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Degradation of [8,9-^{14}C]Endosulfan by Soil Microorganisms

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Twenty-eight soil fungi, 49 soil bacteria, and 10 actinomycetes were tested as to their ability to degrade the insecticide endosulfan. Using ^{14}C-labeled material, the qualitative as well as the quantitative formation of metabolites, as well as of $^{14}CO_2$, could be followed. Sixteen fungi, 15 bacteria, and 3 actinomycetes were found capable of metabolizing more than 30% of the applied endosulfan. The major metabolites detected were endosulfate, formed by oxidation of the sulfite group, and endodiol, formed by hydrolysis of the ester bond. The majority of highly active fungi formed endosulfate as the major metabolite, whereas the majority of active bacteria formed endodiol. In addition to endosulfate and endodiol, individual cultures contained small quantities of endohydroxyether and two unidentified products. The very small quantities of $^{14}CO_2$ evolved from cultures indicated that an extensive mineralization of the carbon skeleton of endosulfan did not occur.

Endosulfan is the common name of the insecticidal compound 1,2,3,4,7,7-hexachlorobicyclo-(2.2.1)-hepten-2,3-bisoxymethylene-5,6-sulfite. The synthesis of this compound results in production of the two stereoisomers α- and β-endosulfan, which are contained in the insecticidal formulation Thiodan in an approximate ratio of 70:30. Because of its chemical formula, endosulfan is sometimes called a "chlorinated hydrocarbon of the cyclodiene group." However, its chemical peculiarities and its physiological behavior in higher organisms make it uncomparable to the other cyclodiene insecticides (6). In residue and metabolism investigations carried out in the past (1, 2, 4, 5, 8), metabolites that originated through a stepwise oxidative degradation of the heterocyclic ring were found; according to Schuphan et al. (8), these could also be formed by various strongly oxidizing chemical agents. These metabolites were the same as those described in the present work. Recently, experiments that deal with the microbial degradation of endosulfan have also been published. Perscheid et al. (7) could identify endodiol as the major degradation product in an undefined mixture of microorganisms obtained from a soil suspension. In a culture of the fungus *Aspergillus niger*, El Zorgani and Omer (3) found endodiol to be the only metabolite. Approximately 30% of the initially applied insecticide (1 μg/1 ml) was degraded within 18 days. All of the data discussed above indicate that endosulfan, in comparison with the majority of the other chlorinated hydrocarbon pesticides, is readily degraded in both plant and animal systems. In view of this, the objectives of the present study were to expand the general knowledge of the behavior of endosulfan in biological systems by investigating the capability of soil microorganisms to degrade and partially mineralize the pesticide in vitro. Since endosulfan is extremly toxic to fish, it would be helpful to find microorganisms that could be used to detoxicate wastes from pesticide production plants.

MATERIALS AND METHODS

Insecticide and metabolites. [8,9-^{14}C]endosulfan (specific activity, 8.3 to 15.2 μCi/mg; see Fig. 1), unlabeled endosulfan (α- and β-endosulfan), and the known metabolites endosulfate, endodiol, endolactone, endohydroxyether, and endoether were obtained from Farbwerke Hoechst AG, Frankfurt/Main.

Microorganisms. The fungi were isolated from three agricultural soils, which originated from Herefordshire, England, the Kampangsaen Province, Thailand, and Braunschweig, West Germany. A soil suspension (1 g/100 ml) was prepared in Czapek-Dox nutrient solution treated with 100, 200, 500, or 1,000 μg of the commercial formulation of endosulfan per ml. For isolations, the fungi that grew as small colonies on the surface of the nutrient solution were further cultivated on Czapek-Dox agar and then purified. A total of 28 different fungal species were isolated and identified.

The 49 bacteria were supplied by G. Jagnow, Institut für Bodenbiologie, Forschungsanstalt für Landwirtschaft, Braunschweig, and the 10 actinomycetes were received from the collection of S. Szabo, Research Institute of Soil Science and Agri-

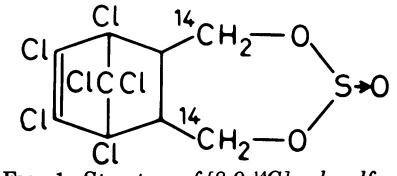

FIG. 1. *Structure of [8,9-^{14}C]endosulfan.*

cultural Chemistry, Hungarian Academy of Science, Budapest.

