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EFFECTS OF PETROLEUM PRODUCTS POLLUTED SOIL ON GROUND BEETLE *Harpalus rufipes*

EFEKT DZIAŁANIA PRODUKTÓW ROPOPOCHODNYCH NA CHRZĄSZCZA *Harpalus rufipes*

Abstract: The effects of soil contamination of petroleum products: unleaded petrol, diesel oil and used engine oil on ground beetle *Harpalus rufipes* De Geer were investigated. We measured development parameters (the survival and growth rate) and biochemical defense system parameters (the activity of the cytosolic fraction enzymes: superoxide dismutase, catalase, glutathione transferase, heat shock proteins HSP70, carboxyloesterase and acetylcholinesterase and microsomal fraction enzymes: cytochrome c (P450) reductase and ethoxyresorufin-O-deethylase) in the ground beetle *H. rufipes.* Animals were reared on contaminated soil (6 g of each petroleum product per kg of dry soil weight) through four week.

There was no difference in growth rate among animals from different experimental groups. The negative impact was revealed on survival rate of the ground beetles exposed to diesel oil (*ca* 30 % lower) at the end of the rearing in comparison with animals from control group. In turn, for animals exposed to other petroleum products, the inhibition of some examined enzymes was measured. The effects of each petroleum product were specific. The animals kept through four weeks on soil contaminated with diesel oil had only higher glutathione transferase activity than control ones. The decrease of catalase activity, HSP70 protein in animals exposed to used engine oil and the decrease of acetylcholinesterase, glutathione transferase activity in animals exposed to unleaded petrol were noted.

Keywords: petroleum contamination, biochemical defence, *Harpalus rufipes*

Introduction

Petroleum derivatives contamination in soil of agroecosystems may be toxic for organisms inhabited these areas [1, 2]. Petroleum products contamination is usually mixture of several substances with complicated fate in surrounding. Their composition

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depends on source, refinery process and product specificity [3]. Petroleum contaminants, among them *polycyclic aromatic hydrocarbons* (PAHs), modify properties of soil, evaporating to air spaces of soil [4]. They may also affect negatively organisms, in time, mixture type and concentration exposure dependent modes [5, 6].

Usage of the epigeic animals was effective for monitoring of soil contamination with heavy metals [7]. In case of soil contamination with petroleum derivatives, there are some informations about effects on webworms or isopods [8]. There is a scarcity of information about petroleum derivatives effect on epigeic insect. Carabidae frequency appeared to be negative affected through 4 month after soil contamination with diesel oil, engine oil and petrol (in concentration 2 dm^3/m^2) [9].

Examination of epigeic animals biochemical response may be important for early monitoring of soil contamination with petroleum derivatives and in assessment of the pollutants toxicity. The early step of PAHs metabolism in organisms are connected with microsomal enzymes activity catalyzing oxygenation reactions and esterases activity. Several of PAHs may induce or inhibit these enzymes. It is important because several of them take part in key processes connected with development, growth of animals and also with resistance to pesticides. Thus, these enzymes seem to be effective indicators of sublethal PAHs action [10, 11].

The reactions catalyzed by the microsomal enzymes may lead to production of reactive oxygen forms. It enhances an oxidative stress and affects antioxidant enzymes activity, which are sensible biomarkers of diesel hydrocarbon contamination. Enhanced oxidative stress may be also due to lipid peroxidation. Among products of lipid peroxidation, *4-hydroxynonenal* (HNE), can be metabolized by *glutathione transferases* (GST) isozymes, which as enzymes of the 2^{nd} phase of detoxification catalyze conversions of intermediates generated during microsomal reactions [12–15].

HSP proteins are also potentially valuable biomarker of petroleum contamination. The HSP proteins cellular responses may manifested non-specific mechanism of toxicity as a consequence of increased production of abnormal proteins and alteration of their functions in cells [15].

