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Sprejeto (accepted):

The Sensitivity of two biochemical biomarkers in berrestrial isopods after short-term copper exposure

Občutljivost dveh biokemijskih biomarkerjev v kopenskih rakih po kratkotrajni izpostavitvi bakru

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Abstract: Biochemical biomarkers, e.g. enzyme activities, have been traditionally considered a very sensitive and specific tool to characterize the hazard of pollutants to organisms. Among them, considerable attention was given to antioxidant enzymes catalase and glutathione S-transferase, which respond to changes in the quantity of reactive oxygen species. In the present study, the two enzymes were assessed in terrestrial isopods *Porcellio scaber* acutely (3 days) exposed to redox active copper and compared to their whole-organism responses, such as feeding, weight change and survival. The animals were fed with copper contaminated food for 3 days and afterwards for another 3 and 6 days with uncontaminated food. In contrast to expectations, no changes of antioxidant enzymes were found throughout the experiment, while feeding parameters were already decreased after 3 days of exposure at the highest exposure concentration 5000 μg/g dry food. The concentrations tested were not acutely lethal for isopods and did not affect their weight change. These findings imply that biochemical biomarkers in some cases are not a fast and sensitive measure to characterise the hazard of chemicals. The observed finding is probably the result of interplay between a very short time of exposure and the type of chemical chosen. Namely, a special relationship exists between isopods and copper, since it is an essential element for *P. scaber*. It is recommended that more data on the relationship between lower and higher-level biomarkers in isopods after different exposure periods is needed and this knowledge will increase their relevance in future studies on the hazard of new emerging contaminants.

Keywords: antioxidant enzymes, biomarker; catalase; environmental risk assessment; glutathione S-transferase; hazard, *Porcellio scaber*

Introduction

The number of environmental studies employing biomarkers (e.g. biological responses that provide a measure of exposure and/or effect of chemicals) as indicators of chemical stress has increased significantly since the beginning of 1990's. Pursuant to the assumption that pollutants have an impact on cellular levels before their effects are observed at the whole-organism level, biochemical biomarkers (e.g. the activities of enzymes) were considered one of the most promising (Adams 2002). Since biochemical response is dependent upon the interaction of the toxicant with a molecular target, it was expected that biochemical biomarkers could be used to determine the bioavailability of absorbed toxicant and could help to identify causal mechanisms potentially responsible for effects realized at higher levels of organization (Peakall & Walker 1994). Biochemical biomarkers were considered a very sensitive (responding already at very low concentration of a toxicant) and specific measure (responding only to a specific class of chemicals).

Also, it is expected, that the changes of biochemical biomarkers are preceded by effects at a higher level of biological organization including processes such as growth, reproduction and mortality (Depledge & Fossi 1994).

In the present paper, biochemical biomarkers catalase and glutathione-S-transferase and whole-organism responses of adult terrestrial isopods *Porcellio scaber* Latreille 1804 (Isopoda, Crustacea) exposed to copper were investigated. Toxicity testing with these invertebrates has been recognized as fast, routine and inexpensive and has been commonly applied to identify hazard of different metals and pesticides (Drobne & Hopkin 1995, Drobne & al. 2008). Terrestrial isopods are commonly regarded as suitable test organisms for metal exposure, because they are able to accumulate high amounts of metal ions (Hopkin 1989). Their main metal storage and metabolic organ is digestive midgut gland (hepatopancreas), where most metal is deposited in intracellular granules. Several responses along biological complexity of these animals were proposed as toxicological end-points: moult frequency (Drobne & Štrus 1996), food consumption, food selection, behavioural responses (Zidar & al. 2003), gut microflora (Drobne & al. 2002), and epithelial thickness (Lešer & al. 2008). However, despite the wide applicability of isopods in toxicity testing, biochemical biomarkers have not been sufficiently investigated and employed after different exposure periods.

The two biochemical biomarkers chosen in the present work were antioxidant enzymes catalase (CAT) and glutathione-S-transferase (GST). Catalase is a very highly conserved enzyme that has been identified in most organisms, including vertebrates, invertebrates, plants, fungi and bacteria. It is primarily localized in peroxisomes, where many enzymes generate hydrogen peroxide, which is further degraded by CAT. Glutatione-S-transferases constitute a large family of multifunctional enzymes involved in the cellular detoxification of many physiological and xenobiotic substances. Their role is to render water-soluble glutathione conjugates, thus facilitating their elimination. Besides biotransformation of xenobiotics, they also detoxify endogenous products formed during lipid peroxidation and are therefore also regarded as antioxidant enzymes (Halliwell & Gutteridge 2007).