Nutrient solutions. Czapek-Dox solution (pH 4.9) was used for soil fungi. The medium for bacteria consisted of: K_2PO_4, 200 mg; $MgSO_4 \cdot 7H_2O$, 50 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; yeast extract (Oxoid), 2 g; tryptone (Oxoid), 2 g; mannitol, 5 g; and 2 g of sucrose per liter of distilled water (pH 7.2). For actinomycetes, a solution containing the following was used: KH_2PO_4, 0.5 g; L(−)-asparagin, 0.5 g; yeast extract (Oxoid), 2 g; and 10 g of glucose per liter of distilled water (pH 6.7).

Design of degradation experiments. Fungi were incubated at 22 C in 300-ml Erlenmeyer flasks containing 100 ml of nutrient solution. After formation of mycelial mats, media in culture flasks were replaced with glucose-free Czapek-Dox nutrient solution. After exchanging the media, 100 μg of [8,9-^{14}C]endosulfan (1 μCi), dissolved in 1 ml of ethanol, was introduced into each culture. During the following 6-week incubation period, cultures were aerated daily for 1 h. The gas that was generated was first led through a cotton filter, then through a wash flask containing 100 ml of a toluene-paraffin (liquid) mixture (1:1, vol/vol), and finally through 100 ml of a 1 N NaOH solution to trap metabolic CO_2.

The bacteria and actinomycetes were incubated, without preincubation, in 50 ml of nutrient solution to which 50 μg of [8,9-^{14}C]endosulfan (0.5 μCi) dissolved in 500 μl of ethanol had been added. Culture flasks, which were connected to absorption flasks as described above, were agitated at 120 rpm on a shaker. During the following 10 days of incubation, the flasks were aerated four times daily for 1 h. The incubation temperature was 27 C.

To determine the influence of hydrogen ion concentration on the chemical hydrolysis of endosulfan, sterile bacterial nutrient solutions, having pH values of 4.3, 5.5, 6.3, 7.0, 7.9, and 8.6, were treated with [8,9-^{14}C]endosulfan and incubated as described for the degradation experiments.

Extraction of cultures. After incubation, organisms were separated from the culture medium for extraction: the fungi were separated by filtration, and the bacteria and the actinomycetes were separated by centrifugation (6,000 rpm). After separation, mycelia were first ground in a mixer (Omni-Mix, Ivan Sorvall, Inc., Norwalk, Conn.) with 50 ml of methanol and then, after addition of 50 ml of benzene, were further disintegrated by sonification (Sonifier B-12, Bronson Sonic Power Co., Danbury, Conn.) for 10 min at 100 W. After filtration of the sonicate, a clear extract was obtained. Bacteria and actinomycetes were suspended in 100 ml of benzene-methanol solution (1:1, vol/vol) and sonified as above. The cell parts were separated from the organic solvent by membrane filtration. After measurement of the pH values, the separated nutrient solutions were extracted by shaking three times with benzene.

Analyses. Further experiments dealt with analyses of extracts in regard to the content and composition of their radioactivity. The quantities of non-extractable activity in the organisms and in the nutrient solutions, as well as the $^{14}CO_2$ evolved, and the amount of pesticide volatilized were determined. Aliquots of extracts from the organisms or from the nutrient media were mixed with a toluene-based scintillation solvent, and their radioactivity was counted in a liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill., model Mark II). All counts were corrected for both background and counting efficiency by using the external standard. For thin-layer chromatography, samples of the extracts were concentrated on a flash evaporator and then applied, in the form of 3-cm-wide bands, to silica gel-coated, thin-layer plates (Kieselgel G, Merck AG, Darmstadt; 0.3 mm). Spotting was done with a Chromatocharger (Camag, Berlin). The plates were developed in hexane-chloroform-acetone (9:3:1, vol/vol/vol). For localization of the radioactive substances after development of the plates, a thin-layer chromatography radioscanner (Berthold und Friseke GmbH, Karlsruhe) was used. Guided by the activity distribution curve drawn by the scanner, the areas of radioactivity could be located, scratched out, and directly measured in the toluene scintillation fluid.

