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Long-term exposure of the isopod *Porcellionides pruinosus* to nickel: Costs in the energy budget and detoxification enzymes

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HIGHLIGHTS

- Exposure to the metal nickel in concentrations up to 250 mg/kg soil causes low toxicity to isopods.
- Nickel causes oxidative stress that still persists after 14 days of recovery.
- Terrestrial isopods exposed to nickel show an impairment of the moult cycle.

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ABSTRACT

Terrestrial isopods from the species *Porcellionides pruinosus* were exposed to the maximum allowed nickel concentration in the Canadian framework guideline (50 mg Ni/kg soil) and to 5× this concentration (250 mg Ni/kg soil). The exposure lasted for 28 days and was followed by a recovery period of 14 days where organisms were changed to clean soil.

Organisms were sampled after 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, and 28 days of exposure, and at days 35 and 42 during the recovery period. For each sampling time the acetylcholinesterase (AChE), glutathione-*S*-transferases (GST), catalase (CAT), lactate dehydrogenase (LDH) activities were determined as well as lipid peroxidation rate (LPO) along with lipids, carbohydrates, proteins content, energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA). The integrated biomarker response (IBR) was calculated for each sampling time as well as for each one of the above parameters. In addition, mortality was also recorded throughout the assay.

The results obtained showed that nickel induced oxidative stress, evidenced by results on GST, GPx, CAT or LPO, but also on changes in the energy reserves content of these organisms. In addition, this study showed that these organisms possess a specific strategy to handle nickel toxicity. In this case, biomarkers were associated with costs in the energy budget, and the increase of energy reserves has a compensation for that cost.

1. Introduction

In order to infer about the impact of stressors in natural ecosystems, ecotoxicological studies have been traditionally based on individual to population level effects, like mortality or reproduction. Nevertheless, it has been lately suggested that a full insight into these effects, with a comprehensive understanding of their causes, is only possible if different/lower levels of biological organization are assessed (Moore et al., 2004). The measurement of biomarkers at a sub-organismal level have already proved to be an effective tool to improve these procedures since they detect earlier signs of stress, possess more sensitivity than the traditional ecotoxicological tests, and show an accurate relationship between toxicant exposure and the biological response (Morgan et al., 1999). In addition they can provide crucial information on stressors' modes of action, which improves the knowledge on their related effects. Along with the measurement of biomarkers, the quantification of energy reserves and the metabolic costs associated to hand the stressor have already proved to be a good parameter to use in ecotoxicology (e.g. Vink and Kurniawati, 1996; de Coen and Janssen, 1997). In this way, the depletion of energy reserves can be probably associated with negative impacts in growth, reproduction and consequent impairment of population structure and dynamics (de Coen and Janssen, 2003).

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Although being largely unknown, the soil compartment is one of the most important environments regarding structure, function, taxonomic diversity and trophic relations. In addition, and not less relevant, soils are crucial resources to man and its quality is dependent of its proper functioning. Soil functioning has been suggested to be hierarchically controlled by multiple natural factors, of which edaphic organisms constitute an important component (Lavelle, 1996). Nevertheless, by influencing each one of these functional levels, human activity can constitute the key factor affecting soil, frequently impairing these ecosystems' health (Lavelle, 1996). One of the most ubiquitous human-induced problems is the release of xenobiotics, particularly metals. The exposure to xenobiotics not only affect organisms, but may also change the overall soil functions, causing a decrease in soil quality and soil services (MEA, 2005).

Terrestrial isopods are macroinvertebrates involved in decomposition processes, vegetal litter fragmentation and re-cycling of nutrients (Zimmer, 2002; Zimmer et al., 2003; Loureiro et al., 2006; Ferreira et al., 2010). The species *Porcellionides pruinosus* has been described as a good test-organism to evaluate soil contamination or changes in their habitat, and was therefore the one selected for the present study (Takeda, 1980; Vink et al., 1995; Jansch et al., 2005; Loureiro et al., 2005, 2009). Isopods have particular value when studying the stress caused by metals since they display exceptional environmental ion assimilation (Donker et al., 1990; Hopkin, 1990) together with a unique homeostatic metallo-sequestration processes in the hepatopancreas' cells (Morgan et al., 1999; Tarnawska et al., 2007). Both these processes display this group's ability to target individual metals to highly specific cell types and into specific intra-cellular compartments and constitute the main reason to show some of the highest internal metal concentrations among soil organisms (Heikens et al., 2001; Vijver et al., 2004). Given their position in the food chain, they can be considered critical regarding biomagnification of metals for higher trophic levels. Therefore studying how metals impair and role their homeostasis is of crucial importance. Another important feature present is their hard exoskeleton that prevents the metal exposure through direct absorption whilst placing the organism under physiological requirement for calcium to support the moult cycle (Vijver et al., 2005). Nevertheless, exposure routes include the pleopods structures that are responsible for the absorption of pore water from soil by capillary action (Sutton, 1980) or through the ingestion of soil particles or food (Diamond, 1999).

