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12 APR 1995
FAXED

Monday, 10 April 1995

Mr Shulze
Executive Director
Cotton Research and Development Corporation
PO Box 282
NARRABRI NSW 2390

Dear Mr Shulze,

RE THE EFFECTS OF ENDOSULFAN SPRAYED ON COTTON:

THE RELATIVE SIGNIFICANCE OF BIODEGRADATION AND PHYSICO-CHEMICAL DISSIPATION OF ENDOSULFAN FROM WATER AND SOIL AND IMPLICATIONS FOR THE COTTON INDUSTRY

Enclosed is a copy of a revised/updated final report to the CRDC on research conducted with funding from the first Industry Development Award - DA Q43C. The title of the project was originally titled "*The Effects of Endosulfan Sprayed on Cotton: The Biodegradation of Aldrin, Dieldrin and Endosulfan*". This document includes copies of further publications arising from the research, a summary of the findings, and the completed survey of "Past CRDC Funded Projects. Please accept my apologies for the late completion of this survey. Please note that I have re-titled this report to more closely reflect the extent of the work conducted.

This research was conducted by the undersigned, T. F. Guerin formerly from the Department of Agricultural Chemistry and Soil Science, University of Sydney.

The findings from the research highlight a number of key issues which have already been outlined in my previous reports, however, these are repeated in summary as follows;

- First, the results have confirmed the results of previous local and international studies which indicated that endosulfan I is the more volatile isomer. However, the study has shown that both isomers readily volatilise from a free water surface.

- Secondly, the principal isomer of endosulfan (endosulfan I), can be rapidly degraded by naturally occurring soil microorganisms from tailwater drain sediments when these organisms are encouraged to grow in anaerobic growth media in laboratory liquid cultures. This finding indicates the potential of microorganisms to degrade endosulfan in environments where oxygen is limiting.

- Thirdly, endosulfan is dissipated at a slow rate from both sullage pit soils and soils from a cotton growing field under aerated conditions in the laboratory. This occurs regardless of whether the soil is sterilised or biologically active. It is likely that the absence of readily available forms of carbon in these soils limited the degradation rates.

These research findings have implications for the Cotton Industry.

Sullage pit soils pose an on-going environmental problem and remediation strategies should be considered for their treatment.

Given the potential of naturally occurring soil microorganisms to degrade endosulfan, an application of bioremediation technology to these sites would be a timely undertaking for the industry.

From the research reported here, and on the basis of the field-scale bioremediation projects the author has been involved with since the completion of the research reported here, there is substantial evidence to suggest that bioremediation is an option for the treatment of endosulfan and related wastes at these sites.

EXECUTIVE SUMMARY AND RECOMMENDATIONS TO THE CRDC

The key findings from the research, which are of direct relevance to the cotton industry, are as follows:

1. Anaerobic Biodegradation of Endosulfan

Endosulfan can be degraded to a non-toxic compound by indigenous soil microorganisms when these are incubated with endosulfan under conditions where organic matter, is not limiting. Indigenous soil microorganisms isolated from freshwater sediments including tailwater retention drains were cultured in the laboratory in liquid medium. These cultures could degrade the principal isomer of endosulfan at high rates, to the relatively non-toxic degradation product, endosulfan diol.

These results indicate that endosulfan may be degraded under environmental conditions where there are low oxygen concentrations.

3. The Degradation of Endosulfan in Soil

Endosulfan dissipated from contaminated soil from a sullage pit, under both sterilised and non-sterilised conditions.

However, biodegradation could only be distinguished from non-biological losses in the cotton-growing soil where endosulfan was present at low concentrations (<2 ppm).

In sullage pit soils, where endosulfan was present at relatively high concentrations (approximately 400 ppm), losses of endosulfan were primary due to volatilisation. As indicated previously, endosulfan has been shown to readily volatilise from free water surfaces.

The volatility of endosulfan demonstrated in the current study indicates that this will be a major route for its loss in the field and probably accounts for its observed high rates of loss immediately after application.

4. Prospects for Full-Scale Remediation of Sullage Pit Wastes

Full-scale remediation projects have recently been conducted by Minenco Pty Ltd on a number of industrial sites that have soil contaminated with chlorinated hydrocarbons. A pilot-scale trial determining the rate of biodegradation of pesticide and herbicide wastes in sullage pit soil could be conducted and would provide sufficient information for the full-scale design and implementation of a biological process to treat these wastes.

It is likely that a bioremediation process using a composting procedure would be most suitable for the treatment of sullage pit wastes.

Further details of CRA's capability in the area is available if the CRDC is interested.

RESEARCH REPORT - REVISED FINAL REPORT

1. Summary of Findings

The research results presented here complement current knowledge on the fate and biodegradability of endosulfan. Endosulfan can be degraded under aerobic conditions in soil. Furthermore the results demonstrate that endosulfan can be biodegraded under anaerobic conditions in the presence of naturally occurring microorganisms. These findings are significant in that microorganisms already present in the soil are capable of biodegrading endosulfan and genetic manipulation or augmentation of contaminated environments is unlikely to be necessary for establishing a biodegradation potential in environments contaminated with endosulfan.

2. Analytical Achievements

As part of a general study into the environmental fate of cyclodiene organochlorines, an efficient method for their extraction from small samples of aqueous microbial growth media was developed. The degradation products of endosulfan and aldrin, together with their parent compounds (some of which are current environmental pollutants), can be detected on the electron capture detector (ECD) gas chromatograph without derivatisation. This has been achieved with prior column conditioning, except for the water soluble degradation product of aldrin, dihydrochlordene dicarboxylic acid (DHCDA). Furthermore, separation of endosulfan and its degradation products was achieved on a single 3m packed column. A detailed comparison was made between the above compounds and aldrin and its major degradation products, and the ECD's relative sensitivity to these compounds. The limits of detection and the extent of linearity of the response of the ECD toward these compounds have been reported. The importance of adequate column conditioning, as well as other problems likely to be encountered with the analysis of these cyclodiene compounds, is described. These results have been published and are available to other researching in the field.

3. Degradation of Endosulfan Under Aquatic Conditions

In laboratory studies addressing effects of non-biological processes, endosulfans I and II were found to dissipate from simple aqueous media in glass incubation vessels sealed with Teflon (PTFE) with apparent half-lives of 46 and 24 days at 30°C, respectively. These values were considerably larger than many reported in the literature. Endosulfan sulfate, the major oxidation product of endosulfan, was much more persistent than either of its parent isomers under the same incubation conditions, and the data and did not fit this same model of decay. However, these rates of degradation of the parent endosulfan isomers were considerably faster than those obtained for the related compounds, aldrin and dieldrin. The data show that these compounds had half-lives greater than 200 days under the same conditions.

By contrast, in unsealed and butyl rubber sealed vessels, both conditions which allowed high rates of dissipation, the endosulfan isomers demonstrated half-lives of only four and seven days for endosulfans I and II, respectively. These latter values were obtained by fitting the early time data only to the first order exponential decay model, a model which clearly did not explain the data from all the sampling times. It seems likely that two independent processes of volatilisation and chemical degradation are required to explain this data.

4. Degradation of Endosulfan in Soil

Experiments using soil were conducted in the laboratory (in PTFE-sealed vessels) to determine whether indigenous microorganisms were involved in the degradation of radioactively labelled endosulfan I, the principal isomer of endosulfan in commercial sprays, and its two major degradation products, endosulfan diol and endosulfan sulfate. An objective of this study was to show to what extent this biological degradation could be distinguished from dissipation by non-biological processes. Hexane-acetone extractable residues of endosulfan I (radioactively labelled in the chlorinated ring), in a non-sterilised cotton farming soil and a soil contaminated with high levels of technical endosulfan, decreased by 35 and 16% over a nine week period, respectively. When the cotton farming soil was sterilised, this decline was reduced to 20% over the same period, indicating that soil microorganisms were contributing a large proportion to the total loss of this compound. However, there was no measurable difference between the sterile and non-sterile treatments containing contaminated soil. This indicated that in this highly contaminated soil, losses due to non-biological processes, such as volatilisation and chemical hydrolysis, masked any contribution by microorganisms. Small increases in the level of methanol extractable radioactivity were observed in the non-sterile treatments indicating that microorganisms were active, but only to a relatively small extent in the contaminated soil treatments.

In further treatments with the cotton farming soil, hexane-acetone extractable residues of applied endosulfan sulfate and endosulfan diol (both of which were radioactively labelled in the non-chlorinated portion of the molecule), decreased at a much higher rate in the non-sterile treatments than those treatments that were sterilised. The increase in $^{14}CO_2$, and radioactivity in the methanol extracts of soil, and the increase in the radioactivity in the water re-extractions of the hexane-acetone phase, was greater in the non-sterile soil containing endosulfan diol, compared to sterilised soil. This indicated that endosulfan diol undergoes further transformation in the presence of indigenous soil microorganisms. These results indicated that although endosulfan I, was dissipated from the cotton farming soil when either sterilised or not sterilised, the endosulfan sulfate and diol degradation products were more readily observed to undergo loss through biodegradation. This is likely to be due to the reduced volatility and resistance to chemical hydrolysis of endosulfan sulfate and diol compared with the parent endosulfan I isomer.

The importance of volatilisation has been demonstrated as the primary source of loss of the major endosulfan isomer, where endosulfan is present at relatively high concentrations in soils.

Only trace amounts of radioactive carbon dioxide were released from applied endosulfan compounds during their incubation in soil. These results demonstrated that while the cyclodiene ring of endosulfan is relatively recalcitrant to microbiological attack, the non-chlorinated portion of the molecule is considerably more susceptible to microbial degradation. Furthermore, the soil experiments indicated that in this study, there was no detectable difference between the rates of loss of endosulfan compounds in sterile and non-sterile soil that had been contaminated with high levels of endosulfan. The suggested reason for this was that the processes of volatilisation and chemical degradation were considerably more dominant than biodegradation at high levels of endosulfan. Conversely, at low levels of applied endosulfan, biodegradation could be distinguished from losses by non-biological processes. These results indicate that endosulfan is only cometabolised in soil and therefore any soil bioremediation process for clean up will require the addition of addition of organic matter to the soil.

5. Anaerobic Biodegradation of Endosulfan

When the major isomer of endosulfan, endosulfan I (1, 2 and 10 ppm), was incubated with indigenous soil microflora from low oxygen environments in anaerobic liquid culture, endosulfan

diol was the principal degradation product formed. Furthermore, endosulfan sulfate was not formed under these conditions, nor was it found as a degradation product when endosulfan was added to mixed aerobic cultures from a wide range of soils. It is unlikely that endosulfan sulfate is formed under conditions of low oxygen in the environment and its presence in sediments is probably due to transport on colloidal material originating from its place of formation. Losses due to non-biological processes were eliminated in the biodegradation experiments by adopting protocols developed early in this study. These included (1) sealing the incubation vessels with Teflon (2), extracting the entire vessel contents when determining degradation rates in sterile controls, and (3) improving the buffering capacity of the anaerobic medium. Buffering the media ensured that the growing cultures did not increase the pH of the medium, as endosulfan is susceptible to alkaline hydrolysis.

These findings demonstrate the capacity of indigenous microorganisms to biodegrade endosulfan to the less toxic degradation product, endosulfan diol, in anaerobic environments. This is in contrast to aerobic environments, where the degradation product formed is usually reported in the literature to be the more toxic pollutant, endosulfan sulfate.

6. Concluding Remarks

I hope this updated and revised final report is of value to you and please let me know if there are any issues that the CRDC would like me to pursue further with respect to this document. Thank you again for your support during my research and I sincerely hope that the industry has found the findings to be valuable and supportive of the goals of the CRDC.

Yours Sincerely,

Dr. Turlough F. Guerin
Senior Environmental Scientist
Minenco Pty Ltd. Environmental Services
(Part of the CRA Group of Companies)

Enclosed and to be forwarded by mail

CRDC Survey
Copies of Publications

Survey of Past CRDC Funded Projects

Project Code: DAQ43C

Project Title: Industry Development Award - Biodegradation of Endosulfan

Principal Researcher: Turlough F. Guerin

Attention: Bruce Pyke/CRDC

Outcome of Research

Have there been any Scientific Journal Publications (published or in preparation) from this research? Please Specify.

Guerin, T.F., Kimber, S.W.L. and Kennedy, I.R. (1992) Efficient one-step method for the extraction of cyclodiene pesticides from aqueous media and the analysis of their metabolites. *Journal of Agricultural and Food Chemistry*, 40, 2309-2314.

Guerin, T.F. and Kennedy, I.R. (1992) Distribution and dissipation of endosulfan and related cyclodienes in sterile aqueous systems: Implications for studies on biodegradation. *Journal of Agricultural and Food Chemistry*, 40, 2315-2323.

Guerin, L.J. and Guerin, T.F. (1994) Constraints to the adoption of agricultural and environmental innovations and technologies: a review. *Australian Journal of Experimental Agriculture.* Vol 34, Issue 4, 549-571.

Have Articles Been Published in Any Magazines Like the Australian Cotton Grower?

Guerin, T.F. and Kennedy, I.R. (1991). The biodegradation of endosulfan in cotton growing soils. *The Australian Cotton Grower*, 12, 13-15.

Guerin, T.F. and Kennedy, I.R. (1991). Reducing the environmental residues of agricultural chemicals. *Agricultural Science*, 4, 37-41.

Have There Been Papers Presented at Conferences?

Guerin T.F. (1994) *"The potential of indigenous micro-organisms from soils and sediments to biodegrade organochlorine pesticides in anaerobic liquid culture".* 2nd National Hazardous and Solid Waste Convention and Trade Exhibition - Waste Management Achievements and Challenges. AWWA, May 8-12, pp 569-575.

Guerin, T.F. (1994) *"Distinguishing between biological and non-biological losses of recalcitrant organic compounds: implications for bioremediation trials".* 2nd National Hazardous and Solid Waste Convention and Trade Exhibition - Waste Management Achievements and Challenges. AWWA, May 8-12, pp 561-568.

Guerin, T.F. and Kennedy, I.R. (1992). The anaerobic biodegradation of endosulfan by mixed cultures of soil microorganisms isolated from sediments. In, "Soil Decontamination Using Biological Processes" Ed. J. Klein, DECHEMA, Karlsruhe, Germany, pp 317-323.

Guerin, T.F. and Kennedy, I.R. (1992). The biodegradation of endosulfan in cotton farming soils and contaminated agricultural waste sites. In, "Soil Decontamination Using Biological Processes". Ed. J. Klein, DECHEMA, Karlesruhe, Germany, pp 324-332.

Guerin, T.F. and Kennedy, I.R. (1992). Environmental biodegradation of endosulfan, "Proceedings of the Australian Biotechnology Conference". Ed. I.G Prince, 10, 135-13.

Have all or Some of the Results of this Research Been Presented at Grower Meetings, Field Days, Shed Meetings, etc?

The findings have not been formally presented at these fora. This was primarily because of the limited budget which did not allow for travel to these venues.

It would be advisable in future projects that each potential project that is undergoing review for funding incorporates a component in its budget to include technology transfer to growers. Refer to recommendations given in Guerin and Guerin (1994) regarding this issue.

Have the Results Been Presented in Other Ways e.g. Videos, Agfacts, Radio, TV interviews?

Yes. These include seminars and invited lectures and these have been summarised in the following entires.

Guerin, T.F. (1992) *"Modes of Biodegradation of Endosulfan in the Environment".* Convenor: Ian P. Anderson, Technical Manager Agrivet, Hoechst Australia Ltd, Melbourne, Australia.

Guerin, T.F. (1992) *"The Degradation of Endosulfan in Sterile Aqueous Systems".* Convenor: Robert, R. Michel. Hoechst Atkiengesellschaft. Marketing Plant Protection, Frankfurt Am Main, Germany.

Guerin, T.F. (1992) *"Further Developments in Endosulfan Research"*. 1. Improved analyses by packed column gas chromatography II. Anaerobic biodegradation, III. Soil biodegradation of the parent isomer and the major metabolites. Convenor: Robert R. Michel. Marketing Plant Protection, Hoechst AG, Atkiengesellschaft, Frankfurt Am Main, Germany.

Guerin, T.F. (1992) A series of 5 lectures on the analysis of biodegradation of endosulfan in Anhui Province at the Anhui Agricultural University and Academy of Sciences, upon invitation from the Chinese Government, Convenor and Organiser: Professor Chang Ben Ying. Director of the Commission for Science and Technology, Anhui Province, China.

Do You Have Plans for any Future Publications etc of the Outcomes of this Research?

Further scientific articles are being prepared on the bioremediation of endosulfan in soil and the environmental fate of this compound under anaerobic conditions. The CRDC will be notified of their publication in due course.

Is there any Follow-up Work Underway?

Although not directly arising from the current research, the awardee has become involved in the field-scale application of bioremediation to contaminated soil. This has involved the conduct of industry research and environmental consulting.

Some of the bioremediation treatability and pilot-scale studies have addressed the bioremediation of chlorinated organic compounds and other agricultural chemicals. Other projects have involved the full-scale bioremediation of a range of toxic organic compounds.

Further information is available on the awardee's expertise in this area upon request.

The Movement and Biodegradation Of Endosulfan In Soils and Implications On Its Toxicology

Turlough F. Guerin

Department of Agricultural Chemistry, and St. John's College, The University of Sydney, New South Wales, 2006 Australia.

Abstract

Endosulfan is used extensively in Australia for the control of a wide range of insect pests, and as a consequence, its fate in the environment has become a priority area for rural research organisations, chemical manufacturers and regulatory authorities. Endosulfan (trade names Thiodan®, Endosan®) is not a classic organochlorine insecticide and consequently does not behave like one, but rather demonstrates properties of a much more labile compound. Some of the physical properties of endosulfan and its metabolites are briefly discussed. Areas requiring further research include the fate of endosulfan under anaerobic conditions and the role of indigenous soil microorganisms in its degradation in the field. Toxicological aspects of endosulfan are discussed, including reference to its degradation products.

