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Impact of Biotransformation and Bioavailability on the Toxicity of the Insecticides α-Cypermethrin and Chlorfenvinphos in Earthworm

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Knowledge about the bioavailability and metabolism of pesticides in soil organisms facilitates interpretation of its toxicity in soil. The present study relates uptake kinetics and metabolism of two insecticides, the pyrethroid α-cypermethrin (α-CYP) and the organophosphate chlorfenvinphos (CFVP), in the earthworm *Eisenia fetida* to their lethal and sublethal toxicity. Experiments were conducted in two soils with different organic matter contents to provide media with contrasting sorption capacity for the insecticides. The results showed that organophosphate CFVP was, when taken up by earthworms, rapidly and irreversibly bound to biomolecules and the fraction of extractable parent insecticide and metabolites was low. In contrast, α-CYP was rapidly metabolized by earthworms but did not form conjugates. It seems that the phase II metabolism of α-CYP is inhibited in earthworms, resulting in an increasing accumulation of its metabolites. Instantaneous binding of non-altered CFVP to the target site presumably resulted in a higher toxicity compared to α-CYP and explains the small difference between lethal and reproduction toxicity. For α-CYP, however, accumulation of α-CYP metabolites in earthworms during chronic exposure may explain the large observed difference between lethal and sublethal toxicity. Bioaccumulation and toxicity of either insecticide decreased with increasing organic matter content in soil, emphasizing the role of compound sorption on bioavailability and toxicity for soil organisms.

KEYWORDS: Metabolism; pyrethroid; organophosphate; reproduction; lethality; soil

INTRODUCTION

Both organophosphorus insecticides and synthetic pyrethroids are used to control insect pests. They are not only used as pesticides in agriculture but also as veterinary pharmaceuticals to treat ectoparastic diseases, as repellents, and in household products. During the last 2 decades, the use of synthetic pyrethroids has increased at the expense of organophosphorus insecticides for certain crops, such as cotton (*1*−*3*). Organophosphorus insecticides tend to be more toxic to mammals than the pyrethroids (*2, 4*), often explained by the effective and rapid biotransformation of pyrethroids in mammals (*5*). Nevertheless, both classes of insecticides are extremely toxic for aquatic invertebrates and fish. For cypermethrin, one of the most potent pyrethroids, Solomon et al. (*6*) showed that the median lethal concentration (LC_{50}) for the 10% most sensitive aquatic species was lower than 10 ng L^{-1}, whereas the respective value for the organophosphate chlorfenvinphos is approximately 30 μg L^{-1} [on the basis of data from the PAN pesticide database (http://www.pesticideinfo.org/)]. Pesticides are applied to agricultural soils, and terrestrial ecosystems are thus more directly exposed to these chemicals than are aquatic ecosystems. Furthermore, hydrophobic pesticides, such as the above-mentioned compounds, sorb strongly to solid phases and are predominantly found in upper soil layers and sediments and not in the water (*7*). Sensitivity of soil organisms to many organophosphorus insecticides and pyrethroids is usually described in terms of acute toxicity because acute toxicity data are sufficient to fulfill the requirements for pesticide authorization. Data on sublethal toxicity are scarce, although sublethal effects on fertility, together with long-term survival, are both important factors for the population growth and maintenance (*8*). Hence, the lack of information about reproduction toxicity makes it difficult to assess the effect of a pesticide for populations of soil organisms.

Toxicity is strongly influenced by the bioavailability of the compound in question. In soil, bioavailability depends upon two factors: physicochemically driven desorption processes, where a compound is released from soil into the aqueous phase, and a physiologically driven uptake process, where the compound is absorbed from the aqueous phase into the organism.

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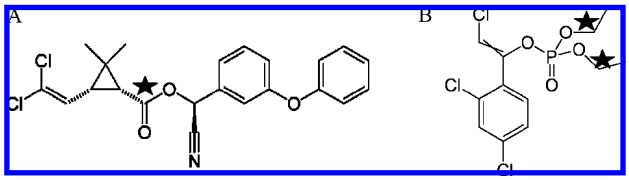

Figure 1. Molecular structure of (A) α-CYP and (B) CFVP. Labeling sites are marked with black stars.

Once taken up, toxicity depends upon the fate of the compound inside the organism, including processes such as the transport of the compound to sensitive organs/tissues, biotransformation, and excretion. These processes may also explain variability in sensitivity between species (*9*). Metabolites may have different toxicity and different half-lives in organisms and may contribute to the toxicity of a compound. To interpret toxicity, it is thus necessary to describe the dynamics of uptake and transformation of the compound and correlate these observations with observed toxic effects. Little is known about metabolism of pyrethroids and organophosphates in soil organisms, and knowledge of the metabolic fate of these compounds is scarce, although it is crucial for understanding their toxicity.

Data on lethal and sublethal toxicity of chlorfenvinphos to earthworms are not available in the literature, and for α-cypermethrin, the difference between lethal and sublethal toxicity appears to be large (*9*). This might be due to metabolism and the formation of a toxic metabolite during chronic exposure. The sorption-related aspects of bioavailability is mainly described in a companion paper (*10*), while the present paper investigates uptake, fate, and toxicity of the compounds in the earthworm *Eisenia fetida*.

The principal objective of this study was to relate the uptake and biotransformation of α-CYP and CFVP in earthworms to the toxic effects that these compounds exert. By following uptake and metabolism of radiolabeled compounds in earthworms during a prolonged exposure period, we intended to detect and quantify intermediate metabolites and relate toxicity to concentrations of parent and transformed compounds. In addition, the influence of bioavailability was assessed by using different soil types with different binding properties.