Therefore the aim of this study was to assess the effects of nickel in the terrestrial isopod *P. pruinosus* upon exposure, but also to evaluate their potential recovery after exposure. Nickel is an essential trace element that, although naturally occurring, can be found at higher levels due to anthropogenic activities (Phipps et al., 2002). Its effects on soil invertebrates is still very scarce, but overall it is considered a carcinogenic metal, that impacts gene transcription, translation processes and even the phosphate cycle (Lee et al., 1995; Pane et al., 2003; Vandenbrouck et al., 2009).

A set of biomarkers was assessed in order to include and study several pathways of stress: neurotoxicity (acetylcholinesterase), detoxification (glutathione-*S*-transferases), oxidative stress (catalase, glutathione peroxidase, lipid peroxidation), energy related enzymes (lactate dehydrogenase) and energy reserves (lipids, carbohydrates and protein contents), along with energy related parameters such as energy consumption, energy available and cellular energy allocation. In addition, mortality was also recorded as a parameter transposing results from individuals to populations.

As previously stated, the use of terrestrial isopods in ecotoxicology is not new and considerable work has been already described, including different procedures and test durations, established for evaluating different endpoints (Loureiro et al., 2002, 2006; Santos et al., 2010; Calhôa et al., 2012; Morgado et al., 2013; Tourinho et al., 2013). Despite the duration required to evaluate different live trait parameters, the use of long term exposures are not frequently applied (Ferreira et al., 2015), although they are advisable if a more detailed and comprehensive effects of a stressor is aimed. In these cases it is possible to observe chemical's fate, bioavailability to organisms and even track possible time changes, along with recovery scenarios. Such long experiments are particularly relevant for metal toxicity given their inherent persistence and low mobility generally and possible chronic effects. For this reason a long-term assay was carried out to test the effects of nickel in the terrestrial isopod *P. pruinosus* during a 28 days exposure period followed by a 14 days recovery period, using the Integrated Biomarker Response to unify results/effects.

The results obtained will help to understand the process that undergoes exposure to nickel and posterior recovery by isopods, how are they coping with the need of reaching homeostasis and also to understand in a general way the pathways affected by the toxicity mode of action (MoA) of this metal to terrestrial isopods.

2. Materials and methods

2.1. Test organism and culture procedure

Organisms from the species *P. pruinosus* Brandt (1833) that were previously collected from a horse manure heap and maintained for several generations in laboratory cultures were used as test organisms. Terrestrial isopods were fed *ad libitum* with alder leaves (*Alnus glutinosa*) and maintained at 22 ± 1 °C, with a 16:8 h (light:dark) photoperiod. Culture's maintenance were performed twice a week, sprayed with water and food provided. Adult organisms (15–25 mg wet weight) were used in the experiments from both genders and organisms with abnormalities, moulting characteristics or pregnant females were excluded.

2.2. Soil spiking

Tests were performed using the natural 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/100 g), 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.

Soil was spiked with nickel in the concentrations of 50 and 250 mg Ni/kg soil, with a final moisture content equivalent to 50% of the soil water holding capacity (WHC). The concentration of 50 mg Ni/kg soil represents the maximum concentration allowed by the Canadian framework guideline (CBP, 2010).

2.3. Experimental procedure

Exposures were performed in plastic boxes (26 length × 18 width × 7.5 height cm), containing approx. a 2 cm layer height of Lufa 2.2 soil and 40 isopods (per box). Test organisms were collect from culture boxes, weighted and placed in each test-box. Food was supplied *ad libitum* (alder leaf disks – ∅ 10 mm) in small quantities but throughout the test period whenever necessary. This allowed a continue food availability and a small soil coverage by leaves that could influence isopod's exposure to contaminated soil. Organisms were exposed to 50 and 250 mg Ni/kg soil in a 16:8 h (light:dark) photoperiod, at 20 °C. A total of five replicates were performed for each concentration. Four organism from each box/replicate were collected at time 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days (exposure period) and 35 days, 42 days (recovery period). In the results section, the 35 and 42 days of test duration will be denominated as 7 and 14 days of post-exposure.

The enzymatic biomarkers glutathione-*S*-transferases (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) were measured using a pool of two full-body organisms per replicate. From another organism, the head was separated and used to analyse acetylcholinesterase (AChE), corresponding to one replicate. All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany), and hydrogen peroxide from Fluka.

For the energy related parameters (total lipids, carbohydrates and proteins and electron transport system), only one organism was used as a replicate. At each sampling time, the number of dead organisms were counted and removed from the test boxes.

2.4. Biochemical biomarkers, energy reserves and IBR

The protocol used is extensively described in the supplementary data, and was previously described by Ferreira et al. (2010) and Ferreira et al. (2015). The lipid peroxidation (LPO) assay was adapted on the methods described by Bird and Draper (1984) and Ohkawa et al. (1979) and adapted to microplate format. The other oxidative stress related biomarkers glutathione-*S*-transferases (GST), glutathione peroxidase (GPx) and catalase (CAT) activity were determined based on the method described Habig et al. (1974), Mohandas et al. (1984) and Clairborne (1985) respectively. The acetylcholinesterase (AChE) activity was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate format by 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.