Introduction

Endosulfan has become an important component of insect control primarily in cotton but also increasingly in other crops both within, and outside, Australia. This is mainly because of its characteristics of being persistent and effective long enough to selectively control the insect pests of concern without discussing beneficial populations of predatory insects. Since 1989, its use in Australian cotton growing has almost doubled, whereas the amount of synthetic pyrethroid sprayed has increased at about one and a half times over the same period (Table 1). This increase in the use of endosulfan has partly been due to the increase in the area of cotton grown, but also because of the need of the cotton industry for an effective insecticide.

Endosulfan if not used with care and vigilance may cause environmental problems. Drift of endosulfan to wooded areas occupied by wildlife, and other land areas not intended for treatment and fish bearing waters, are the main problems associated with the current use of endosulfan. There are other areas of concern with regard to endosulfan use, namely the problems of run-off or wash-off by rain from treated areas to fish-bearing waters. Other problems arise where applications are made too often or in excess of the recommended amounts, or where they are made at the wrong times. However, the problem of operator carelessness is one of the major problems associated with the pollution of the environment with endosulfan and related chemicals (Shaw, 1991).

As a result of its increased use for cotton production, it is important that its fate in the Australian soil environment be understood, particularly in view of its potential for environmental toxicity. In fact, very few studies on its environmental fate have been conducted under Australian conditions. This article describes the background and aims of a current study on the role of microorganisms in the degradation of endosulfan in cotton growing soils north-west of New South Wales, Australia. It also brings together some of the key literature describing the toxicology of endosulfan relevant to cotton farming.

Chemical and physical properties of endosulfan

Since the first synthesis of endosulfan in the early 1950s, the compound has sometimes been loosely classified in the group of chemicals called chlorinated hydrocarbons, of the cyclodiene group. However, it is not simply a cyclodiene organochlorine. Endosulfan is a hexachlorocyclic sulfite diester, as designated by the IUPAC, the world authority on chemical classification. In the technical formulation, endosulfan exists in two forms; the α-and β-isomers (also commonly referred to as endosulfan I and II) in a ratio of 70:30. Trade names for the technical formulations of endosulfan are given in Table 2. The most common brands sold in Australia are Thiodan® and Endosan®.

The presence of the reactive sulfite functional group in both these isomers gives endosulfan properties which strongly differentiates it from the cyclodienes eg. aldrin, dieldrin, endrin, isodrin, telodrin, and heptachlor, so much so that it cannot be grouped with them. The presence of this sulfite functional group in endosulfan enhances its biodegradation and its susceptibility to chemical hydrolysis. Some of the physical properties of endosulfan and its metabolites are presented in Table 3 and compared with aldrin and dieldrin.

Endosulfan, like aldrin and dieldrin, has a low water solubility. The α- and β-isomers have solid-phase solubilities of 0.33 and 0.41 ppm respectively. These are considerably higher than the lower values of 0.06 and 0.18 ppm for aldrin and dieldrin, respectively. The endosulfan metabolites endosulfan sulfate, endosulfan diol and endosulfan ether have values of 0.5, 4.8 and 14 ppm respectively (Guerin and Kennedy, 1992). One of the major environmental implications of the low solubilities for endosulfan and endosulfan sulfate is that these compounds will tend to partition into lipids and lipid-containing bodies. They may also be absorbed onto non-polar surfaces and soil particles. However, there are very few reports describing the accumulation of endosulfan residues in the bodies of grazing and other animals (Noble, 1991). This is unlike the situation reported for the classic cyclodienes, aldrin and dieldrin, which also have low water solubilities (Noble, 1991; Albertson et al., 1990; Mitchell, 1990). The reason for this low accumulation of endosulfan residues in animals is probably due to their susceptibility to hydrolysis, imparted by the reactive sulfite group.

Toxicological properties of endosulfan

Because endosulfan is the most commonly used insecticide in cotton production and in combination with its relatively high application rates and potential toxicity, it is necessary that its levels and effects be closely monitored in the environment. Mammals, birds and the majority of soil and water microorganisms can tolerate relatively high concentrations of endosulfan when ingested or in their environment. Fish, however, are acutely sensitive to endosulfan residues (Table 4). Recent studies have shown that the growth of the blue-green algae is adversely affected in the presence of endosulfan (Tandon et al., 1988). There are a number of studies describing the effect of endosulfan on microorganisms, and all of these indicate that endosulfan has no adverse effect, even at high concentrations (Goebel et al., 1982).

Table 1. Total usage of endosulfan and pyrethroids in cotton growing areas of New South Wales and Queensland, Australia[a].

Region	Area (ha)	Endosulfan		Synthetic Pyrethroids	
		K litres	Av. No. Sprays	K litres	Av. No. S
1989/90					
Emerald	11800	133	3.76	71	2.0
Central Qld	6500	127	6.5	11	1.7
Darling Downs	12700	193	5.08	124	3.25
St George	10930	116	3.55	62	1.9
Mcintyre	22070	189	2.85	199	3.0
Gwydir	55100	588	3.56	364	2.2
Namoi	41300	504	4.07	292	2.36
Macquarie Valley/Bourke	24600	225	3.05	92	1.25
TOTAL	190000	2595	4.53	1215	2.13
1990/91					
Emerald	13600	294	7.2	100	2.45
Central Qld	6100	110	6.04	16	0.9
Darling Downs	32000	700	7.3	181	2.08
St George	11500	237	6.88	64	1.93
Mcintyre	24000	462	6.42	162	2.3
Gwydir	72000	1454	6.73	584	2.7
Namoi	49000	923	6.28	315	2.15
Macquarie Valley/Bourke	36500	679	6.2	229	2.09
TOTAL	244700	4828	6.58	1651	2.25

[a] Estimate of total endosulfan and synthetic pyrethroid usage, at 3.0 L/ha. Source: Hoechst Australia Limited in Barrett et al.(1991).

Table 2. Common names and trade names of technical formulations of endosulfan[a]

ENT 23979	Thiodan	Malix	Thimul
OM 570	Thionex	SD 4314	Benzoepin
HOE 2671	Thiotox	Endosulfan 35EC	Endosan
BIOl 5462	Tiovel	Chlorthiepin	Hidan
Niagara 5462	Tionex	Endosulphan	Endocel
		Beosit	Endotaff

[a] compiled from Chemical Abstracts, American Chemical Society and Worthing (1987)

Table 3. Some physical properties of endosulfan, aldrin, dieldrin and the endosulfan metabolites.

Common name	Empirical formula	M_w(g.mol^{-1})	mp °C (°K)	Molecular[a] volume
aldrin	$C_{12}H_8Cl_6$	364.9	104-104.5 (377.4)	316.8
dieldrin	$C_{12}H_8Cl_6O$	380.9	175-176 (448.7)	318.2
endosulfan I	$C_9H_6Cl_6O_3S$	406.9	109 (382.1)	318.2
endosulfan II	$C_9H_6Cl_6O_3S$	406.9	213 (486.2)	-
endosulfan sulfate	$C_9H_6Cl_6O_4S$	422.9	181 (454.2)	-
endosulfan diol	$C_9H_8Cl_6O_2$	360.9	204 (477.2)	-
endosulfan ether	$C_9H_6Cl_6O$	342.8	225 (498.2)	-
endosulfan hydroxy-ether	$C_9H_6Cl_6O_2$	358.9	235 (508.2)	-
endosulfan lactone	$C_9H_4Cl_6O_2$	356.8	264.5 (537.7)	-

[a] determined using the Le-Bas method (Suntio et al., 1991)

Endosulfan toxicity is reduced considerably once the sulfite group has been removed, that is, after its residues have undergone hydrolysis to its diol form. The β-isomer is approximately half as toxic to mammals than the α-isomer and the sulfate derivate. However, during the period of maximum insecticidal activity, the few days immediately after application, fish are very susceptible to endosulfan poisoning, from the three major residues, particularly if there are heavy rains during this period. Table 5 records the toxicity of endosulfan in a range of aquatic vertebrates. In a recent study by Nowak and Ahmad (1989), three species of fish exposed to sublethal doses of technical endosulfan were found to concentrate endosulfan sulfate and metabolise it to various products. In the same report field sampling during 1986-87 from the Gwydir river showed that catfish could accumulate high levels of endosulfan. Birds, bees and numerous beneficial insects remain unaffected at practical application rates. Whyte and Conlon (1990) and Goebel et al. (1982) have reviewed the acute and sublethal effects of endosulfan on wildlife and its disruption to terrestrial ecosystems.

Table 4. Acute toxicity of endosulfan and its metabolites[a]

	Toxicity LD_{50} (mg/kg)			
Compound	insects[b]	fish[c]	birds[d]	mammals[e]
endosulfan I	5.5	0.001-0.01	26-243	76, 359[f]
endosulfan II	9.0	-	-	240
endosulfan sulfate	9.5	-	-	76
endosulfan diol	>500	1-10	-	>15,000
endosulfan ether	>500	1-10	-	>15,000
endosulfan hydroxy ether	>500	1-10	-	1,750
endosulfan lactone	-	1-10	-	105-290

[a] single values and ranges compiled from Goebel et al. (1982) and Brooks (1974). [b] LD_{50} to housefly (topical toxicity). [c] LC_{50} for 13 genera for Thiodan®. [d] LD_{50} for 3 genera (oral toxicity) for Thiodan®. [e] rats (oral toxicity). [f] rabbits (dermal toxicity) for Thiodan®.

The movement and persistence of endosulfan in the environment

Over the past 40 years, many laboratory and field studies on endosulfan degradation have been performed and these have been reviewed by Goebel et al. (1982), and Barrett et al. (1991). These studies can be further classified into their movement in the soil and their fate and degradation in the environment.

Table 5. The toxicity of endosulfan to a range of aquatic species[a]

Species	LC_{50} (mg/kg)
Rainbow trout	0.0014
Cladocerans	0.0162
Freshwater crayfish	0.423
Mummichog	0.012
Mosquito fish	0.0013-0.037
Cyprinus carpio	0.011
Guppy	0.0015

[a] modified from Barret et al. (1991)

Movement of endosulfan in soil

Between application and complete degradation, endosulfan residues penetrate the soil profile to a depth of 15cm or less. This has recently been confirmed in a leaching experiment with a sandy loam (Kimber 1990). Residue analyses on soils from temperate climates, which have been treated for several years and more, show that endosulfan is not accumulated, unlike the classic cyclodiene compounds. Preliminary results from a survey conducted in the central and northwestern regions of New South Wales, Australia, have shown that only low levels of endosulfan and endosulfan sulfate reach waterways after being sprayed on the growing cotton crop (Otton 1991). In this study 10 sites throughout these regions were sampled at various times throughout the year and the levels of endosulfan residues were measured in water (Table 6). These sites were the major water ways likely to be affected from spraying of endosulfan for the control of insects in cotton in those regions. The data indicate that there were only low levels of endosulfan and endosulfan sulfate in the sampling sites and that there was a peak of endosulfan residues during February. December and January would generally be considered to be the main months of endosulfan usage. These preliminary results tend to suggest that is a lag period before the sprayed residues reach nearby waterways, and in Otton's study, this lag is approximately one month.

Fate and degradation in the environment

From a range of overseas studies it appears that the α-endosulfan isomer has a half life of 50-150 days in aerated temperate climate soils, the β and sulfate forms in the same soils for up to 1-1.5 years, and some 10-20% longer for all residues in flooded fields (Goebel et al., 1982 and references therein). Kimber (1990) found that the half life of β-endosulfan was 35 days in a Narrabri clay soil (from the north-west of New South Wales), in a laboratory experiment when incubated in a column, at 37°C. Although there is some variation in these results between Australia and overseas, the estimates of the half lives are all very much lower than those for the classic cyclodienes such as dieldrin and heptachlor in soil.

Table 6. Mean levels of endosulfan and endosulfan sulphate detected at the 10 sites in samples taken on the nominated dates

Sampling date	Endosulfan (μg/L)	Endosulfan Sulfate (μg/L)
15/10	ND	0.002
23/11	0.011	0.071
14/1	0.010	0.139
4/2	0.004	0.071
25/2	0.022	0.199
25/3	ND	0.047
23/4	ND	0.015
20/5	ND	ND

ND indicates Not Detected (<0.01 μg/L) (Otton, 1991)

There are a number of factors influencing the residual behaviour of endosulfan in the environment. Although the α-isomer normally degrades at a faster rate than the β-isomer in soil, in water and aqueous media, the β-isomer is the more labile of the two

(Guerin and Kennedy, 1992). The main degradation product in soil, endosulfan sulfate, is formed by oxidation of the parent endosulfan isomers and is subsequently hydrolysed, as are the parent isomers, to the non-toxic diol. The rate of formation of endosulfan sulfate from endosulfan is usually faster than its degradation, and for this reason it is included in the total endosulfan residue level. This is the major metabolite formed in aerated soils, from the action of microorganisms, but also in the roots and on the surfaces of plants. (Goebel et al., 1982; Singh et al., 1991). The biological formation of the sulfate derivative is likely to be the result of the action of mixed function oxidase enzymes and may be similar to the system that converts aldrin to dieldrin (Kennedy et al., 1990). The more volatile α-isomer tends to disappear from plant and soil surfaces faster than the β-isomer, sulfate and diol derivatives, leaving the latter as the predominant residues. Endosulfan can be degraded by chemical hydrolysis in the soil. Under alkaline conditions, both isomers are hydrolysed to the diol form in either aerated or waterlogged soils. While it is known that the diol and sulfate derivatives are sensitive to breakdown by light (photolysis), there is some evidence that the parent isomers are also degraded by this process (Goebel et al., 1982). This could be significant where endosulfan enters waterways.

Role of microorganisms in endosulfan degradation

A significant factor affecting the degradation of endosulfan in soil is the action of soil microflora, particularly in the black earths of the cotton growing areas. In a study by Martens (1976) in Germany, 28 soil fungi, 49 soil bacteria and 10 actinomycetes were tested to see whether they could degrade endosulfan. Sixteen fungi, 15 bacteria and three actinomycetes were found to metabolise more than 30% of originally applied endosulfan in pure culture. Although the major metabolites were endosulfan sulfate and endosulfan diol, small quantities of other metabolites were detected, and only a small amount of pesticide-derived carbon dioxide was released, indicating that extensive mineralisation of the carbon skeleton did not occur. This study suggests that the ability of microorganisms to co-metabolise (i.e. only partly degrade and not use as an energy or nutrient source) endosulfan may be ubiquitous among soil microorganisms and that soil bacteria tend to form the less toxic endosulfan diol derivative while fungi, although with a few exceptions, oxidise endosulfan to the sulfate derivative. This latter transformation is less desirable as the sulfate is equally toxic to mammals as the α-isomer, and more persistent in soil (Table 4). Although there is little evidence, it is thought that both the α- and the β-isomers can be dechlorinated to form the pentachloroderivatives, under anaerobic conditions. The pentachloroderivatives of endosulfan, like the pentachloroderivatives of other cyclodiene insecticides, are significantly less toxic to mammals than the parent compounds. So the formation of the diol, β-isomer, and the partially dechlorinated endosulfan forms, constitute significant steps in endosulfan detoxification. From laboratory studies with mixed cultures of microorganisms from soil, it is suggested that endosulfan can be completely degraded, via the diol and subsequently the ether and lactone derivatives (Miles and Moy 1979). This particular study was conducted under aerobic conditions in liquid culture at low levels of added endosulfan. Similar experiments have been conducted under anaerobic conditions to indicate if indigenous microflora from low-oxygen environments, are also capable of transforming endosulfan, and preliminary results suggest that such organisms do (Guerin and Kennedy, 1992).

Research priorities

Although some research has been done on endosulfan biodegradation in soils, and by pure and mixed microbial cultures in overseas studies, little has been performed in Australia (Barrett et al., 1991 and references therein; Hoechst Australia, 1991). With the extensive use of endosulfan as an integral part of cotton growing in Australia, a number of pertinent questions need to be addressed as to the fate of this chemical and its primary metabolite, endosulfan sulfate. For example, to what extent is endosulfan degraded by soil microorganisms and what percent of the total microorganisms are involved? Is endosulfan sulfate degraded by the same mechanism as that of the parent isomers? Do soil microorganisms degrade endosulfan and endosulfan sulfate in flooded soils and highly contaminated sites? Do individual species bring about a significant detoxification or is a consortium of microorganisms required? Are there special conditions in which endosulfan may accumulate and escape into waterways, or is this primarily a result of poor management of the insecticide, which can be controlled by more vigilance in its use? At a more basic level, what are the biochemical processes involved in endosulfan biodegradation; which enzymes for example, if any, catalyse endosulfan hydrolysis to form the non-toxic diol metabolite? What happens to the bridged organochlorine rings? If the organochlorine ring goes to chloride ion, as in common salt, the environmental concerns could be significantly reduced. These questions need to be addressed if the environmental fate of endosulfan is to be predicted.

The current study attempts to address some of these questions. In particular, to determine the potential for biodegradation of endosulfan by various New South Wales soils, and of mixed and pure cultures of microorganisms from these soils.

Summary

Endosulfan is a very important for the cotton grower in controlling harmful insect pests, and its use for this purpose is likely to continue into the foreseeable future. Given the potential ecological hazards posed by the use of such a compound, prudence requires that a number of questions be addressed regarding its behaviour in Australian soils. The current study aims to address some of the soil biodegradation issues and the outcome of the study should provide a clearer picture of the fate of endosulfan in cotton growing soils and of the role of soil microorganisms involved in its degradation, perhaps suggesting a strategy to minimise the potential for environmental impacts.

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THE CLEAN-UP OF ORGANOCHLORINE PESTICIDES FROM SOIL USING BIOLOGICAL PROCESSES.

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SUMMARY

Bioremediation provides an approach to the clean-up of soil contaminated with persistent pollutants. Successful case studies on the decontamination of organochlorines from soil using bioremediation have been examined and show that bioremediation should be considered in developing soil decontamination strategies. Developing a strategy for the clean-up of organochlorine contaminated soil using biological systems requires that degrading microorganisms are made available, and secondly that these microorganisms be established, with their degrading capabilities, in the contaminated soil. Laboratory biodegradation studies, along with ecological studies, are necessary components of any successful research project addressing bioremediation. The biodegradation potential of the indigenous soil microorganisms will also need to be addressed. Other approaches to the clean-up of organochlorine contaminated soil are briefly examined.

