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Highlighted article

Effects of short-chain chlorinated paraffins on soil organisms

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Abstract

Despite the fact that chlorinated paraffins have been produced in relatively large amounts, and high concentrations have been found in sewage sludge applied to soils, there is little information on their concentrations in soils and the effect on soil organisms. The aim of this study was to investigate the toxicity of chlorinated paraffins in soils. The effects of short-chain chlorinated paraffins (64% chlorine content) on invertebrates (*Eisenia fetida*, *Folsomia candida*, *Enchytraeus albidus*, *Enchytraeus crypticus*, *Caenorhabditis elegans*) and substrate-induced respiration of indigenous microorganisms were studied. Differences were found in the sensitivity of the tested organisms to short-chain chlorinated paraffins. *F. candida* was identified as the most sensitive organism with LC_{50} and EC_{50} values of 5733 and 1230 mg/kg, respectively. Toxicity results were compared with available studies and the predicted no effect concentration (PNEC) of 5.28 mg/kg was estimated for the soil environment, based on our data.

Keywords: Soil invertebrates; Soil microorganisms; Short-chain chlorinated paraffins; Soil pollution; Risk assessment

1. Introduction

Chlorinated paraffins (CPs; chlorinated *n*-alkanes) are industrial chemicals comprising of chlorinated straight chain hydrocarbons. These chemicals have been produced since the 1930s and usually exist as mixtures. They can be subdivided into three main groups according to the length (the number of carbons) of the alkane chain: short (SCCPs; $C_{10–13}$), medium (MCCPs; $C_{14–17}$) and long (LCCPs; $C_{>17}$; European Commission, 1999). Chlorinated paraffins are widely used as additives in metal working fluids, flame retardants in rubbers, additives in paints, coatings, sealants and adhesives. Due to their high chemical and thermal stability, they were used to replace polychlorinated biphenyls in the mid 1980s. In the last decade, the annual world-wide production of chlorinated paraffins was estimated to be 300 ktons (European Commission, 1999). Due to the widespread distribution in the environment and high log K_{ow} values (5–12.6; Tomy et al., 1998), CPs exert a high potential for bioaccumulation, with strong sorption on sewage sludge, soils and sediments and very low mobility.

Levels of chlorinated paraffins in some environmental matrices are relatively well known. Levels in water have been detected within the range 0.05–6 μg/l (Tomy et al., 1998). Concentrations of 0.0005–1000 μg/kg (sampling area remote from an industrialized area) and 1.5–18 μg/kg (near an industrial area) were detected in river sediments (Tomy et al., 1998). Additionally, concentrations of short- and medium-chain chlorinated paraffins of 69–431 mg/kg were found in sediments from the North and Baltic seas (Hüttig and Oehme, 2005). Tomy et al. (1998) summarized the available data on the levels of short- and medium-chain chlorinated paraffins in sewage sludge from industrialized areas (0.76–65 mg/kg). Concentrations up to 93 mg/kg were found in UK sewage sludge from an industrial area by Nicholls et al. (2001). It is well known that the application of sewage sludge to agricultural soils improves soil quality but, on the other hand, it may also increase the concentration of contaminants in soils. However, Nicholls et al. (2001) reported that concentrations of SCCPs in farm soils after the application of digested sewage with high levels of chlorinated paraffins were below the limit of the

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detection (0.1 mg/kg). Nevertheless, detectable amounts (up to 1.7 mg/kg wet wt) were found in earthworms collected in these soils, implying that the bioconcentration of chlorinated paraffins may occur in sewage sludge-treated soils. No other studies are available on chlorinated paraffin levels in soils under sewage sludge application.