MATERIALS AND METHODS

Chemicals. Radiolabeled α-CYP (α-cyano-3 phenoxybenzyl-(1*R*)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) and CFVP (*O*,*O*-diethyl-*O*-1-(2′,4′-dichloro-phenyl)-2-chlorovinyl-phosphate) was purchased from the Institute of Isotopes, Budapest, Hungary, and had a specific activity of 35.5 and 8.3 kBq μmol^{-1}, respectively. α-CYP was labeled at the acid moiety, and CFVP was labeled at the ethyl group of the molecule (**Figure 1**). Unlabeled α-CYP (technical-grade, 97.6% purity) was provided by Inter-Trade Denmark (Bindslev, Denmark). CFVP (technical grade, 94% purity), was provided by BASF (Wädenswil/Au, Switzerland). Acetone, 2-propanol, and cyclohexane (all Merck LiChroSolv with a purity >99.7%) were purchased from VWR Norway (Oslo, Norway). Sample oxidizer cocktails (Carbosorb-E, Permaflour-E, and Combustaid), liquid scintillation cocktail Optima Gold, and oxidizer sample trays were obtained from Perkin-Elmer (Hvidovre, Denmark).

Soils and Organisms. The experiments were performed with two different soils: the A_h-horizon of a forest soil from Steinskogen, Norway (59° 55′ N, 10° 31′ E) and the A_p-horizon of an agricultural soil from Askov, Denmark (55° 28′ N, 09° 07′ E). The soils were dried at 40 °C for 48 h, sieved (<2 mm), and stored at 4 °C until used. Selected soil properties are listed in **Table 1**.

Adult earthworms (*E. fetida*) with a body weight >350 mg and a well-developed clitellum were used in the experiments. Prior to the experiments, the earthworms were washed with tap water and placed on wet filter paper for 24 h to purge their guts.

Table 1. Physical−Chemical Properties of the Test Soils

	Askov	Steinskogen
soil type	sandy loam	sandy loam
organic carbon (%)	1.39	5.5
pH	6.2	5.5
sand (%)	68.4	54.2
silt (%)	18.8	36.2
clay (%)	10.4	9.6
CEC (meq kg^{-1})	106	
C/N ratio		21.8
water holding capacity (%)	33.8	44.0

Uptake Experiments. Glass jars (1 L) were filled with 400 g of soil (dw) and individually spiked with a mixture of ^{14}C-labeled and unlabeled compounds. Solutions of labeled and unlabeled compounds were prepared in acetone to achieve final soil concentrations of 5, 20, and 100 mg kg^{-1} α-CYP or 0.4, 1.6, 8.0, and 16 mg kg^{-1} CFVP. ^{14}C acitivity was 555 and 333 kBq kg^{-1} for α-CYP and CFVP, respectively. A total of 10 replicates were prepared for each concentration. The soils were thoroughly mixed with a spatula and left overnight for solvent evaporation under a fume hood before water was added, to achieve a water content in soil corresponding to 60% of their respective water holding capacity.

The following day, 10 preweighed earthworms were added to each of the 10 replicates and fed with up to 5 g (air-dried) of grounded cow manure once a week. The amount of manure was dependent upon the feeding activity of the worms. The single replicates were sampled, and earthworms were recaptured after 1, 2, 3, 4, 5, 7, 9, 12, 19, and 28 days of exposure. For the uptake experiment with α-CYP in Askov soil, uptake was also measured after 50 days of exposure by omitting sampling after 12 days. During sampling, earthworms were placed on wet filter paper for 24 h for purging, weighed, and immediately frozen at −20 °C. Soil from the harvested jars was air-dried and mixed thoroughly before a representative soil sample was collected and frozen at −20 °C for further analyses of radioactivity and degradation products. The relative accumulation of pesticides in soil was expressed as the biota-to-soil-accumulation factor (BSAF) and was calculated as the ratio of total ^{14}C activity in earthworms to the total ^{14}C activity in soil. Subsamples were taken to ensure homogeneity of the distribution of the radiolabeled compound in the soil. The activities varied on average 3 and 5% for the treatments with α-CYP and CFVP, respectively.

^{14}C Measurements. Concentrations of the compounds in soil and earthworms were measured as ^{14}C activity. The total amount of pesticides (both extractable and residual) in soil and earthworms was measured in triplicate by combusting single earthworms (300−500 mg in weight) or 0.5 g of soil in a sample oxidizer, Packard-model 307 (Berkshire, U.K.). Produced $^{14}CO_2$ was captured in the scintillation cocktails Carbosorb-E and Permaflour-E, and ^{14}C activity was measured by liquid scintillation counting (LSC; Tri-Carb 2300TR liquid scintillation counter, Canberra Packard, Zellik, Belgium). Pesticide concentrations in the soils were determined by multiplying the ratio of unlabeled compound and ^{14}C-labeled compound with the ^{14}C acitivity in soils or earthworms. For the uptake studies, accumulated radioactivity is expressed as μg g^{-1} equivalent of α-CYP or CFVP.

Chemical Analyses. To identify parent compounds, metabolites, and bound residues, further studies were conducted for the treatments with 20 mg kg^{-1} α-CYP or 8 mg kg^{-1} CFVP. For this purpose, soils and earthworms were exhaustively extracted and the extracts were further fractionated on a high-performance liquid chromatograph (HPLC).