INTRODUCTION

There are many contaminated waste sites throughout the world originating from a wide range of sources. In Australia and New Zealand, these are associated largely with agriculture and the manufacturing industry. In agriculture, for example, old cattle tick dip sites and sullage pits pose a considerable problem, in that they contain high levels of DDT and related persistent compounds. Sullage pits contain mixtures of agricultural chemicals which are concentrated in small and confined evaporation dams. They may also contain inorganic pollutants such as arsenic and its derivatives. In the manufacturing industry there are a number of problems caused by the presence of waste dumps and these are often located in the vicinity of residential areas. In some instances, there are contaminated soils associated with practices in the mining industry. These soils often have high levels of heavy metals and other inorganics such as cyanide and acids. Soil pollution problems in China arise from industrial waste water, pig waste, domestic waste water, gases and organic pollutants. Although the major pollutants resulting from the above sources are heavy metals, there is a problem with organochlorine pesticide residues in soil (Chien and Yao, 1990).

In cattle dip sites, the organochlorines are usually confined proximal to the site of original application, and therefore represent a point-source type of soil pollution. The potential of these compounds to pollute groundwater through leaching from these point sources is low, as indicated from the results of studies of organochlorine movement in soil

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(Gerritse et al., 1991 and references therein; Kimber, 1990). Significant environmental contamination problems are more likely to arise from the mass transfer of soil from these sites such as during a flood. These point sources of contamination nevertheless represent a challenge for environmental chemists and microbiologists.

Reducing the residues in the environment from agricultural chemicals is important from the points of view of industry, human health, and the general well being of the environment. Prospects for reducing these residues encompass a number of disciplines within the general field of pesticide science, but one of the most promising is that of the field of biodegradation (Guerin and Kennedy, 1991; Munnecke, 1979). Biodegradation studies are of primary importance in developing strategies for degrading various pollutants. These studies provide a means of making effective pollutant degrading microorganisms available for further research. Such microorganisms may provide the degradative genes which can be manipulated into more suitable host organisms and in particular those better adapted to survival in contaminated soils.

There is a good deal of literature describing the biodegradation of pesticides (Alexander, 1981; Macrae, 1990; Wallnöfer and Engelhardt, 1990). As pointed out by Kearney et al. (1986), in many cases the responsible degrading enzymes have been identified and characterised, but only in a relatively few cases have the genes encoding these enzymes been described. In some cases these genes have been localised on a plasmid, but more often they are partially encoded by chromosomal DNA. Some have been reported to be located on transposons, discrete pieces of DNA that move between bacterial chromosomes and plasmids, within a particular cell. A number of naturally occurring, as well as engineered, microorganisms have been employed in the clean-up of pesticide wastes, and most success has been achieved with relatively labile pesticides such as organophosphates.

This paper introduces some of the aspects of the biodegradation of the chlorinated pesticide DDT and the concept of applying bioremediation to the clean-up of organochlorine pesticide contaminated soil. It also describes the constraints in developing bioremediation stategies for polluted soils and discusses key areas requiring further research.

THE BIODEGRADABILITY AND ENVIRONMENTAL FATE OF DDT

DDT is a persistent organochlorine pesticide. The recalcitrant nature of DDT is related to its highly chlorinated structure, which renders it less susceptible to enzymic attack and subsequent chemical modification. There are however, a number of reports describing the bio-conversion of DDT in the environment. Some of these conversions occur under reducing conditions to form dechlorinated derivatives, while others require both reductive and oxidative conditions for their formation. The formation and detection of various metabolites indicates the potential of microorganisms to biodegrade DDT. Many of these biotransformations have been reviewed by Metcalf (1973), Johnsen (1976) and Golovleva and Skryabin (1981). Specific microorganisms involved in these transformations are outlined in Table 1. In laboratory liquid culture studies, *Psuedomonas sp* and *Aerobacter sp* have been found to be the most active bacteria in degrading DDT. A number of soil fungi demonstrate an ability to degrade DDT, particularly the white rot fungi, and many of these can bring about complete degradation of DDT to CO_2.

Table 1 Microorganisms involved in the degradation of DDT

microorganism	products formed[a]	degradation conditions[a]	reference
Pseudomonas aeruginosa	benzyl hydrol, DDD, DDE, DDA, benzophenone	complex sequence of conditions in laboratory cultures	Golovleva and Skryabin (1981)
Phaerochaete chrysosporium	DDD, dicofol, 2,2-dichloro-1,1-bis (4-chlorophenyl) ethanol, 4,4'-dichlorobenzophenone, CO_2	liquid culture	Bumpus and Aust (1987)
Pleurotus ostreatus, Phellinus weirii, Polyporus versicolor	CO_2	liquid culture	Bumpus and Aust (1987)
Norcardia, Streptomyces Spp Escherichia coli, yeast, rumen fluid	DDD	liquid culture	Johnsen (1976)
Trichoderma viride	DDA	liquid culture	Johnsen (1976)
Aerobacter aerogenes	DDD,DDE,DDMU,DDMS,DDA,DDNU,DBP, DDM,DBH	liquid culture, cell-free extracts	Johnsen (1976) Wedemeyer (1966)

[a] abbreviations for the products formed are given in the appendix table in this article. [b] liquid culture degradation conditions was where DDT degradation was monitored in batch (or flask) studies. Cell-free extracts indicated that there was activity when the cultures were harvested and broken into sub-cellular fragments. This activity may be due to the action of co-metabolic enzymes.

THE CONCEPT OF BIOREMEDIATION

Bioremediation can be defined as the application of biological systems to the clean up of soils contaminated with foreign compounds. A site is considered contaminated when it contains these toxic compounds at levels higher than background or acceptable levels. Such a system generally involves the introduction of specific microorganisms to the contaminated soil. These microorganisms have been isolated or engineered to degrade the specific contaminant. Although bioremediation generally involves the introduction of microorganisms which have been isolated or modified to degrade the specific contaminant, in a broader sense, bioremediation may also include the encouragement of indigenous soil microflora by various management practices.

The major components of a typical bioremediation strategy can be considered as follows. First, it is necessary to obtain microbes capable of detoxifying the pollutant, or to determine whether indigenous microbes can bring about degradation in sites. Secondly, these microorganisms must be inoculated and established in the contaminated soil with their degradation capabilities. Thirdly, careful monitoring of the site, involving field sampling and laboratory analyses, will be required to determine if the process is effective. Kelley et al. (1992) have defined these stages of a typical bioremediation process and these have been compiled in Table 2.

Table 2. Components of a typical bioremediation strategy[a]

1. Site assessment
2. Microbiological assessment
3. Treatability study
4. Process design
5. Treatment
6. Process monitoring and control
7. Validation
8. Decommissioning

[a] Compiled by Kelley et al. (1992)

Much work has been conducted in the field of bioremediation in a number of European countries. A recent conference in Karlesruhe, Germany, has addressed many general aspects of bioremediation, as well as providing evidence for a number of successful clean-up programs (Arendt et al. 1990). While there has been much work reported on the bioremediation of hydrocarbon contaminated soil, there has been relatively little reported on the clean-up of soil contaminated with highly chlorinated organic compounds such as DDT.

SUCCESSFUL APPLICATION OF BIOREMEDIATION TO THE CLEAN-UP OF ORGANOCHLORINE CONTAMINATED SOIL

There are a few reports in the literature which describe the successful clean-up of organochlorine contaminated soil using this biotechnology. Chatterjee et al. (1982) have reported the biodegradation of 2,4,5-T (an organochlorine herbicide) in soil by a pure bacterial culture. This bacteria, *Pseudomonas cepacia*, was selected in a biodegradation study where it was shown to use 2,4,5-T as a sole source of carbon and energy (Kilbane et al. 1982). When added to soil it was shown to degrade, and grow in the presence of 2,4,5-T. The optimum temperature for the degradation of this compound was found to be 30°C with a soil moisture content of 15 to 50% wt/vol. This culture was found to degrade as much as 95% of the applied 2,4,5-T at the high concentration of 1mg/g soil (1000 ppm) within 1 week of introducing the culture to the soil. Over a 6 week period, repeated applications of the bacterium degraded more than 90% of the chemical from soil containing very high levels of 2,4,5-T, i.e. 20 mg/g soil (20,000 ppm).

Kearney et al. (1969) have reported the accelerated biodegradation of DDT in a range of soils contaminated with DDT . Although the levels of DDT in these studies were between 5-20 ppm, they are considerably higher than those expected to be found in most farming soils. However, these levels are similar to the minimum levels required for the clean up of contaminated sites. Using a sandy loam, a silty clay and a clay, as representative soils, a comparison was made between flooded soils (to simulate anaerobic conditions) with or without having a facultative anaerobic bacterium added, known to degrade DDT. This particular bacteria, *Aerobacter aerogenes*, available from the American Type Culture Collection (ATCC 13048), can apparently co-metabolise DDT extensively (Wedemeyer, 1966). The trends in these results were that DDT disappearance was fastest in the inoculated soils and especially in the clay soil, referred to as Sharkey in Figure 1.; the metabolism was primarily co-metabolic, with little mineralisation occurring; and DDD (or TDE) was shown to be formed in most soils, with no other degradation products. Kearney et al. (1969) concluded that although the DDT reduction in the contaminated soil was real and reproducible, the mechanism involved was not fully understood. The results of this study are shown in Figure 1.

Johnsen (1976) has summarised the results of a number of other successful laboratory experiments where DDT residues in soil were significantly reduced, by encouraging the growth of particular soil microorganisms. Recent researchers have reported on the clean-up of other organochlorine contaminated soil, such as those impregnated with the industrial pollutant PCB and its various congeners, using bioremediation techniques. Unterman (1990) and Bourquin (1990), have had success with decontaminating soil containing organochlorine residues under aerobic conditions. Stevens et al. (1990) have been successful in implementing the release of organochlorine degraders into anaerobic environments. These studies indicate that bioremediation of organochlorine contaminated soil may be achieved under a range of environmental conditions.

CONSTRAINTS TO BIOREMEDIATION

There are a number of limitations in implementing a bioremediation strategy. These can be considered under the three major components of a bioremediation strategy, i.e., 1. The isolation of degrading microorganisms and the determination of the biodegradation potential of the indigenous site microflora, 2. The treatment stage, and 3. Site monitoring. Economic constraints, although important in considering bioremediation as

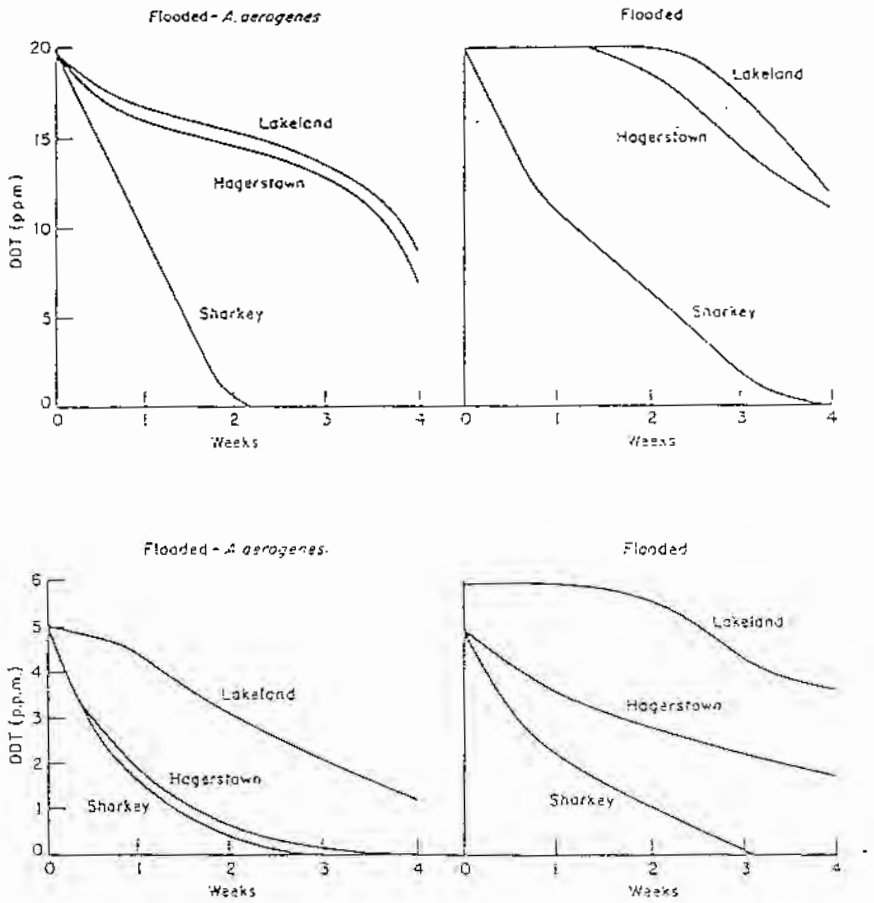


Figure 1. DDT decomposition in three soils at 5 and 20 ppm (Kearney et al. 1969)

a clean-up alternative, have not been detailed in this report. A summary of these constraints is given in Table 3. In general, there appears to be very little information available on determining the cost of using biological systems for soil clean-up. Some aspects of the economic constraints have been described by European specialists in the field (Arendt et al.,1990) and by Kelley et al.(1992) in Australia.

Table 3. Economic constraints to bioremediation

Cost of pilot study
Cost of implementing clean-up process (engineering, fermenter operation)
Length of remediation period
Monitoring and demonstration of bioremediation (validation)
Need for basic research

1. Isolation of DDT degrading microorganisms and determination of the biodegradation potential of the contaminated site microorganisms

The first stage in the development of a bioremediation process involves isolating organisms capable of transforming the contaminant to relatively simple and readily metabolisable forms or at least in such a way that renders the contaminant less or non-toxic to most organisms. Also, in this initial stage of developing a process, the biodegradation potential of the indigenous microorganisms needs to be addressed.

Isolating DDT-degrading microorganisms is a difficult task. The compound is usually only co-metabolised, if at all and is not generally used as a source of carbon and energy. Unfortunately, this means that attempts to use selective media, formulated with DDT as the sole source of carbon to isolate DDT degraders, are unproductive. Microorganisms capable of partially degrading DDT however, have been isolated from soil and sewerage. There are a number of such reports and Wallnöfer and Engelhardt (1990) Macrae (1990) and Johnsen (1976) have reviewed the work in this field. Some individual reports include that of Wedemeyer (1966), Pfaender and Alexander (1973) and more recently that by Bumpus and Aust (1987). The latter researchers have described the biodegradation of DDT by the white rot fungi.

Basic isolation studies provide microorganisms for laboratory biodegradation studies. These laboratory studies allow the effectiveness of pure and mixed cultures, to be compared, to determine their optimum nutrient requirement, pH, and temperature conditions and other factors for the degradation. Although laboratory studies usually do not reflect what occurs in the field, it does provide some indication of the potential success of the degradative organisms. Most of the reports to date describe the partial degradation (co-metabolism) of DDT, with only a few studies showing complete degradation (mineralisation) at low rates. Like many other organochlorine compounds such as aldrin and dieldrin, DDT is a relatively unattractive source of carbon or energy for microbial growth. In order to bring about significant degradation of the DDT

molecule, it is necessary to remove some of the chlorine atoms to allow oxidation of the biphenyl ring carbons, or to cleave the biphenyl ring system, to allow the formation and subsequent degradation of benzene derivatives by ubiquitous soil microorganisms. Wedemeyer (1966) has demonstrated the mono-dechlorination of DDT to DDD by a pure bacterium, *Aerobacter aerogenes* and Table 1 describes other examples of DDT biodegradation. In the later work of Pfaender and Alexander (1973), it was demonstrated, using pure cultures of *Hydrogenomonas sp.* isolated from sewerage, that DDT and related structural analogues could be converted to ring cleavage products primarily p-chlorophenylacetic acid. Another bacterium was isolated, *Arthrobacter sp.*, that was able to grow on p-chlorophenylacetic acid i.e. using this primary DDT metabolite as a source of energy. This study indicates that detoxification of DDT may occur through the activities of a consortium of soil microorganisms in natural environments and particularly in those having reduced oxygen levels such as sludges and sediments. Whether such indigenous microbial activity occurs in dip sites in Australian soils is yet to be demonstrated.

2. Treatment of the site and environmental constraints

Although in theory it is possible to release DDT degrading microorganisms into contaminated sites, the success of their establishment in these soils is generally hampered by a host of environmental constraints (Alexander 1984, 1990). Not only are levels of DDT variable from site to site, but there are usually a range of other pollutants present including arsenic, ethion, BHC, chlorpyriphos and dioxathion, and their residue levels also vary. Such a diversity of organic compounds reduces the probability of any one of these compounds to provide an energy source for the growth of an introduced pesticide-degrading species or microbial consortium. In addition, DDT is relatively resistant to microbial attack, i.e., it is rarely mineralised to CO_2 and is usually only co-metabolised by microorganisms to different forms (Alexander 1979), some of which are less toxic. Other ecological limitations include the inability of inoculated microorganisms to locate the chemical to be degraded, and that there are low, as well as variable, levels of pollutant at the contaminated site. Of greatest importance, however, is that the indigenous soil microflora have a competitive advantage for the majority of food sources and other nutrients over those introduced.

Testing the efficacy of pesticide degraders *in situ*, whether directly isolated from soil or genetically engineered in the laboratory, requires that a number of questions relating to microbial ecology be addressed. Inoculation of soil with alien (non-indigenous microorganisms), or with those taken from the same soil is often an exercise in futility. Organic compounds which are not usually found in nature, for example DDT, are potential sources of energy for microbial growth. But on many occasions it has been shown that such compounds are not always utilised by the indigenous microflora in contaminated soils. So in theory, if a pollutant is not being metabolised by the indigenous population of microorganisms, then the inoculation of that soil with isolates capable of utilising the compound, would be successful because there is no competition for that particular energy resource (Focht, 1986). This reasoning assumes that there are no adverse conditions in the contaminated soil environment which would hamper the establishment of the degrading inoculants. Unfortunately, such idealistic conditions do not exist in soils.