Copper (Cu^{2+}) was selected as a model toxicant due to its presumed production of reactive oxygen species and induction of oxidative stress damages, such as the effects on the DNA integrity and the induction of lipid peroxidation (Stohs & Bagchi 1995). It is expected, that the activities of both antioxidant enzymes CAT and GST will increase upon exposure to Cu^{2+}. Copper is a common environmental soil pollutant, although recent concentrations detected in topsoil of different European towns (up to 290 µg/g) (Poggio & al. 2008) were not as high as in the past (industrial site; up to 15000 µg/g) (Bengtsson & Tranvik 1989). Very high concentrations of Cu^{2+} up to 2700 µg/g are still being reported in mine wastes (Perez-Lopez & al. 2008).

The accumulation, metabolism, storage, detoxification, excretion and toxicity of Cu^{2+} in isopods are well documented (Hopkin 1989). Terrestrial isopods exhibit high tolerance towards copper, since they are able to accumulate vast amounts of this element in cuprosomes, vesicles of the lysosomal system and epithelial cells of hepatopancreas (reviewed in Weissenburg & Zimmer 2003). The reason for storage lies in the fact, that copper is an essential metal in isopods, because it is a part of the oxygen carrying protein haemocyanin, it promotes digestive processes of leaf litter and is involved in detoxification processes and immune response of isopods (Irmak & al. 2005).

While copper is essential for isopods, it may become toxic at high concentrations (Hassall & Rushton 1982, Hopkin 1989, Farkas & al. 1996, Zidar & al. 2003). In these toxicity studies mainly long exposure periods up to 6 weeks were applied. In the present paper, a very short exposure period (3 days) was selected due to presumably quick response of biochemical biomarkers. It has been previously shown, that this period is long enough to cause sublethal effects of heavy metals on digestive glands in isopods (Nolde & al. 2006).

The aim of the present paper was to assess the short-term effects of Cu^{2+} on biochemical biomarkers and whole-organism (higher-level) responses, such as feeding, weight change and survival of isopods *P. scaber*. The sensitivity of biochemical biomarkers in comparison to higher-level responses is discussed.

Materials and methods

Chemicals

The following chemicals were purchased from Sigma-Aldrich (Munich, Germany): dibasic and monobasic potassium phosphate, hydrogen peroxide (30%), 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form). Protein Assay Reagents A and B were purchased from Pierce (Rockford, IL, USA). The source of Cu^{2+} ions was $Cu(NO_3)_2$ (Merck, New Jersey, USA). All chemicals were of the highest commercially available grade, typically 99% or higher.

Test organisms

Terrestrial isopods (*Porcellio scaber*, Latreille 1804) were collected under the litter layer in an uncontaminated location in the vicinity of Ljubljana. The experiments were conducted within 11 days after the collection of animals from the field, as previously proposed by Jemec & al. (2008). In the laboratory, the animals were kept in a terrarium (20 × 35 × 20 cm) filled with a 2 to 5 cm layer of moistened sand and soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*). The substratum in the terrarium was heated to 80°C for several hours to destroy predators (spiders) before the introduction of the isopods. The culture was kept at controlled room temperature (21±1°C), 16:8 h light/dark regime and high humidity.

Feeding experiments

The adults of *P. scaber* with body weights ranging from 30 to 80 mg, of both sexes and all moult stages, were used in the experiment. Also, females with brood chamber were used in the experiments, but these specimens were not included in the analyses of whole-organism endpoints, because both the moult and the presence of broods might influence the feeding and animal mass change. However, all animals were included in the analysis of CAT and GST, since these biomarkers were previously found independent on these endogenous characteristics of isopods (Jemec & al. 2008). Each animal was placed individually in a Petri dish, to which individual pieces of dry leaves dosed with Cu^{2+} were added. Humidity in the Petri dishes was maintained by regular spraying with tap water on the internal side of the lids. All Petri dishes were placed in a large plastic-covered glass container maintained at relative humidity close to 100%, and a 16:8 h light/dark regime.