Soils were extracted by shaking 10 g of soil with 20 mL of cyclohexane and 20 mL of acetone on a horizontal shaker (150 rpm, 24 h), adding 30 mL of demineralized water, and continue shaking for another 15 min. Phase separation was achieved by centrifugation at 670*g* for 5 min, and the cyclohexane phase was then collected for further analyses. The procedure performed after the addition of water was repeated with another 20 mL of cyclohexane. The two cyclohexane phases were combined, and the volume was reduced to 3−8 mL under a flow of nitrogen. This extraction procedure was compared to Soxtec extraction using a 60 mL cyclohexane/acetone mixture (1:1) and a boiling period of 1.5 h, followed by a rinsing period of 1.5 h. For CFVP,

Table 2. ^{14}C Distribution (Percentage of Initially Added Compound) in Askov and Steinskogen Soils Spiked with 20 mg kg^{-1} α-CYP or 8 mg kg^{-1} CFVP during 28 Days of Incubation[a]

	soil	parent compound (%)	metabolite (%)	parent compound plus metabolite (%)	non-extractable residues (%)
α-cypermethrin	Askov	83.6 ± 3.0	6.7 ± 1.3	90.0 ± 2.4	9.9 ± 2.0
	Steinskogen	84.1 ± 2.3	5.1 ± 2.3	88.7 ± 1.5	11.0 ± 2.0
chlorfenvinphos	Askov	94.7 ± 0.8	1.5 ± 0.5	97.1 ± 0.3	3.6 ± 0.8
	Steinskogen	95.6 ± 1.1	1.3 ± 1.0	95.5 ± 0.5	3.4 ± 0.5

[a] Additionally, soils with different α-CYP concentrations (5 and 100 mg kg^{-1}) or CFVF concentrations (0.4, 1.6, and 20 mg kg^{-1}) were included. Results are presented as means ± SD in a percentage of the total ^{14}C activity.

the extraction efficiencies were similar for both methods. For α-CYP, Soxtec extraction was ≤10% more efficient than the shaking method, which was used subsequently. The ratio of parent compound to degradation products was not expected to vary significantly between the two methods.

To extract the recalcitrant fractions of the pesticides, the extracted soil was extracted a second time after alkaline hydrolysis. A total of 50 mL of methanolic KOH (1:9, 3 M KOH/methanol) was added to the extracted soil, and the suspension was shaken in a water bath at 70 °C for 2 h. Thereafter, the suspension was centrifuged at 670*g* for 5 min, and the supernatant was decanted. After washing with 20 mL of methanolic KOH and centrifugation, the soil was dried at 105 °C and an aliquot was analyzed with the sample oxidizer, as described above.

Pesticides were extracted from earthworm samples using Soxtec extraction. Because of small amounts of sample material, HPLC fractionation of pesticides in earthworms was performed on pooled samples. Technical problems during extraction of CFVP and metabolites from earthworms in Askov soil prevented fractionation for all exposure times. Before extraction, the worms were homogenized with an Ultra turrax T25 (IKA, Staufen, Germany). About 1 g of homogenized tissue was mixed with 4 g of waterfree Na_2SO_4 and extracted with 60 mL of acetone/cyclohexane (1:1) using a Soxtec Avanti 2050 (Tecator AB, Höganäs, Sweden), with a boiling cycle of 2.5 h and a rinsing cycle of 1.5 h. The extraction temperature was 195 °C. The extract was reduced to 3−5 mL under a stream of nitrogen.

To measure the amount of hydrolyzable conjugates, the extracted worm tissue was re-extracted by saponification in 50 mL of methanolic KOH at 235 °C for 1 h using the Soxtec extractor.

Fractionation of Soil and Earthworm Extracts. Fractionation of the acetone/hexane extracts of soil and earthworms were carried out by preparative HPLC, using a Beckman System Gold HPLC (Beckman, Fullerton, CA), with an analogue integrator module 406, two DE126 pumps, a DE268 UV detector, and a LKB SuperFrac fraction collector. A total of 1 mL of the extract was manually injected onto a 10 × 250 mm LiChrospher Si 60 column with 10 μm particle size (Merck, Darmstadt, Germany). The separation was performed at a constant flow rate of 5 mL min^{-1}, with cyclohexane and 2-propanol as mobile phases. Solvents and gradient of mobile phases were selected to give a satisfying separation of α-CYP and its predominant degradation products, as well as CFVP and its main degradation products.

For α-CYP, the best separation was achieved with cyclohexane as a mobile phase during the first 6 min, followed by a linear gradient to 2-propanol within 7 min. For CFVP, cyclohexane was used as a mobile phase during the first 5 min, followed by a linear gradient to 2-propanol within 6 min. The eluate was monitored with a UV detector at 254 nm. Fractions were collected in 24 mL scintillation vials containing 8 mL of Ultima Gold scintillation cocktail, at a rate of one fraction per 1.5 min during a 16.5 min run for soil extracts and a 21 min run for earthworm samples. In some cases, fractions was collected every 3 min. The resulting fractions were measured using liquid scintillation counting.

Elution times of parent compounds and known metabolites were established from separate HPLC runs with only one compound at a time.

Toxicity Tests. Lethal and sublethal toxicity of α-CYP and CFVP was measured in both test soils according to an adapted version of the standardized OECD guideline 222 (*11*). Adult mortality and cocoon production after 28 days of experimentation were used as end points.

The toxicity test was conducted with two replicates and nine concentrations between 2.5 and 1000 mg kg^{-1} dw for α-CYP and 0.4 and 250 mg kg^{-1} dw for CFVP. For the controls, eight replicates were used. This experimental design was chosen to determine lethal and effective concentrations (LC_{50} and EC_x) as recommended in the guideline. The concentrations in soil were measured by a gas chromatography−electron capture detector (GC−ECD) according to Hartnik et al. (*9*) and deviated less than 15% from nominal concentrations.

The software GraphPad Prism (version 4.0; San Diego, CA) was used to perform nonlinear regression analysis of the dose−response functions. For the sublethal end points, the 10 and 50% effective concentration (EC_{10} and EC_{50}) were calculated using normal sigmoid functions. For acute toxicity, the 50% lethal concentration (LC_{50}) was determined.

