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EFFECT OF SOIL CONTAMINATION WITH PETROLEUM PRODUCTS ON ESTERASE ACTIVITY IN EARTHWORM *Lumbricus terrestris*

WPŁYW ROPOPOCHODNYCH ZANIECZYSZCZEŃ GLEBOWYCH NA AKTYWNOŚĆ ESTERAZOWĄ DŻDŻOWNIC *Lumbricus terrestris*

Abstract: Among biomarkers of exposure the suitability of esterases especially carboxylesterases (CarE), metabolizing wide array of xenobiotics being important for organismal functioning, was very often underlined. Moreover, neurotoxic effects of constituents of petroleum products may be exert by inhibition of cholinesterase (ChE) isozymes in animals. Here, we examined esterase activity toward acetylthiocholine iodide and *p*-nitrophenyl acetate in earthworms *Lumbricus terrestris* reared through four weeks on loamy sand soil contaminated with petroleum products (unleaded petrol, used engine oil), in two weeks intervals. Such contamination was simulated in one m^3 volume soil containers, at a dose of 6 $g \cdot kg^{-1}$ of dry soil (experimental field in Mydlniki, Krakow suburb, Poland; 50.0815ºN, 19.84730ºE). We compared the effects of these contaminants action in soil collected in the year of treatment and one year later. We evaluated also effects of animals rearing on soil unremediated and remediated with "cocktail" of microorganism, and also checked the enzymatic activities response in two weeks intervals. We found lower ChE activity in animals reared on petroleum derivatives contaminated soil when compared with control animals, but only in the case of four weeks rearing on soil collected in the year of treatment. Similar response of ChE in animals reared on soil collected a year after the treatment, but estimated in earlier period of time – after two weeks exposure to petrol unleaded contamination was observed. CarE activity characterized higher variability with time than ChE activity. Generally, the activity of CarE lowered from the beginning to the end of the rearing (in all experimental groups). Animals reared on remediated soil, in most cases, had higher ChE activity than those reared on unremediated soil (control and diesel oil exposed animals).

Keywords: esterase, petroleum contamination, earthworm *Lumbricus terrestris*

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Contamination of agroecosystems by petroleum derivatives may be a threat for their invertebrate inhabitants [1, 2]. Among soil inhabitants, earthworms seems to be important in assessment of negative effect of soil contamination. Earthworms as a common standard organisms have a notable place in soil toxicology. The earthworms are an important factor in mixing the soil, increase the contact of microbials with toxins. They are presented as a model organism in respect to its role in removal of polycyclic aromatic hydrocarbons (PAHs) from soil. They may reduce time of remediation process in petroleum derivatives contaminated sites [3, 4].

There are plenty of petroleum contamination sources, like leakages from: tanks, pipes during distribution, petrol stations, different types of transport, industry [5]. In industrial soil, the concentrations of PAHs may go up even to 6 g · kg^{-1} of soil [6]. Serious soil pollution by PAHs in Poland was reported by Wcislo [7]. Petroleum products are usually mixtures of several substances having complicated fate in soil surrounding. Their compositions depend on source, refinery process and product specificity [8]. Petroleum contaminants, among them PAHs, modify properties of soil, evaporating to its air spaces. Most hydrocarbons are quickly removed from soil as volatile compounds or degraded readily by microorganisms, but some of them are more resistant and may accumulate in fat tissues of animals [9, 10]. The effects of soil petroleum contaminants might be typical for local sources of contamination. Schaefer and Juliane [4] measuring effects of different representatives of earthworms, observed their high mortality (above 58 %) when exposed for 28 days of rearing to soil contaminated with total petroleum hydrocarbons, with concentration higher than 4 g · kg^{-1} of soil.

Remediation of contaminated soil may make it less destructive. According to Salanitro et al [11] after contamination with total petroleum hydrocarbons (4–27 g · kg^{-1} of soil), alkanes, alkenes, aromatic and polar constituents of petrol derivatives were degraded continuously during remediation process, being less toxic for earthworm after 5 to 12 months after contamination (it took longer with lower organic contents in soils).

Natural remediation process, especially due to absence of suitable endogenous bacteria, may be enhanced by inoculation with PAH specific bacteria. Additional input of non indigenous bacteria not always lead to significant biomagnifications of the remediation process. Their compatibility to the site application is very important, however some of them are capable to metabolize wide spectrum of substances [9, 12–13].