The present study examined the effects of different petroleum contaminants on beetles *H. rufipes* reared during four weeks period. The soil used in the experiment was contaminated with: unleaded petrol, used engine oil, diesel oil or uncontaminated (control). We measured: survival rate, body mass, detoxifying enzymes of cytosolic and microsomal fraction of the beetles. *Harpalus rufipes* De Geer individuals commonly inhabited different agroecosystems in Europe [16]. We suspected different sensitivity of adult beetles to petroleum derivatives, revealed by changes in performance of the adult individuals during rearing period and by variation in selected biochemical defense systems.

Materials and methods

The contaminated soil used in the experiment, was originated from cubic containers of 1 m^3 volume with drainage and system of evaporation kept in the field (Mydlniki, Krakow, Poland). The containers had an attest to use petrol substances. They were

placed into the ground and the soil inside them was up to the same level as that outside. Their walls in the upper part were perforated to allow the penetration by the field organisms. In June 2010, the soil was contaminated with petroleum products: unleaded petrol, diesel oil or used engine oil, in concentration of 6 g per kg of dry soil weight. In August 2010, the upper part of soil (ca 5 cm layer) was collected and air dried just prior experiment provided in laboratory conditions.

Animals were collected in Mydlniki area and acclimated to laboratory conditions. The animals were randomly divided to experimental groups. They were reared in cages (2 dm^3 volume) on thin layer of different contaminated soil (0.1 kg of soil in each cage) at 25 °C. The beetles were offered the larvae of houseflies as a food *ad libitum*. Every second day, water was spread on the layer of soil. The experimental groups were due to soil contamination: control group, unleaded petrol, diesel oil and used engine oil groups. Animals from each experimental group were reared in two cages (18 adult individuals of both sexes were kept in each cage).

During four weeks of rearing, we measured the gained mass of the alive beetles and recorded their number, in a week intervals. At the end of the experiment, male beetles were anaesthetized on ice, and homogenized without legs and wing cases, in 0.05 M buffer Tris-HCl, pH 7.4 with 1 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM DTT, 1 mM PMSF, 1 mM PTU and 20 % sucrose. Homogenates were centrifuged at 1000 g 10 min at 4 °C. The supernatants were centrifuged at 15,000 g 10 min at 4 °C. Next supernatants were centrifuged 100,000 g 1 h at 4 ^oC. Finally obtained supernatants were used for cytosolic enzymes measurements, while microsomal enzymes were measured in suspension of pellet (in homogenization buffer contained 20 % glycerol). The enzyme assays were carried using a microplate spectrophotometer Infinite M200 and fluorescence spectrophotmeter f-7000. There was six replicates used for each biochemical measurement (for each replicate we used 2–3 animals).

CAT activity toward H_2O_2 in 0.05 M phosphate buffer (pH 7.0) was measured at $\lambda = 240$ nm [17]. Blanks were registered in the absence of H_2O_2. The enzyme assay was carried during first half of minute, within the linear range of reaction rate. Exctinction coefficient was equal to 40 $M^{-1} \cdot cm^{-1}$. One unit of the enzyme activity was defined as the decomposition of 1 nmol of H_2O_2 $min^{-1} \cdot$ mg $protein^{-1}$.

GST activity was measured as described by Yu [18] using ethanol solution of *1-chloro-2,4-dinitrobenzene* (CDNB). 1 cm^3 of the reaction mixture contained: 0.1 mM Tris-HCl buffer (pH 7.5) consisted of 1.5 mM GSH in buffer, 15 mM CDNB and 0.01 cm^3 sample. Blank values (in the absence of sample) were subtracted to yield the final absorbance values. The enzyme assay was carried during first three minutes, within the linear range of reaction rate. The measurements were done at $\lambda = 340$ nm. Enzyme activity was expressed as nmol of GSH conjugates $min^{-1} \cdot$ mg $protein^{-1}$. The extinction coefficient was equal to 9.6 $mM^{-1} \cdot dm^{-3} \cdot cm^{-1}$.