RESULTS

Mass Balances. The mass balance conducted for soil showed that summed ^{14}C activities of the acetone/cyclohexane extract, the saponification extract, and the non-extractable residue accounted for 99.9 ± 4.3% of the total activity in soil that was measured by combustion. Mass balances for worms were not performed because of the high amounts of sodium sulfate used in the extractions, which made it difficult to quantify the non-extractable residues in extracted worms.

For worms, the HPLC fractionation of the acetone/cyclohexane extracts had a mean [±standard deviation (SD)] recovery of 95.8 ± 5.9% of the total activity for α-CYP and 78.5 ± 26.8% for CFVP. The lower recoveries in the case of CFVP might be due to highly polar metabolites that are not collected during fractionation. However, extensive elution for 40 min, corresponding to a 200 mL mobile phase did not result in higher recoveries (results not shown).

Fate of the Test Compounds in Soil. For both pesticides, no significant reduction in ^{14}C activity was measured in either soil for any test concentration during 28 days of incubation. This demonstrates that neither of the two pesticides were mineralized to CO_2 during this period. **Table 2** shows the distribution of ^{14}C activity for α-CYP, CFVP, and their metabolites in the Askov and Steinskogen soils for all 10 sampling times up to 28 days. The activity of extractable parent compound, metabolites, and non-extractable residues did not change considerably over the experimental period, and no significant difference was observed between the soils. This indicates that little parent compound was transformed in soil. For α-CYP, the parent compound accounted for approximately 84% of the activity originally added to the soil, whereas metabolites accounted for approximately 6%. The remaining 10% was non-extractable residues. For CFVP, ca. 95% of the total activity was parent compound, only 1.5% was metabolites, and the remaining 3.5% was non-extractable residues.

Uptake in Earthworms. The accumulation of α-CYP and CFVP in earthworms during exposure is shown in **Figure 2**.

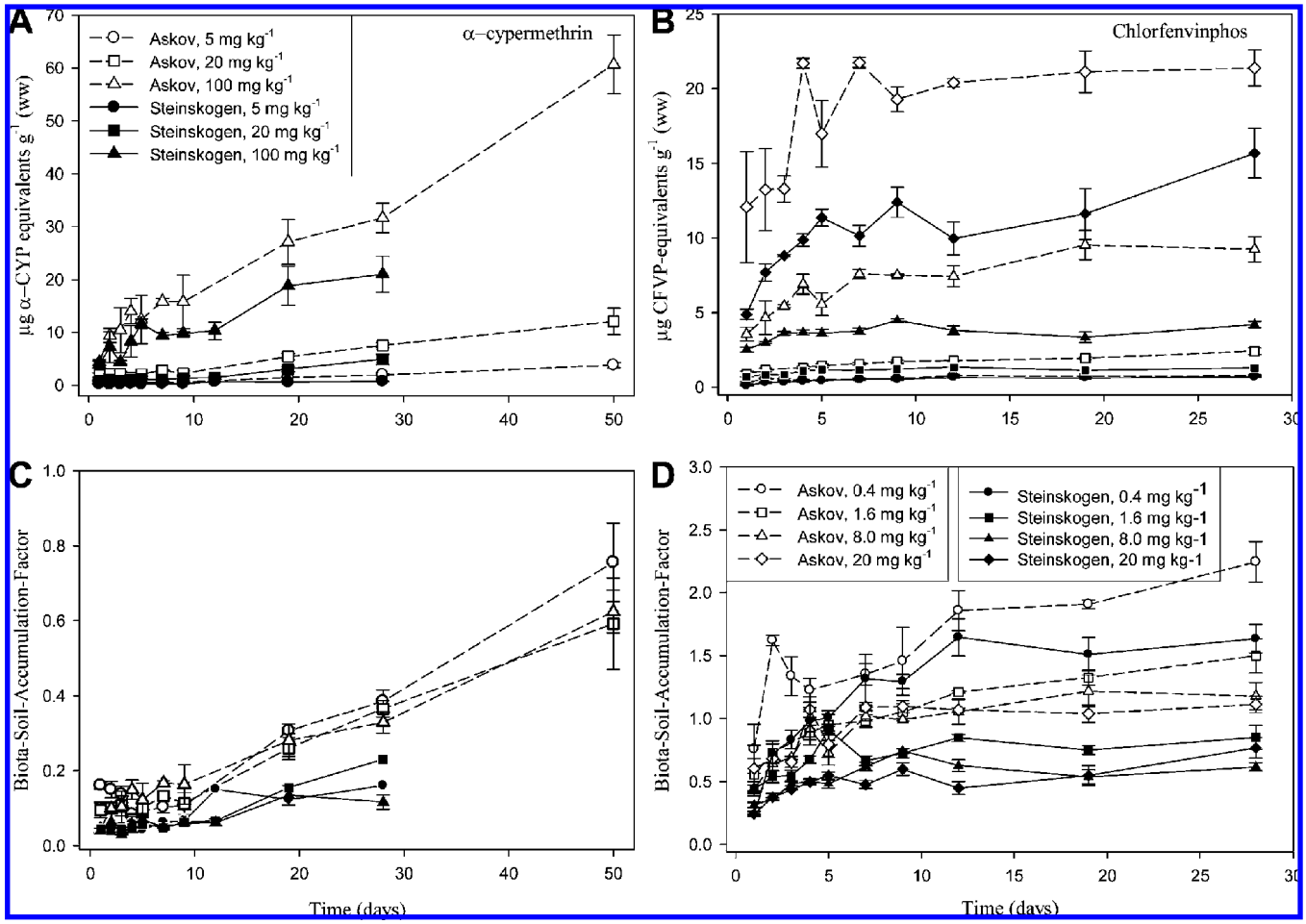

Figure 2. ^{14}C residues in earthworms during the experimental period of up to 50 days. Graphs A and B show the accumulated concentration of the two compounds (μg of pesticide equivalents g^{-1}, ww) in earthworms, and graphs C and D show the BSAF for α-CYP (A and C) and CFVP (B and D), respectively.