Esterases of various organisms inhabited contaminated areas have a promising role as biomarkers. Wheelock et al [14] emphasised, next to cholinesterases, the usefulness of carboxyloesterases in that matter, especially, when recording toward *p*-nitrophenyl acetate as a substrate. Vejares et al [15] depicted the suitability measuring the effects of organophosphate pesticides on both enzymes: CarE – important as enzymatic barrier against organophosphate uptake during ingestion of contaminated soil, ChE activity – connected with proper neuronal transmission.

ChE activity was measured in earthworms toward different substrates, but dominant isozymes, as was revealed, had an affinity to acetylthiocholine iodide [16].

The present study examined the effects of different petroleum contaminants: unleaded petrol and diesel oil on cholinesterases and carboxylesterases activity in

earthworms *Lumbricus terrestris.* The animals were reared on soil well-mixed with these products during four weeks period, firstly using soil collected two month after its contamination from containers located in the field and secondly, using soil collected a year after. We compared the effects using soil not only contaminated but also being mixed with cocktail of microorganisms. The main question was whether these esterases were universal and suitable biomarkers of such contamination.

Material and methods

Earthworms *Lumbricus terrestris* Linnaeus 1758 were purchased from a commercial sportfishing supply and were reared thought four weeks on loamy-sand soil contaminated with unleaded petrol or used engine oil. The used mature clitellate animals weight, at the beginning of the experiment, was 3–6 g. Twenty individuals were reared in each containers of 3L volume, filled with soil contaminated with unleaded petrol or used engine oil. Soil was collected on the surface layer (0–10 cm), dried on air and sieved (sieve with 1 mm diameter) and after 78–80 % rH moisturing was used for earthworms rearing (soil hygrometer TFH-100-E, Ebro GmbH). Every week, wet soil was mixed with 4 g dried powder of horse manure.

The contaminated soil used in the experiment, was originated from cubic containers of one m^3 volume with attest to use petrol substances. The containers, equipped with drainage and evaporation system, were kept in the field (Mydlniki, Krakow, Poland; 50.0815^{o}N, 19.84730^{o}E). They were placed into the ground and the soil inside them was filled up to the same level as that outside. Their walls in the upper part were perforated to allow the penetration by the field organisms. For better soil remediation process, the soil was aerated in gravity system using perforated tubes, digged to a depth of *ca* 70 cm. Before remediation mixture was applied to the top soil layer in the containers, multi-component fertilizer (Azofoska; 13.6 % N, 2.8 % P and 15.8 % K) in a dose of 100 g $\cdot$ container^{-1} was applied. Leachates from containers were pumped regularly not to allow their flooding. In the June of 2010, the soil was contaminated with petroleum products: unleaded petrol or used engine oil, in concentration of 6 g $\cdot$ kg^{-1} of dry soil weight. In the August of 2010 and next, in the August of 2011, the upper part of the soil (*ca* 10 cm layer) was collected and air dried prior experiment provided in laboratory conditions.

The experiment were provided twice, firstly with usage of soil collected two months after contamination. Secondly, with the usage of soil collected one year later (fourteen months after its contamination).

The experimental group names are connected with petroleum derivatives contamination of soil, used in the experiment, on which earthworms were reared.

– Group C – control group – earthworms reared on soil without petrol contaminations,

– Group P – earthworms reared on soil contaminated with petroleum unleaded,

– Group EO – earthworms reared on soil contaminated with used engine oil,

– Group C + R – earthworms reared on soil uncontaminated and bioremediated with "cocktail" of microorganisms,

– Group P + R – earthworms reared on soil contaminated with petroleum unleaded and bioremediated,

– Group EO + R –earthworms reared on soil contaminated with used engine oil and bioremediated.

The loamy-sand soil, which was used in this study was characterized by concentration of 75 % of sand particles (mainly fine sand), 21 % of silt particles (mainly coarse silt) and 4 % of clay particles. The measured soil pH was: pH (KCl) 8.5 and pH (H_2O) 7.1. The soil contained 1.04 % of $C_{organic}$. The soil was also characterized in the respect of nitrogen and carbon concentration with VARIO MAX CNS elemental analyser. Nitrogen concentration in the control soil was 1.134 $g \cdot kg^{-1}$ dry weight of soil, and comparable values were found in the soil from other experimental groups. Only in the cases of groups P + R and EO + R higher concentrations of this element were found. Carbon concentration in control soil samples was 10.035 $g \cdot kg^{-1}$ dry weight of soil, and in the case of EO + R group it was three times higher than in the controls. The EO + R group significantly differentiated higher proportion of carbon to nitrogen than other experimental groups (Kafel, unpublished).