Despite a high bioconcentration potential and high levels of chlorinated paraffins in sewage sludge applied to soils, there is limited data on the toxicity of CPs to soil organisms. Hence, in this study, the toxicity of an SCCP mixture (64% chlorine content) was studied using a battery of soil toxicity tests. Effects on survival and reproduction of *Folsomia candida*, *Eisenia fetida*, *Enchytraeus albidus*, *Enchytraeus crypticus*, *Caenorhabditis elegans* and on substrate-induced respiration of microorganisms were determined and compared with the results from Sverdrup et al. (2006). The predicted no effect concentration (PNEC) was characterized according to the European Commission (2003) guideline based on our results and available data from the literature.

2. Materials and methods

2.1. Test organisms

All invertebrates (*F. candida, E. fetida, E. crypticus, E. albidus, and C. elegans*) have been cultured at RECETOX laboratories (Brno, Czech Republic). *F. candida* (Insecta: Collembola) was kept on plaster of Paris and pulverized activated charcoal in ratio of 9:1 (w:w) at 18±2 °C in darkness. Once a week, dry baker's yeasts were added as food. *E. fetida* (Annelida: Oligochaeta) was cultured in the mixture of sphagnum peat and horse manure in the ratio of 1:1 (w:w) that was adjusted to pH of 6–7 with calcium carbonate ($CaCO_3$). The water content was ~80% of the maximum water holding capacity (WHC_{max}). Additional feeding was not necessary. The culture was maintained at 20±2 °C in the dark. *E. crypticus* (Annelida: Oligochaeta) was cultured in OECD artificial soil (OECD, 2000a; 50% of WHC_{max}) and *E. albidus* (Annelida: Oligochaeta) was kept in commercial garden substrate (80% of WHC_{max}; 1% $CaCO_3$). Both species were maintained at 18±2 °C in darkness and were fed with oat flakes weekly. *C. elegans* (Nematoda), wild type strain N2, var. Bristol, culture was kept on NGM agar with bacterial lawn of uracil-deficient strain of *Escherichia coli* (OP50) as a food source and was maintained at 20±2 °C in darkness. The indigenous microbial community of a natural soil was used in microbial test.

2.2. Preparation of soil

OECD artificial soil was prepared for *F. candida, E. fetida, E. albidus* and *E. crypticus* tests. The soil composition was 70% sand, 20% kaolin clay and 10% finely ground sphagnum peat (OECD, 2000a). The organic matter content was 4.7%. The pH_{KCl} was set to 6.0±0.5 with $CaCO_3$ at the beginning of tests and was found to increase to 6.5±0.5 at the end of the tests. The artificial soil was found to be an unsuitable substrate in the *C. elegans* test because the firm peat floated on the surface during the extraction procedure and disabled counting of surviving worms. Hence, the natural soil was used in this test and in the microbial test. This soil was collected from the top layer of a field near Brno (Czech Republic). The soil was a loamy sand cambisol with the following particle size distribution: sand (>50 μm) 64.4%, silt (2–50 μm) 29.1%, clay (<2 μm) 6.5%. The total cation exchange capacity was 20 meq/100 g and the pH_{KCl} was 6.48. Organic carbon and total nitrogen contents were 2.35% and 0.27%, respectively. Organic pollutants and heavy metals contents were comparable to the background levels according to the Czech Republic guideline (Ministry of Environment of Czech Republic, 1996). For *C. elegans* test, the soil was air-dried at room temperature and then sieved (<2 mm), defaunated (deep freezing) and stored under dry conditions and darkness. For the microbial test, the fresh soil was sieved (<2 mm) after sampling and stored in 4 °C in darkness. The water content was adjusted to 50% of WHC_{max} (artificial soil) and ~80% of WHC_{max} (*C. elegans* test). The water decrease in invertebrate tests was checked weekly by weighing and water was replenished if necessary. The water content in the microbial test is described thereinafter.