The radioactivity remaining in the fungi after their extraction was determined by ashing 5 mg of the dried and powdered organisms. For ashing, a half-automatic apparatus (Micro-Mat BF 5010, Friseke und Höpfner GmbH, Erlangen) was used. The radioactive CO_2 formed during ashing was collected in ethanolamine; this was mixed with methanol and then measured in the toluene scintillation fluid. The bacteria and actinomycetes that had been collected on the cellulose membrane filters were hydrolyzed to a clear or slightly cloudy solution within 20 min in 2.5 ml of 50 C concentrated HCl. After dilution of this solution to 50 ml with distilled water, 2 ml was measured in a dioxane scintillation solvent.

The CO_2 adsorbed in 1 N NaOH was transferred to ethanolamine for counting. For this purpose, the alkali was acidlified with dilute H_2SO_4 in a round-bottomed flask, and the solution was refluxed for 10 min. Using a stream of N_2, the CO_2 was driven through the side arm on the flask into a glass column (1,000 mm long; 10-mm internal diameter) containing 50 ml of ethanolamine-methanol (30:70, vol/vol). To guarantee total absorption of the CO_2, the gas was introduced into the absorption mixture by passage through a D2 fritted-glass filter. Ten milliliters of the ethanolamine-methanol solution was mixed with 10 ml of the toluene scintillation solvent for measurement.

To determine the amount of insecticide volatilized, the cotton plug behind the culture was measured in 20 ml of toluene scintillation solvent. An aliquot of toluene-liquid paraffin mixture was also measured.

RESULTS

Metabolism by soil fungi. The radioactivity originally introduced into the nutrient solution was, for the most part, recoverable from the mycelia after 6 weeks of incubation. The mycelia of six fungi were found to contain over 90% of the applied radioactivity; 16 others incorporated 80 to 90%, and six others contained 60 to 80%. Correspondingly, little activity was detected in the nutrient solutions. The radioscanthin-layer chromatography investigations of the mycelial extracts and nutrient media extracts allowed detection of two major metabolites: endodiol and endosulfate. Great differences were found between the fungal species in regard to the type and the amount of the major degradation products they formed. In Fig. 2 the percentage of participation of the species (28 species = 100%) in the formation of quantities of the metabolites is shown.

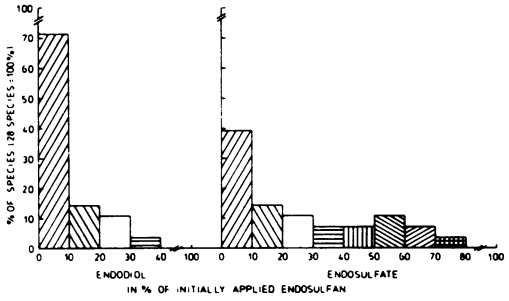

FIG. 2. *Participation of fungal species in formation of metabolites from [8,9-^{14}C]endosulfan.*

In single instances, the endohydroxyether and two unknown metabolites, as well as endodiol and endosulfate, could be detected (Fig. 3).

The fungi are listed in Table 1, in which a total of more than 30% of the intially applied radioactive endosulfan was found as metabolites in the extracts of media plus mycelia.

Metabolism by soil bacteria. After 10 days of incubation, the amounts of recovered radioac-

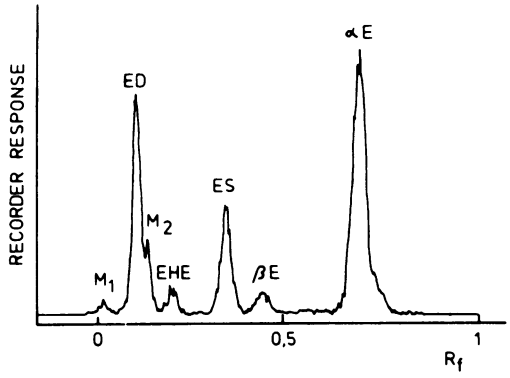

FIG. 3. *Separation of [8,9-^{14}C]endosulfan and its degradation products in an extract of the fungus Arthrinium urticae by thin-layer chromatography as determined by using a radiochromatogram scanner. Abbreviations: M_1/M_2, unknown metabolites; ED, endodiol; EHE, endohydroxyether; ES, endosulfate; α-, βE = α-, β- endosulfan.*