The food for the feeding experiment was prepared following the protocol proposed by Drobne & Hopkin (1995) and in detail described by Jemec & al. (2008). Different Cu^{2+} solutions were spread over the dry leaves using a brush in a way that they contained 100, 1000 or 5000 µg of Cu^{2+} per gram dry weight of leaves The amount of copper on leaves was not measured in this particular study, since our previous experience with this kind of food preparation show, that the actual concentrations of Cu^{2+} on leaves are within 5 % of the nominal ones (Zidar & al. 2003; Zidar & al. 2004). The concentrations were selected based on previous toxicity testing with this organism (Zidar & al. 2003). The control was prepared by applying distilled water instead of Cu^{2+} dispersion.

The feeding experiment consisted of 3 days feeding with Cu^{2+} contaminated food, followed by 3 days and 6 days period of feeding without contaminated food (e.g. recovery). In each exposure group 18 animals was exposed at the beginning of the experiment. After 3 days of feeding 6 animals were removed for enzyme analysis, 12 of them were fed on non-contaminated food. After 3 days of recovery 6 animals were removed and after 6 days of recovery the remaining 6 were used to measure the enzymes. This experiment was repeated twice, so altogether 12 animals per concentration and a certain time exposure (3 days feeding, 3 days recovery, 6 days recovery) were exposed. After each exposure period whole-organism responses were evaluated. The leaves were weighed after drying at room temperature for 24 h, and the faecal pellets were counted and weighted after drying in the exsiccator for 48 h. The animals were weighted right after the experiment, they were dissected and the digestive glands were isolated for measurements of CAT and GST activities. Animal mortality was also recorded.

Measurements of biochemical biomarkers

The whole digestive gland of each animal was homogenized in 800 µL of 50 mM phosphate buffer pH 7.0 for 3 min. The homogenate was centrifuged for 15 min at 15000 *g* and 4 °C. The GST activity was measured on microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) using 1-chloro-2.4-dinitrobenzene as a substrate (Jemec & al. 2008). The GST activity was expressed in nmoles of conjugated GSH/min/mg protein (extinction coefficient ε_{340} = 9600 $M^{-1}cm^{-1}$). The CAT activity was determined as described in Jemec & al. 2008. The reaction was followed spectrophotometrically on a Shimadzu UV-2101PC spectrophotometer (Japan). The CAT activity was expressed in µmoles of degraded hydrogen peroxide/min/mg protein (extinction coefficient ε_{240} = 43.6 $M^{-1}cm^{-1}$). Protein concentration was measured using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

Data analysis

The feeding rate and a defecation rate of isopods were calculated as the mass of consumed leaf and mass of faecal pellets per animal wet weight and per day, respectively. The animal mass change was determined as the difference in animal mass at the beginning and at the end of the experiment.

For statistical analysis and the presentation of results, both experiments were combined, because no differences between the controls and corresponding concentrations of both experiments were observed. In Fig.1 data for 12 animals are shown at each concentration. In result on feeding parameters (Fig.2.) the total number of animals included at each concentration is: 36 after 3 days of feeding, 24 after 3 days of recovery and 12 after 6 days of recovery. Different numbers of animals are due to gradual animal removal for enzyme analysis. The significant differences between the control and exposed groups of animals were determined by Kruskal-Wallis analysis and Mann-Whitney *U* test ($p<0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA). Homogeneity of variance was tested using Levene's test.

Results

The activities of antioxidant enzymes

The activities of both antioxidant enzymes CAT and GST remained unchanged in digestive glands of isopods exposed up to 5000 µg Cu^{2+}/g dry food for 3 days. Also, no changes in comparison to control were observed after 3 days and 6 days of recovery without contaminated food (**Fig. 1 a-f**).

The activities of CAT and GST in control animals after 6 days of recovery were significantly lower as in controls after 3 days of feeding, which is in line with our previous observation that the activities of both enzymes decrease while the animals are kept in the laboratory (please see the explanation in Jemec & al. 2008). In our case, the animals investigated after 3 days of feeding were kept in the laboratory for 3 days, while the ones dissected after 6 days of recovery were kept for 9 days. Since all the specimens exposed within a certain experimental group (3 days feeding, 3 days recovery, or 6 days recovery) were kept in the laboratory for the same period of time, the comparisons between the control and exposure concentrations in each experimental group were possible.