In the field of microbial ecology probably the single most important question to be

addressed with regard to DDT bioremediation is whether or not DDT degrading microorganisms survive, let alone proliferate and degrade the contaminant, when added to soil. Thorough tests in this regard are yet to be performed on DDT degrading isolates. Some of the reasons why survival may be hampered are detailed in the following sections, and they have been classified into three key ecological components and other general environmental constraints.

i) The concentration of the contaminant

The distribution of DDT at dip sites is predominantly within a relatively small area, although its concentration is often high. This point source type of pollution is of consequence to the *in situ* biodegradation of this compound. For example, if a theoretical a DDT-utilising bacterium (or consortium) is added to that soil, then the amount and distribution of the DDT will dictate largely the survival of the inoculum. In many cases pollutants are not degraded at very low concentrations (<10 ppb), levels which are not uncommon in natural waters and agricultural soils. Dip sites present different problems however, including the possibility of toxicity to the innocula because of the high levels and wide range of agricultural chemicals present. Therefore studies would be necessary to determine at what pesticide concentration range the degraders are capable of bringing about a decrease of residues in the contaminated soil.

ii) The problem of microbial predation

The problem of microbial predation as an environmental constraint should also be considered. Many ecosystems, in particular soils, are endowed with a range of microbial predators such as protozoa, metazoa, myco-bacteria and single cell slime moulds. These are known to predate a range of bacteria, however there is scant information on the effect of predators on modifying the microbial activity of microorganisms that degrade pollutants (Alexander, 1984). Although a protozoan in a particular contaminated soil may enhance the activity of a pollutant-degrading bacteria, specific studies would need to be conducted in order to characterise these effects for a specific degrading bacterium or population intended for release. The impact of predators may be overcome by using bacteria (that can withstand predator grazing), as pollutant degraders *per se*, or as vehicles for the genes encoding the pesticide degradative capabilities (Alexander, 1984).

iii) Pollutant distribution and bioavailability

Organochlorines are water insoluble compounds and their movement in soils is generally restricted to slow diffusion or with the movement of organic matter and colloids. Consequently, compounds such as DDT tend to remain concentrated near the top of the soil profile where they were initially deposited. So although the potential problem of DDT leaching through the profile and therefore away from the added degrading populations is minimal, the problem of binding and microbial availability need to be considered. The presence of particular enzymes in the introduced innocula may be necessary for the release of bound forms of organochlorines from colloids, such as those capable of degrading humic acids. Experiments are therefore necessary to determine the capability of the potential degrading inoculants, to release soil bound forms of the pollutant. Experiments in our laboratory where soils containing high levels of the organochlorine endosulfan (approximately 8000 ppm in soil), are incubated in media which encourages the growth of the indigenous soil microorganisms, show that after approximately 8 days that there is a significant increase in the parent isomers detected in the medium. Such an increase does not occur in sterile media, and incubations

containing innocula from uncontaminated soil, and can only be explained in terms of release of bound forms from the soil carried over in the inoculum by the action of microorganisms (Guerin and Kennedy, 1992).

iv) Other environmental constraints

Environmental constraints can be classified into three broad groups, namely ecological, microbial/genetic/physiological and soil-geophysical factors (Tables 4 and 5). Other ecological constraints are the mobility of the degrading populating in the soil matrix, the susceptibility of this population to attack by other native microorganisms, and the water and nutrient requirements of the added innocula. Degrading populations of microorganisms must be isolated or engineered, and they may need to be able to solubilise the pollutants so that they may be made available for transformation. Therefore the solubility of the pollutant and its sorption onto the soil particle surfaces are going to be important constraints to the success of a bioremediation project.

Table 4 Environmental constraints to bioremediation of contaminated soils

Ecological	Microbial/Genetic/Physiological
microbial movement	availability of degraders
oxygen requirement	type of metabolism leading to degradation
microbial competition and predation	toxicity of pollutants to innocula
pH optimum	ability of innocula to solubilise pollutants
water requirement	
starvation tolerance	soil biodegradation potential

Soil-geophysical factors include the bedrock composition, whose porosity will affect infiltration rates, and mineral composition, which will influence microbial absorption. Another aspect is the nature of the drainage basin area at the contaminated site. Thus the drainage basin characteristics will influence water flow through and across the site. The presence of fault and fracture lines will also influence the movement of ground water, which in turn will modify pollutant and/or microbial movement. Ground water pH levels will affect the mobility and precipitation of metals and other compounds as well as the conditions for microbial survival.

Table 5 Soil-geophysical constraints to applying pollutant-degrading organisms to soil

water table movement and soil moisture potential
solubility of pollutants in water
site topography, soil type and microclimate
pollutant sorption
pollutant type, distribution and movement throughout the site profile
adsorption and movement of microorganisms throughout the site profile
soil mineralogy and bedrock composition
drainage basin area
fault and fracture zones
ground water pH

Although there are many constraints to be considered, study of the geological limitations will be necessary prior to developing a site-specific bioremediation process. Data on the specific geological characteristics likely to affect the bioremediation process will need to be gathered, using previous drilling or sampling records, and their relative significance determined. Geologists will play a vital role in developing bioremediation strategies in providing this information to microbiologists and engineers. This information will include rock and mineral types, distribution, and their relative proportions at specific sites. These factors will influence the survival and proliferation of added microorganisms. For example, the presence of specific mineral types in both the soil and rocks, are likely to influence the binding of both pollutants and soil microorganisms. Further experimental data may need to be acquired on the influence of various rocks and minerals on pollutant and microbial adsorption.

The mining industry can provide expertise in obtaining these answers. Some mining companies have already started putting their efforts into bioremediation projects. A case in point is CRA's Bioremediation Group now located in Melbourne (Kelley et al., 1992). They have reported a number of successful in-house and commercial clean-ups during their relatively short period in this field. In some cases, bioremediation technologists have overcome a number of the environmental constraints discussed earlier, and particularly the geological constraints, through a technique termed "land farming". This is where contaminated soil is moved from the contaminated site, to a neighbouring site where it can be farmed to bring about further aeration for example, for improved oxidation of the pollutants. This method is relatively expensive, because of transport and the excavation costs involved. However, since the soil is moved away from its geological environment to a new site, (as a top soil), these geological constraints are largely removed. As the technology improves in this new field, such costs may be reduced.

3. Monitoring, demonstration and validation of bioremediation

The measurement of pollutant residues at the contaminated site is necessary for monitoring the effectiveness of the introduced microorganisms. For this, a sampling technique will need to be decided upon and analytical equipment will need to be available for the determination of the remaining soil residues. An electron capture gas chromatograph would be appropriate for DDT and preferably in conjunction with a mass

spectrophotometer for confirming the formation of metabolites. If the monitoring shows that the introduced microorganisms are ineffective in degrading the pollutant *in situ*, then changes can be made at the site, one at a time, until the biodegradation is optimised.

FUTURE PROSPECTS FOR BIOREMEDIATION

Applications of molecular biology

One of the ultimate objectives in microbial degradation studies is to combine the degradative capabilities of two or more microorganisms. Pemberton (1981) outlines the potential for combining the cometabolic activities of two different microorganisms, into a single microorganism, using the concept of Sommerville (1978) (Figure 2). The hypothetical DDT degrader produced by this approach now contains degradative genes from a number of organisms which would otherwise have been rare or non-existent in nature. Using this method the co-metabolic activities of several microorganisms can be combined within a single organism which has a degradative pathway for a novel substrate, in this case DDT. Often the evolution of new microorganisms containing the combined degradative pathways is very slow. This is particularly the case where a number of different metabolic steps in a pathway are required such as with DDT. Timmis et al. (1986) therefore describe three experimental approaches to the laboratory evolution of degradative pathways in microorganisms.

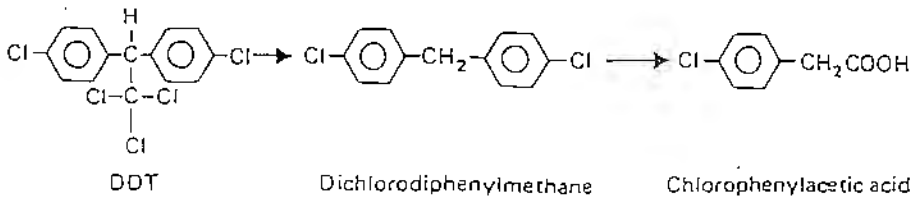
These are:

i) Long term chemostat selection, which often involves progressive replacement of a mineralisable analogue by the actual compound to be degraded. In the case of DDT this could be the dechlorinated DDT analogue, diphenylmethane, being replaced by DDT.

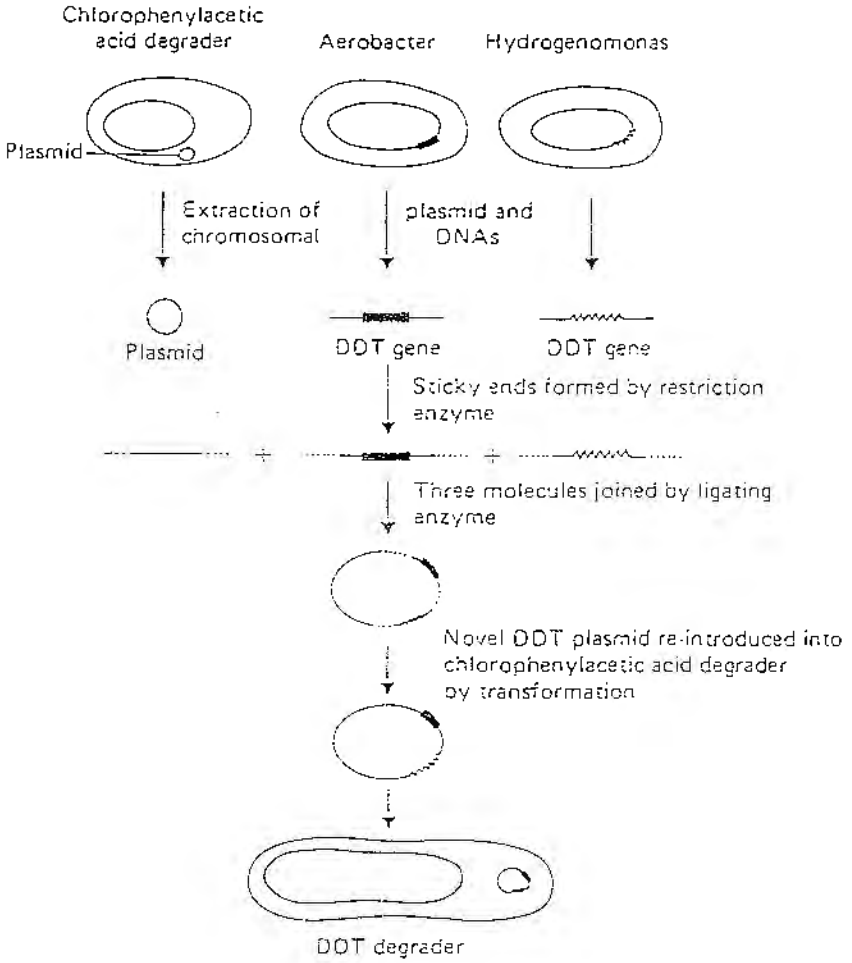
ii) In vivo genetic transfers, in which genes of critical enzymes of one organism are recruited into a pathway of another through experiments involving natural genetic transfer processes.

iii) In vitro evolution, in which known and well characterised genes are selectively transfered into different organisms to evolve new pathways. Pemberton (1981) has proposed such a pathway for the degradation of DDT.

Another approach in molecular biology is the use of protein engineering to develop the more active degradative enzymes. Assuming enzymes for the co-metabolism of DDT can be made available, it is possible to engineer these enzymes so as to endow them a greater capacity to degrade DDT and its primary metabolites. Wackett (1990) and Jannsen et al. (1990) have reported examples of engineering of enzymes which dehalogenate relatively simple organochlorines. This approach requires that pure enzymes and the gene(s) for the particular enzyme(s) be available. Given that further basic research is carried out in order to isolate potential DDT degrading microorganisms, then enzymes from those isolates that are most active, could be extracted and purified to homogeneity using various column chromatography configurations. The pure enzyme could then be characterised (sequenced) and used in protein engineering studies, to develop enzymes with a greater capacity for degrading DDT and its primary co-metabolites. The genes encoding the enzyme with a lower K_m towards DDT (i.e. a greater affinity and thus a



Co-metabolism of DDT by the action of two bacteria, *Aerobacter* and *Hydrogenomonas* (Somerville 1978).



Production of a DDT-degrading bacterium by *in vitro* genetic engineering; note the co-metabolic activities of *Aerobacter* and *Hydrogenomonas* are added to an organism capable of chlorophenyl acetic acid degradation (Somerville 1978, Pfaender and Alexander 1973).

Figure 2. Construction of potential DDT degrading microorganisms (Pemberton 1981).

faster rate of degradation) could then be introduced into a host microorganism in the manner outlined earlier by Pemberton (1981).

Removal by plants and plant-microbial systems

The possibility of using plants having high lipid content and extensive root penetration capabilities has been mentioned by Kearney et al., (1969). Earl and Kennedy (1972), Guerin (1988), Hwang (1990), have demonstrated that soybean (*Glycine max*) and a range of vegetable crops, are capable of removing organochlorines from perlite, a defined plant growth medium. This concept of using plants as 'sinks' for absorbing soil residues of organochlorines has received little attention. Lipophilicity is likely to be a key characteristic in determining the usefulness of plants in soil clean-up. Phytotoxicity will need to be considered particularly where chlorinated phenoxy compounds are present. Another potential role of plants in developing bioremediation is through encouraging microbial populations that can degrade the compound of concern (Van Zwieten et al., 1992). Such an interaction may provide a specialised niche for specific soil microorganisms to grow in, through the exudation of various nutrients and co-factors. The microorganisms that flourish in these environments may then demonstrate the capacity to degrade pesticides, and thereby accelerating the biodegradation of the contaminant.

OTHER CLEAN-UP PROCEDURES

There are a number of non-biological decontamination procedures currently being investigated and developed for the large scale remediation of soils containing persistent compounds. These include the reductive dechlorination of organochlorines catalysed by porphyrins, i.e. tetrapyrrole derived compounds of biological origin including vitamin B_{12}. This process, although not developed for field scale application at this stage, has the potential to provide a low cost and effective method for dechlorinating DDT, and is effective even at very low concentrations of organochlorines (Miskus et al., 1965; Zoro et al., 1974; Marks et al., 1989). Using an iron-redox system in a water saturated soil, Glass (1972) reported that DDT could be degraded to TDE and the rate of conversion was related to the rate of ferrous iron formation. The DDT degradation was also observed in soil-free iron redox systems. The Sydox Process, currently being developed for bench top application by Beattie et al. (1989) may find application for the clean-up of relatively small soil samples containing very high levels of DDT. The Sydox process, and two other methods, namely Plasma Arc Technology and Supercritical Fluid Technology, are being developed in Australia for the destruction of intractable wastes on a commercial scale and these have been briefly reviewed by Beder (1991). The limitations in these methods at present are that they require the organochlorine compounds to be in extremely high concentrations, otherwise the cost of treatment would be too high.

CONCLUSIONS

DDT can undergo biodegradation in the environment by a range of soil bacteria and fungi. Many of the degradation products of DDT, produced by these naturally occurring microorganisms, are less toxic than the parent compound. This research has found application in the development of bioremediation for DDT-contaminated soil through the release of DDT degrading bacteria into contaminated soil. The potential role of bioremediation as a means of reducing soil residues of DDT therefore needs to be considered in cleaning up DDT contaminated soil such as that present at cattle dip sites.

There are a number of reports in the literature describing the biodegradation of DDT in laboratory culture. There is however, very little information describing the release and establishment of these microorganisms in contaminated soil environments. Aspects of this field which require further investigation have been detailed, and in conclusion further research needs to be conducted to determine optimum conditions for microbial DDT degradation in contaminated soil.

Abbreviations, chemical formulae and names of DDT and its degradation products (Johnsen, 1976).