Animals from each experimental group were reared in two containers. After two and four weeks of rearing the earthworms were anaesthetized on ice, freezed in nitrogen liquid and homogenized in 0.05 M buffer Tris-HCl, pH 7.4 with 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM phenythiourea and 20 % sucrose. Homogenates were centrifuged for 10 minutes at 1000 g at 4 ^{o}C, and the obtained supernatants were centrifuged for 15 minutes at 15,000 g at 4 ^{o}C. Next, the supernatants were centrifuged at 100,000 g for 1 h at 4 ^{o}C. After that centrifugation, the supernatants were used for esterase enzymes measurements.

Carboxylesterase (CarE) activity was measured in presence of *p*-nitrophenyl acetate as a substrate. The measurement was taken at 400 nm for 3 min. Results were corrected by subtracting blanks contained buffer instead of sample. To calculate CarE activity, the extinction coefficient of 9.25 $mM^{-1} \cdot cm^{-1}$ was used [17].

Acetylcholinesterase (AChE) activity was determined according to Ellman et al [18]. The reaction mixture contained buffer, sample and acetylthiocholine iodide (a substrate) and 5,5'-dithio-bis(2-nitrobenzoic acid). The linear changes in absorbance were measured at 412 nm through 5 min. The activity was presented as nmol mg $protein^{-1} \cdot min^{-1}$.

The protein concentrations were assessed as in Bradford [19], using bovine serum albumin as a standard.

The assumption of variance homogeneity and normal distribute were tested before statistical analysis. If necessary, the data were log transformed. ANOVA was performed with LSD post-hoc test. Furthermore, linear regression analysis were done to evaluate the putative association among biochemical variables but we did not found any relationships. The data in the tables are presented as mean values ± SD of six-seven replicates in each experimental groups (each replicate was composed from homogenate from one individual).

Results

The animals reared through four weeks on soil collected two months after its contamination with petroleum derivatives characterized significant lower ChE activity than in control animals. In the case of animals from P group it was *ca* 28 % lower and in the case of animals from group EO it was almost 50 % lower in comparison with control values (Table 1). ChE and CarE activity was examined in individuals from experimental groups, which were reared on soil collected fourteen months after its contamination. At the start of the experiment, animals had lower activity ChE than in the control groups but higher in the animals from the other groups, when consider effects after two weeks exposure. After next two weeks exposure, control animals had lower activity than in previous measurement (measured after two week exposure) and opposite trend was found for animals from the other experimental groups.

Table 1

ChE activity [nmol · min^{-1} · mg · $protein^{-1}$] in earthworms *L. terrestris* reared through four weeks on the soil collected two months after contamination with petroleum derivatives: petrol unleaded and used engine oil (first year of the experiment). Different letters denotes significant differences between experimental groups (LSD, $p < 0.05$)

Experimental groups	ChE (Mean ± SD)	
Group C	50.44 ± 15.97	a
Group P	36.48 ± 7.82	b
Group EO	26.58 ± 5.87	b

When compared effects of petroleum derivatives contamination with proper (measured after the same period of rearing) control, the differences were found only after two weeks rearing but not after four weeks rearing. The earthworms analysed after two weeks of exposure derived from P and EO groups had more than 5 times lower activity than earthworms from the proper control group.

Regarding CarE activity changes, we found the lowering with time tendency. The highest activity was measured in animals at the start of the experiment. And then, in animals reared through two weeks from almost all examined groups, the activity was higher than in animals reared through four weeks. The only exception was found in the case of animals derived from EO + R group. In animals from this group, similar activity of the enzyme was measured in animals after two and four weeks of rearing (13.16 *vs* 11.53 nmol/min/mg protein, respectively). When compared effects of petroleum derivatives contamination with proper (measured after the same period of rearing) control, in the case of CarE, we found lower activity in animals from P groups (those being after two weeks of exposure) and in the case of animals from EO group (those being after four weeks of exposure). Moreover, animals reared through two weeks on remediated soil and exposed to petrol unleaded or used engine oil differentiated lower CarE activity than in control (Table 2).

Table 2

ChE and CarE activity [nmol · min^{-1} · mg · protein^{-1}] in earthworms *L. terrestris* reared through two and four weeks on the soil collected fourteen months after its contamination with petroleum derivatives: petrol unleaded and used engine oil, and additionally after bioremediation process. Different letters denote significant differences among experimental groups after the same time of treatment, star denotes differences with control values gained at the beginning of the experiment (from C group), dash denotes differences between the same groups in different time of rearing: two and four weeks (LSD, $p < 0.05$).