To determine total protein, carbohydrate and lipid contents, energy consumption (Ec), energy available (Ea) and cellular energy allocation (CEA), the protocol was adapted from the one described for daphnids from de Coen and Janssen (1997). The Ea, Ec and CEA values were calculated as described by Verslycke et al. (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ/mg org.).
Ec (energy consumption) = Electron Transport System activity (mJ/mg org./h).
CEA (cellular energy allocation) = Ea/Ec (h).

To integrate all results from the different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002) which is also extensively described in the supplementary data. The IBR calculations were always performed with the same order of parameters for all sampling times: the neurotoxicity biomarker AChE (the enzyme for which the pesticide was designed to inhibit), followed by the detoxification and oxidative stress biomarkers GST, LPO and CAT, then the energy related biomarker LDH to serve as transition between biomarkers and energy related parameters and finally the lipids, carbohydrates and proteins content, the energy available (Ea), the energy consumption (Ec) and the CEA that integrates the last two parameters. Further details can also be found in the supplementary data.

2.5. Data analysis

To compare differences between treatments the one-way analysis of variance (ANOVA) was performed followed by a Dunnett's comparison test in order to discriminate statistically different treatments from the control (SPSS 1999). Whenever necessary data transformation was used to achieve normality, however when data did not show a normal distribution or homoscedasticity, the non-parametric test Kruskal–Wallis One Way Analysis of Variance on Ranks was used.

Data values that were higher or lower than the mean value, plus or minus two times the standard deviation, were considered outliers, and withdrawn from analysis (Rousseeuw and Croux, 1993). Whenever there was enough data ($n \geqslant 3$, due to high mortality rates), a two-way analysis of variance (two-way ANOVA) was performed to check for interactions between time and concentration. The two-way ANOVA was performed separately for the exposure and the recovery period. The one-way ANOVA and two-way ANOVA with significance of $\alpha = 0.05$. Due to the mortality observed, the last sampling time (14 days of recovery) was excluded from the analysis.

3. Results

In Fig. 1 it is represented the number of dead organisms by the cumulative number at a given sampling time. Due to high mortality observed at 14 days of recovery under nickel exposure, no sampling or analysis could be performed.

3.1. Enzymatic biomarkers activity

The detailed biomarkers activity and energy reserves content of organisms exposed to nickel during the exposure and recovery periods are presented in Fig. 1SD (Supplementary Data).

The neurotoxicity biomarker AChE showed a significant increase for the highest concentration after 14 days of exposure (One Way ANOVA, $F_{2,11} = 4.557$; Dunnett's test $p = 0.036$). For the oxidative stress related biomarkers significant increases were also observed for LPO (highest concentration, 28 days of exposure; One Way ANOVA, *reciprocal transformation*, $F_{2,10} = 7.810$; Dunnett's test $p = 0.009$), for GST (highest concentration, 7 days of recovery; One Way ANOVA, $F_{2,10} = 7.561$; Dunnett's test $p = 0.010$). The biomarker CAT showed a significant increase at the highest concentration after 24 h of exposure (One Way ANOVA, $F_{2,9} = 16.301$; Dunnett's test $p = 0.001$) and a significant decrease after 7 days of recovery (One Way ANOVA, *reciprocal transformation*, $F_{2,9} = 9.392$; Dunnett's test $p = 0.019$).

For the energy related parameters significant increases were observed for carbohydrates for the lowest concentration after 24 h of exposure (One Way ANOVA, $F_{2,10} = 21.160$; Dunnett's test $p < 0.001$) and for the highest concentration after 96 h of exposure (One Way ANOVA, $F_{2,11} = 5.096$; Dunnett's test $p = 0.027$). The

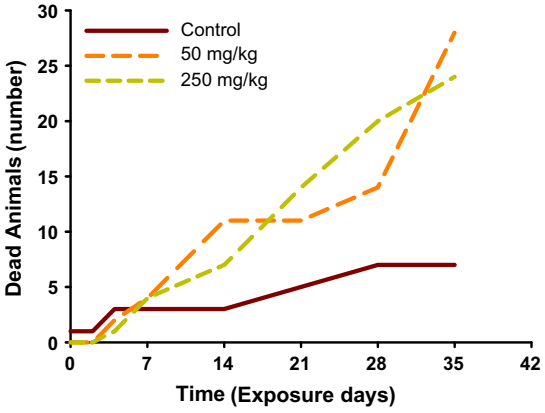

Fig. 1. Cumulative total number of dead organism from the species *Porcellionides pruinosus* during the exposure and recovery period in the control and exposed to 50 mg Ni/kg soil and 250 mg Ni/kg soil. A total of 200 organisms were exposed per treatment.