Abbreviations	Formula[a]	Name
p,p'-DDT	$(R)_2$ - CH - CCL_3	1,1'-bis(*p*-chlorophenyl)-2,2,2-trichloroethane
p,p'-DDD (TDE)	$(R)_2$ - CH - $CHCl_2$	1,1'-bis(*p*-chlorophenyl)-2,2-dichloroethane
p,p'-DDE	$(R)_2$ - C = CCl_2	1,1'-bis(*p*-chlorophenyl)-2,2-dichloroethylene
p,p'-DDMU	$(R)_2$ - C = CHCl	1,1'-bis(*p*-chlorophenyl)-2-chloroethylene
p,p'-DDMS	$(R)_2$ - CH - CH_2Cl	1,1'-bis(*p*-chlorophenyl)-2-chloroethane
p,p'-DDNU	$(R)_2$ - C = CH_2	1,1'-bis(*p*-chlorophenyl)-ethylene
p,p'-DDNS	$(R)_2$ - CH - CH_3	1,1'-bis(*p*-chlorophenyl)ethane
p,p'-DDOH	$(R)_2$ - CH - CH_2OH	1,1'-bis (*p*-chloroplienyl)-2-hydroxyethane
p,p'-DDA	$(R)_2$ - CH - COOH	bis (*p*-chlorophenyl) acetic acid
p,p'-DBH	$(R)_2$ - CHOH	4,4'-dichlorobenzhydrol
p,p'-DBP	$(R)_2$ - C = O	4,4'-dichlorobenzophenone
p,p'-DDM	$(R)_2$ - CH_2	bis (*p*-chlorophenyl) methane
p,p'-dicofol	$(R)_2$ - COH - CCl_2	1,1'-bis (*p*-chlorophenyl)-2,2,2-trichloroethanol
o,p'-DDT	R(R^1) - CH - CCl_3	1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl) -2,2,2-trichloroelthane

[a] R = p-chlorophenyl and R^1 = o-chlorophenyl

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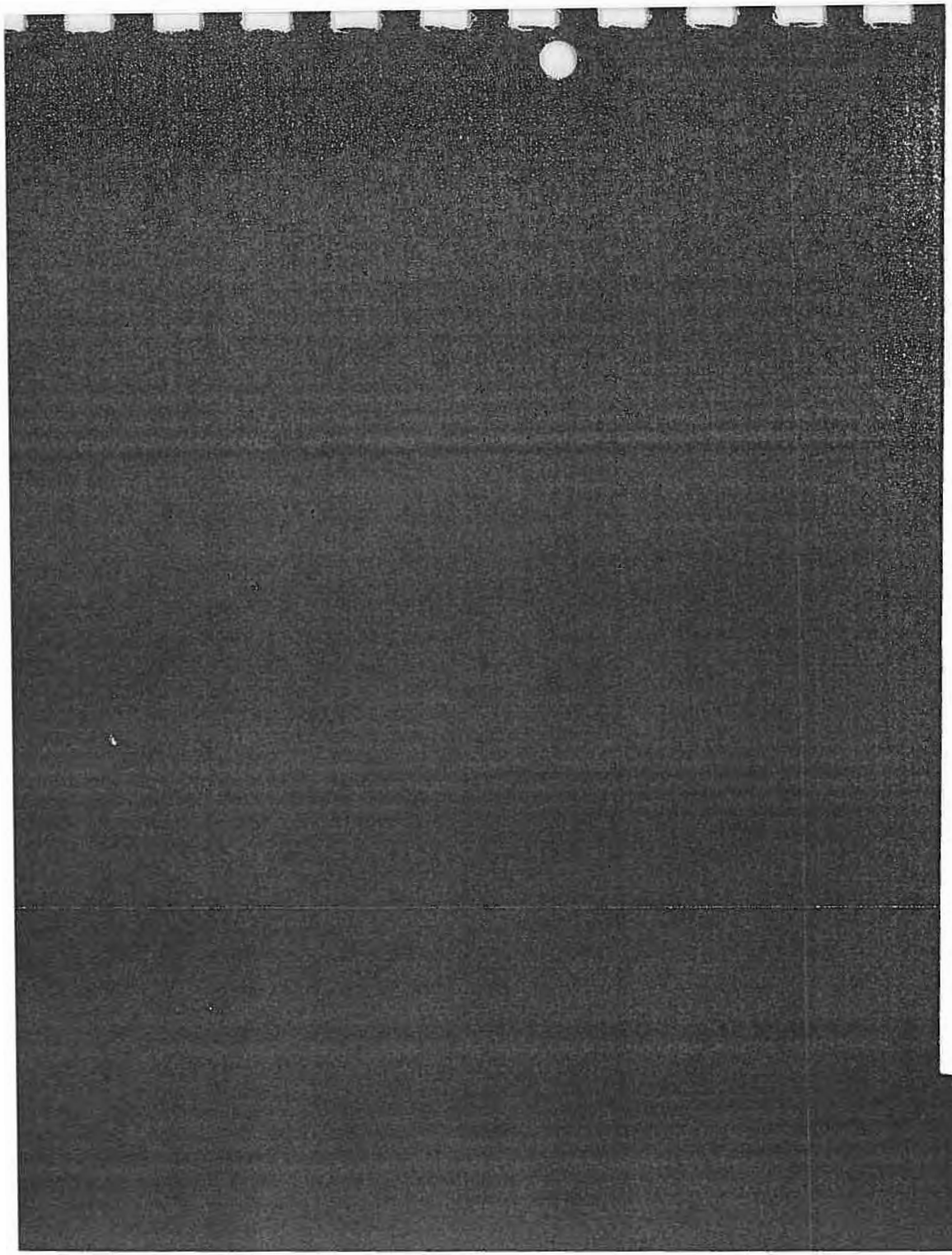

THE PARLIAMENT OF THE COMMONWEALTH OF AUSTRALIA

WASTE DISPOSAL

A report from the Senate Standing Committee on Environment, Recreation and the Arts

SEPTEMBER 1994

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developing a process to decontaminate soil and sludges, which enabled the residue to be treated in a liquid BCD plant.[74]

5.51 The Technosafe Waste Disposal Company is also operating a BCD process in Melbourne. The company has operated a pilot plant for over 18 months and is commissioning a 5 tonne per day plant.[75] This company does not subscribe to mobile nnits because of the problems that can be experienced in decommissioning and recommissioning plants and the high costs involved.[76]

Bioremediation

5.52 Bioremediation can be used in the treatment of contaminated soils. Biological treatment is non-intrusive and can carry out profound degradation of intractable materials under the right conditions.[77]

5.53 This process uses bacteria or other micro-organisms to accelerate the natural breakdown of contaminants.[78] The method of approach depends on soil type, structure, moisture content and the nature and depth of the contamination.[79] Fungi can also be used when the soil is stored in a bunker so that environmental conditions can he controlled.[80]

5.54 Some species of land plants can accumulate large quantities of heavy metals, which may be a nseful attribute for soil remediation.[81]

[74] Ibid, p.iv.

[75] Technosafe Waste Disposal, Submission No.21, p.1.

[76] Isherwood, Evidence, p.682.

[77] Mainwaring, Evidence, p.764.

[78] Australian Maritime Safety Authority, Submission No.34, p.2.

[79] Makhteshim-Agan (Australia) Pty Ltd, Submission No.14, p.12.

[80] Guerin, Evidence, p.634.

[81] Kelly and Guerin, Submission No.74, p.2-3.

Plants can uptake both inorganic and organic compounds and are aesthetically pleasing.[82]

5.55 Bioremediation can be used to treat sites such as petrol stations, army bases, coal gasification works, oil refineries, power stations, beaches, docks and wharves contaminated with oil, sludge ponds, storage tanks and sites, and industrial waste sites.[83] This technology may be adapted to solve other environmental problems, such as the degradation of polyaromatic hydrocarbons or halogenated hydrocarbons.[84]

5.56 Bioremediation is particularly suited to motor-vehicle service stations and gas works sites.[85] The Committee was told that 20 per cent of service stations have leakages problems and that there are a total of 10 000 stations in Australia.[86] Bioremediation of gas work sites is being used in Melbourne and Sydney; however, there are over 100 contaminated gas works sites in Australia.[87]

5.57 The remediation of 160 contaminated sites in New South Wales was being monitored by the EPA, but as many as 7000 sites may need treatment at an estimated cost of $2 billion.[88] Mr Gurvitz told the Committee that restrictions should be placed on the time for the achievement of set degradation levels.[89] The tineframes with microbes

[82] Guerin, Evidence, p.631.

[83] Makhteshim-Agan (Australia) Pty Ltd, Submission No.14, p.12.

[84] Ibid, p.12-13.

[85] Ibid, p.4.

[86] Ibid, p.4.

[87] Makhteshim-Agan (Australia) Pty Ltd, Submission No.14, p.4 cites Knight M (1991) *Organic chemical contamination of groundwater in Australia, an overview.* Short course publication 2/91, CGMH. pp.1.1-1.35.

[88] Guerin, Submission No.74a, p.8.

[89] Gurvitz, Evidence, p.315.

:hin a couple of years depending on the chemicals and other
·s, and plants take a longer time.[90]

Bioremediation is being used to treat contaminated sites on Coode
i by the Minenco company in conjunction with CRA's Advanced
ıical Development laboratory in Perth.[91]

:ment of Oil Spills

In the USA, bioremediation is also used for oil-spill catastrophe
ngency planning, and it was suggested that Commonwealth and
governments be encouraged to consider this option.[92]
ıteshim-Agan (Australia) Pty Ltd considered that:

> Advances in bioremediation technologies using water insoluble fertilisers resulted in efficient and inexpensive systems with degradation rates far in excess of those afforded naturally. This means that the accelerated restoration of the environment minimises the ecological damage, and ravaged populations can recuperate sooner, reducing the threat of species extinction and the loss of biodiversity.[93]

Oiled debris from oil spills is currently treated by burial or land
ing.[94] The Australian Maritime Safety Authority told the
mittee that Australia is most interested in bioremediation of oil
; but does not have the capacity to undertake the appropriate
ng.[95]

The Committee was told that biological remediation of oil spills
not included in advisory documents on oil spill responses prepared
he NSW State Pollution Control Commission, the Australian

Guerin, Evidence, p.632-633.

CRA Limited and the University of Western Australia, Submission No.16, p.7.

Makhteshim-Agan (Australia) Pty Ltd, Submission No.14, p.10.

Ibid, p.23.

Australian Maritime Safety Authority, Submission No.34, p.1.

Ibid, p.2.

Maritime Safety Authority and NSW EPA, although the important role of biological agents was recognised by US authorities.[96] The potential ecological impact of an oil spill on areas such as the Great Barrier Reef would warrant the consideration of all available technologies.

5.62 The major limitation of bioremediation of hydrocarbon-contaminated water and soil is the availability of nitrogen and phosphorus.[97] Concern was expressed in relation to the use of water-soluble nutrients which can be used to enhance or augment biodegradation due to their potentially harmful effect on the environment[98] particularly in sites which may result in the eutrophication of adjacent water bodies. This situation can be overcome by the use of new controlled-release, hydrophobic fertilisers.[99] This type of fertiliser is locked within a matrix and is available only to the degrading bacteria, not the other bacteria within the system.[100]

5.63 Not all biological transformations of hazardous wastes are positive, as some toxic products may be formed such as Aldrin being converted to Dieldrin and Heptachlor to Heptachlor epoxide.[101]

5.64 There may also be other problems associated with the presence of fauna which may ingest contaminated plants.[102] Contaminated sites should be fenced off and it should be made clear that the public is not welcome on that land.[103]

[96] Makhteshim-Agan (Australia) Pty Ltd, Submission No.14, p.25.

[97] Ibid, p.11.

[98] Ibid, p.10.

[99] Ibid, p.11.

[100] Gurvitz, Evidence, p.317.

[101] Guerin, Evidence, p.630.

[102] Kelly and Guerin, Submission No.74, p.5.

[103] Guerin, Evidence, p.632.

5.65 Micro-organisms may not be able to move through the soil to reach the chemicals to be degraded.[104] There may be problems relating to the bioavailability of soil-bound chemicals.[105] The competitive advantage of indigenous micro-organisms may inhibit the growth of the degrading organisms.[106] The low concentration of contaminants in the soil may be insufficient for the growth of introduced micro-organisms.[107] There is also a problem of the disposal of the plants which have accumulated toxic materials.[108]

Catalytic Extraction Processing

5.66 This is a molten metal technology (MMT) which converts wastes, residuals and by-products into valuable products.[109] This process can be used to treat a range of waste products, including halogenated compounds.[110] MMT has a number of commercial-scale, pilot-scale, bench-scale and lab-scale demonstrations.[111] MMT has been funded through customer paid research, private placements and public offerings for more than US$142 million since 1990.

Cement Kilns

5.67 Cement kilns have been used internationally for the destruction of scheduled waste, and this technology is discussed in more detail in Chapter 6.

[104] Guerin, Submission No.74a, p.7.

[105] Ibid, p.7.

[106] Ibid, p.7.

[107] Ibid, p.7.

[108] Guerin, Evidence, p.633.

[109] Molten Metal Technology, Submission No.25, p.1.

[110] Ibid, p.2.

[111] Ibid, p.1.

CSIRO /Pacific Power Invention

5.68 CSIRO and Pacific Power have developed a process which not only destroys the PCBs but also restores the dielectric properties of the oil, thus providing an entirely new approach to the recycling of transformer oils.[112] This process could also be used for the destruction of other chlorinated materials, such as DDT, dioxins and HCB.[113] This process can recover 99 per cent of the oil and is transportable so can be moved to the major transformers.[114]

ECO LOGIC Process

5.69 The Thermal Gas Phase Reduction Process has been developed for treatment of organic hazardous waste, particularly in an aqueous form, including pure chemicals, and wastes such as harbour sediments, lagoon sludges and landfill leachate.[115] Environmental Solutions International Ltd considered that unlike almost every other technology, it can handle almost any kind of waste.[116] This technology has been proven in Canada and the United States and has undergone extensive auditing by the US EPA.[117]

5.70 The main features of the process include the high destruction efficiency; no formation of dioxins or furans; continuous process monitoring and process control; suitability for aqueous wastes; and reasonable costs.[118] This process is mobile and has on-line monitoring

[112] CSIRO Division of Coal and Energy Technology, Submission No.23, p.2.

[113] Ibid, p.2.

[114] Ekstrom, Evidence, p.426.

[115] Campbell, K R, Hallett, D J *Report: Demonstration Testing of a Thermal Gas Phase Reduction Process*, Abstract, p.1 provided as part of Submission No.3.

[116] Ibid, p.124.

[117] Bridle, Evidence, p.122.

[118] Environmental Solutions International Ltd, *The ECO LOGIC Thermal Gas-phase Reduction Process*, November 1993, p.1.

Biodegradation of endosulfan in cotton growing soils

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Endosulfan has become an important component of insect control — primarily in cotton (see Forrester and Cox, The Australian Cottongrower, Vol 11, No. 2, May–July, 1990) — but also increasingly in the production of other other crops. This is mainly because it is persistent and effective long enough to selectively control the insects of concern

Also it is reported to be sufficiently biodegradable to avoid the accumulation of residues in soils and animal tissues.

As a result of its increased use for cotton production, it is important that its fate in the Australian soil environment be understood, particularly in view of its potential for environmental toxicity. In fact, very few studies on its environmental fate have been conducted under Australian conditions

This article describes the background and aims of the current study on the role of micro-organisms in the degradation of endosulfan in cotton growing soils, at the Department of Agricultural Chemistry, University of Sydney.

ENVIRONMENTAL CONCERNS

Endosulfan is the most commonly used insecticide in cotton production and in combination with its relatively high application rates and potential toxicity, it is necessary that its levels and effects be closely monitored in the environment. Although soil and water micro-organisms can tolerate high concentrations (>>10 parts per million) and only a few aquatic invertebrates are susceptible at practical application rates, fish are acutely sensitive to endosulfan residues (1–20 parts per billion).

This is reduced 100–1000 times once the sulfite group has been removed, that is after its residues have undergone hydrolysis to its diol form (see Figure 1). However during the period of maximum insecticidal activity, that is the few days immediately after application, fish are very susceptible to endosulfan poisoning, particularly if there are heavy rains during this period.

In a recent study by Nowak and Ahmad (1989), three species of fish exposed to sublethal doses of technical endosulfan were found to concentrate endosulfate and metabolise it to various products. In the same report, field sampling during 1986–87 from the Gwydir River showed that catfish could accumulate high levels of endosulfan.

Birds, bees and numerous beneficial insects remain unaffected at practical application rates. Whyte and Conlon (1990) have extensively reviewed the acute and sublethal effects of endosulfan on wildlife and its disruption to terrestial ecosystems.

ENDOSULFAN PERSISTENCE

Since the first synthesis of endosulfan in the early 1950s, the compound has sometimes been loosely classified in the group of chemicals called chlorinated hydrocarbons, of the cyclodiene group, although it is not simply an organochlorine. Endosulfan is a hexachlorocyclic sulfite diester, as designated by the IUPAC, the world authority on chemical classification.

In the technical formulation, endosulfan exists in two forms: the alpha and beta isomers. The presence of the reactive sulfite functional group gives the endosulfan properties which strongly differentiates it from the cyclodienes, for example aldrin, dieldrin, endrin, isodrin, telodrin, and heptachlor, so much so that it cannot be grouped with them.

The presence of this sulfite functional group in endosulfan enhances its biodegradation. Over the past twenty-five years, many laboratory and field studies on endosulfan degradation have been performed (see Goebel et. al. 1982, for an extensive review and references therein). In summary, between application and complete degradation, endosulfan residues penetrate the soil profile to a depth of 15 cm or less

This has recently been confirmed in our laboratory in a leaching experiment with a sandy loam (Kimber 1990). Residue analyses on soils from temperate climates, which have been treated

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The authors, Dr Ivan Kennedy (centre) and Turlough Guerin (right), pictured with Stephen Kimber (left).

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for several years and more, show that endosulfan is not accumulated, unlike the classic cyclodiene compounds.

From the range of overseas studies it appears that the alpha- endosulfan residue has a half life of 50–150 days in aerated temperate climate soils, while the beta and sulfate forms in the same soils lasts for up to 1–1.5 years, and some 10–20 per cent longer for all residues in flooded fields.

However in Australia, Kimber (1990) found that the half life of beta endosulfan was only 35 days in a Narrabri clay soil. This finding was made in a laboratory experiment at 37°C.

Although there is some variation in these results between Australia and overseas, all estimates of the half life of endosulfan are very much lower than those for the classic cyclodienes such as dieldrin and heptachlor in the soil.

RESIDUAL BEHAVIOUR

There are a number of factors influencing the residual behaviour of endosulfan in the environment.

The alpha isomer normally degrades at a faster rate than the beta isomer. The main degradation product in soil, endosulfan sulfate, is formed by oxidation of the parent endosulfan isomers and is subsequently hydrolysed, as are the parent isomers, to non-toxic compounds.