2.3. Preparation of test substance and spiking procedure

Chlorinated paraffins (labeled as C_{12}, 64% chlorine content by weight; a viscous honey-like liquid) were provided by Novácké závody Inc. (Slovakia) as an industrial product. Based on chemical analysis by SCGC/LRMS-ECNI (HP 5890 Series II gas chromatograph) described in Stejnarova et al. (2005), this mixture included all short-chain paraffin fractions ($C_{10–13}$) with a composition of C_{10} 6%, C_{11} 37%, C_{12} 32% and C_{13} 25%.

Stock solutions were prepared by dissolving paraffins in acetone (HPLC purity, Merck, Czech Republic) for tests with invertebrates and cyclohexane (HPLC purity, Merck, Czech Republic) for tests with microorganisms. Stock solutions corresponding to the highest concentrations were mixed in all tests separately. Dilutions from the stock solution were made using acetone or cyclohexane. Range-finding tests found low SCCP toxicity and the maximum concentration required by the respective guidelines (1000 mg/kg) did not affect most of the organisms. Hence, the tested concentration series in the tests were set from 100 to 10 000 mg/kg. The soil surface in each container was sprinkled with the acetone/cyclohexane solution to reach the appropriate concentration in the soil. Solutions were applied to dry or moist soil (40% of WHC_{max}) for invertebrate or microbial tests, respectively. The volume of solutions corresponded to 10% of soil dry wt in all tests. All containers were kept in the fume hood (24 h) to evaporate the solvent. After the evaporation, soils were mixed thoroughly, distilled water was added at an appropriate volume and soils were mixed again. Pure acetone/cyclohexane controls to check solvent toxicity and water controls (without solvent application) were prepared in each test. Volumes of the chemical in soils were analyzed at the beginning of the test according to Stejnarova et al. (2005) by SCGC/LRMS-ECNI (HP 5890 Series II gas chromatograph) and initial soil concentrations corresponded well to nominal concentrations. All concentrations were expressed on a soil dry weight basis as nominal (initially added) concentrations.

2.4. Folsomia candida test

The test was performed according to ISO 11267 (1999). Each test container contained 30 g dry wt of the soil. Exposure concentrations were 78.125, 156.25, 312.5, 625, 1250, 2500, 5000, 10 000 mg/kg. Five replicates were used for each concentration. Ten synchronized, 10–12 d old, organisms were introduced into each test container. The exposure period was 28 d and containers were maintained at 20 °C in 16/8 light–dark cycle. Dry baker's yeasts were added as food at the beginning of the test and after 14 d. The mortality of adults and number of juveniles were determined at the end of the test. Organisms were extracted by flotation and animals floated on the surface were manually counted.

2.5. Eisenia fetida test

The toxicity test was performed according to OECD guideline 222 (OECD, 2000a). Five exposure concentrations (100, 320, 1000, 3200, 10 000 mg/kg) were used. Three containers (1 l volume) per each concentration with 500 g dry wt of the soil were prepared and 10 adult worms with clitellum (300–400 mg) were introduced into each container. Food (5 g of dry ground horse manure) was added weekly under the soil surface. The survival and reproduction (the number of cocoons) were

evaluated after 4 weeks by manual counting. The test was kept in 20±2 °C and under 16/8 light–dark cycle.

2.6. Enchytraeid tests

Tests with both enchytraeid species were performed according to OECD guideline 220 (OECD, 2000b). Ten and 20 g dry wt of the soil was prepared for tests with *E. crypticus* and *E. albidus*, respectively. Exposure concentrations were equal in both tests (500, 1000, 3000, 6000, 10 000 mg/kg). Five replicates for treatment and eight replicates for the controls were prepared. Ten adult worms with clitellum were added to each container in both tests. Animals were exposed for 42 d (*E. albidus*) and 28 d (*E. crypticus*). Tests were kept in 20±2 °C and under 16/8 light–dark cycle. Approximately 50 mg of dry ground oat flakes per container were added every week. At the end of the exposure periods, the worms were killed by 5 ml of ethanol applied to the soil and dyed by Bengal red. The survival of adults and the number of juveniles were manually counted.