Whole-organism parameters

After 3 days of exposure, the feeding rate (W = 308; p=0.0039) and defecation rate (W=359, p=0.0270) were decreased at 5000 µg Cu^{2+}/g dry food. After 3 days of recovery feeding and defecation rates were not changed, but they were increased at all three exposure concentrations of Cu^{2+} after 6 days of recovery (Feeding rate: W=77, p=0.0403 at 100 µg/g; W = 81, p=0.0185 at 1000 µg/g; W=53, p=0.0262 at 5000 µg/g and defecation rate: W=73, p=0.0186 at 100 µg/g; W=79, p=0.0044 at 1000 µg/g; W=55, p=0.0181 at 5000 µg/g) (**Fig. 2 a-f).** No weight change of isopods or their mortality was observed after 3 days feeding with Cu^{2+} dosed food (p>0.05), and 3 days or 6 days of feeding with uncontaminated food.

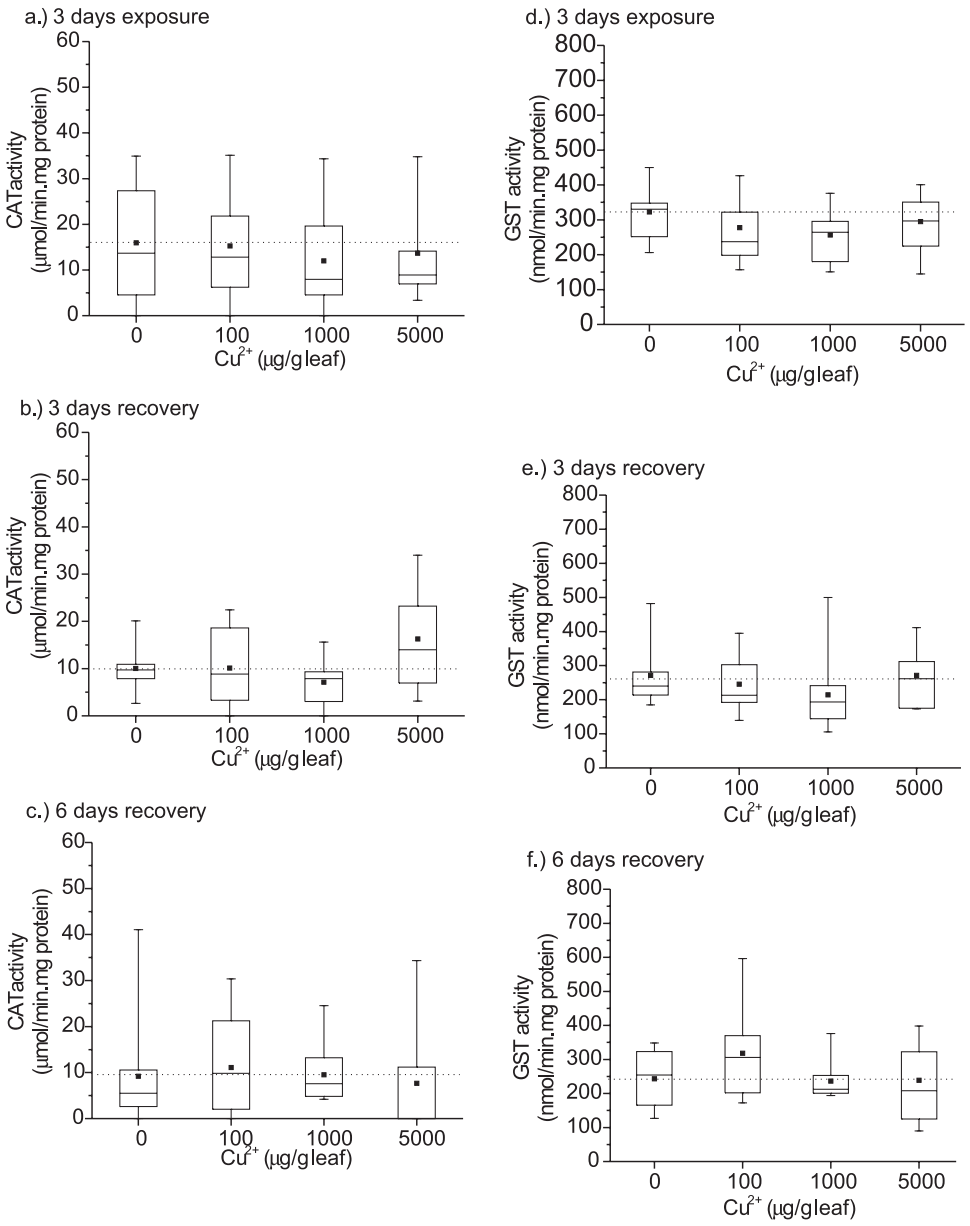

Fig. 1: The effect of Cu^{2+} on the activities of catalase and glutathione-S-transferase after 3 days of exposure, 3 days recovery and 6 days recovery. Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (■). The dashed line represents the mean value of the control.

Slika 1: Učinki Cu^{2+} na aktivnosti katalaze in glutathione S- transferaze po 3 dneh hranjenja z onesnaženo hrano ter 3 in 6 dneh hranjenja z neonesnaženo hrano. Simboli pomenijo: maksimalna in minimalna vrednost (⊥), srednja vrednost (■). Črtkana črta predstavlja srednjo vrednost kontrole.