The uptake of both compounds increased with increasing concentrations in soil. For α-CYP, the ^{14}C activity in earthworms increased during the whole exposure period of 28 days (for Steinskogen soil) or 50 days (for Askov soil) without reaching a steady state. For CFVP, ^{14}C activity in earthworms increased with increasing exposure time and reached a steady state after 5 days. Thereafter, ^{14}C activity did not change significantly with increasing exposure time. In all cases, the accumulation of α-CYP and CFVP was significantly higher in Askov soil than in Steinskogen soil ($p < 0.001$). Biota-to-soil accumulation factors were almost a factor 3 and 2 higher in Askov soil than in Steinskogen soil for α-CYP and CFVP, respectively. Interestingly, the biota-to-soil accumulation factors of both chemicals showed an increasing trend with decreasing soil concentrations. This trend was weak and non-significant for α-CYP but significant for CFVP.

Metabolism in Earthworms. Figure 3 shows the HPLC elution profiles for treatments with 20 mg kg^{-1} α-CYP and 8 mg kg^{-1} CFVP and their main metabolites in earthworms as a function of exposure time. Elution profiles for Steinskogen and Askov soils were similar (results for Askov soil not shown).

For α-CYP, the acetone/hexane extracts contained 84 ± 12% (mean ± SD) of the total activity in the earthworms. Three main peaks were observed in the chromatograms. The first peak was α-CYP, with a retention time (RT) of 3.8 min. The other two peaks were metabolites derived from the breakdown of α-CYP. One of them with a RT of 7.8 min could not be identified. The other was 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCVA) with a RT of 11.2 min. The figure demonstrates a large fraction of α-CYP and unidentified metabolite during the first 1−5 days of exposure, which gradually decreased during the following 24 days. In contrast, the concentration of the second metabolite, DCVA, was low during the first 5 days of exposure but steadily increased during the experiment, constituting more than 30% of the total activity after 28 days of exposure.

For CFVP, the acetone/hexane extractable fraction constituted considerably less than for α-CYP and contained only 7 ± 4% of the total activity in earthworms. In the chromatograms for CFVP, two main peaks were found. The first compound with a RT of 9.9 min was CFVP. The other, which was eluted already after 6.6 min, could not be identified. Throughout the experiment, most activity was found in the parent compound. The unidentified metabolite constituted approximately 25% of the total activity in the earthworms after 15 days of exposure and onward.

The ^{14}C activity of non-extractable residues, hydrolyzable conjugates, parent compound, and metabolites (Bq g^{-1}, ww) as a function of the exposure time is shown in **Figure 4**. A comparison of metabolic patterns in earthworm from Askov and Steinskogen soils did not reveal distinct differences in metabolism in the two soils.

For α-CYP, the activity of the parent compound and conjugates remained low during the entire experiment. However, the metabolites derived from the breakdown of α-CYP and the non-extractable residues increased during the entire experiment,

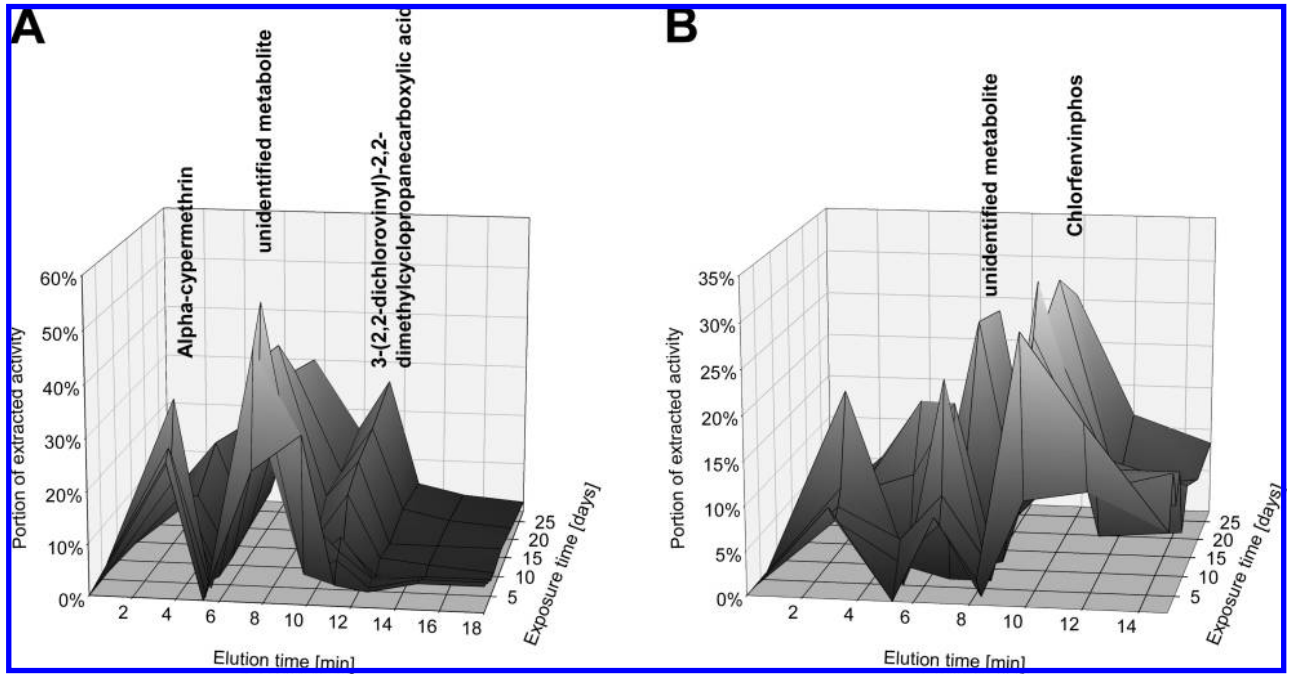

Figure 3. HPLC elution profiles of hexane/acetone extracts of earthworms exposed to (A) α-CYP and (B) CFVP in Steinskogen soil. The horizontal axis shows retention time (min), and the vertical axis shows exposure time of earthworms in soil (days). The color corresponds to the magnitude of the fraction (percentage of the total ^{14}C activity).