2.7. Caenorhabditis elegans test

The test was performed according to ASTM (2001) guideline. Three replicates with 2.33 g dry wt of the soil were prepared. Five exposure concentrations (500, 1000, 3000, 6000 and 10 000 mg/kg) were used for the test. Ten age-synchronized adults (3–4 d) were introduced into each test container. The test took 48 h and was maintained at 20 °C in darkness. After the exposure period, the adult survival was evaluated by LUDOX extraction and counts of animals were determined under microscope.

2.8. Microbial test

The test was performed according to OECD (1999) guideline draft 217. Ten grams of the soil adjusted to 40% of WHC_{max} were prepared and three replicates were designed for all treated levels. The preincubation was carried out during 4 d. After that, exposure concentrations (500, 1000, 5000, 10 000 mg/kg) were prepared as described above. Water loss after the evaporation was replenished and total water content was adjusted to 60% of WHC_{max}. Bottles with soil were covered by rubber caps and incubated at 22 °C in darkness. Soil samples were analyzed for glucose-induced respiration rate after 14 and 28 d of the incubation. The respiration was measured as CO_2 production within the first 6 h after the addition of glucose (5 mg $C_{glucose}$/g dry wt soil) (ISO 14240-1, 1997). Gas samples (1 ml) were withdrawn from the bottles by a syringe, and CO_2 was quantified by GC with H_2 mobile phase, Porapack Q stationary phase, and thermal conductivity detector.

2.9. Data analysis

All statistical analyses were done with STATISTICA 6.0 software (StatSoft, Inc., 2004). The concentration at which 50% of adult survival was observed (LC_{50}) and 50% effect concentration for the reproductive output (EC_{10} and EC_{50}) were calculated according to Haanstra et al. (1985) using logistic regression analysis. The standard error was used for the expression of the data variation. No observed effect concentrations (NOECs) and lowest effect concentrations (LOECs) were determined by analysis of variance (ANOVA) and Dunnett's procedure at a 5% significance level.

3. Results

Results obtained from all the tests were valid according to the criteria defined in the test guidelines. No significant differences were observed between solvent and water controls (ANOVA; $p<0.05$). No significant mortality of *E. fetida* and *E. albidus* adults (ANOVA; Dunnett's test; $p<0.05$) occurred at any exposure concentration. Short-chain chlorinated paraffins had significant effect (ANOVA; Dunnett's test; $p<0.05$) on *E. crypticus* mortality at the highest exposure concentration (70% survival in comparison with solvent control), however, a dose–response curve could not be established and only NOEC and LOEC values were determined for this endpoint. Dose–response curves of adult mortality were established for *F. candida* and *C. elegans* and LC_{50} values were calculated. The reproduction proved to be a more sensitive endpoint that allowed the creation of dose–response curves, and the calculation of EC_{50} values for all species, except *C. elegans* where the reproduction was not evaluated. EC_{10} values were calculated only for the reproduction of *F. candida* and *E. fetida*. Results of invertebrate tests are summarized in Table 1. Effects on substrate-induced respiration of microorganisms were evaluated after 2 and 4 weeks. This endpoint was not affected at 14 d of the incubation at any exposure concentration. Hence, the highest exposure concentration (10 000 mg/kg) was established as the NOEC value. After 28 d of the incubation, a significant decrease of microbial

Table 1
Summary of toxicity test results of SCCPs (64% chlorine content)

Test organism	Adult survival				Reproduction			
	Test duration	NOEC (mg/kg)	LOEC (mg/kg)	LC_{50} (95% CI)[a] (mg/kg)	NOEC (mg/kg)	LOEC (mg/kg)	EC_{50} (95% CI)[a] (mg/kg)	EC_{10} (95% CI)[a] (mg/kg)
*Folsomia candida**	28 d	1250	2500	5733 (4483–7149)	625	1250	1230 (1009–1451)	660 (341–870)
*Eisenia fetida**	28 d	10 000[b]	ND	ND	1000	3200	2849 (2170–3529)	1158 (336–1980)
*Enchytraeus albidus**	42 d	10 000[b]	ND	ND	3000	6000	6027 (3576–8478)	ND
*Enchytraeus crypticus**	28 d	6000	10 000[b]	ND	6000	10 000[b]	7809 (4381–11 237)	ND
*Caenorhabditis elegans***	48 hrs	1000	3000	8836 (6003–11 668)				

