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Enchytraeid *Enchytraeus crypticus* as a Test Organism for Crude Oil Contamination of Soil

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It is difficult to estimate the direct impact of crude oil contamination on soil biota at oil mining sites. Human activities such as building, trampling, and flooding by mining water change soil and influence soil biota around mining sites. A possible way for the assessment of crude oil contamination is using of sensitive species and parameters of soil biota as biotests.

Among soil animals, mites, springtails, earthworms, and enchytraeids are susceptible to crude oil contamination (Pirhonen and Huhta 1984; Artem'eva 1989) and earthworm *Eisenia fetida* was recommended as the biotest of crude oil contamination (Saterbak et al. 1999). However, earthworms, who live in soil as in a whole body, are exposed to contaminants in other manner than dwellers of soil pores such as microarthropods or enchytraeids. Enchytraeids, who inhabit a variety of substrates (soils, sediments, organic remains), were suggested as a model test object for organic chemical testing (Römbke 1989; Westheide and Bethge-Beilfuss 1991, Römbke et al. 1998). They were examined also as a biotest of soil contamination by sewage sludge (Achazi et al. 1996) and in the paper we consider them as a biotest of toxicity of soils contaminated by crude oil.

MATERIALS AND METHODS

The enchytraeid *Enchytraeus crypticus* Graefe et Westheide were taken from laboratory culture of Lab. Bioindication of the Institute of Ecology and Evolution of the Russian Academy of Sciences. This species had been used in laboratory examination of toxicity of chemicals and contaminated soils (Römbke et al. 1998). It has short life cycle and breeds and develops well in different substrates as artificial OECD soil (OECD 1984), agar media, soils and sediments at temperatures up to 30^{O} C.

A set of samples of the surface (0-10 cm) layer of podzolic soil was collected at Luginetzky mining field (58^{O} 10'05"N and 78^{O} 53'10"E, Tomsk Region, Russian Federation) around a well and places of accidental oil releases. Some soil parameters and concentrations of contaminants in the samples are given in Table 1. Samples F1 and F2 were taken under flare of the well. Samples P16, P17 and P54 were collected in the places of crude oil accidental releases. F2 and P 54 looked like humus layer of soil. F1 and P16 were similar gley soils due to flooding by water. P17 looked like mineral layer of gley soil.

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Samples were air-dried at 40° C for 1 week and milled in a grain mill. Plastic jars (100 mL) were applied as experimental vessels. Soil (10 g at dry mass basis) was put into a jar and moistened to 60% of maximal WHC. Six replicates for every soil sample were used. For some degraded soils, two parallel sets of jars were prepared. The first set contained the original soil and the other set the soil with 1% $CaCO_3$ added to increase soil pH. The OECD soil (70% of fine sand, 20% of caolinite and 10% of fine ground sphagnum peat) was considered as a reference substrate.

Table 1. Parameters of contaminated soils.

Parameters	OECD	F1	F2	P16	P17	P54
pH $CaCl_2$	5.37	6.35	4.80	4.72	4.69	4.68
pH after adding 1% $CaCO_3$			6.04	5.46	5.48	5.43
LAI%	15.0	5.0	10.2	4.7	8.7	9.9
Max. WHC %	94.1	72.3	127.8	90.1	65.4	108.4
C %		1.84	3.63	1.76	3.1	4.41
N %		0.11	0.27	0.09	0.12	0.28
S %		0.014	0.043	0.013	0.028	0.044
P_2O_5 %		0.13	0.41	0.07	0.11	0.24
Phenols µg/g		0.4	0.6	0.1	1.0	2.0
PAH µg/g		0.4	0.1	0.1	4.0	0.1
TCH mg/g		0.3	0.3	0.4	10	0.4
SAC, µg/g		0	0*	0*	3.5	0*

F1 – near the well; F2 – 1 km from the well; P16 – P54 – accidental releases; LAI – losses after ignition; WHC – water holding capacity; PAH – polycyclic aromatic hydrocarbons; TCH – total crude oil carbohydrates; SAC – surface active compounds. * - matrix prevents determination of SAC although they were in samples

Ten adult specimens were put in every jar and 25 mg of oat flakes was added for food. All jars were covered by plastic food foil to prevent water losses. The jars were kept in an incubator at 20±2° C, 80% relative humidity and 16:8 h of light:dark period during 4 weeks. Water was added every week up to 60% of max. WHC and food was added only the first two weeks. After two weeks, adult animals were removed from all jars to estimate mortality. At the end of the experiment, 10 mL of 96% alcohol were put into every jar and after 10 min some

drops of 1% alcohol solution of Bengal Rose (Acros) dye were added in every jar to stain enchytraeids for enumeration as described by Wim de Coen (Römbke et al. 1998).