Experimental groups	ChE (Mean ± SD)		CarE (Mean ± SD)	
At the beginning of the experiment				
Group C	20.97 ± 10.67		32.82 ± 17.56	
After two weeks of the experiment				
Group C	48.09 ± 20.35	a *#	26.57 ± 10.49	a #
Group P	8.75 ± 4.17	c *#	16.52 ± 2.58	b *#
Group EO	6.85 ± 2.28	c *#	17.95 ± 5.49	a #
Group C + R	17.14 ± 7.98	b #	16.64 ± 3.12	b #
Group P + R	8.10 ± 3.97	ab *#	13.68 ± 5.45	b *#
Group EO + R	6.67 ± 1.67	ab *#	13.16 ± 2.69	b *
After four weeks of the experiment				
Group C	23.83 ± 3.97	b #	9.74 ± 2.37	b *#
Group P	18.23 ± 6.17	b #	10.72 ± 3.18	ab *#
Group EO	25.62 ± 6.33	b #	13.47 ± 2.15	a *#
Group C + R	38.80 ± 5.33	a *#	10.85 ± 3.54	ab *#
Group P + R	34.23 ± 13.66	ab *#	7.46 ± 1.81	b *#
Group EO + R	24.40 ± 8.81	ab #	11.53 ± 0.41	b *

Discussion

Responses of the earthworms' examined enzymes to soil contaminants: after treatment with petrol unleaded or used engine oil, if noted, generally, were connected with a decrease of animals activity in comparison with proper controls. Such reaction was revealed in the case of ChE activity in earthworms reared through four weeks on soil collected two months after its treatment. In the case of ChE and CarE in the earthworms reared on soil collected fourteen months after its treatment with petrol unleaded (but just after two weeks of rearing) and CarE activity in those treated with soil contaminated with used engine oil (after four weeks of rearing), the lower activity (in comparison with control) of both enzymes was recorded (Tables 1 and 2). Both enzymes are presented as effective biomarkers of different kind of contamination: with heavy metals or pesticides (organophosphate and carbamate) and PAHs [20–23]. In highly stressful situation the mentioned enzymes were inhibited by different xenobiotics. Such situation was shown by Alpuche-Goal and Gold-Bouchot [23] in the case of *H. lumieri* fish, for which lowered ChE activity was measured in brain and liver

under benzo[a]pyrene and chlorpyrifos exposure. But, sometimes, the enzymes could also be an ineffective as biomarkers, as it was in the case of acetylcholinesterase (predominant ChE) activity measured in brains *of Sparus aurata*. A lack of effects on the enzyme to exposure of different PAHs present in petroleum contaminated environments (phenanthrene, pyrene and fluorine) were shown [24]. The ways of petroleum constituents action may be uptaken mainly by alimentary tract of the earthworms and also by their skin [25]. Their detoxifying responses may be important, because they fulfil a role as a barrier, bio-filter and bio-remediator of petroleum derivatives contamination. Sinha et al [26] presented, on the example of the earthworm *Eisenia fetida,* high reduction (above 99 %) of highly toxic hydrocarbons (C10-C14, C15-C28 and C29-C36) in soil of vermifilter bed.

But, regarding suitability esterase as biomarkers, it should be also analysed: the duration of exposure, the level of petroleum constituents in the soil in the following years after contamination and remediation process. Impact of petroleum substances on organisms in relation to time and concentration of petroleum derivatives mixtures was presented by Baggi [27], Stroomberg et al [28] and Singh et al [29].

Generally, CarE activity lowered with time of rearing, and in groups reared on control and unleaded petrol contaminated (remediated and unremediated) soil, there were noted significant differences among records done in animals being exposed through two weeks and four weeks. The highest activity of CarE was measured in animals at the start of the experiment (Table 2). This might be connected with lowering of the level of contaminants in the soil (unpublished data). These ubiquitous enzymes, have still not well established physiological functions, with wide range of substrate, apart from their participation in cellular metabolism, are important in detoxifying or inactivating toxins process [30, 31]. Sanchez-Hernandez and Wheelock [25] shown high relevance between level of carboxylesterase in different parts of alimentary tract of *L. terrestris* and in located there ingested soil. Their activity may be important for hydrogen constituents limitation.

In turn, in the case of ChE activity changes it is difficult to describe a general tendency. The changes were specific in each experimental group. When reactions are related to changes with time, general unspecific response of CarE and specific ChE are seen. Some confounding results may be connected with variation of the enzymes activity in specific tissues [15].

We have done measurements of ChE, twice, in the first year of experiment and in the next year, accordingly to time of current soil collection. It was seen a lower enzyme activity after four weeks of rearing on contaminated soils in the first year of exposure in comparison to control (Table 1). In the second year such duration of exposure (four weeks) did not exert impact on the enzyme activity (Table 2). It could be connected with, mentioned earlier in the Introduction part, lowering amounts of hydrocarbons with time.