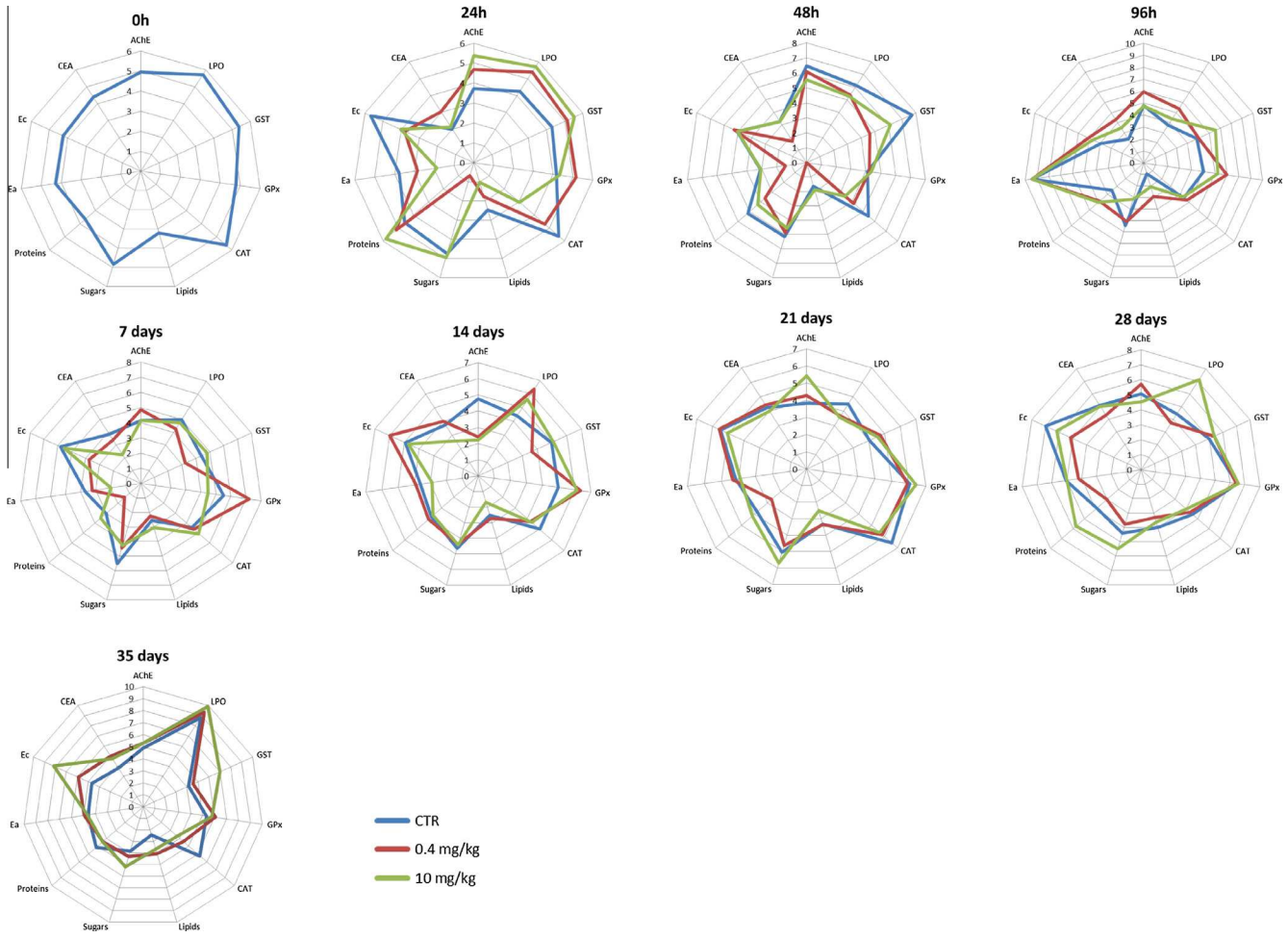

Fig. 2. Star plots for each sampling time of *Porcellionides pruinosus* exposed to 50 mg and 250 mg Ni/kg soil (exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days, 42 days). AChE = acetylcholinesterase, GST = glutatione-*S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

other parameter that also showed significant differences was Ec, where a significant decreases were observed after 24 h, 7 and 28 days of exposure for the lower concentration (respectively ANOVA on Ranks, $H = 6.806$, d.f. = 2; Dunn's test $p = 0.033$; One Way ANOVA, $F_{2,11} = 10.681$; Dunnett's test $p = 0.003$; One Way ANOVA, $F_{2,9} = 7.013$; Dunnett's test $p = 0.015$). Significant increases were also observed for Ec for both exposure concentrations after 96 h of exposure (One Way ANOVA, $F_{2,10} = 8.800$; Dunnett's test $p = 0.006$) and for the higher concentration after 7 days of recovery (One Way ANOVA, $F_{2,11} = 7.6586$; Dunnett's test $p = 0.011$).