Soil pH was determined after 2 h shaking of soil suspension in 0.01 M $CaCl_2$ (1:2.5). Losses of ignition were estimated after 6 h soil ignition at 450^{O}C in a muffle oven. Water holding capacity was determined by weighing water-saturated soil samples before and after 24 drying in an oven at 105^{O}C. Soil carbon, nitrogen and sulfur was determined by CNS analyzer (Carlo Erba 1106) modified for soil samples up to 200 mg, and phosphates were determined by the colorimetric method with ammonium molybdate and tin chloride. Data on soil contamination were put at our disposal by Dr. Eugeny.Voznesensky.

To compare effects on enchytraeids and soil microorganisms, soil respiration was measured in conditioned soil samples. Fifty g of air-dried soil (4 replicates for every soil) was moistened by 25 mL of demineralized water and kept at 20°C for 7 days. After 7 days, 6 g per portion (25 portions per soil) were taken for each of 5 substrates and 2 mL of a substrate were added to a portion. The next substrates were selected: demineralized water, glucose solution 12 g/L, α-ketoglutaric acid solution 2.5 g/L, asparagine solution 2 g/L and Tween 80 suspension 40 g/L according to Degens and Harris (1996). The portions were incubated at 26°C for 1.5 hour. CO_2 evolution was measured in every portion during 5 minute accumulation at 18^{O} C in Van Wensem et al (1992) microcosms by Infra Red Gas Analyser (Type 225 Mk3, Plant Physiology, Analytical Development Co. UK) with Unit (WA 161 Mk3/12, Analytical Development Co. UK).

Descriptive statistics, t-test for independent samples, Pearson, Spearman and Kendall tau rank correlation were applied for data examination by using the statistical package Statistica 5.0 for Windows.

RESULTS AND DISCUSSION

The results of soil testing are shown in Fig. 1-2. Enchytraeids reacted to crude oil contamination of soil, and in the most contaminated point (P17), enchytraeid survival was very low with and without $CaCO_3$ addition. In the case of the less contaminated soil F2 an effect of survival is only visible after $CaCO_3$ addition. There was linear correlation between adult survival and PAH and TCH content in soil (r = -0.95 and -0.93 respectively). Spearman and Kendall tau rank correlations were significant only between survival and PAH content. Enchytraeid survival in OECD soil did not differ from survival in most soils under examinations (except P17).

Reproduction (number of juveniles and juvenile/adult ratio) was influenced possibly by other factors such as pH. It is partially confirmed by addition of $CaCO_3$ to some soils. It increased breeding success in the P17 and F2 soils but not in the P16 soil. Number of juveniles and the ratio in OECD soil was similar with the same parameters in F1 and P54 soils. There was no correlation between reproduction parameters and contaminant content or measured soil characters. Nonetheless, number of juveniles and juvenile/adult ratio correlated linearly with

asparagine-induced respiration. Spearman and Kendall tau rank correlation revealed a connection between breeding success and basal and substrate-induced respiration, except respiration induced by α-ketoglutaric acid.

Effects of crude oil contamination on adult enchytraeids were noticeable at concentration of TCH 10 mg/g of soil and PAH 0,4 μg/g. Such effects on enchytraeids were observed at experimental contamination 6.25 l of light fuel oil per m^2 (Pirhonen, Huhta 1984) and 10 l of crude oil per m^2 (Artem'eva 1989). Earthworms did not reacted on concentration of total petroleum hydrocarbons up to 8.6 mg/g of soil (Salanitro et al 1997).

It is appear that the survival of adult enchytraeids may be used as an index of heavy crude oil contamination of soil because correlated linearly with PAH and TCH content. Similar correlation with total petroleum hydrocarbon content in soil was found for earthworm (*Eisenia fetida*) survival during 14-day test (Saterbak et al. 1999).