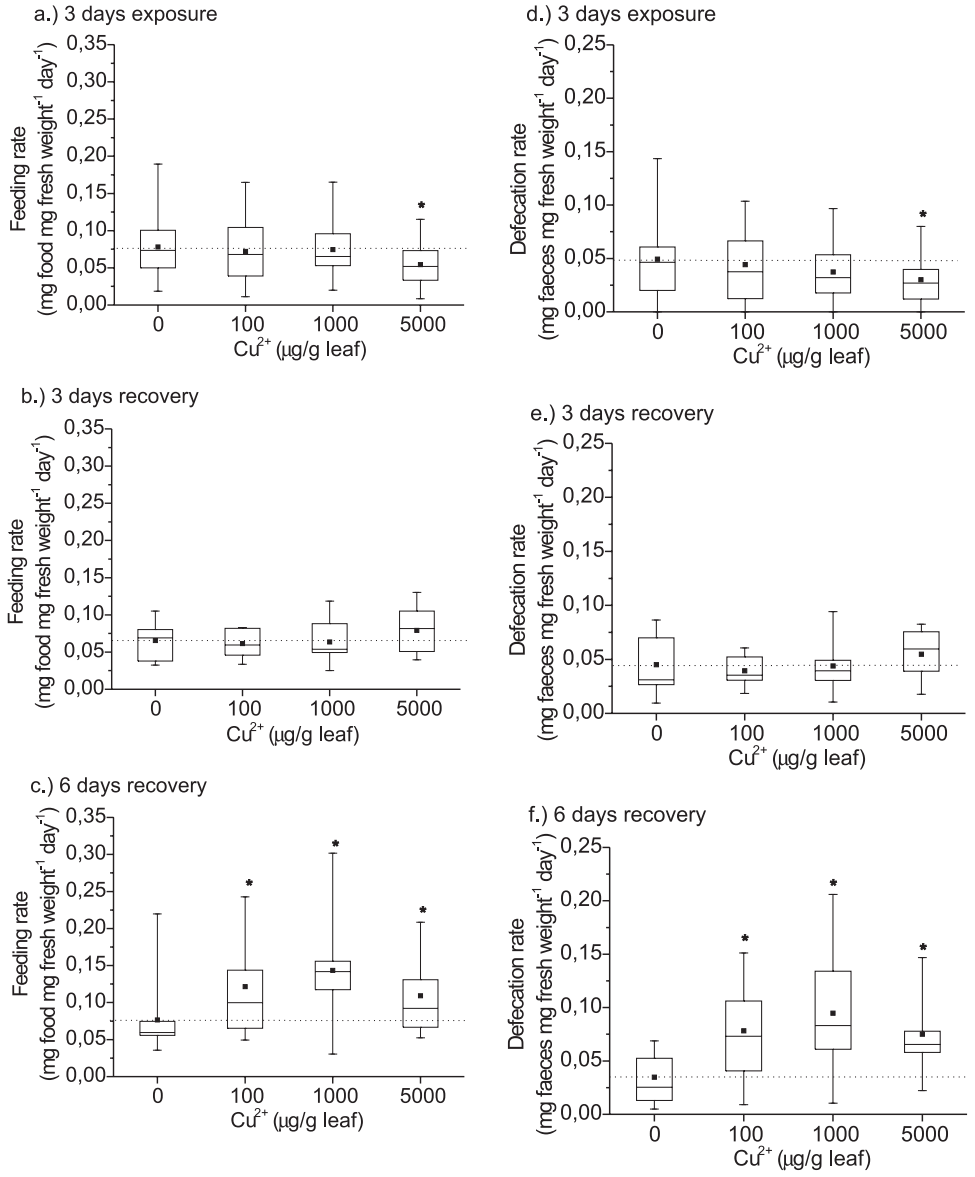

Fig. 2: The effect of Cu^{2+} on feeding rate and defecation rate after 3 days exposure, 3 days and 6 days recovery. Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (■). The dashed line represents the mean value of the control. The effects at a certain exposure concentration, which are significantly different in comparison to control, are shown (*) $p<0.05$.

Slika 2: Učinki Cu^{2+} na hranjenje in iztrebljanje kopenskih rakov po 3 dneh hranjenja z onesnaženo hrano ter 3 in 6 dneh hranjenja z neonesnaženo hrano. Simboli pomenijo: maksimalna in minimalna vrednost (⊥), srednja vrednost (■). Črtkana črta predstavlja srednjo vrednost kontrole. Statistično značilni različni učinki glede na kontrolo so označeni z (*) ($p<0.05$).

Discussion

Biochemical biomarkers are generally considered to be more sensitive than whole-organism responses which means, that the changes at the sub-cellular level should be detected at lower concentrations than the ones at the whole-organism level. However in this study no changes of biochemical biomarkers were observed, while changes in whole-organism parameters, such as feeding behaviour, were noticed. The literature contains many such examples where biomarker responses did not become detectable until the exposure levels at which already reductions in survival, growth and reproduction became apparent (Brown & al. 2004; Forbes & al. 2006). It has previously been suggested, that this is probably due to transient nature of biomarker response and their high response variability, which depends on the species being investigated, the periods of exposure, and the type of chemicals chosen (Barata & al. 2005).

The relationship between biochemical biomarkers and whole-organism responses obtained in this paper is therefore most probably a result of interplay between a very short period of exposure and the type of chemical chosen. Namely, a special relationship exists between isopods and copper, since it is an essential element for *P. scaber*. A very complex process appears to be involved in balancing between copper nutritional requirements and mitigation from copper toxic effects. Apart from the physiological mechanisms that enable assimilation and excretion of this