Interactions between time of exposure and nickel concentrations were significant in the exposure period only for catalase (Two Way ANOVA, $F_{2,83} = 2.006$; $p = 0.027$). In the recovery period significant interactions were observed for: GST (Two Way ANOVA, $F_{2,21} = 5.949$; $p = 0.009$), GPx (Two Way ANOVA, $F_{4,24} = 2.237$; $p = 0.012$), Ec (Two Way ANOVA, $F_{2,18} = 9.924$; $p = 0.001$) and lipids (Two Way ANOVA, *reciprocal transformation*, $F_{2,20} = 3.634$; $p = 0.045$).

3.2. Integrated biomarkers response (IBR)

The IBR starplots are presented in Figs. 2–5. They include the scores of each parameter and each sampling time during the exposure and recovery period. Better or worse scores (respectively lower or higher values) obtained for each parameter are summarized in Table 1SD. Better scores represent healthier organisms whilst worse scores represent more stressed organisms.

The analysis of the IBR values according to sampling time (Fig. 4) showed better scores (lower values) for the control exposures for 24 h, 96 h, 28 days of exposure and 7 days of recovery. Unexpectedly, the other sampling times showed worse scores for the control rather than Ni exposures. It also shows that frequently the lower Ni concentration had better values (lower values) than the higher concentration of exposure. The sampling time that reports to the recovery period (7 days of recovery) was the only one showing an expected result with values increasing with the increasing of concentrations.

Looking at the IBR values according to the measured parameter (Fig. 5), the results showed a different scenario. Control presented the better or second better values (lower values) for all biomarkers except CAT. On the contrary for the energy related parameters control presented an opposite trend.

A comparison between the mean IBR/n values for all sampling times did not show any statistically different from the control, regarding the exposure concentrations (One Way ANOVA, $F_{2,26} = 0.182$; $p = 0.835$).

4. Discussion

In the present study the mechanisms involved in nickel toxicity to the terrestrial isopod *P. pruinosus* were assessed. This analysis,

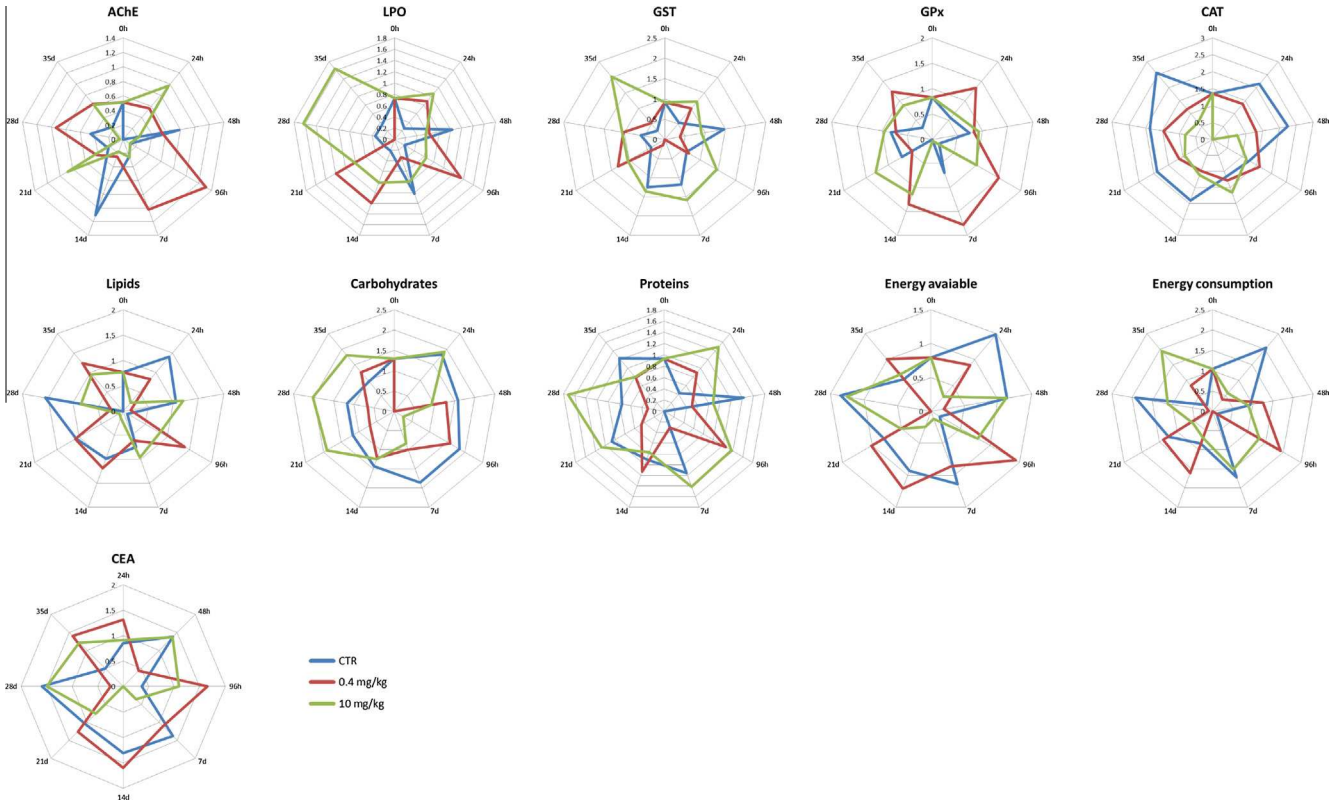

Fig. 3. Integrated Biomarker Response of *Porcellionides pruinosus* in control and exposed to nickel (50 and 250 mg/kg soil) during the exposure and recovery period. (AChE = acetylcholinesterase, GST = glutathione-*S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation).

