F=1.218; p=0.313	F=2.411; p=0.078	F=0.48; p=0.698	F=0.753; p=0.526	F=4.972; p=0.004	F=5.804; p=0.002	F=1.854; p=0.150	F=0.591; p=0.624	F=0.165; p=0.919	F=5.072; p=0.004
	MCZ	F=4.113; p=0.011	F=1.631; p=0.195	F=3.249; p=0.031	F=3.617; p=0.020	F=1.171; p=0.331	F=1.302; p=0.285	F=1.91; p=0.141	F=2.592; p=0.064	F=1.104; p=0.357	F=1.628; p=0.195
	CPF*MCZ	F=2.528; p=0.041	F=3.340; p=0.011	F=3.153; p=0.016	F=0.427; p=0.828	F=4.833; p=0.001	F=6.067; p<0.001	F=4.42; p=0.002	F=3.175; p=0.015	F=0.488; p=0.784	F=5.092; p<0.0001
7d	CPF	F=26.86; p<0.001	F=3.463; p=0.023	F=0.864; p=0.466	F=1.187; p=0.325	F=0.455; p=0.715	F=0.394; p=0.758	F=3.854; p=0.015	F=0.919; p=0.439	F=0.598; p=0.620	F=0.372; p=0.774
	MCZ	F=20.46; p<0.001	F=1.978; p=0.130	F=4.969; p=0.004	F=1.159; p=0.222	F=9.577; p<0.001	F=7.195; p<0.001	F=12.092; p<0.001	F=6.511; p<0.001	F=9.194; p<0.001	F=9.191; p<0.001
	CPF*MCZ	F=13.67; p<0.001	F=1.861; p=0.119	F=0.37; p=0.867	F=1.188; p=0.329	F=0.911; p=0.482	F=0.824; p=0.539	F=2.218; p=0.068	F=1.073; p=0.387	F=3.506; p=0.009	F=1.445; p=0.225
Juveniles											
48h	CPF	F=1.017; p=0.394	F=1.176; p=0.329	F=6.29; p=0.001	F=0.22; p=0.882	F=1.082; p=0.366	F=1.078; p=0.368	F=1.092; p=0.362	F=2.388; p=0.081	F=3.247; p=0.030	F=0.991; p=0.405
	MCZ	F=3.091; p=0.036	F=0.689; p=0.563	F=3.512; p=0.022	F=2.781; p=0.051	F=0.777; p=0.513	F=0.777; p=0.512	F=3.846; p=0.015	F=3.545; p=0.021	F=2.28; p=0.092	F=0.749; p=0.528
	CPF*MCZ	F=1.884; p=0.115	F=1.694; p=0.155	F=1.533; p=0.198	F=1.038; p=0.407	F=4.114; p=0.004	F=6.498; p<0.001	F=4.563; p=0.002	F=3.537; p=0.009	F=2.508; p=0.043	F=3.921; p=0.005
96h	CPF	F=2.516; p=0.069	F=0.854; p=0.471	F=0.047; p=0.986	F=0.998; p=0.402	F=1.905; p=0.141	F=1.598; p=0.202	F=1.925; p=0.138	F=0.823; p=0.488	F=1.109; p=0.355	F=3.531; p=0.063
	MCZ	F=13.669; p<0.001	F=4.991; p=0.004	F=0.18; p=0.910	F=2.436; p=0.076	F=3.284; p=0.029	F=2.997; p=0.040	F=7.139; p<0.001	F=0.053; p=0.984	F=3.123; p=0.034	F=3.531; p=0.022
	CPF*MCZ	F=12.35; p<0.001	F=3.011; p=0.019	F=0.396; p=0.849	F=0.737; p=0.599	F=3.011; p=0.019	F=2.91; p=0.023	F=3.914; p=0.005	F=1.665; p=0.162	F=0.452; p=0.810	F=4.18; p=0.003
7d	CPF	F=14.14; p<0.001	F=6.514; p<0.001	F=5.693; p=0.002	F=0.105; p=0.957	F=1.163; p=0.334	F=1.019; p=0.393	F=0.579; p=0.632	F=0.864; p=0.466	F=3.168; p=0.033	F=1.481; p=0.231
	MCZ	F=14.14; p<0.001	F=8.351; p<0.001	F=11.377; p<0.001	F=1.475; p=0.233	F=1.180; p=0.327	F=1.246; p=0.303	F=1.986; p=0.129	F=0.247; p=0.863	F=5.965; p=0.002	F=1.719; p=0.176
	CPF*MCZ	F=14.14; p<0.001	F=5.135; p<0.001	F=3.552; p=0.008	F=0.709; p=0.620	F=1.628; p=0.171	F=1.545; p=0.194	F=0.992; p=0.433	F=3.216; p=0.014	F=2.133; p=0.077	F=2.668; p=0.033

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