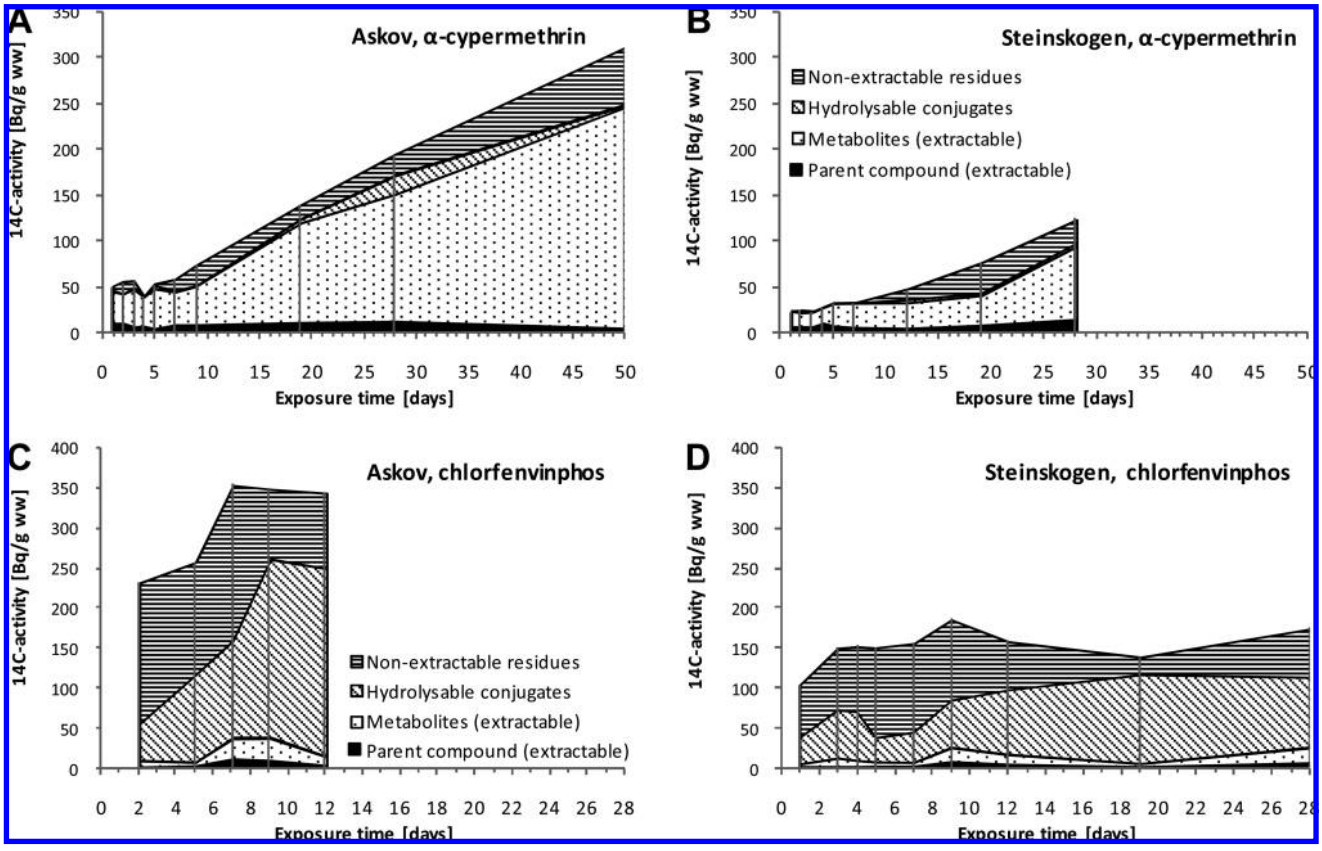

Figure 4. Distribution of ^{14}C activity in earthworms in (A and C) Askov soil and (B and D) Steinskogen soil during the experimental period. Parent compound, metabolites, hydrolyzable conjugates, and non-extractable residues for earthworms exposed to (A and B) α-CYP and (C and D) CFVP.

indicating that α-CYP is easily metabolized and degraded by the worms. The percentage of parent compound in earthworm from either soil was on an average 20% of the activity in earthworm at the start of the experiment and decreased to approximately 10% after 28 days. The fraction of extractable metabolites was about 70% for both soils, with a slight trend to increase with increasing exposure time. The non-extractable fraction varied considerably in earthworms from both soils (between 0 and 40% of activity in earthworms), with a tendency to increase with increasing exposure time.