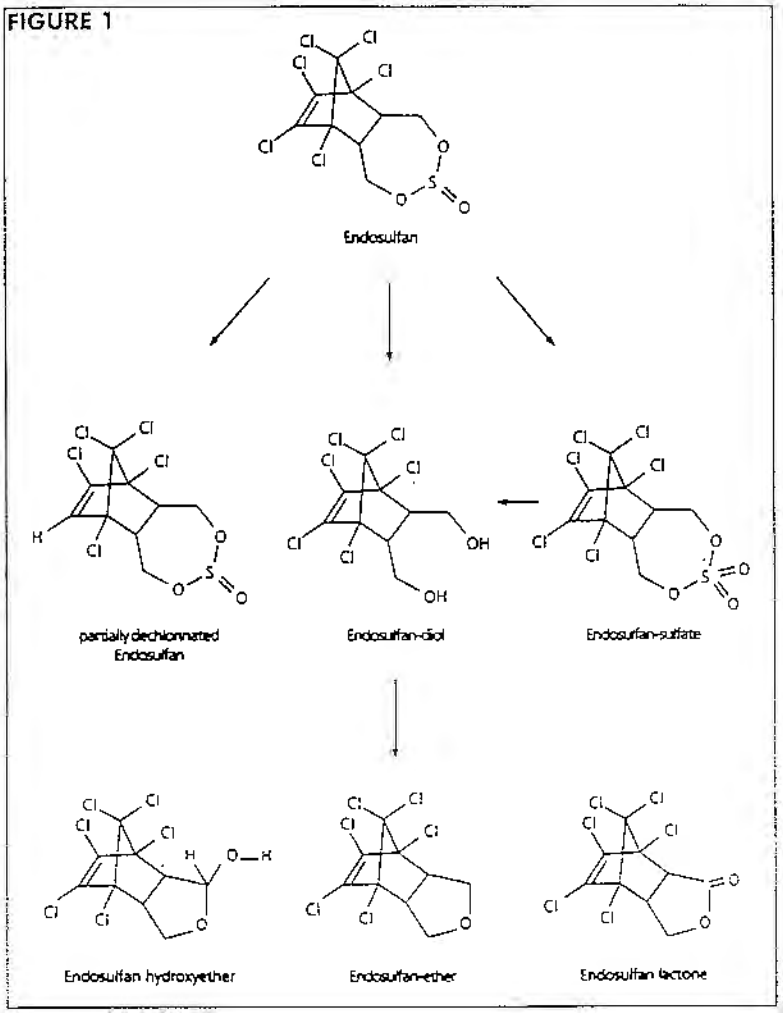
The rate of formation of endosulfan sulfate from endosulfan is usually faster than its degradation, and for this reason it is included in the total endosulfan residue level. The sulfate is the major metabolite formed in aerated soils, from the action of micro-organisms, but also on the roots and on the surfaces of plants.

The biological formation of the sulfate derivative is a result of the action of mixed function oxidase enzymes and probably similar to the system that converts aldrin to dieldrin (Kennedy, Dennis and Guerin 1990). The more volatile alpha isomer tends to disappear from plant and soil surfaces faster than the beta isomer, sulfate and diol derivatives, leaving the latter as the predominant residues (see Figure 1).

Endosulfan can be degraded by chemical hydrolysis in the soil. Under extreme acidic or alkaline conditions, both isomers are hydrolysed to the diol form in either aerated or waterlogged soils. While it is known that the diol and sulfate derivatives are sensitive to breakdown by light (photolysis), there is some evidence that the parent isomers are also degraded by this process (Goebel et. al. 1982).

If so, this could be important if endosulfan enters waterways and should be studied.

FIGURE 1



ROLE OF MICRO-ORGANISMS

The most significant factor affecting the degradation of endosulfan in soil is the action of soil microflora, particularly in the microbiologically active black earths of the cotton growing areas. In a study by Martens (1976) in Germany, twenty-eight soil fungi, forty-nine soil bacteria and ten actinomycetes were tested to see whether they could degrade endosulfan.

Sixteen fungi, fifteen bacteria and three actinomycetes were found to metabolise more than 30 per cent of originally applied endosulfan in pure culture. Apart from the major metabolites of endosulfan sulfate and endosulfan diol, small quantities of other metabolites were detected, and only a small amount of pesticide-derived carbon dioxide was released, indicating that extensive mineralisation of the carbon skeleton did not occur.

This study suggests that the ability of micro-organisms to co-metabolise (that is, only partly degrade and not use as an energy or nutrient source) endosulfan is fairly ubiquitous among soil micro-organisms and that soil bacteria tend to form the less toxic endosulfan diol derivative while fungi, although with a few exceptions, oxidise endosulfan to the sulfate derivative.

This latter transformation is less desirable as the sulfate is equally toxic to mammals as the alpha isomer and more persistent in the soil. Endosulfan-diol is approximately 200 times less toxic to mammals than the alpha isomer, and non persistent in the soil.

The beta isomer is approximately half as toxic to mammals as the alpha isomer and the sulfate derivative. Although there is little evidence, it is thought that both the alpha and the beta isomers can be dechlorinated to form the pentachloroderivates, under anaerobic conditions.

The pentachloroderivatives of endosulfan, like the pentachloroderivates of the pure cyclodeine insecticides, are significantly less toxic to mammals than the parent compounds.

So the formation of the diol, beta isomer, and the partially dechlorinated endosulfan forms, constitute significant steps in endosulfan detoxification. From laboratory studies with mixed cultures of micro-organisms from soil, it is suggested that endosulfan can be completely degraded, via the diol and subsequently the ether and lactone derivatives (Miles and Moy 1979).

RESEARCH PRIORITIES

Although much work has been done on endosulfan biodegradation in soils, and by pure and mixed microbial cultures in overseas studies, little research has been performed in Australia. With the extensive use of endosulfan as an integral part of cotton growing in Australia, a number of pertinent questions need to be addressed as to the fate of this chemical and its primary metabolite, endosulfan sulphate.

For example, to what extent is endosulfan degraded by soil micro-organisms and what percent of the total micro-organisms are involved? Is endosulfan sulfate degraded by the same mechanisms as that of the parent isomers? Do soil micro-organisms degrade endosulfan and endosulfan sulfate in flooded soils and highly contaminated sites?

Do individual species bring about a significant detoxification or is a consortium of micro-organisms required? Are there special conditions in which endosulfan may accumulate and escape into waterways, or is this primarily a result of poor management of the insecticide, which can be controlled by more vigilance in its use?

From a more basic level, what are the biochemical processes involved in endosulfan biodegradation; which enzymes for example, if any, catalyse endosulfan hydrolysis to form the non-toxic diol metabolite? What happens to the bridged organochlorine rings? If the organochlorine ring goes to chloride ion, as in common salt, the environmental concerns could be significantly reduced.

The current study attempts to address some of these questions. In particular, to determine the potential for biodegradation of endosulfan by various NSW soils, and of mixed and pure cultures from these soils. Individual micro-organisms capable of rapid rates of endosulfan transformation will provide cell material for a laboratory study of endosulfan degradation which may aid our understanding of what is happening in the field.

SUMMARY

Endosulfan is a very important tool for the cotton grower in controlling harmful insect pests, and its use for this purpose is likely to continue into the foreseeable future. Given the potential ecological hazards posed by the use of such a compound, prudence requires that a number of questions be addressed regarding its behaviour in Australian soils.

The current study aims to address some issues of soil biodegradation. The outcome of the study should provide a clearer picture of the fate of endosulfan in cotton growing soils and of the role of soil micro-organisms involved in its degradation, perhaps suggesting a strategy to minimise the potential for environmental impacts.

ACKNOWLEDGEMENTS

We are grateful to the Cotton Research and Development Corporation for their financial support.

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Distribution and Dissipation of Endosulfan and Related Cyclodienes in Sterile Aqueous Systems: Implications for Studies on Biodegradation

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Endosulfans I (α) and II (β) dissipated in simple aqueous media in glass incubation vessels sealed with Teflon show apparent half-lives of 46 and 24 days at 30 °C, respectively. These values are significantly greater than others reported in the literature, because of precautions taken to prevent volatilization and biodegradation. Endosulfan sulfate, the major oxidation product of endosulfan, is much more persistent than either of the parent isomers under the same incubation conditions. A study of the distribution of the cyclodienes endosulfans I and II, aldrin, dieldrin, and endosulfan sulfate in a sterile aqueous microbial broth showed that these compounds were distributed to the glass/medium interface. The importance of the Henry's law constant and fugacity in the nonbiological loss of these compounds and precautions that need to be taken in studies of microbial degradation are discussed.

INTRODUCTION

The cyclodiene insecticides are of considerable significance to Australian agriculture and the environment. Although aldrin and dieldrin are no longer permitted for general use, dieldrin, in particular, is present in many waterways and cropping soils (Ang et al., 1989; Gilbert et al., 1990; McDougall et al., 1987). If pastures are grown on these soils and grazed, then livestock may become contaminated (Gilbert and Lewis, 1982). Endosulfan, on the other hand, does not bioaccumulate in mammals. It is currently registered for the control of insects in a range of crops, particularly cotton and canola, and has a vital role in the Pyrethroid Resistance Strategy which is currently proving successful in Australia for cotton production (Forrester, 1989).

Endosulfan is commonly classified as a chlorinated hydrocarbon of the cyclodiene group, on the basis of its chemical structure. Its physical, chemical, and physiological properties, however, are markedly different from those of aldrin and dieldrin (Maier-Bode, 1968). Endosulfan is readily metabolized in nontarget organisms, and this property arises from its relatively reactive sulfite group (Van Woerden, 1963). Endosulfan is considered to have a low level of persistence in the environment and a low toxicity to most higher organisms with the exception of fish, which are extremely sensitive to it (Goebel et al., 1982).

There are a number of papers describing the conditions favoring the chemical modification of endosulfan, and these have been reviewed by Goebel et al. (1982). There are, however, relatively few studies describing the fate of endosulfan in simple, well-defined, nonbiological aqueous systems (Greve, 1971; Martens, 1976; Miles and Moy, 1978; Singh et al., 1991). Thus, there is a need for a greater understanding of the fate of endosulfan in aqueous media, particularly in relation to biodegradation studies, where it is important to distinguish between the mechanisms of nonbiological and biological loss. It has not been conclusively determined to what extent losses of endosulfan result from degradation as against dissipation by other means, such as volatilization, in aqueous incubation systems. The primary aim of this study is to describe the nonbiological dissipation of endosulfan in aqueous media under well-defined conditions, preparatory to a study of the microbial degradation of endosulfan. The ways in which nonbiological losses of endosulfan can be reduced in biodegradation systems have been addressed. Consideration has also been given to the reasons for the observed physical behavior of the pesticides in these experiments.

MATERIALS AND METHODS

Chemicals. Endosulfan and its metabolites were a gift from Dr. Klaus Stumpf, Hoechst AG, Frankfurt am Main, FRG, and Hoechst Limited Australia. Aldrin and dieldrin were provided by Shell Chemicals, Australia. *cis*- and *trans*-aldrin diol were kindly provided by Dr. David H. Hutson, Shell Chemicals Research, Sittingbourne, Kent, U.K. Hexane (Nanograde) and methanol (ChromAR) were purchased from Mallinckrodt Chemicals. Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Sigma Chemical Co. All other chemicals used were of analytical grade.

Incubation Conditions. Pesticides and their metabolites (100–500 ppm in methanol) were added to either Nanopure-filtered, sterile, distilled, and deionized water or sterile 100 mM potassium phosphate buffered soil extract yeast mannitol medium in 4-mL Wheaton vials, to give final amounts of 1–5 μg mL^{-1}. The level of added pesticide exceeded the limits of the solubility for these compounds in the aqueous phase. The pesticides were delivered in 10 μL of methanol. Duplicate vials were made of unsilanized borosilicate glass and prepared by washing in concentrated sulfuric acid followed by rinsing in acetone prior to use. The medium contained K_2HPO_4 (anhydrous) 6.79 g L^{-1}, KH_2PO_4 (anhydrous) 8.30 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.05 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g L^{-1}, yeast extract (Difco) 2.00 g L^{-1}, mannitol (Sigma) 2.00 g L^{-1}, sucrose (Pronalysis) 2.00 g L^{-1}, and soil extract 100 mL L^{-1} (Allen, 1957). Vessels were sealed with either poly(tetrafluoroethylene) (PTFE)-lined silicone rubber seals (Wheaton, Millville, NJ, supplied by Edwards Instrument Co., Narellan, Australia) or non-PTFE-lined butyl rubber stoppers. Duplicate vessels were kept at 30.0 ± 0.5 °C, in an air flow controlled incubator for 30 days. No further attempt was made to artificially aerate the incubation flasks during the course of the experiment, and sterile air at atmospheric pressure was used as the gas atmosphere, from a laminar flow cabinet. To maintain the incubations sterile throughout the experiment, sodium azide was added at 0.5% (w/v). The pH of all the media was adjusted to 7.00 ± 0.05 with KOH or HCl prior to dispensing into the incubation vessels.

Extraction and Recovery of Parent Pesticides and Metabolites. Duplicate incubation vessels containing the aqueous media (500–1000 μL) had their contents quantitatively transferred

(Mixxor by Genex) and were extracted according to the method previously developed (Guerin et al., 1992). Samples were taken on days 0, 2, 4, 6, 8, 15, and 30. A total solvent volume of 10 mL of hexane/acetone/methanol/medium (15:5:2:2) was also added to the Mixxor reservoirs. The piston of the Mixxor was moved 60 times in its reservoir to partition the pesticides into the solvent phase. After the phases were allowed to separate (approximately 1 min), the solvent layer was decanted off the aqueous phase directly from the Mixxors into volumetric flasks and the total volumes were made to either 10.0 or 25.0 mL with hexane. Subsamples were dried with anhydrous sodium sulfate prior to analysis. Cleanup in the experiment was not necessary as there were no interfering peaks recorded on the chromatograms. Recoveries (percent) of the parent compounds and their standard deviations at the 1 ppm level were as follows; aldrin 85 ± 2.9; dieldrin, 88.5 ± 4; endosulfan I, 89.4 ± 4.1; and endosulfan II, 82.7 ± 4.1. The recovery of endosulfan sulfate at the 5 ppm level was 83.9 ± 6.6. Recovery of the aldrin diols and endosulfan diol was 70 ± 7.1 at the 0.5–1.0 ppm level of spiking. Residue levels were plotted as log $100 \times C/C_0$, where C is the concentration at time (t) and C_0 is the concentration at t_0. The values for C_0 were 1 ppm for aldrin, dieldrin, and endosulfans I and II and 5 ppm for endosulfan sulfate. An assumption was made that the degradation and disappearance of the compounds followed first-order kinetics at constant pH. The equation used to plot the degradation in this study was that used by Cotham and Bidleman (1989). Half-lives were determined by plotting a line of best fit through the data points calculated using the above equation.

Gas Chromatographic Analysis. The analytical conditions were as previously described (Guerin et al., 1992). Extracts were analyzed on a Shimadzu GC 8A ^{63}Ni ECD gas chromatograph. Columns used were an OV-210 (5%):SE-30 (5%) (7:3) on Chromosorb W-HP, 80/100 mesh, and an SE-30 (5%):DC-200 (5%) (3:1) on Gaschrom Q 80/100 mesh packed into 3 m × 2.0 mm custom-made unsilanized borosilicate glass columns. The former column was used for confirmation of pesticides and their metabolites only. The N_2 gas flow rates on both columns were adjusted to 25 mL min^{-1}. The column and injector/detector temperatures were set at 210 and 290 °C, respectively. Chromatograms were recorded on a Shimadzu CR-3A integrator.

Metabolite Identification. Metabolites were identified by comparing retention times of metabolite standards, prepared in hexane, with the degradation product peaks appearing on the treatment chromatograms. Peak identity was confirmed by consistent retention time and coelution with standards on two different chromatographic columns under the conditions previously described (Guerin et al., 1992). All of the compounds analyzed were measured at concentrations within their linear ange on the ECD gas chromatograph.

Determination of Pesticide Distribution. Three types of media were employed in determining the distribution of pesticides in the incubation vessels, which were duplicate PTFE-sealed Wheaton vials (4 mL). These media were as follows: (1) Nanopure-filtered, sterile, distilled, and deionized water; (2) growth medium with pesticide (1–5 μg mL^{-1} added in 10 μL of methanol); and (3) growth medium and pesticide and 0.1% Tween 80. In all three treatments, half the total amount of medium originally added was carefully subsampled by removing the liquid with a narrow-bore pipet from the bulk after 4 h of incubation at 30 °C. Losses of pesticide during this equilibrium period were found to be negligible, so there was not measurable degradation or dissipation from the vessels over the 4-h period. The subsampled fractions were termed the medium bulk. The remaining medium, which contained the medium at the interfaces, i.e., on the glass walls and on the liquid/air interface, was termed the interface medium, and all of these remaining residues were collected by rinsing the incubation vessels into the extraction chambers of the Mixxors. Both the subsamples and rinsed fractions were extracted individually and analyzed for the cyclodiene pesticides, using the method described under Gas Chromatographic Analysis and further described in Guerin et al. (1992).

Surface microlayer subsamples in triplicate were also taken after 4 h (30 °C) from vessels set aside especially to determine whether there was any difference in pesticide concentration at volume of 100 μL from the surface, an amount equivalent to the top 0.9 mm of the medium, using a pipet. The pipet was rinsed with solvent and the medium extracted as described under Extraction and Recovery of Parent Pesticides and Metabolites. An equivalent volume removed from the medium bulk was also analyzed for pesticides.

RESULTS AND DISCUSSION

Losses of Aldrin and Dieldrin. Initial experiments were performed to indicate how much pesticide loss could occur by escape from the aqueous phase from unsealed flasks or flasks sealed with butyl rubber during 4 weeks of incubation at 30 °C. These are the conditions under which studies of microbial degradation of pesticides are often performed (Wallnofer and Engelhardt, 1990; MacRae, 1989, and references cited therein). In aerobic microbial degradation studies, cotton wool has often been employed as a stopper to keep the flask contents sterile or prevent contamination by other microorganisms. In anaerobic studies, butyl rubber is often used to seal the flasks to prevent contamination by oxygen. In these initial experiments, there were high rates of loss of aldrin and dieldrin from both unsealed incubation vessels and flasks sealed with butyl rubber (Figure 1A,B). In the butyl rubber sealed vessels, aldrin and dieldrin had largely disappeared at the 30th day, with apparent half-lives of 0.4 and 1.8 days, respectively. These results were similar to the values of 3.7 and 3.5 days for half-lives in the open flasks. The difference between the two systems was that the initial rate of dieldrin disappearance in the unsealed vessels was slightly higher than in the butyl rubber sealed vessels. In contrast, when aldrin and dieldrin were incubated in similar sterile media, but with PTFE-lined silicone rubber seals, there was no apparent loss (Figure 1C,D).