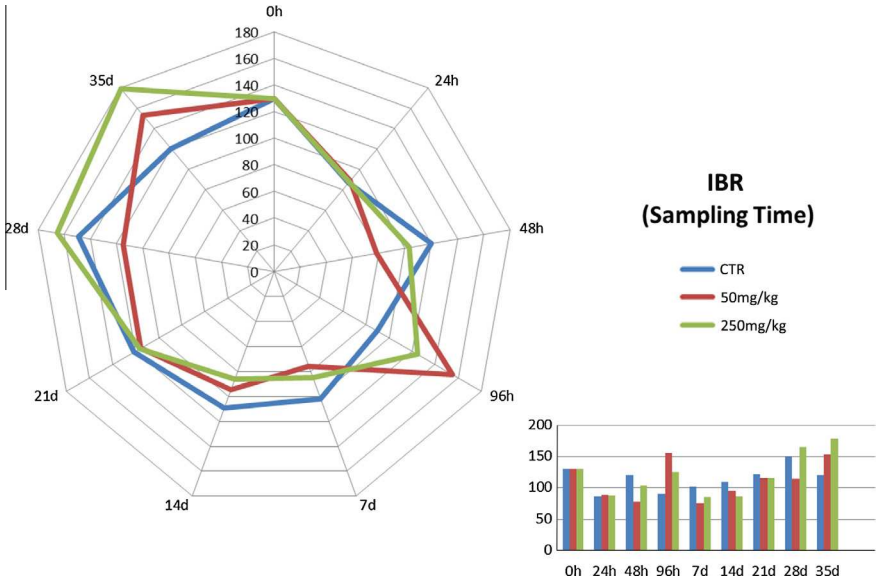

Fig. 4. Star plots for each sampling time (integrating all the measured endpoints) of *Porcellionides pruinosus* in the control and exposed to nickel (50 and 250 mg/kg soil). Exposure period: 0 h, 24 h, 48 h, 96 h, 7 days, 14 days, 21 days, 28 days; recovery period: 35 days.

not only provided information about the toxicity inherent to the nickel concentrations used, but also the patterns observed at the different sampling times, and the differences between the exposure and recovery period.

The first highlight from the present study was a possibly increase on mortality when this long term exposure to nickel was carried out, especially at the highest concentration. But we should regard this carefully as we cannot state accurately a mortality rate as organisms were sampled in several points in time. Even though, at the end of the test 20 organisms were recorded dead at the highest concentration (250 mg Ni/kg), when comparing to the 7 dead organisms from the control. In a 28 day study with the earthworm *Eisenia veneta*, nickel induced a reduction of 10% on the cocoon production at 85 mg Ni/kg, and adult survival was only

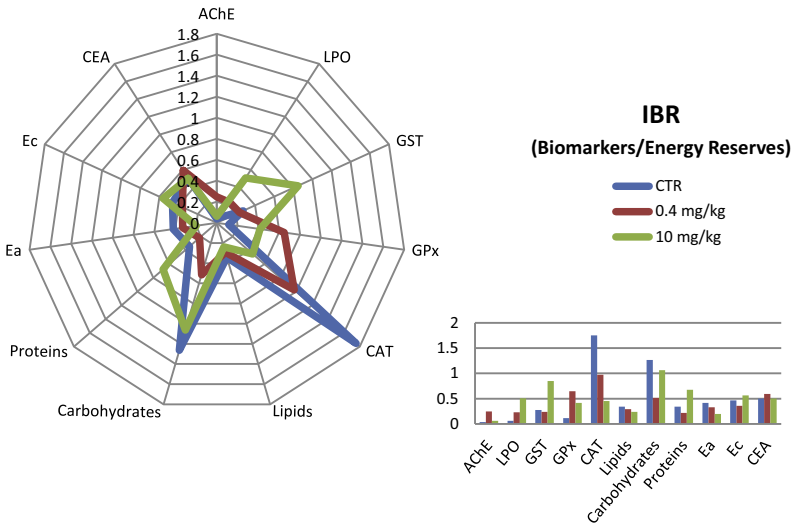

Fig. 5. Star plots for each measured endpoint (integrating multiple sampling times) of *Porcellionides pruinosus* in the control and exposed to nickel (50 and 250 mg/kg soil). AChE = acetylcholinesterase, GST = glutathione-*S*-transferases, LPO = lipid peroxidation, CAT = catalase, LDH = lactate dehydrogenase, Ea = available energy, Ec = energy consumption, CEA = cellular energy allocation.

reduced at concentrations above 245 mg Ni/kg (Scott-Fordsmand et al., 1998). In another study with *Folsomia candida*, the LC50 for nickel exposure was 279 mg Ni/kg dry soil (Broerse and van Gestel, 2010).