*Artificial soil.
**Loamy sand soil.
a, CI confidence interval; b, the highest tested concentration; ND, could not be estimated.
Estimated values for 50% effect on adult survival (LC_{50}), 10% and 50% effect on reproduction (EC_{10} and EC_{50}) and no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for both endpoints are reported for *F. candida*, *E. fetida*, *E. albidus*, *E. crypticus* and *C. elegans*.

respiration (ANOVA; Dunnett's test; $p<0.05$) was found at the highest exposure concentration (72% of solvent control), and concentrations of 10 000 and 5000 mg/kg were designated as NOEC and LOEC values, respectively. All results and dose–response relationships are presented in Fig. 1.

4. Discussion

4.1. Sensitivity of species

Differences in ecological strategy, life cycle history and/or exposure route make the comparison of organisms difficult. Moreover, experimental designs were not identical for all tests. Since *F. candida*, *E. fetida*, *E. albidus* and *E. crypticus* were tested under similar conditions (artificial soil, moisture content, temperature, test duration, endpoints), results of these tests may be compared. *F. candida* was shown to be the most sensitive organism in both evaluated endpoints. This finding is in accordance with previously published studies (Sverdrup et al., 2002; Lock and Janssen, 2002). The lethality endpoint showed similarly low sensitivity in all *Oligochaeta* tests. However, earthworms were shown to be relatively sensitive to SCCPs when the reproduction was taken as the endpoint. Enchytraeid species are taxonomically close to earthworms and some studies reported similar sensitivity of these species (Römbke and Moser, 2002). However, results obtained in our study are in accordance with other studies reporting lower sensitivity of enchytraeid species to some chemicals (e.g. PAHs and mercury reported by Sverdrup et al., 2002 and Lock and Janssen, 2001). Within this group