A completely different pattern was observed for CFVP. The activity of extractable metabolites remained low throughout the

Table 3. EC_{10}, EC_{50}, and NOEC Values for *E. fetida* Reproduction (Number of Cocoons Produced) and LC_{50} Values for Adult *E. fetida* in Askov and Steinskogen Soils Spiked with α-CYP and CFVP[a]

	LC_{50} (mg kg^{-1})		EC_{50} (mg kg^{-1})		EC_{10} (mg kg^{-1})		NOEC (mg kg^{-1})	
chemical	Askov	Steinskogen	Askov	Steinskogen	Askov	Steinskogen	Askov	Stein-skogen
α-CYP	>1000	>1000	43 (34−59)	80 (67−95)	2.6 (1.1−4.1)	28 (17−40)	5.0	50
CFVP	204 (108−230)	>250	29 (25−35)	57 (43−75)	12 (8.7−16)	21 (4.9−37)	20	100

[a] Numbers in parenthesis are 95% confidence intervals.

experiment. Also, the activity of the parent compound was low. The main fraction of the activity was found in hydrolyzable conjugates and as non-extractable residues. The activity of the conjugates increased during the exposure period, whereas there was a decrease in the non-extractable fraction.

Lethality and Reproduction Toxicity. Results of the toxicity tests conducted with α-CYP and CFVP are shown in **Table 3**. No lethality was observed for α-CYP up to 1000 mg kg^{-1}, and for CFVP, earthworm lethality was only observed in Askov soil at concentrations ≤250 mg kg^{-1}. It is interesting to note that EC_{50} values (with cocoon production as the end point) for α-CYP in both soils were only 40−50% higher than for CFVP, while LC_{50} values were more than 4 times higher. This shows that differences between lethal and sublethal toxicity are higher for α-CYP than CFVP.

DISCUSSION

Uptake in Earthworm *E. fetida*. Both α-CYP and CFVP predominantly existed as non-metabolized insecticides in the test soils, and less than 5% was biotransformed as measured by ^{14}C analysis. This means that the accumulated ^{14}C activity in earthworm was taken up as parent compound and that metabolism primarily occurred in the earthworms. The low degradation rates for both insecticides in soil are in contrast to a review report published by the European Commission (*12*), which estimated a half-life of 14 weeks at 20 °C for α-CYP, and a study performed by Miles et al. (*13*), who determined a half-life of 6 weeks at 15 °C for CFVP. The lower degradation of the pesticides in the present study is probably due to drying at 40 °C and spiking of the soil where chemical dissolved in acetone was added to the soil. This decimates the bacterial flora in the soil considerably and most likely inhibits degradation of the compounds. However, because the present study did not aim at investigating the degradation of the pesticides but their metabolism and toxicity, the low degradation was not unfavorable.

The considerably higher uptake of pesticides in earthworms from Askov soil than from Steinskogen soil was most likely due to lower soil organic matter in Askov soil, leading to a higher bioavailability of the pesticides. In soils with low organic matter content, hydrophobic pesticides, such as α-CYP or CFVP, bind less strongly to soil particles than in soils with high organic matter content, and consequently, the pore water will have higher pesticide concentrations (*14*). α-CYP with an organic carbon corrected sorption coefficient (log K_{OC}) of 6.14 sorbs much stronger to organic matter than CFVP, with a log K_{OC} of 3.42 (*10*), and therefore, the pore water concentration of α-CYP is expected to be considerably lower than that of CFVP. However, lower α-CYP concentrations in pore water do not necessarily result in lower bioaccumulation by earthworm because α-CYP has a much higher lipophilicity and thereby a higher bioconcentration factor (partition coefficient between earthworm and water) than CFVP. Uptake via soil pore water is believed to be the main uptake route for earthworms, and uptake via the gut can be estimated by partitioning of the compound between the solid and aqueous phases (*15*). The feeding behavior of the earthworms might affect accumulation of the pesticides in natural environments and varies between different earthworm species. However, in soil with a homogeneous distribution of chemical, differences in feeding behavior are not expected to influence significantly on bioaccumulation. It can be concluded that differences in bioaccumulation between the two soils were indeed related to different sorption of α-CYP and CFVP to soil particles. However, differences in bioaccumulation between compounds cannot necessarily be associated with differences in sorption characteristics and are most likely due to different metabolic fate in the organisms. Further information on the relationship between sorption and earthworm uptake is provided in a companion paper (*10*).

Metabolism of α-CYP. Research on pyrethroid metabolism has demonstrated that pyrethroids are predominantly metabolized by carboxyl esterases, while oxidases are merely responsible for further degradation of α-CYP metabolites (*16*). Glutathione-*S*-transferases are apparently not involved in direct detoxification of pyrethroids (*17*). The first stage of α-CYP metabolism is usually a cleavage of the ester bond generating 3-phenoxybenzaldehyd, 3-phenoxybenzoic acid, and DCVA as major metabolites (*16, 18*). Metabolism of [^{14}C-cyclopropyl]-cypermethrin in soil arthropods have also generated different isomers of hydroxyl-cypermethrin as primary metabolites (*19*).

Because of the labeling of α-CYP at the acid moiety in the present study, both parent α-CYP, hydroxylated α-CYP, and, after cleavage, DCVA and its metabolic products could be traced.

The unidentified metabolite of α-CYP had a RT of 7.8 min, indicating that it was more hydrophobic than DCVA and its conjugates and metabolites. Possibly, the unidentified metabolite was hydroxy-cypemethrin that has a log K_{ow} of 5.89, according to the Syracuse log K_{ow} estimation software (http://www.syr-res.com/esc/kowdemo.htm, Syracuse Research Corporation, New York). Its hydrophobicity falls between that of α-cypermethrin (log K_{ow} = 6.60) and DCVA (log K_{ow} = 3.38) and would probably also give a RT between these two compounds.