metal, isopods are known to regulate copper intake at the behavioural level by discriminating between highly copper contaminated food and uncontaminated diet (Weissenburg & Zimmer 2003, Zidar & al. 2004). Several mechanisms, by which isopods could discriminate copper, were proposed. Among them are the existence of contact-chemoreception of copper (Weissenburg & Zimmer 2003) and it was also suggested that this discrimination might not necessarily be directly related to copper, but to copper induced changes in odour of metabolites released by microorganisms that colonise food particles. On the other hand, ingested copper might have adverse metabolic effects on isopods, which might reduce their consumption. Most probably both mechanisms are involved in the copper avoidance behaviour (Zidar & al. 2004).

The results of the present study show that the concentrations up to 5000 µg Cu^{2+}/g dry food were not lethal for isopods after 3 days of exposure and did not affect their weight change. However, feeding and defecation rate were already decreased at the highest concentration tested. These data cannot be directly compared to previous studies since there are not much literature data available after such a short exposure period. Another isopod species *Porcellionides pruinosus* exhibited avoidance behaviour when applied on soil contaminated with Cu^{2+} for 48 h (EC_{50} = 802 µg/g dry soil) (Loureiro & al. 2005). Isopods *P. scaber* consumed significantly less food as unexposed animals when exposed to 1200 µg of Cu^{2+}/g dry food weight for two weeks (Zidar & al. 2003), to 500 µg of Cu^{2+}/g dry food weight for 4 weeks (Farkas & al. 1996), and to 282 µg of Cu/g dry food weight for 6 weeks (Hassall & Rushton 1982). The specimens of *Porcellionides pruinosus* decreased food consumption after two weeks of feeding on 13710 µg of Cu^{2+}/g dry food and decreased their egestion rate at 6310 µg of Cu^{2+}/g dry food (Loureiro & al. 2006). After 6 days of recovery on non-contaminated food we noticed an increase of feeding at all three exposure concentrations of Cu^{2+}. The enhanced feeding of isopods after cessation of stress has been observed before (Drobne 1996) and is most probably a compensation for reduced consumption during copper exposure.