Carboxylesterase (CarE) was measured in presence of *p*-nitrophenyl acetate as a substrate. The measurement was taken at $\lambda = 400$ nm for 3 min. Results were corrected by subtracting blanks contained buffer instead of sample. To calculate CarE activity, the extinction coefficient 9.25 $mM^{-1} \cdot dm^{-3} \cdot cm^{-1}$ was used [19].

Acetylcholinesterase (AChE) activity was determined using acetylthiocholine iodide as a substrate [20]. The linear changes in absorbance were measured at λ = 412 nm through 5 min.

NADPH dependent cytochrome c (P450) reductase activity was measured in 0.3 M potassium phosphate buffer with 0.1 mM EDTA, pH 7.4 with freshly prepared cytochrome c (5 mg/cm^3) and 1 mM KCN. After addition of NADPH to the mixture, the changes in absorbance were recorded at 550 nm.

Ethoxyresorufin o-deethylase (EROD) activity was measured at excitation wavelength λ = 560 nm and emission wavelength λ = 589 nm. The measurement of the activity started after addition 7-etoxyresorufine and NADPH. The measurement of progressive increase in fluorescence lasted 40 min. The activity was defined as pmol of resorufine min^{-1} · mg protein^{-1} and calculated upon product (resorufine) curve basis. The protein concentrations were assessed using *bovine serum albumin* (BSA) as a standard [21].

To evaluate HSP70 proteins and HNE levels we used ELISA procedure. Cytosol portions contained 20 µg protein were transferred to wells of the plate and the following primery antibodies: anti-HSP 70 Cayman (No. cat. 19015) (amounts of the antibody in proportion with *physiological saline buffer* (PBS), pH 7.4 (PBS), 1:250) and Rabbit anti-HNE 11S (Alpha diagnostic) (amounts of the antibody in proportion with PBS, 1 : 250) were applied, and before evaluation of the examined products the goat anti-rabbit IgG-AP (Stressgen SAB 301) (amounts of the antibody in proportion with PBS, 1:5000) as a secondary antibody was added. Firstly, microtitre plates were coated with samples and, dependently on measurement, with HSC70 (evaluation of HSP protein level) or HNE (evaluation of HNE product level) as standards. Plates were incubated overnight at 4 °C. Active sites remaining on the plate were blocked by adding 3 % BSA solution in PBS. Afterwards, anti-HSP or anti-HNE antibodies were respectively added to wells, and the plates were incubated at 37 °C for 2 h, afterwards the secondary antibody was added. Finally, to induce the colour reaction, a solution of *p*-nitrophenyl phosphate was added to each well. In between addition of BSA as well as primary or secondary antibodies, the wells were washed three times with PBS-0.1 % Tween solution. The product of the final reaction was measured at λ = 405 nm, and amounts were determined using a standard curve of proper standards.

Data of HSP70 and HNE levels (three replicates) were analysed with the nonparametric Kruskal-Wallis test and presented as mediane and range of quartiles (25–75 %). The other data are presented as the mean ± SD of six-seven replicates in each experimental groups (each replicate was combined homogenates of two-three males). If necessary, the data were log transformed and checked for homogeneity and normality. ANOVA one-way analysis and correlation analysis were carried out to establish significant relationship between measured biochemical parameters in beetles from experimental groups. *F* values having $p < 0.05$ were considered as significant.

Results and discussion

The most affected were beetles inhabited soil contaminated with diesel oil compared with those from other experimental treatments. They started to die after two weeks of

exposure, and finally from 36 survived only 25 individuals. In others examined groups survival was not changed (Table 1). The parallel study provided that diesel oil contamination was highly toxic for other representatives of coleopteran *Pterostichus cupreus* (Kafel, unpublished). The different sensitivity of soil organisms to petroleum hydrocarbons was due to quantity and quality of contaminants mixtures [22]. The examination of *Drosophila melanogaster* performance being exposed to benzene, toluene or xylene (single or in mixture) shown significant differences between single and combinatory effects [15]. But, the possibility of different performance of different arthropod representatives was also suggested.