TABLE 1. *Soil fungi with a high capacity for degrading endosulfan*

Fungi	% of initial activity						
	Metabolites[a] in extracts of media plus organisms					Unextractable activity in:	
	M_1	ED	EHE	ES	$^{14}CO_2$	Mycelia	Medium
Penicillium sp. 3	1.0	3.0	ND[b]	75.0	0.1	3.6	1.5
Aspergillus sp. 1	4.9	1.0	0.2	66.0	0.1	1.9	3.1
Botrytis cinerea	3.0	0.4	ND	64.4	0.7	7.4	3.0
Aspergillus terricola	7.2	0.5	ND	59.9	0.1	5.7	5.2
Alternaria alternata	1.0	0.2	ND	53.0	0.1	4.8	2.9
Aspergillus sp. 2	ND	3.9	ND	52.4	0.1	1.4	6.3
Penicillium nigricans	1.6	3.5	1.5	47.5	0.1	4.0	0.2
Mucor hiemalis	5.6	7.4	2.0	43.2	0.2	7.4	8.1
Zygorrhynchus moelleri	0.7	1.0	0.6	38.5	0.1	28.1	2.3
Fusarium graminearum	ND	2.7	2.4	32.9	ND	1.2	0.8
Rhizopus nigricans	2.4	1.2	0.6	30.2	0.1	5.4	11.1
Circinella simplex	3.5	2.3	4.3	25.3	0.1	20.0	13.3
Aspergillus niger	2.4	2.8	0.3	23.4	0.2	16.2	4.4
Penicillium commune	1.9	32.9[c]	ND	11.5	ND	1.7	1.2
Sterile mycelia	0.8	26.9	1.8	9.8	ND	1.0	2.1
Arthrinium urticae	0.9	25.7[c]	2.6	10.5	0.1	21.5	4.9

[a] M_1, Unknown metabolite; ED, endodiol; EHE, endohydroxyether; ES, endosulfate.
[b] ND, None detected.
[c] This result is based on the double peak ED + M_2 (see Fig. 3).

tivity in the cell masses of the bacteria were quite different from species to species. Thirty-two of the 49 species investigated contained 40 to 70% of the activity. With only two organisms, it was over 80%. In all of the remaining species, less that 30% could be detected; in some of the species, even less than 20% was found in the cell masses.

The 49 bacteria tested also degraded endosulfan to the two major metabolites, endodiol and endosulfate. A noteworthy contrast between bacteria and the soil fungi was that the bacteria with a high degradation capacity hydrolyzed endosulfan to endodiol; for the most part the fungi with a high degradation capacity oxidized endosulfan to endosulfate (Fig. 2 and 4).

In addition to the two major metabolites, small quantities of three further metabolites were detected by thin-layer chromatography analyses. These were the same as those found in the fungal cultures, namely, the endohydroxyether and two unknown metabolites. The bacteria that degraded more than 30% of the initial [8,9-^{14}C]endosulfan are shown in Table 2. Since the residual activity in the bacteria (<1%), as well as in the nutrient solution (1 to 3%), was very small, the data are not given in Table 2.

Metabolism by actinomycetes. The distribution of activity in the organisms at the end of the experiments corresponded to the relationship found with the majority of bacteria; that is, 40 to 70% was found in the organisms. Of the 10 actinomycetes investigated, three genera (*Streptomyces albidoflavus*, *Streptomyces griseus*, and *Streptomyces levoris*) metabolized more than 30% of the applied endosulfan. It was therefore remarkable that the simultaneous formation of the previously mentioned metabolites, in respect to both their quality and quantity, was pronounced. Figure 5 shows an example of this. As with the fungi and the bacteria, only small quantities (≤0.1%) of $^{14}CO_2$ could be detected.