When looking at the biomarkers and energy reserves data, two main results can be highlighted: (1) the IBR analysis showed that the control was surprisingly presenting worse values in some of the sampling times (48 h, 7 days, 14 days and 21 days) than the exposure concentrations; (2) but when looking individually at each biomarker or energy-related parameters, organisms from the control showed to be in better conditions than treatments for the measured biomarkers, but in worse conditions regarding energy related parameters.

In order to better understand the previous results, a general characterization of the effects of metals in organisms is necessary. In several studies, the exposure to low concentrations of metals lead to hormetic responses (e.g. Calabrese and Baldwin, 2001; Nascarella et al., 2003; Calabrese and Blain, 2005; Lefcort et al., 2008). If the same hormetic response was present, we would expect that the higher concentration presented higher toxicity values (higher IBR values) and the lower concentration lower toxicity values than our control, which is not the case. Therefore, isopods were using several enzymatic related processes to avoid oxidative stress, and the patterns of response seem to indicate that organisms were dealing positively with exposure. On the other hand, mortality showed nickel concentrations were inducing high toxicity, which can be indicative of another possible mode of action present within Ni exposure rather than just oxidative stress or neurotoxicity.

The analysis of the IBR values for each measured parameter showed different patterns between biomarkers and energy related parameters. The majority of the measured biomarkers showed that nickel exposures were inducing more toxicity than the control, and lipids, carbohydrates and proteins were always lower in the control.

Regarding biomarkers, it is possible to observe that nickel affected oxidative stress biomarkers as expected for metals, but could also target the neurotoxicity pathway signalised by the biomarker AChE. Although an inhibition of AChE would be expected when organisms are exposed to stressors (e.g. Ribeiro et al., 1999; Stanek et al., 2006; Ferreira et al., 2015), a significant increase was observed (14 days of exposure). This has been also reported for the same species under stress exposures (Santos et al., 2010; Morgado et al., 2013).

Nevertheless results should still be analysed with caution as in a previous study, where Frasco et al. (2005) evaluated the activity of AChE *in vitro*, after exposure to five metals (nickel, copper, zinc, cadmium and mercury) showed that with the exception of nickel, all other metals inhibited AChE. This study also showed that the technique used to measure AChE activity can strongly influence the obtained results, since complexes between the metals and the reaction buffers were formed. Although the previous study was based on *in vitro* techniques, the results obtained in our study should not be discarded or observed has an artefact.

Regarding the oxidative stress biomarkers (LPO, GST, GPx and CAT), the results showed a clear connection between each other. The exposure of isopods to both nickel concentrations showed an increase in LPO rates with the increase of nickel concentrations, which indicates that cells are being damaged. In the review of Valko et al. (2005) metals such as iron, copper, cadmium, chromium, mercury, vanadium and nickel had the ability to produce reactive radical species (that include oxygen-, carbon-, sulphur-radicals) that may result, between other effects, on lipid peroxidation. These effects observed for LPO in this study were also reported in human lymphocytes *in vitro*, where a concentration-level dependency of lipid peroxidation was observed (Chen et al., 2003 in Valko et al., 2005). Likewise, a concentration-dependency and time-dependency was observed for plasma of human blood *in vitro* (Chen et al., 2002 in Valko et al., 2005) or even in the intestinal mucosa of broilers fed with corn-soybean contaminated diet (Wu et al., 2013). The activity of the other biomarkers (GST, GPx and CAT) apparently followed a pattern within the oxidative stress detoxification pathway. For example, whenever GST and GPx are inhibited, to deal with the oxidative stress, CAT activities are induced. This response within biomarkers can be seen using the IBR values, although when looking at them isolated, low or no changes can be depicted just by comparing to the control. Some of them suggested the oxidative stress process as being possibly important for the induction of carcinogenesis by nickel, despite a direct correlation between its ability to produce oxidative stress and its carcinogenicity is yet to be proved. Moreover, several other metals, such as copper and iron, are known to strongly

induce oxidative stress but were not found to be carcinogenic (Gilman, 1962 in Valko et al., 2005). In other studies, Wu et al. (2013) similar results were reported, with inhibitions of GST, GPx and CAT in the intestinal mucosa of broilers, while in the study of Sunderman et al. (1985) no effects on CAT or GPx activities were observed in rats' blood. The observed patterns in our study, where GST and GPx appeared inhibited, are supported by the GSH depletion effect already reported for nickel (Valko et al., 2005). The enzymes GST and GPx are known to be strongly dependent of glutathione (GSH) for properly performing their functions. For instance, GPx transforms GSH in GSSG, a process necessary to handle the transformation of H_2O_2 into H_2O and O_2 (Cadenas and Davies, 2000). Similarly GST depends on the presence of GSH in order to act as catalysts for the conjugation of various electrophilic compounds (Armstrong, 1987; Gulick and Fahl, 1995).