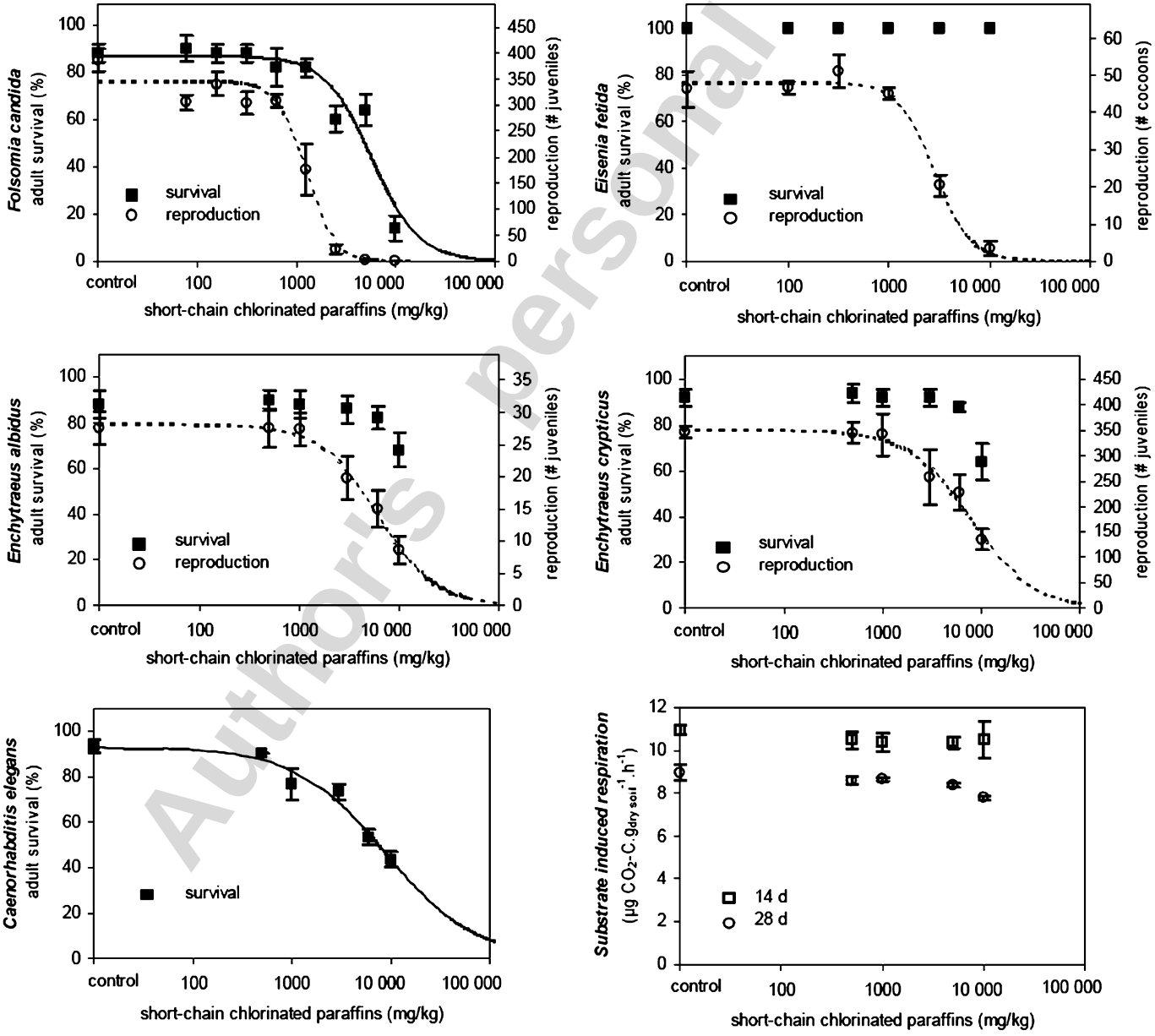

Fig. 1.

of tests with similar test conditions, we can determine the following order of species sensitivity to SCCPs ranked from the most to least sensitive: *F. candida* > *E. fetida* > *E. albidus* ~*E. crypticus*.

Nematoda *C. elegans* was not compared to other organisms with regard to different test conditions. Despite this fact, *C. elegans* values of the NOEC and LC_{50} did not differ from others markedly. Substrate-induced respiration of microorganisms was relatively insensitive to SCCP toxicity. This endpoint describes the activity of whole soil microbial community as a "black box" and possible changes in the structure of microbial community induced by contaminant might be not recognized. Hence, other parameters (e.g. nitrification; van Beelen and Doelman, 1997; Sverdrup et al., 2006) describing a sensitive part of the microbial community, should be selected for the toxicity testing.

Considering all of these facts, *F. candida* may be recommended for the toxicity testing of organic chemicals for its high sensitivity in both endpoints and low test demands. The reproduction of *E. fetida* is also sensitive in our study and may be recommended. However, the low sensitivity of the mortality of adults as well as the high test demands make this organism less suitable. Other organisms proved the low sensitivity and high data variability in our study. However, since other chemicals may differ from SCCPs in bioavailability, exposure routes, mechanisms of action, etc., this ranking of sensitivity of organisms cannot be generalized for study of other chemicals. Therefore, a battery of organisms should be used for testing and obtaining data for risk assessment as recommended in many studies (e.g. Cortet et al., 1999; Bierkens et al., 1998; Hund and Traunspurger, 1994).