The pattern of loss for aldrin from butyl rubber sealed flask and that of dieldrin from unsealed flasks appeared to be biphasic, i.e., high early in the experiment, decreasing to a much lower rate of loss at 30 days. A possible reason for this non-first-order loss could have been that a large proportion of pesticide was concentrated at the liquid–air interface (surface microlayer) and, as a consequence, is volatilized early in the experiment. After this surface quantity had all volatilized, further losses must come from the solution bulk through the process of diffusion. Another possible reason for this biphasic loss may be because of a rim effect, where the more rapid diffusion occurs at high adsorptions (i.e., at concentrations higher than the compound's solid-phase solubility early in the experiment) across the glass to the surface. The latter explanation best explains the pattern in this experiment, because there was no detectable concentration of insecticides at the liquid–air interface (see Distribution of Pesticides in the Aqueous Incubation System).

The inclusion of the very stable cyclodienes aldrin and dieldrin in this study provided internal controls that indicatod disappearance predominantly from physical losses, thereby providing the maximum limits of these processes in the system. The experimental conditions were too mild and the incubation period was too short to allow any chemical degradation of these compounds (Worthing and Walker, 1987; Singh et al., 1991). The persistence of aldrin and dieldrin in these incubations therefore represents the maximum limits for either slow volatilization or other processes such as irreversible binding to glass. Thus, any differences between the persistence of the internal controls and that of endosulfan represent the actual disappearance owing to chemical reaction. The very slow

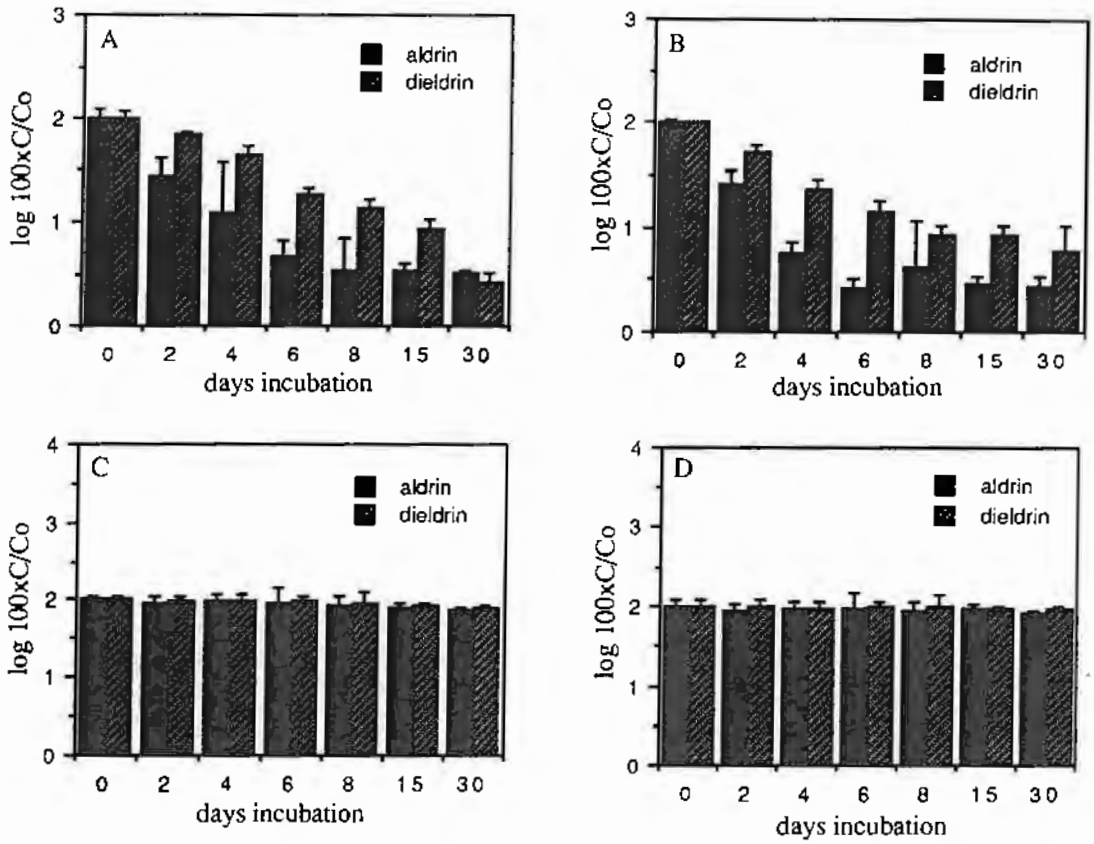

Figure 1. Dissipation of aldrin and dieldrin from (A) growth medium in butyl rubber sealed vessels, (B) growth medium in unsealed vessels, (C) growth medium in PTFE-sealed vessels, and (D) water in PTFE-sealed vessels.

sealed vessels in both water and growth medium confirmed that the system was well sealed.

Loases of Endosulfan. After the losses of the cyclodienes from the incubation vessels had been minimized, the persistence of endosulfan was examined. In both the butyl rubber sealed and the unsealed vessels, the endosulfan isomers were lost at high rates. After 30 days of incubation, the calculated half-lives of endosulfans I and II in the butyl rubber sealed flasks were 2.9 and 9.5 days, respectively. When endosulfans I and II were incubated in unsealed vessels, the half-lives were 2.0 and 11.2 days, respectively (Figure 2A,B).

When the vessels were sealed with PTFE, rates of disappearance for both isomers were much lower in both water and microbial growth medium (Figure 2C,D). In the growth medium, this corresponded to half-lives of 46 and 24 days, respectively, for endosulfans I and II. In water, the half-lives were 47 and 28 days for endosulfans I and II, respectively. The PTFE treatment presumably reduced the volatilization of the parent compound from the flasks. Endosulfan I, however, dissipated at a slower rate than endosulfan II in both media under these conditions.

The higher rate of dissipation of endosulfan II from both water and microbial medium in PTFE-sealed vessels reinforces the conclusion that endosulfan II is the more chemically labile of the two isomers (Goebel et al., 1982). Given the relative chemical inertness of the PTFE-sealed systems and that traces of endosulfan diol were detected in the same system, it is reasonable to conclude that both endosulfan isomers were chemically degraded in the aqueous incubations. There was very little difference in the degradation rates of either isomer between the water and the microbial growth medium incubations. This the isomers. Van Woerden (1963) reports that the hydrolysis of organic sulfites is not affected by the presence of metal ions when dissolved in aqueous solution. One exception has been reported with *o*-phenylene sulfite, which can be hydrolyzed at a faster rate in the presence of bisulfite ions (Van Woerden, 1963).

In previous studies a general trend has been that endosulfan II disappears at a faster rate than endosulfan I, and these have been summarized in Table I. Miles and Moy (1979) reported that under aerobic conditions at a lower temperature of 22 °C the half-lives of endosulfans I and II in a potassium phosphate buffered, minimal salts medium (pH 6.5) were 88 and 40 days, respectively. In their paper no mention was made on how the vessels were sealed. Cotham and Bidleman (1989) described the degradation of endosulfan in seawater and seabed sediments incubated in flasks sealed with polyurethane. The half-lives of endosulfans I and II were 4.9 and 2.2 days, respectively, in nonsterile seawater (pH 8.0). These incubations were carried out aerobically and at 20 °C under laboratory lighting. In another study, incubations in lake water (Greve and Wit, 1971) showed that the half-life of endosulfan I was 35 days at pH 7 and 105 days at pH 5.5. It was shown in the same study that when iron hydroxide gel is mixed with water, the rate of hydrolysis is considerably accelerated. The data in Table I show that both isomers of endosulfan are much more chemically stable than previous papers would suggest. The shorter half-lives reported in previous studies are presumably a result of either nonsterile conditions or volatilization.

When the different investigator's half-lives are compared, the role of pH is important. This is because the endosulfan isomers are susceptible to alkaline hydrolysis (Goebel et al., 1982). Therefore, rates of hydrolysis at pH

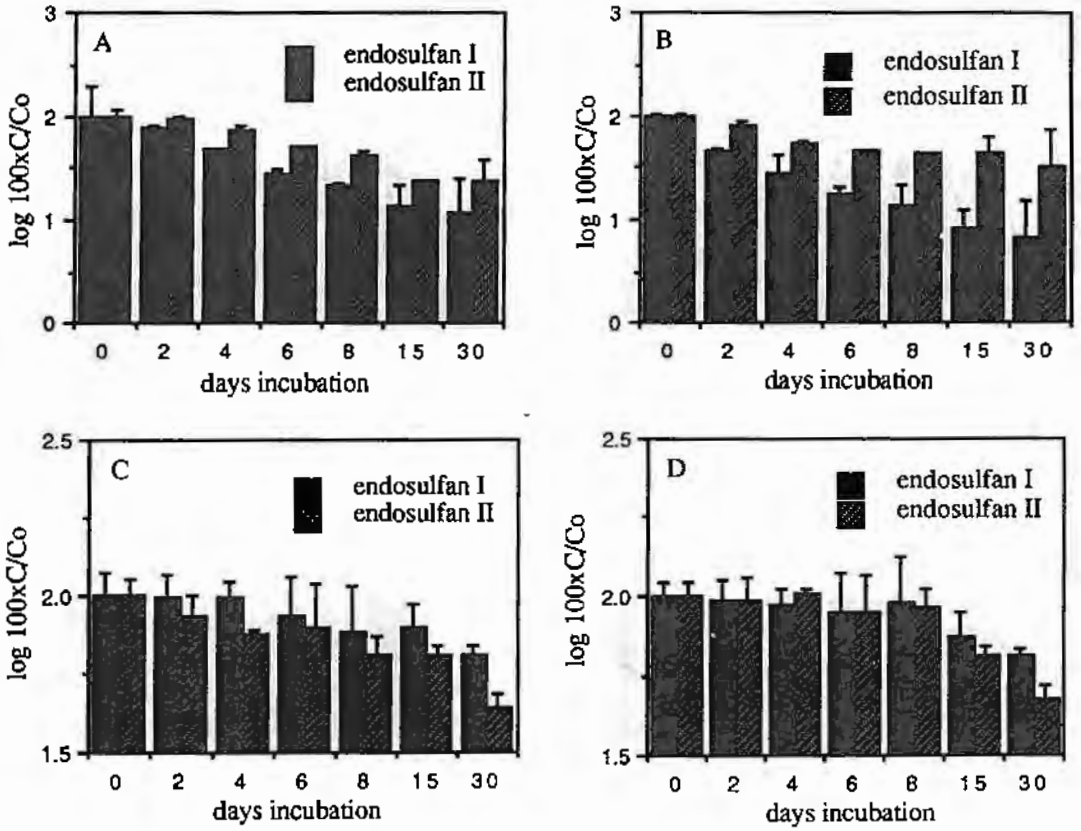

Figure 2. Dissipation of endosulfan isomers from (A) growth medium in butyl rubber sealed vessels, (B) growth medium in unsealed vessels, (C) water in PTFE-sealed vessels, and (D) growth medium in PTFE-sealed vessels.

Table I. Comparison of the Current Study with Reported Half-Lives of Endosulfan in Sterile Aqueous Media[a]

type of aqueous medium	vessel type and seal	pH	temp, °C	subsampling[b]	apparent half-life, days	
					endosulfan I	endosulfan II
lake water[c]	–	7	–	–	35	–
river water[d]	–	7.3–8.0	room temp	–	4.0[e]	–
phosphate medium[f]	glass and cotton wool	7.0	27	–	5.5[e,g]	–
phosphate medium[h]	–	6.5	22	yes	88	40
seawater[i]	glass and polyurethane	8.0	20	no	4.9	2.2
distilled water[j]	glass vessel	6–7	25	–	5.2	4.2
river water[k]	glass and foil	7.2	room temp	no	49	22
phosphate–citrate KCl buffer[l]	–	7.0	30	yes	27.5	23.5
phosphate medium,[m] current study	glass and Teflon	7.0	30	no	46	24
distilled water, current study	glass and Teflon	7.0	30	no	47	28

[a] Data from the literature were chosen from experiments conducted with simple aqeous media at pH 7 ± 1. [b] Indicates whether an aliquot of media was removed from a larger total volume at each sampling time. [c] Greve and Wit (1971). [d] Eichelberger and Lichtenberg (1971). [e] Assuming first-order kinetics. [f] Martens (1976). [g] Formation of endosulfan diol was used as an indicator of loss of endosulfan. [h] Miles and Moy (1978). [i] Cotham and Bidleman (1989). [j] Meenatchi-Sunderam (1990). [k] Peterson and Batley (1991). [l] Singh et al. (1991). [m] Allen (1957). A dash indicates there were no specific information reported.

differences in temperature, which may also affect the hydrolysis rates of pesticides. The values from the literature used in Table I were those in which the studies were conducted within the pH range 6–8 and the temperature range 20–30 °C. Thus, not all of the studies reported in Table I are directly comparable. However, the specific values for these parameters are given, where reported in the literature, in Table II.

Losses of Endosulfan Sulfate. In all of the experiments carried out, endosulfan sulfate was very stable and considerably more persistent than the parent isomers (Figure 3A,B). The apparent half-life of endosulfan sulfate in the sterile aerobic vessels was 770 days when sealed with PTFE and 30 days in the unsealed vessels. Its persistence was only slightly higher in the vessel sealed with butyl rubber, compared to that in the unsealed vessels. Miles and Moy (1979) have also reported on the persistence for its half-life, under the previously described conditions, as greater than 140 days. The persistence of endosulfan sulfate in the aqueous systems studied in the current paper indicates that this endosulfan transformation product may remain in some water environments longer than the parent isomers.

Role of Volatilization in Pesticide Disappearance. From the increased losses in the open-vessel incubations, it is evident that the endosulfan dissipation in these experiments was primarily owing to volatilization. Endosulfan I disappeared at a much faster rate than endosulfan II, as expected, since the former is more volatile and less water soluble. The relatively high volatilization rate of endosulfan I has previously been reported from solid surfaces as well as aqueous systems (Beard and Ware, 1969; Singh et al., 1991). The rates of volatilization of the

Table II. Liquid-Phase Physical Properties of Endosulfan and a Comparison with Related Compounds

compound	water solubility		log K_{ow}[a]	vp,[b] Pa	H,[c] Pa mol^{-1} m^3
	ppm	mol m^3			
aldrin	0.37 ± 0.49[d]	1.01×10^{-3}	3	0.005 ± 0.004	4.95
dieldrin	6.0 ± 0.67[e]	1.58×10^{-2}	3.7	0.0083 ± 0.006	0.53
endosulfan I	2.29 ± 1.32[f]	5.63×10^{-3}	3.6	0.006	1.07
endosulfan II	31.1 ± 7.58[g]	7.6×10^{-2}	–	0.003	0.04
endosulfan sulfate[h]	18.14	4.39×10^{-2}	–	–	–
endosulfan diol[h]	300.0	8.31×10^{-1}	–	–	–
endosulfan ether[h]	1392.8	4.06	–	–	–

[a] Octanol/water coefficient at 20 °C in the aqueous phase from Suntio et al. (1988). [b] Vapor pressure at 20 °C from Suntio et al. (1988) and Cotham and Bidleman (1989). [c] Henry's constant calculated from the vspor pressure divided by water solubility. [d] At 20–25 °C from Park and Bruce (1968), Weil et al. (1974), Biggar and Riggs (1974), and Kenaga (1980a,b). [e] 25 °C from Biggar and Riggs (1974), Eye (1968), Gunther et al. (1968), Weil et al. (1974). [f] 22–25 °C from Phillips (1975), Weil et al. (1974), and Worthing and Walker (1987). [g] 20–25 °C from Phillips (1975), Weil et al. (1974), Worthing (1987), Bowman and Sans (1983a). [h] 20 °C from Hoechst (1986). A dash indicates that the authors could oot obtain data for those compounds.

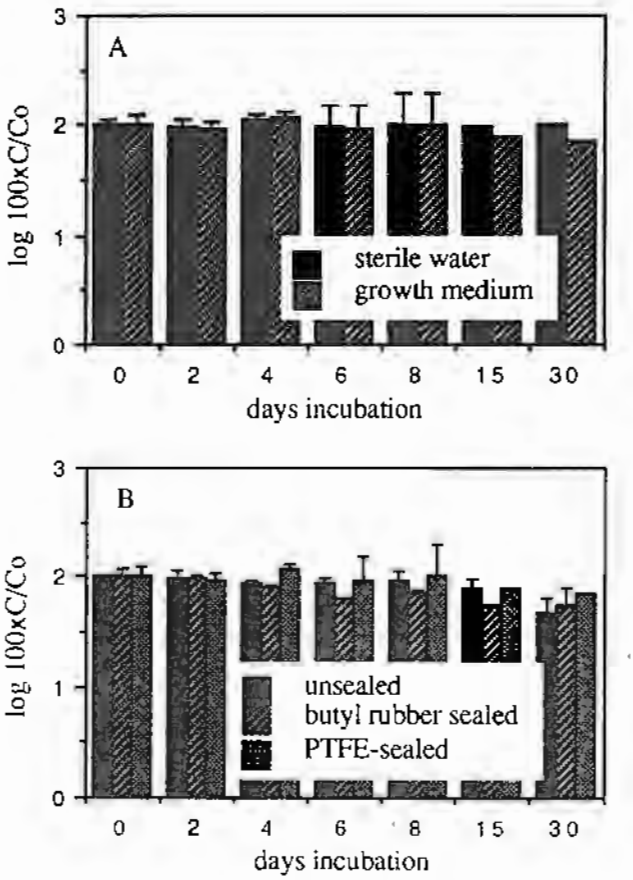

Figure 3. Dissipation of endosulfan sulfate in (A) water and growth medium in PTFE-sealed vessels and (B) growth medium with various types of sealing.

indicating that volatilization of these compounds from the aqueous media is not reduced by the presence of the butyl rubber seal. Aldrin and dieldrin were also lost at fast rates in similar incubations, confirming that either solubility in, or volatilization through, the butyl rubber seals was the major cause of loss. No hydrolysis products of endosulfan, endosulfan sulfate, aldrin, or dieldrin were detected in hexane/acetone extracts from the open or butyl rubber sealed treatments, thereby providing evidence that the dissipation of all compounds in the non-PTFE-sealed flasks was primarily a result of volatilization and not chemical degradation.