Reproduction of enchytraeids is more sensitive to the contamination although there are no correlations with measured parameters of contamination. For earthworms, mortality during 14-day test was the more sensitive index than reproduction due to high variation of cocoon and juvenile production in control soils (Saterbak et al. 1999). Absence of any correlation between enchytraeid breeding parameters and the contamination is possibly connected with short period of maintaining juveniles in contaminated soils or with impact of other crude oil compounds then measured ones or with invisible changes of soil quality under conditions of crude oil mining. Sulfur compounds from flare of the well may impact on young enchytraeids in F2 soil. High phosphorus content in F2 soil may be also responsible for low breeding of enchytraeids. For earthworms, toxicity of petroleum hydrocarbons was higher in soils amended with phosphorus (Knoke et al. 1999). The chemicals were possibly neutralized in soil with neutral pH (F1) but were bioavailable in acid soil (F2). Addition of $CaCO_3$ to F2 soil neutralized them and increased breeding success. $CaCO_3$ has also ameliorating effect in gley soil and due it number of juveniles and ratio juveniles/adult increased in P17 soil after liming. Soil pH was the main factor determined enchytraeid reproduction failure in acid soils contaminated by sewage sludge (Achazi et al. 1996). The high variability of number of juveniles and ratio juveniles/adult in P16 soil might be an indication for the high variability of soil features in the point under the contamination. The observed variability may also be an index of contamination impact at least of low level impact. The standard deviation/mean ratio for number of juveniles in P16 soil is 183.3% while in F2 soil it is 132.7% and for other soils lowers than 86% (minimal for OECD soil – 20.2%). Addition of $CaCO_3$ leads to a decrease of the ratio but it is 105% for P16 soil and lowers than 51% for all other soils. Skewness and kurtosis values also show that there are significant departures from normality for number of juveniles in P16 and P54 soil. Calcium carbonate addition sharply decreased the value for both soils. It also says on influence of crude soil contamination in these soils nonetheless that there are now significant differences between mean values for OECD and P54 soils.

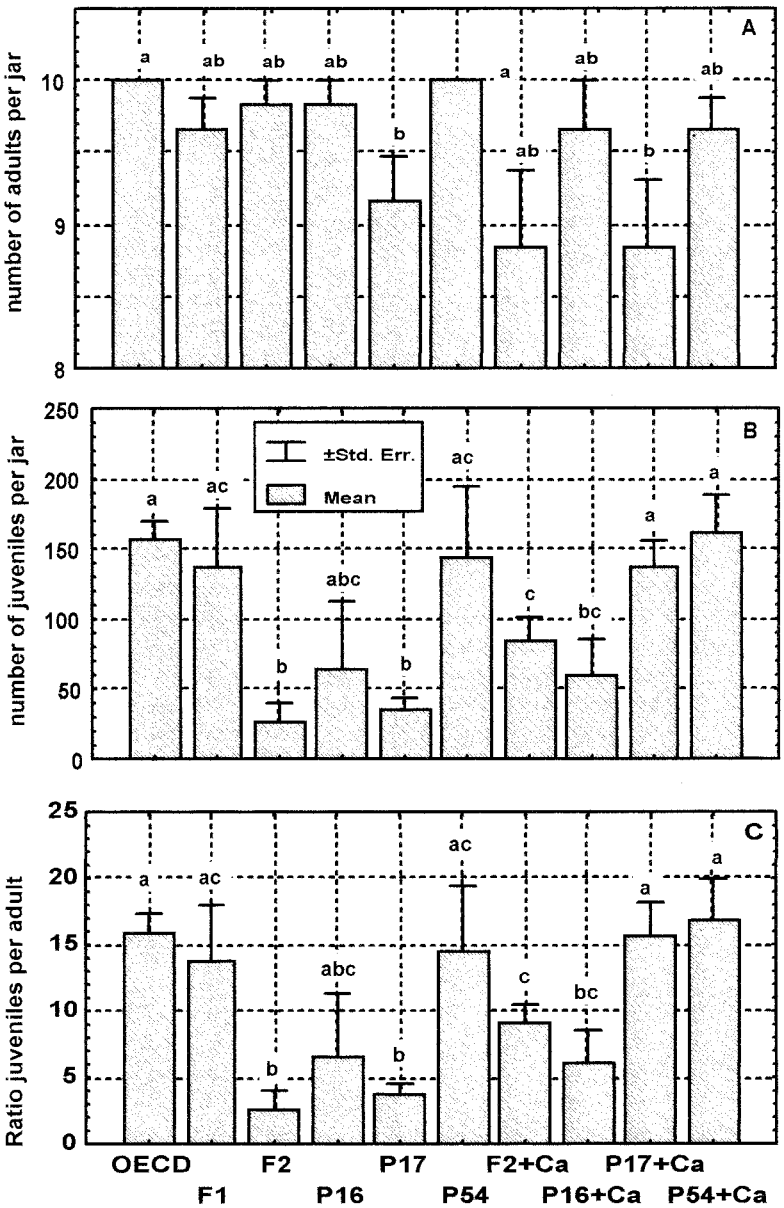

Figure 1. Adult animal survival (A), and breeding (B) of enchytraeids, and ratio juveniles per adult (C) in different soils under study. Letters above bars show differences at 95% level. Number of replicates is 6.