Table 1

Survival of the beetles of *H. rufipes* during following weeks of rearing, presented in % of alive animals, 100 % was equal to the 36 animals provided at the beginning of the experiment in each experimental group, living on soil contaminated with petroleum products: unleaded petrol, diesel oil, used engine oil and on uncontaminated soil (control)

	Control	Unleaded petrol	Diesel oil	Used engine oil
At the beginning of rearing	100	100	100	100
After one week of rearing	100	100	100	100
After two weeks of rearing	100	100	100	100
After three weeks of rearing	95	100	97	100
After four weeks of rearing	92	100	67	100

The body mass of animals did not changed significantly during rearing period, and did not differ between animals from experimental groups (Table 2).

Table 2

The body mass of beetle *H. rufipes* [mg of the alive individual] living on soil contaminated with petroleum products: unleaded petrol, diesel oil, used engine oil and on uncontaminated soil (control) in the following four weeks of rearing. There was no significant differences among the experimental groups and in the following weeks of the rearing. (LSD, $p < 0.05$)

	Control	Unleaded petrol	Diesel oil	Used engine oil
At the beginning of rearing	93 ± 20	94 ± 15	89 ± 19	87 ± 18
After one week of rearing	95 ± 24	93 ± 15	90 ± 18	84 ± 19
After two weeks of rearing	88 ± 14	91 ± 21	91 ± 18	85 ± 17
After three weeks of rearing	93 ± 13	97 ± 18	94 ± 18	86 ± 17

Looking for suitable indicators of petroleum products contamination, among microsomal enzymes activity: EROD and NADPH-dependent reductase cytochrome c we did not find any (Table 3). In the parallel study provided on *P. cupreus* males, the decrease of the microsomal enzyme activity was registered for animals exposed to petroleum contaminants when compared with the controls (Kafel, unpublished). The response of

the microsomal enzymes may depend on time of contaminants exposure. On example of *Abarenicola pacific* exposed to PAHs in sediment, the increase of the enzymes during first two weeks and the decrease after longer time of exposure was found [23]. Differences in metabolic biotransformation of PAHs between terrestrial invertebrates: isopods and springtails were presented [6]. The increasing response might be due to detoxification *via* single P450 enzyme. The responses may depend on bioavailability of PAHs. There are plenty of factors affected substances availability in soil like: pH, organic matter content, moisture, chemical properties, time of presence in the soil [24–26]. It should be also underlined the discrepancies in microsomal enzymes assessment. They might be connected with preparation of samples, as sources of group P450 enzymes activity.

Table 3

The activity of cytosolic and microsomal enzymes in the beetle *H. rufipes* living on soil contaminated with petroleum products: unleaded petrol, diesel oil, used engine oil and on uncontaminated soil (control) after four weeks of rearing presented as mean ± SD. Different letters depicts the significant difference among experimental groups (LSD, $p < 0.05$)

	Control	Unleaded petrol	Diesel oil	Used engine oil
GST [nmol · mg protein^{-1} · min^{-1}]	11.47 ± 1.75 ^{b}	8.78 ± 1.71 ^{a}	17.78 ± 2.97 ^{c}	9.05 ± 1.84 ^{ab}
CarE [nmol · mg protein^{-1} · min^{-1}]	5.45 ± 1.88 ^{a}	4.95 ± 1.74 ^{a}	6.41 ± 3.24 ^{a}	4.90 ± 0.80 ^{a}
AChE [nmol · mg protein^{-1} · min^{-1}]	1.07 ± 0.29 ^{a}	2.39 ± 0.50 ^{b}	1.58 ± 0.59 ^{ab}	1.38 ± 0.54 ^{a}
CAT [nmol · mg protein^{-1} · min^{-1}]	149.5 ± 69.9 ^{b}	125.1 ± 48.4 ^{ab}	116.9 ± 46.7 ^{ab}	89.9 ± 23.9 ^{a}
EROD [pmol · mg protein^{-1} · min^{-1}]	7.79 ± 5.11 ^{a}	5.96 ± 6.61 ^{a}	3.89 ± 2.57 ^{a}	5.37 ± 4.86 ^{a}
NADPH reductase [µmol · mg protein^{-1} · min^{-1}]	65.5 ± 11.1 ^{a}	73.1 ± 15.8 ^{a}	66.3 ± 14.0 ^{a}	71.9 ± 17.3 ^{a}