The fact that 80% of the total activity of α-CYP could be extracted with acetone/hexane but without prior acid or alkaline hydrolysis shows that metabolites in earthworms do not form conjugates with biomolecules, such as amino acids, glutathione, or glucuronic acid. Instead, they accumulate in earthworms and are negligibly excreted. This conclusion is in line with observations of Curl et al. (*20*), who showed that ^{14}C-cypermethrin could not be excreted by the earthworms *Lumbricus terrestris* and *Allolobophora calagillosa*, in contrast to detoxification of cypermethrin in, e.g., rainbow trout (*Salmo gairdneri*), rats, and other organisms (*18, 19, 21*) The elution profile for different exposure periods suggests that α-CYP is rapidly metabolized. However, ester cleavage and DCVA formation mainly occurred after more than 15 days. The results of the present study indicate a suppression of the phase II detoxification of α-CYP with low excretion of polar conjugates and a resulting accumulation of metabolites in earthworms. This result contradicts the conclusions of Curl et al. (*20*) that DCVA is present as conjugates in

earthworms. These authors suggest that DCVA truly forms covalent ester bonds to biomolecules because the main part of ^{14}C activity could not be extracted with hexane but only after hydrolysis with 1 M hydrochloric acid. An important difference between the study of Curl et al. (*20*) and our experiments concerns the methods of earthworm extraction. While Curl et al. (*20*) used non-exhaustive Ultraturrax extraction with hexane, we applied a more stringent Soxtec extraction with a moderately polar organic solvent (acetone/hexane). Thus, differences in the results obtained may be due to methodological differences and not different metabolic processes in earthworms.

Metabolism of CFVP. When taken up by earthworms, CFVP rapidly formed conjugates and non-extractable residues, and the concentration of unmodified CFVP in worms remained low during the whole exposure period. The major routes of detoxification are de-ethylation and hydrolysis, which are in principle similar to those of related vinyl phosphates. The products of dealkylation and hydrolysis are desethylchlorfenvinphos, 2,2′,4′-trichloroacetophenone, and diethyl phosphate, respectively (*18*).

In the present study, CFVP was labeled at the ethyl groups, and therefore, metabolic pathways could only be followed for the diethyl phosphate moiety after cleavage of the phosphoester bond. The ^{14}C activity detected in earthworms is therefore from parent CFVP, desethylchlorfenvinphos, diethyl phosphate, or ethanol, either solely or as conjugates. Both diethyl phosphate and ethanol are highly polar (log K_{ow} of 0.32 and −0.31, respectively) and are rapidly excreted without conjugation. CFVP and desethyl-CFVP form extremely stable phosphoric and phosphonic acid esters with the catalytic center of cholinesterases but also react with other molecules containing the amino acid serine (*22*). The constantly low concentration of extractable non-metabolized CFVP indicates that the sorption of CFVP to enzymes and other biomolecules is a fairly rapid process. Continuous uptake of CFVP in earthworms and a rapid reaction with acetylcholinesterase (AChE) and other biomolecules presumably increased concentrations of hydrolyzable conjugates in earthworms with increasing exposure time. However, after approximately 5 days, a steady state in CFVP accumulation was obtained, where uptake and elimination rates were equal. This indicates that radiolabeled pesticide that is bound to enzymes and other biomolecules is degraded and excreted.

Toxicity in Relation to Metabolism. The toxicity tests clearly demonstrated that CFVP is more toxic to *E. fetida* than α-CYP. Pyrethroids are known to alter the normal function of nerve cells by modifying the kinetics of voltage-sensitive sodium channels. This results in the prolongation of the sodium current, which leads to additional release of transmitters, such as acetylcholine and dopamine, and the development of a variety of toxic symptoms (*5, 23*). The actual mechanism by which pyrethroids affect sodium channel functions remains to be investigated.

The significantly lower toxicity of α-CYP compared to CFVP for earthworms is probably due to the rapid metabolism of α-CYP as shown in **Figure 3**. α-CYP is either metabolized in earthworms before it reaches the nerve cell, where it exerts its toxicity, or is reversibly bound to the receptor and redistributed between receptors and other tissues. In the latter case, extensive metabolism keeps the concentration in the tissue low and, thereby, also the concentration at the target site. Even in soils with concentrations of 1000 mg kg^{-1}, α-CYP concentrations at the target site were not high enough to exert a neurotoxic effect that was lethal to adult earthworms.

Reproduction toxicity of α-CYP, with EC_{50} values of 43 mg kg^{-1} for Askov and 80 mg kg^{-1} for Steinskogen soil, were in the same range as previously published results (*9*). The acute/chronic ratio ($ACR = LC_{50}/NOEC_{reproduction}$) for Askov soil was higher than 200 and indicates a different mode of toxic action for acute and chronic toxicity, as hypothesized by Roex et al. (*24*). It is known that ACRs can be particularly high for compounds that are effectively metabolized by organisms and where one or several metabolites exert sublethal effects on the test organism (*25*). For earthworms that are not able to excrete certain α-CYP metabolites as shown above, it is possible that one or more metabolites reach concentrations that cause a sublethal toxic effect.

CFVP mainly acts by phosphorylating the catalytic center of AChE. This inhibits the hydrolysis of acetylcholine and thus impedes cholinergic neurotransmission. The low concentrations of extractable CFVP and the high concentrations of non-extractable residues and conjugates suggest that a considerable portion of CFVP or desethyl CFVP reaches the target site (cholinesterases), forms stable conjugates, and exerts its neurotoxic effect. This resulted in a more than 5 times lower LC_{50} value in Askov soil (204 mg kg^{-1}). This value is identical to the LC_{50} of 204 mg kg^{-1} for CFVP reported by Weyman (*26*).

The ACR for CFVP determined in Askov soil was approximately 10. This value is considerably lower than that found for α-CYP and is in good agreement to the average ACR of narcotic-acting compounds (*25*). For these compounds, the mode of action between lethal and sublethal toxicity is presumably similar and the toxic effect is most likely caused by the parent compound and not by a metabolite that is produced during chronic exposure.

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