The changes on the energy-related parameters are, as stated before, the endpoints that contribute the most for the control to show higher IBR values than the nickel exposures. However, the higher amounts of energy reserves observed in exposed organisms during most of the sampling times were not expected. In fact, they were mainly expected at 96 h and 7 days of exposure, period where organisms normally show moulting processes (Ferreira et al., 2015), although other physiological processes could also be involved as described below. Previous studies suggest that nickel replaces iron in the oxygen carrier, switching the signal to permanent hypoxia, which in turn activates the hypoxia-inducible factor 1alpha (HIF-1) factor (Goldberg et al. (1988) in Valko et al. (2005)). The HIF-1 is also involved in the regulation of several genes that belong to the glucose transport and glycolysis pathways (Semenza, 1999 in Valko et al., 2005). In addition, as described previously, isopods have unique processes to handle metals that range from metallo-sequestration in the hepatopancreas cells, to structural characteristics, such as the hard exoskeleton that limits the exposure route of metals. The former process involves a daily cycle of cells present in the hepatopancreas. There are two groups of cells: the large and binucleate '*B*' cells and the small uninucleate '*S*' cells. The '*S*' cells contain Cu-thiol vesicles whereas '*B*' cells accumulate Fe rich phosphate granules with a still unknown function. The '*B*' cells undergo a striking diurnal cycle of ultrastructural changes in which the contents of the apical cytoplasm are voided in an apocrine manner over ~11 h, followed by a recovery phase, during which the cytoplasm is recharged with lipids, glycogen and Fe rich inclusions (Hames and Hopkin, 1991). In our study, the collection of organisms was always performed within the same timeline, which excluded the variation of sampling organisms in different periods of the daily cycle. Nevertheless the mechanism involved on the regulation of metals may also be affected and such changes may be the reason for this pattern of high contents of energy reserves. Despite the processes involved, the Ea showed a clear pattern of dose-dependency, where organisms exposed to higher doses of nickel appeared to accumulate more energy reserves than the others from the control. This interesting result, along with a clear strategy of the organism, in order to handle nickel by modifying their behaviour (organisms exposed to the lower concentration showed lowed Ec, whereas the organisms exposed to the higher concentration showed higher Ec), led to CEA values very close to those found in the control. These results can then be supported in some degree by field studies, where populations of terrestrial isopods survive in areas with overall concentrations of nickel much higher than the used in this study (e.g. Alikhan and Storch, 1990; Bayley et al., 1997). In the work of Vandenbrouck et al. (2009) daphnids exposed to Ni, showed a decrease in the energy reserves content along with a decrease in Ec. Although a direct comparison cannot be made, after 96 h of exposure the same decrease was observed for isopods, with higher IBR values for total lipids and proteins, but not for carbohydrates or Ec.

In the present study the exposure period was followed by a recovery period, where only few organisms were available due to the mortality observed during exposure. Anyway, in the first 7 days of recovery it was possible to observe retrieval from organisms. Although some stress could be observed after 7 days that might be confirmed for a 14 days recovery period, with the results obtained for LPO, which showed higher rates at 28 days of exposure, and identical levels in the recovery comes as an evident of this recovery. Once again this fast recovery comes in accordance with the previous studies stated before, that show the terrestrial isopods as species capable of handling environments with high metal contents.

5. Conclusions

Nickel is a commonly used metal that reaches the environment not only through natural sources but mainly from anthropological sources such as mining, alloy production, electroplating, refining or even welding. The effects of nickel in organisms, especially invertebrates are still pretty unknown and this type of studies can bring new insights. The evaluation of several biomarkers and energy-related parameters allowed to confirm oxidative stress induced by nickel exposure but other unknown and more critical modes of action which may have also a role on nickel acute toxicity. In addition, this study also brought new insights on energy budget regulation.

The organisms exposed to the lower concentration (50 mg/kg soil), corresponding to the maximum allowed concentration in fields by the Canadian framework guidelines, generally presented low toxicity, with some biomarkers presenting conditions very similar to the control. Identically to the previous concentration of exposure, organisms exposed to the highest nickel concentration (250 mg/kg soil) also seem to cope with oxidative stress and may present other modes of action, which can be observed by the observed mortality at the end of the experiment.

The inclusion of a recovery period in ecotoxicological studies may be considered very useful regarding the information provided under long-term exposures that may not always cease when organisms are transferred to control conditions after an exposure period.

Although new information was added to previous studies, there is still the need to observe the effects of nickel at a molecular and metabolic level. In the isopods particular case, due to their capacity of living and tolerate environments with high concentrations of nickel, the proposed evaluation will provide even more insight on their regulation mechanisms.

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Appendix A. Supplementary material

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