The remediation process may be effectively augmented by additional input of microorganism into the soil. It supposedly might come to lowering levels of petroleum contamination constituents. Hubalek et al [32] shown that bioremediation process limited negative impact on *Eisenia fetida* development examined after 3 months from

application of hydrocarbons to the soil (around 6 g · kg^{-1}) in the field (such contamination was done with mineral oil, hydraulic fluids and grease). After that period of time, soil contained about 4.5 g hydrocarbons · kg^{-1} of dry weight of soil. But, it is difficult to find any tendency in esterase activity changes in our study which would be connected with such supposing. Moreover, the earthworms responses in control groups having contact with soil treated with remediation cocktail indicated that changes in soil, originated from microbial activity, might be connected with availability of nutrients, tended to changed activity in group C + R in comparison with control group. Simultaneously provided examinations of nitrogen and carbon contents, showed some decline of these elements in soil collected after second week of the earthworms rearing (Kafel, unpublished). Such situations might exert impact on animals condition and these enzymes levels, apart from petroleum contaminants action.

Conclusions

1. CarE activity in *L. terrestris* seems to be a more applicable biomarker of petroleum derivatives contamination soil.
2. Effect of soil contamination with petroleum derivatives (at a dose of 6 g · kg^{-1} of dry soil) on CarE and ChE activity in *L. terrestris* was more pronounced with time of rearing (differences between effects of two or four weeks exposure).
3. Differences in esterases responses were found between animals reared on remediated with "cocktail" of microorganism and unremediated soil.

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WPŁYW ROPOPOCHODNYCH ZANIECZYSZCZEŃ GLEBOWYCH NA AKTYWNOŚĆ ESTERAZOWĄ DŻDŻOWNIC *Lumbricus terrestris*

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Abstract: Spośród biomarkerów ekspozycji szczególnie użyteczne są esterazy, a zwłaszcza karboksyloesterazy (CarE) metabolizujące szeroki zakres ksenobiotyków. Podkreśla się często, że odgrywają one również ważną rolę w funkcjonowaniu organizmów. Co więcej, neurotoksyczne efekty składników substancji ropopochodnych mogą być wywierane również na izoenzymy cholinoesteraz (ChE) u zwierząt. W tym doświadczeniu, w dwutygodniowych interwałach, badaliśmy aktywność esteraz wobec jodku acetylotiocholiny i octanu para-nitrofenylu u dżdżownic *Lumbricus terrestris*, hodowanych przez cztery tygodnie w glebie gliniasto-piaszczystej zanieczyszczonej produktami ropopochodnymi (benzyną bezołowiową, zużytym olejem silnikowym). Takie zanieczyszczenie symulowano w glebie umieszczonej w kontenerach o pojemności jednego m^3 w stężeniu 6 g · kg^{-1} suchej masy gleby (eksperyment prowadzono w Mydlnikach, na przedmieściu Krakowa, 50.0815°N, 19.84730°E). Porównano efekty działania tych zanieczyszczeń w glebie zebranej w roku kontaminacji i rok później. Oceniono także wpływ gleby remediowanej z użyciem „koktajlu" mikroorganizmów i nieremediowanej na hodowane zwierzęta, i sprawdzano u nich zmiany aktywności enzymatycznej, również w dwutygodniowych interwałach. Stwierdzono niższą aktywność ChE u zwierząt hodowanych w glebie skażonej substancjami ropopochodnymi w porównaniu do zwierząt kontrolnych, lecz tylko w przypadku czterotygodniowej hodowli na glebie zebranej w roku kontaminacji. Podobną odpowiedź ChE ustalono u zwierząt hodowanych w glebie skażonej benzyną bezołowiową zebranej rok później, ale w krótszym okresie czasu, już po dwóch tygodniach. Aktywność CarE cechowała się dużą zmiennością w czasie, w porównaniu ze aktywnością AChE. Generalnie, aktywność CarE obniżała się od początku hodowli wraz z upływem czasu (we wszystkich grupach eksperymentalnych). Zwierzęta hodowane w glebie poddanej remediacji w większości przypadków wykazywały się wyższą aktywnością ChE niż te, które były hodowane w glebie nie poddanej remediacji (grupy: kontrolna i eksponowana na zużyty olej silnikowy).

Słowa kluczowe: esterazy, zanieczyszczenia ropopochodne, dżdżownice *Lumbricus terrestris*