Chemical degradation and volatilization of endosulfan. For chemical degradation under the conditions used in the experiments, two reactions are feasible: (i) chemical hydrolyses of the ester linkage, which would yield endo-

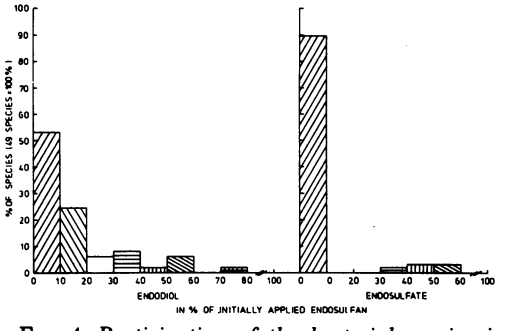

FIG. 4. *Participation of the bacterial species in formation of metabolites from [8,9-^{14}C]endosulfan.*

TABLE 2. *Soil bacteria with a high capacity to degrade endosulfan*

Bacterium	% of initial activity							
	Metabolites[a] in extracts of media plus organisms					Activity at end of expt in:		pH[b]
	M_1	ED	EHE	ES	$^{14}CO_2$	Bacterium	Medium	
Nocardia sp. 4	3.3	73.3	ND[c]	2.5	ND	17.4	70.7	8.3
Corynebacterium sp. 1	ND	58.6	ND	ND	ND	32.4	57.2	8.5
Bacillus polymyxa	1.2	52.8	ND	ND	ND	47.2	42.8	5.7
Mycobacterium sp. 3	1.0	51.6	ND	ND	ND	37.1	53.1	7.2
Corynebacterium sp. 2	0.3	40.2	ND	0.7	ND	70.0	26.0	4.6
Pseudomonas fluorescens	0.6	37.8	0.9	0.8	ND	61.5	33.6	5.5
Corynebacterium sp. 5	0.8	34.4[d]	ND	ND	ND	68.1	22.2	7.2
Arthrobacter sp. 4	0.2	32.1	ND	ND	ND	41.0	49.5	6.8
Gram-negative rods	0.4	30.9	ND	ND	ND	65.1	23.1	4.6
Nocardia sp. 1	0.5	28.1[d]	ND	2.0	ND	43.4	38.7	6.8
Corynebacterium sp. 9	2.6	1.3	1.3	55.8	ND	47.1	40.7	5.0
Mycobacterium sp. 1	2.9	0.6	1.5	50.1	0.1	46.3	38.9	4.9
Corynebacterium sp. 7	2.4	1.2	1.4	47.0	0.1	34.3	33.0	5.0
Corynebacterium sp. 4	2.0	1.6	0.9	44.1	0.1	47.6	39.5	5.2
Nocardia sp. 3	1.4	1.9	1.7	32.4	ND	41.9	40.3	5.0

[a] M_1, Unknown metabolite; ED, endodiol; EHE, endohydroxyether; ES, endosulfate.
[b] pH value at the end of the experiment in the culture medium.
[c] ND, None detected.
[d] This result is based on the double peak ED + M_2 (see Fig. 3).

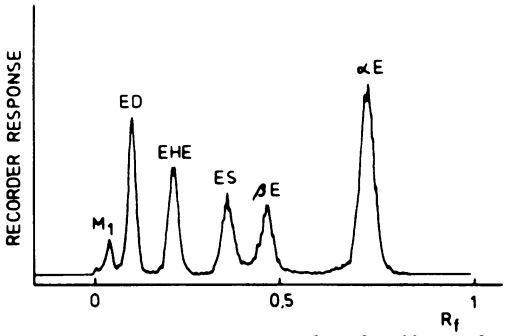

FIG. 5. *Separation of [8,9-^{14}C]endosulfan and its degradation products in an extract of the actinomycete Streptomyces griseus by thin-layer chromatography as determined by using a radiochromatogram scanner. Abbreviations: See Fig. 3.*

diol, and (ii) oxidation of the sulfite group to endosulfate. In experiments in which sterile bacterial nutrient solution was incubated with [8,9-^{14}C]endosulfan for 10 days, only endodiol was formed. Endosulfate was not detected. The quantity of the endodiol formed was directly dependent on the pH of the medium. At pH 4.3 the concentration of endodiol was under 1%; at 5.5 it rose to 2%; at 6.3 it was 8%; and at pH 7 it was 28%. At pH values of 8 or greater, 90% or more of the endosulfan was hydrolyzed.

A further nonbiological factor that led to changes in the quantity of endosulfan in the nutrient solution was the volatility of the compound. This was greatest in the organism-free nutrient cultures (near 30%) and least in the presence of fungi (<1%). With the bacteria and actinomycetes, losses between 2 and 20% were recorded. In these experiments, the known fact that α-endosulfan is more volatile than the β-isomere was confirmed.