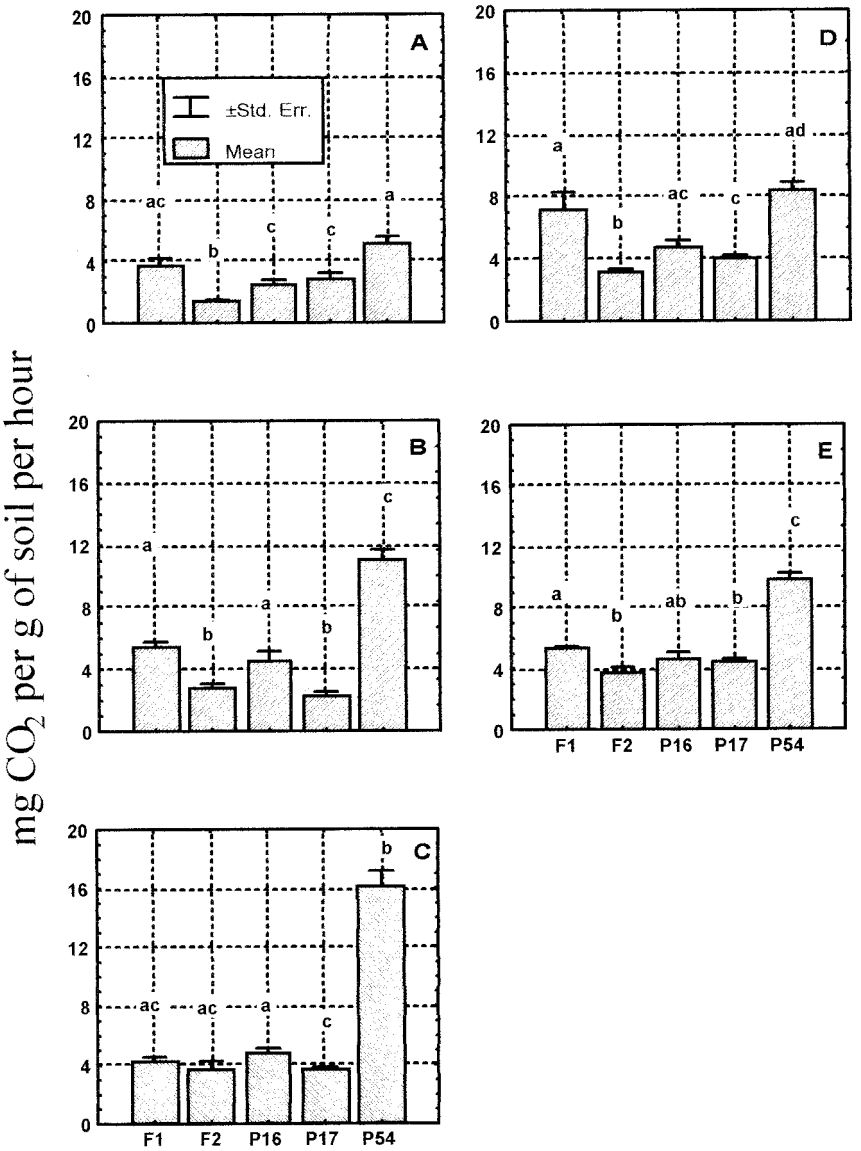

Figure 2. CO_2 production by substrate induced respiration in different soils. A – basal respiration; B – glucose induced; C - α-ketoglutaric acid induced; D – asparagine-induced; E – Tween 80 induced respiration. Letters above bars show differences at 95% level. Number of replicates is 4.

It is more important to note that breeding success correlated with soil respiration, especially with asparagine-induced respiration. It may be a result of independent parallel changes in breeding success of animals and in microbial communities under the contamination, or a result of primary changes in microbial communities under the contamination and following changes in breeding success of enchytraeids, who used microorganisms as food. The first assumption is more realistic because heavy metal contamination does not induce such parallel changes (Pokarzhevskii et al, 1999). Moreover, trends in changes of enchytraeid breeding and substrate-induced respiration under heavy metal contamination were in some extent opposite. It is presumably reflecting differences in availability of toxic compounds in soil. Effects of heavy metals on enchytraeid breeding were dependent on soil organic matter, calcium and phosphorus content (Filimonova et al. 2000). Effects of crude oil on animals were modified by application of $CaCO_3$, however they not depended on soil organic matter. We discussed above effects of $CaCO_3$ addition and it is appear that crude oil compounds may impact on enchytraeids directly while heavy metal contamination through availability of compounds in food. Soil microorganisms have more close relations with toxicants in their environment and hence availability of toxic compounds for them is depended on environment conditions.

The enchytraeid *Enchytraeus crypticus* may be used as a biotest of crude oil contamination of soils, although, only high level of contamination shows obvious depletion in the biotest populations (mortality of adults and decreased breeding success). Low levels of contamination induce a decrease of breeding success or an increase of variability in number of hatchling enchytraeids. The comparison of parameters of enchytraeids in contaminated soils with parameters of substrate-induced respiration exhibits similar reaction of enchytraeid and microbial populations to contamination. It suggests that crude oil contamination affects on enchytraeids and soil microorganisms by similar ways.

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