4.2. Comparison with available studies

Whereas information on the toxicity of SCCPs to aquatic invertebrates and fish were well gathered in the Risk Assessment Report (European Commission, 1999) and effects on mammals are also known (Duncan et al., 1980), information on toxicity in soils is limited. Sverdrup et al. (2006) published data on effects of SCCPs ($C_{10–13}$; 60% chlorine content) on nitrifying microorganisms, *E. crypticus* and red clovers (*Trifolium pratense*). The test was carried out in agricultural soil and exposure levels did not exceed the concentration of 1000 mg/kg. Neither enchytraeid reproduction nor weight of red clover seeds were affected by these concentrations, which corresponded with our results. For nitrifying microorganisms EC_{10} was determined as 570 mg/kg. This value is similar to the EC_{10} value (660 mg/kg) for *F. candida* reproduction in our study, nevertheless very far from 10 000 mg/kg which caused only cca 10% inhibition of substrate induced respiration in our study.

In another study (Thomson et al., 2001), effects of MCCPs ($C_{14–17}$; 54% chlorine content) to *E. fetida* were determined using the artificial soil test. The adult mortality, effects on the growth and reproduction (the number of juveniles) was evaluated with NOEC values of 3200, 1000 and 320 mg/kg (nominal concentrations), respectively. Identical concentrations were used in our study to make the comparison of results possible. MCCPs proved the higher toxicity to *E. fetida* mortality. The reproduction results cannot be compared to data from our study due to differences in evaluated reproduction endpoints (numbers of juveniles and cocoons). Since SCCPs are generally regarded to be more toxic than those with medium- or long-chains (Tomy et al., 1998), it was surprising that the effect of SCCPs to *E. fetida* mortality gathered in our study was lower than effect of MCCPs reported by Thomson et al. (2001). This supports our above-discussed opinion concerning low sensitivity of this endpoint in our work.

4.3. Ecological risk assessment

Ecological risk assessment of SCCPs was elaborated by European Union (European Commission, 1999). The significant risk for the aquatic environment was indicated for some local sources (European Commission, 1999). This evaluation was not done for the terrestrial compartment due to limited knowledge on SCCPs in soils. In that report, the PNEC value for terrestrial compartment was suggested to be 0.8 mg/kg based on the equilibrium partitioning model and levels in soils were modeled using the EUSES model. Recently, Sverdrup et al. (2006) estimated the PNEC as 57 mg/kg where the EC_{10} value for effect on nitrifying microorganisms (570 mg/kg) and assessment factor of 10 (three trophic level toxicity data) according to guideline European Commission (2003) were used for PNEC calculation. In our study, the EC_{10} value of *F. candida* reproduction (660 mg/kg) was applied to the PNEC calculation. Data was extrapolated according to guideline (European Commission, 2003) to standard soil organic carbon content (2%; in the present study, soil organic carbon in *F. candida* test was 5%) and assessment factor of 50 were used (for long-term toxicity tests of two trophic levels). After data extrapolation, the final PNEC value of 5.28 mg/kg was estimated based on our data. This study together with that of Sverdrup et al. (2006) provided PNEC values based on a large data set including three trophic levels. However, more information on environmental levels, bioavailability and fate in the environment should be found to estimate the risk for terrestrial environment.

5. Conclusion

Within the test battery, *F. candida* proved to be the most sensitive organisms to SCCPs (64% chlorine content). Estimations of EC_{10}, EC_{50} and LC_{50} values for all tested organisms were in the concentration range of 600–9000 mg/kg. The lowest EC_{10} value of 660 mg/kg was found for the *F. candida* reproduction. The least sensitive was substrate-induced respiration of soil microorganisms. The compar-

ison of our data with available studies on CPs showed relatively similar results, despite the difficulty of an inter-laboratory comparison. The PNEC for soil organisms was estimated based on our data with resulting value of 5.28 mg/kg for SCCPs (64% chlorine content) but the risk cannot be evaluated because a reliable PEC is missing.

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