Elevation of GST activity was observed among animals exposed to diesel oil contaminations in soil. It was 1.5 times higher than in control animals (Table 3). The enhancement of GST activity under diesel oil exposure was documented for oyster *Crasostrea gigas*, but dependently on level of contamination and environment conditions. It was suggested that GST increase might be correlated with increase of lipid peroxidation [4]. But, in our case, we did not find any changes in level of products of lipid peroxidation – HNE (Fig. 2). A significant increase of some GST isozymes expression activity was shown for Atlantic code larvae (*Gadus morhua*) after crude oil exposure [27]. A correlation between GST activity and PAHs concentration in tissues of mussels was also evaluated [28].

In our experiment the animals with high GST turnover were characterized by low survival (Tables 1 and 3). Increasing energetic demands for higher enzymatic turnover may result in reduced performance of animals [29].

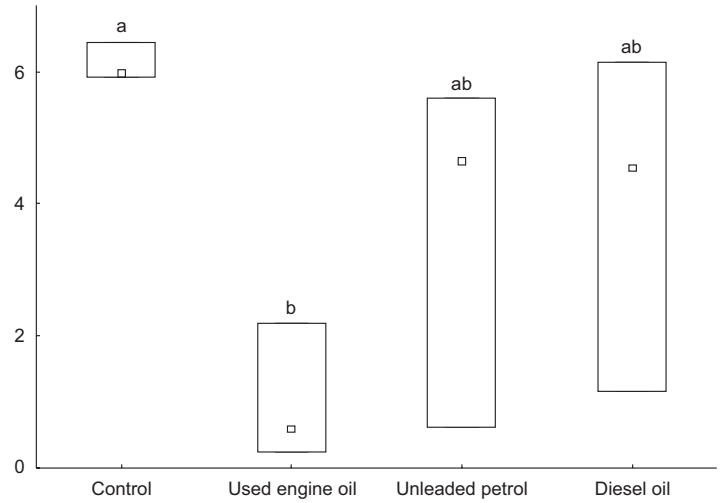

Fig. 1. The level of HSP 70 [ng · (mg · protein)$^{-1}$] in cytosolic fraction of beetle *H. rufipes* living on soil contaminated with petroleum products: unleaded petrol, diesel oil, used engine oil and on uncontaminated soil (control) after four weeks of rearing (Kruskal-Wallis, $p < 0.05$).

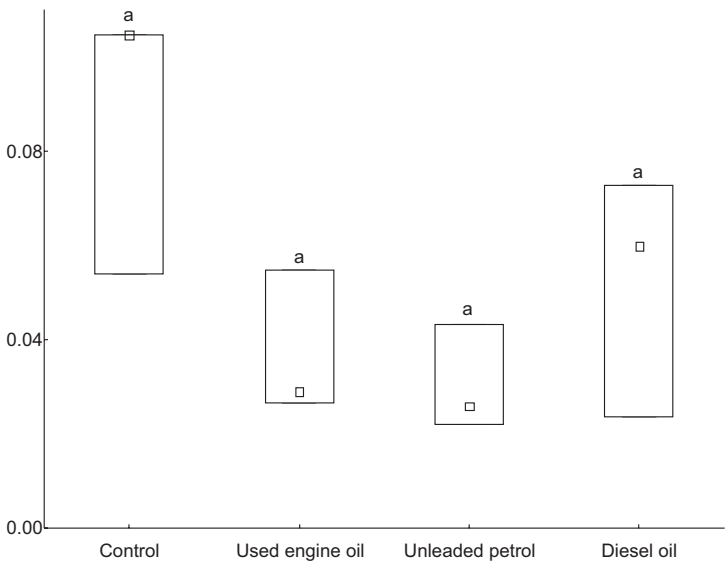

Fig. 2. The level of HNE products [ng · (mg · protein)$^{-1}$] in cytosolic fraction of beetles *H. rufipes* living on soil contaminated with petroleum products: unleaded petrol, diesel oil, used engine oil and on uncontaminated soil (control) after four weeks of rearing (Kruskal-Wallis, $p < 0.05$)