Although the link between the two groups of biomarkers (biochemical and higher level) was not established in this work, we believe that more data on the relationship between lower and higher-level biomarkers in isopods investigated after different exposure periods will increase their relevance in future studies on the hazard of new emerging contaminants, such as nanomaterials. Most probably, other novel biomarkers besides enzyme activities, such as proteomic and genomic profiles, will have a role in assessing risks associated with new contaminants.

Povzetek

Biokemijski biomarkerji, med katere uvrščamo aktivnosti encimov, so domnevno zelo občutljiva in specifična orodja pri določanju vplivov onesnaževal na organizme. Začetke njihove uporabe v okoljskih študijah beležimo okoli leta 1990, število znanstvenih objav z njihovo uporabo pa vse odtlej močno narašča. Med bolj pogosto uporabljenimi biokemijskimi biomarkerji sta antioksidativna encima katalaza in glutation S-transferaza, katerih aktivnost se spremeni v prisotnosti reaktivnih kisikovih zvrsti. V tej raziskavi smo proučevali aktivnosti omenjenih dveh encimov v prebavni žlezi kopenskih rakov enakonožcev *Porcellio scaber* ter spremembe na višjem organizacijskem nivoju kot so prehranjevanje, sprememba mase in preživetje. Ti testni organizmi so pogosto uporabljeni v toksičnih študijah za določanje tveganja različnih kovin in pesticidov, vendar pa je na voljo malo znanja o odzivih biokemijskih biomarkerjev po različnih časih izpostavitve. Kot modelno kemikalijo smo izbrali baker, za katerega je znano, da v organizmu povzroči nastanek reaktivnih kisikovih zvrsti. Dosedanje študije o vplivih bakra na te rake so potekale dalj časa (do 6 tednov), v tej raziskavi pa smo izbrali krajši čas (3 dni) zaradi pričakovanega hitrega odziva te vrste biomarkerjev. Poleg tega so nekatere prejšnje študije pokazale, da je ta čas že dovolj za povzročitev celičnih sprememb v prebavni žlezi kopenskih rakov. Organizme smo 3 dni izpostavljali hrani z dodanim bakrom, nato smo le to nadomestili z neonesnaženo hrano in opazovali živali še 3 in 6 dni po zamenjavi hrane. V nasprotju z našimi pričakovanji tekom poskusa nismo opazili sprememb aktivnosti antioksidativnih encimov, medtem ko so bili parametri na nivoju organizma spremenjeni. Pri najvišji testirani koncentraciji bakra (5000 μg/g suhe teže hrane) smo namreč opazili zmanjšano prehranjevanje in iztrebljanje rakov, ta koncentracija pa ni vplivala na njihovo težo in preživetje. Ti rezultati nakazujejo, da biokemijski biomarkerji v določenih primerih niso enostavna, hitra in nedvomno občutljiva mera za določanje tveganja kemikalij. Opaženi zaključki so najverjetneje posledica skupnega učinka zelo kratke dobe izpostavitve in izbranega tipa kemikalije. Baker je namreč esencialna kovina za rake *P. scaber*, zato imajo le ti kompleksne mehanizme regulacije ravnotežja količin bakra, med tistimi, ki so potrebne za normalno fiziološko delovanje ter tistimi, ki so za organizem že škodljive. Predlagam nadaljnje študije o povezavi med biomarkerji na nižjih in višjih nivojih biološke organizacije v kopenskih rakih pri različno dolgih časih izpostavitve, kar bo v prihodnosti povečalo njihov pomen v raziskavah o tveganjih kemikalij z neznanim delovanjem, kot so npr. produkti nanotehnologij.

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