DISCUSSION

In the present investigation, in which 87 microorganisms were tested, it is noteworthy that, besides the bacteria and fungi that form endodiol, organisms that produce endosulfate were detected. This has not been reported by other authors. Metabolism to endosulfate was particularly notable when endosulfan was metabolized by soil fungi. It must, nevertheless, be taken into consideration that fungi incubated in nutrient-poor medium, such as that selected for use in the present work, would probably have degradation capacities other than those of cultures with optimal nutrient conditions, such as those used by El Zorgani and Omer (3). Bacteria having a high capacity for endosulfan degradation could be clearly divided into two groups: one that could form endodiol and one that could form endosulfate. Figure 6 demonstrates the transformation of the endosulfan molecule by microorganisms in culture.

In the organism-free cultures, chemical formation of endosulfate could not be detected when the pH was between 4.3 and 8.6, so that the formation of this degradation product was exclusively due to the enzymatic reactions of the microorganisms. In contrast to this, classification of endodiol formation as a chemical or an enzymatic reaction of the microorganisms cannot be readily done. First, as previously mentioned, there is the pH-dependent formation of endodiol; second, microorganisms can change the pH of their cultivation medium to higher or lower values by excretion of respiratory metabolites and by lysis. Nonetheless, comparison of endodiol quantities found in bacterial cultures to final H^+ ion concentrations of the media allows estimation of the ratio of chemical to enzymatic hydrolysis. The culture media of the two best degrading bacteria (Table 2) had pH values of 8.3 and 8.5 at the end of the experiment, so that a large part of the total degradation was probably due to chemical hydrolysis. With the remainder of the bacterial cultures, in which over 30% of the insecticide was degraded, it could be calculated, on grounds of the low end pH value, that a large portion of the degradation was enzymatic. Besides changes of pH in the culture medium, the extent of chemical hydrolysis would also be dependent upon the capacity of individual organisms to take up the insecticide. By incorporation and storage in lipoidal parts of the cell, the substance can be removed from the site of deg-

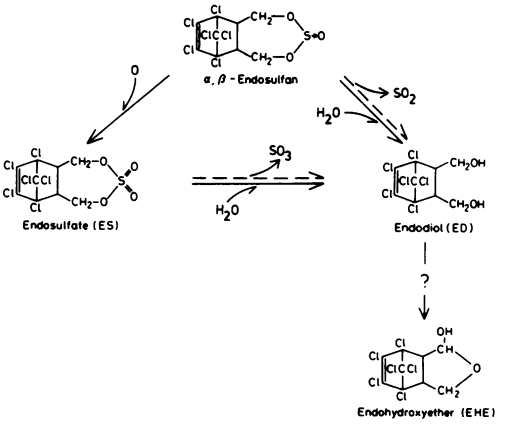

FIG. 6. *Proposed pathway for the degradation of endosulfan in microbial cultures. Symbols: solid arrow, enzymatic transformation; broken arrow, chemical transformation.*

radation in the medium. This gives a possible explanation why in one culture (data not included above), which had an end pH value of 8.2, there was only 10% conversion to endodiol. In this case, the ^{14}C activity found in the bacteria, which was 65%, was relatively high, whereas in the case of the two bacteria mentioned above, which had a high endodiol formation capacity, the activity in the bacterial cell masses was low (17 and 32%).

In the case of endosulfan, and in consideration of the results given for experiments with single organisms, it can be concluded that, with pH values under 7, enzymatic degradation is of importance. With pH values over 7, chemical transformations can also contribute to degradation as long as the insecticide is not incorporated to a great extent by the microorganisms.

In these investigations it was found that microorganisms in culture can metabolize endosulfan as readily as higher organisms. In 37 of the 87 cultures investigated, more than 30% of the applied endosulfan was degraded. It must, thereby, be taken into consideration that endosulfate is just as toxic as endosulfan (6) and that only the formation of endodiol and endohydroxyether constitutes a detoxication. The fact that $^{14}CO_2$ was either absent or only found in small quantities indicates that the carbon skeleton of endosulfan is just as resistant to microbial degradation as the other highly chlorinated cyclodiene hydrocarbons.

ACKNOWLEDGMENTS

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