GST and AChE activity varied between animals exposed to different petroleum derivatives. The action of petrol, may depend on origin of the crude oil and processing technology. Animals from unleaded petrol group had lower activity of GST (1.3 times)

than in animals from control group. We also found an elevation of AChE activity upon unleaded petrol exposure, but not under other petroleum derivatives (Table 3). It is reported that AChE activity can serve as a good exposure biomarker but its response might be species specific [30].

The change in CAT activity was only found for animals exposed to used engine oil in soil. It was 1.7 times lower than in control group (Table 3). Generally, however, the different tendencies might be possible in response of CAT activity, that might be time exposure dependend. Such phenomenon, was presented for marine organisms [4, 10, 31].

The examined enzymes: CarE, EROD and NADPH depended reductase cytochrome c and HNE products level did not varied among different conditions of soil contamination with petroleum constituents (Table 3, Figs. 1 and 2).

Conclusions

1. Diesel oil contamination mostly affected the beetles.
2. Body mass changes are not suitable biomarker of petroleum products contamination.
3. The changes in activity of the examined enzymes were specific for particular exposure of petroleum products.
4. GST activity (however variable) seems to be the most sensitive biomarker of *H. rufipes* exposure to petroleum products.

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EFEKT DZIAŁANIA PRODUKTÓW ROPOPOCHODNYCH NA CHRZĄSZCZA *Harpalus rufipes*

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Abstrakt: W badaniach porównywano wpływ produktów naftowych (benzyna bezołowiowa, olej napędowy i zużyty olej silnikowy) na chrząszcza *Harpalus rufipes* De Geer. Mierzono parametry: rozwojowe (przeżywalność i tempo wzrostu) i parametry biochemiczne związane z funkcjonowaniem systemu obronnego (aktywność enzymów frakcji cytozolowej: dysmutazy ponadtlenkowej, katalazy, transferazy glutationowej, białek szoku cieplnego HSP70, karboksyloesterazy i acetylocholin esterazy oraz aktywność enzymów frakcji mikrosomalnej: reduktazy cytochromu c (P450) i deetylazy etoksy-rezorufiny) w ciele chrząszczy. Do badań wykorzystano zwierzęta hodowane na glebie skażonej dawką 6 g każdego produktu ropopochodnego na kg suchej masy gleby przez okres czterech tygodni.

Nie wykazano istotnych zmian w tempie wzrostu między zwierzętami z różnych grup eksperymentalnych. Ujawniono negatywny wpływ na przeżywalność chrząszczy eksponowanych na olej napędowy (ok. 30 %) pod koniec okresu doświadczalnego, w porównaniu ze zwierzętami z grupy kontrolnej. Z kolei u zwierząt eksponowanych na pozostałe produkty ropopochodne stwierdzono zahamowanie aktywności niektórych badanych enzymów. Efekty działania produktów ropopochodnych były specyficzne. Zwierzęta przetrzymywane na glebie skażonej olejem napędowym cechowała wyższa aktywność transferazy glutationowej w porównaniu do kontroli. Obniżenie aktywności katalazy i poziomu białek szoku cieplnego HSP70 zarejestrowano u zwierząt poddanych działaniu zużytego oleju silnikowego, natomiast obniżenie aktywności acetylocholinesterazy i transferazy glutationowej stwierdzono u zwierząt eksponowanych na działanie zanieczyszczeń benzyny bezołowiowej.

Słowa kluczowe: zanieczyszczenie produktami ropopochodnymi, obrona biochemiczna, *Harpalus rufipes*