Volatilization from uninoculated controls in aerobic microbial degradation studies is likely to be a significant factor in overall pesticide disappearance in unsealed systems, particularly with pesticides such as endosulfan. In the recent study by Cotham and Bidleman (1989), 30% from a sediment/seawater incubation system (sealed with polyurethane) during the first 4 days of the experiment. The current findings therefore confirm the necessity to seal such systems with an inert material. The high volatilization rate of endosulfan I, also illustrated in other studies (Beard and Ware, 1969; Singh et al., 1991), is due to its low water solubility and relatively high vapor pressure, or its high Henry's law constant. The ratio of liquid-phase vapor pressure and solubility, or solid-phase vapor pressure and solubility, provides a value for the Henry's law constant (Suntio et al., 1991; Gerritse et al., 1991). This relationship may be used to show the difference in the relative rates of volatilization of the parent endosulfan isomers (Cotham and Bidleman, 1989; Goebel et al., 1982, and references cited therein) and of the recalcitrant cyclodienes, aldrin and dieldrin (Table II).

In calculating the Henry's law constant, values for water solubility and vapor pressure must be for the same phase, i.e., both for the liquid phase or both for the solid phase. The values presented in Table II are for the liquid phase for each of the pesticides, which were converted from the properties of the solid compounds, reported throughout the literature, using eq 1 (Suntio et al., 1988). In eq 1, F

$$F = P_S/P_L = C_S/C_L = \exp[-0.023(T_M - 298)] \quad (1)$$

is the fugacity ratio of the compound under study, C_S is the solubility of the solid compound, and C_L is the solubility of the liquid compound. P_S is the vapor pressure of the compound as the solid phase, and P_L is the vapor pressure of the liquid compound. T_M is the melting point of the compound in Kelvin. The exponent in the equation takes into account the entropy of fusion of the solid compound, and since there are often no experimental data available, the value of −0.023 is generally adopted (Suntio et al., 1988).

In illustrating the importance of the Henry's law constant of a compound, it is convenient to introduce the concept of fugacity. The fugacity is the escaping tendency of a compound from a particular phase (eq 2). In eq 2, f

$$f = C/Z \quad (2)$$

if the fugacity (units of pressure Pa), C is the concentration (units of mol m^{-3}), and Z is the fugacity capacity (units of mol m^{-3} Pa^{-1}). Each phase has its own fugacity, and at equilibrium, compounds will accumulate in phases with the lowest fugacity, or highest Z values. So in water the fugacity capacity is the inverse of the compound's Henry's constant (H) (eq 3) (Connell, 1991; Barber and Davis, 1991).

$$Z = Z_{water} = 1/H \quad (3)$$

The calculated fugacities of the cyclodiene compounds

Table III. Percentage Distribution of Aldrin, Endosulfan Isomers, and Their Major Oxygenated Metabolites toward the Interfaces and Medium Bulk in Borosilicate Glass Vessels[a]

	growth medium only		growth medium + Tween 80		water + Tween 80		
compound	IM[b]	MB[c]	IM	MB	IM	MB	CV,[d] %
aldrin	39.6 ± 4.3	60.4 ± 6.5	2.8 ± 0.2	97.2 ± 7.7	6.7 ± 0.4	93.3 ± 5.3	8.2
dieldrin	51.3 ± 8.5	48.7 ± 8.1	5.9 ± 0.9	94.1 ± 14.1	6.9 ± 0.3	93.1 ± 3.9	11.9
endosulfan I	38.9 ± 0.9	61.1 ± 1.5	0.0 ± 0.03	100.0 ± 4.9	8.2 ± 0.4	91.8 ± 4.5	4.0
endosulfan II	49.8 ± 4.7	50.2 ± 4.7	0.4 ± 0.04	99.6 ± 9.8	3.8 ± 0.1	96.2 ± 3.5	5.8
endosulfan sulfate	45.2 ± 1.5	54.8 ± 1.8	2.5 ± 0.1	97.5 ± 4.2	2.9 ± 0.1	97.0 ± 3.9	3.9

[a] Conditions of the incubation are described under Materials and Methods. Values are in percentage of total recovered ± standard deviation determined from the duplicate extractions. [b] IM, interface medium, includes both the liquid–glass and liquid–air interfaces of the closed system. [c] MB, medium bulk, subsampled from the total volume of medium. [d] Coefficient of variation determined from the average standard deviation for each of the compounds across all treatments.

in the same phase because the concentration (C) is equal to their water solubilities for the solid compounds. The solubility data used in Table II are those values reported from shake flask experiments conducted at 20–25 °C with analysis by ECD gas chromatography and converted to liquid-phase solubilities using eq 1, many of which were compiled from Shui et al. (1990) and references cited therein. The values for vapor pressure were selected from the data compiled by Suntio et al. (1988) and from Cotham and Bidleman (1989). These values were determined from gas chromatographic analyses where the retention times of the unknowns were compared to structurally related compounds with known and reliable vapor pressures, using nonpolar chromatography columns.

Some of the behavior observed in the butyl rubber sealed and unsealed vessels can be accounted for by differences in their calculated fugacities. The fastest rates of disappearance from both of these treatments was that of aldrin, which also had the greatest fugacity. Endosulfan I has a Henry's law constant approximately 27 times that of endosulfan II, which correlates well with the greater rate of disappearance from the non-PTFE-sealed vessels. The behavior of dieldrin, however, cannot be explained by its calculated fugacity alone, as this value is many times less than that of aldrin, yet its rate of disappearance was high. Direct measurements of the Henry's law constant for dieldrin have been reported (Suntio et al., 1988; Atkins and Eggleton, 1971; Slater and Spedding, 1981). An average value calculated from the literature is 3.4. This is a high value relative to those calculated indirectly in Table II. This may explain why dieldrin appears to be more volatile, in the current study, than expected from the calculated Henry's law constant.

Detection and Analysis of Potential Hydrolysis Products. Trace levels of the hydrolysis product, endosulfan diol, were detected in the incubations containing endosulfan initially. Data from the recovery experiments showed that this potential degradation product, when spiked into zero-time vessels, was being extracted, and its identification was confirmed using two different gas chromatographic conditions (Guerin et al., 1992). The highest levels of endosulfan diol were detected in the PTFE-sealed incubations. With endosulfan I, these levels were 0.08–0.1 ppm of diol after 30 days. This rate of diol formation correlates well for the calculated half-life of endosulfan I of approximately 46 days in the sterile media. Slightly higher levels of 0.1–0.15 of ppm endosulfan diol were detected in the endosulfan II incubations under the same conditions, consistent with its low stability. Only trace levels of endosulfan diol (<0.03 ppm) were detected in the endosulfan sulfate incubations and then only in PTFE-sealed incubations.

The potential hydrolysis products of dieldrin, *cis*- and *trans*-aldrin diol, were not detected in any of the treatment tized standard compounds were chromatographed successfully under the conditions described for analyzing the parent compounds (Guerin et al., 1992). Given the highly recalcitrant nature of dieldrin, and the mild incubation conditions of water and growth medium, no hydrolysis products would be expected to form. Another recent paper confirms this stability of dialdrin in aqueous systems (Singh et al., 1991).

Distribution of Pesticides in the Aqueous Incubation System. Approximately 40–50% of the total amount of pesticides recovered from the growth medium (1–5 μg mL^{-1} originally added) was found to be concentrated at the interfaces of the system (Table III). An attempt was made to subsample the liquid–air interface and determine pesticide concentration. However, there was no detectable difference between the surface sample and that in the bulk of the medium. Therefore, the pesticides must have accumulated at the liquid–glass interface under these conditions. Although it is possible that a slower rate of hydrolysis could occur at this interface, insulated from the effect of the hydroxyl ion, this is unlikely to explain the increased half-lives for the endosulfan isomers reported in this study with effective sealing. A similar increase in half-life was also observed for the more stable compounds such as dieldrin, when the flasks were sealed with PTFE.

This distribution to the glass–medium interface was observed when the compounds under study were added to either water or microbial growth media. An even distribution of pesticide throughout the entire system was achieved by adding 0.1% Tween 80 (Table III). All of the compounds studied were distributed throughout the system in a similar fashion, and all responded similarly to the detergent treatment in both the sterile distilled water and growth medium. There was a greater distribution of all the pesticides to the liquid–glass interface in the vessels containing pure water. An explanation for this is that the solubility of all of the solid compounds in water was much less than the levels which were applied to the vessels (1–5 μg mL^{-1}). These solid-phase values, from which the values in Table II are derived, are as follows: aldrin, 0.06 ppm; dieldrin, 0.18 ppm; endosulfan I, 0.33 ppm; endosulfan II, 0.41 ppm; endosulfan sulfate, 0.5 ppm; endosulfan diol, 4.8 ppm; and endosulfan ether, 14.0 ppm. The more even distribution of the cyclodiene compounds in the treatment vessels containing microbial growth medium compared with that in pure water, after the addition of Tween 80, suggests, however, that there is an additional solubilizing effect contributed by the medium. This effect would also be exerted by lipophilic constituents or dissolved organic matter in the soil, peptone, and yeast extracts. These results illustrate the importance of avoiding subsampling when aqueous extracts containing relatively high concen-

The distribution of pesticides in aqueous systems is of particular importance in microbial degradation studies where the availability of the compound is likely to affect its degradation. Thus, when the pesticide is added in small amounts of solvent to the aqueous phase (as has generally been reported in microbial pesticide degradation studies, often followed by evaporation of solvents with N_2 gas), its distribution in the incubation vessel will tend to be associated with the interfaces. The geometry of the incubation vessel as well as the constituents of the medium will effect the pesticide distribution. In microbial degradation experiments, when the insoluble compounds are added in methanol or similar solvent, an apparent increase in pesticide concentration with time will be observed in the bulk of the medium, once exponential growth commences. This effect may be overcome by completely sacrificing the entire treatment incubation flasks at each sampling time. Maloney et al. (1988) reported on the twofold benefit of using Tween 80 in a study of the microbial degradation of the insoluble pyrethroid pesticides. It was shown to solubilize the compound to be degraded and provided the growing microorganisms with a source of readily utilizable carbon, for the transformation of the pyrethroids. Tween 80 is a mixture of oleic acid (approximately 70%) with the balance comprised of linoleic, palmitic, and stearic acids.

Significance of Water Solubility. The water solubility of the compounds studied and that of some of their metabolites have been compiled in Table II. These values, in conjunction with the octanol/water distribution coefficient, vapor-phase pressure, and pesticide polarity, are important in understanding the distribution and reactivity of these compounds in the environment (Seiber, 1987; Shui et al., 1990a,b). The relatively low solubility of all the compounds studied can account for the general distribution patterns of the pesticides observed in the incubation vessels. The slightly higher water solubilities of endosulfan II and endosulfan sulfate as compared to that of endosulfan I are a consequence of the differences in the compound's polarity. The dipole moment, which provides an indicator of a compound's polarity, has been determined by Forman et al. (1965) as 1.02, 3.18, and 4.10 Debye units for endosulfans I and II and the sulfate, respectively. The higher values for endosulfan sulfate and endosulfan II compared to that of endosulfan I correlate well with the differences in water solubility of these compounds.

The environmental significance of the low solubilities of these compounds is that they have the potential to be readily taken up by biological and other lipid-containing systems present in waters polluted with them. Accounts of the bioaccumulation of aldrin and dieldrin in a number of aquatic species are numerous, but with endosulfan, also of low water solubility, the extent of the problem is primarily with initial acute toxicity, not accumulation. The labile sulfite group necessitates a relatively rapid metabolism of the parent endosulfan isomers, to the nontoxic diol, and thereby reduces the potential of these compounds to accumulate in biological systems.

CONCLUSION

This study reports that the endosulfan isomers can be dissipated in simple aqueous systems at neutral pH in the absence of biological material or chemical catalysts. Endosulfan II is more readily degraded then endosulfan I, a phenomenon already observed in various aqueous systems. Apparent half-lives determined from the data indicate that the [illegible] the major oxidation product of endosulfans I and II, endosulfan sulfate, is less volatile and can persist longer than either of the parent isomers in the same system. Given that endosulfan sulfate is formed in many natural environments through biological oxidation and that it is only slowly degraded, both chemically and biologically (Miles and Moy, 1978), it may represent a predominant residue of endosulfan in aerobic aqueous environments.

Endosulfan sulfate was not formed in any of the treatments. This suggests that endosulfan sulfate would not be formed in aerated waters in the absence of microbial activity or strong chemical oxidants.

The current study illustrates the importance of understanding the distribution and physical behavior of insoluble xenobiotics in aqueous systems, so that meaningful conclusions on the relative role of biological degradation in the disappearance of these compounds can be made. Even in a simple aqueous system such as that used in the current study, the apparent half-lives of the compounds studied are a function of many interacting factors. Since the compounds have very low water solubilities, their percentage distribution to the interfaces of the system will affect their rate of disappearance. The effect of the composition on the vessel's sealing material on cyclodiene disappearance was significant in all of the experiments conducted. Both endosulfan isomers disappeared from the incubation vessels at faster rates when the vessel was sealed with butyl rubber, compared with PTFE. The relatively inert PTFE seals greatly reduced losses from volatilization, thus providing the necessary conditions for studying the chemical degradation of endosulfan.

The fact that there was very little difference in the rates of dissipation of aldrin and dieldrin from media sealed with butyl rubber or unsealed showed that butyl rubber sealing was ineffective. Such a rubber seal would therefore be unsuitable for microbial degradation studies when endosulfan or other volatile cyclodienes are being examined. Although butyl rubber has a very low permeability toward O_2, it has a high affinity for organic compounds (e.g., hexane and organochlorine pesticides). On the other hand, PTFE, because of its very low coefficient of friction, has an extremely low porosity to both gases and organic volatiles (Schlanger and Baumgartner, 1980). PTFE-lined silicone rubber provides an ideal seal for anaerobic degradation studies with compounds of high volatility, and such seals have been used in anaerobic microbial degradation studies (Grbic-Galic et al., 1990).

Once there is significant microbial growth in the incubation vessel, the effect of volatilization on overall pesticide disappearance of these volatile pesticides is greatly reduced (Anderson et al., 1970; Lichtenstein et al., 1968; Wheeler, 1969). However, during the first few days of incubation or in uninoculated controls, volatilization may be significant, particularly if the system is unsealed. Therefore, one effect of the microbial growth is to stabilize the cyclodienes, reducing their fugacity in the liquid phase and thus their tendency to escape from the incubation vessel.

The concentration of 1–5 ppm of pesticide used in this study reflects the levels commonly used in studies of the microbial degradation of pesticides and in aquatic toxicology, where bioassays are performed. The relatively high proportion of pesticides distributed to the glass–media interfaces is a consequence of the small volume of the incubation vessels, as well as the low solubility of the

The results of this study are applicable to the design of studies on the microbial degradation of volatile compounds. For example, in anaerobic incubations, it is necessary to have seals which do not allow volatilization of pesticides from the growth medium, which are pliable enough to receive several punctures, and which, at the same time, prevent oxygen from entering. PTFE-lined rubber provides such a seal. Uninoculated controls could then indicate the loss from chemical degradation and not artifacts of volatilization or adsorption. In studies of aerobic biodegradation, volatilization of pesticides presents particular difficulties, because of the need to introduce air or oxygen for the respiratory needs of the cultures. If it is desired to prevent volatilization by using Teflon seals, monitoring of the oxygen concentration would be necessary, with internal absorption of carbon dioxide in alkali. In such a system a low-oxygen indicator could be included in the medium, to show if the system is becoming anaerobic, or oxygen concentration could be monitored with a suitable probe.

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Registry No. Endosulfan I, 959-98-8; endosulfan II, 33213-65-9; endosulfan sulfate, 1031-07-8; aldrin, 309-00-2; dieldrin, 60-57-1.

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Efficient One-Step Method for the Extraction of Cyclodiene Pesticides from Aqueous Media and the Analysis of Their Metabolites

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An efficient method for the extraction of cyclodiene organochlorines from small samples of aqueous microbial growth media is described. Metabolites of endosulfan and aldrin, some of which are current environmental pollutants, can be detected on the electron capture detector (ECD) gas chromatograph with separation on a packed column. This has been done with their underivatized forms with prior column conditioning, with the exception of dihydrochlordenedicarboxylic acid (DHCDA). Separation of endosulfan and all of its metabolites has been achieved on a single packed column, and a comparison has been made of the relative sensitivity of the ECD to these compounds, aldrin, and its major metabolites. The limits of detection and the extent of linearity of the response of the ECD toward these compounds are reported. The importance of adequate column conditioning and likely problems encountered with the analysis of these cyclodiene compounds are deacribed.

INTRODUCTION

The cyclodiene pesticides, endosulfan, aldrin, and their metabolites, are pollutants often detected in the natural environment. The presence of the two isomers of endosulfan and their biologically active oxidation product, endosulfan sulfate, in waterways (Otton, 1991) is of particular concern, as they are extremely toxic to fish (Goebel et al., 1982). In the environment, aldrin is converted to its epoxide, dieldrin, and both aldrin and dieldrin may undergo various transformations at slow rates (Scheunert, 1989) to compounds that may be more or less toxic than these parent compounds. In determining the environmental fate of cyclodienes, it is therefore necessary to detect all metabolites, taking into account their toxicity, their ability to bioaccumulate in animal tissue (in the case of dieldrin and endosulfan sulfate) (Noble, 1991; Albertson et al., 1990; Mitchell, 1990), and their ability to move from the places of initial formation to other parts of the environment (Day, 1991).

In laboratory studies it is desirable to have an extraction system that is effective in extracting pesticides in a fast and reproducible way. There are many methods currently available for extracting pesticides from liquid media. The most popular procedures include use of the separating funnel and various liquid/liquid partitioning systems; these methods have been reviewed by Beroza et al. (1969). Other methods for pesticide extraction include solid-phase minicleanup columns, which are applications of the principles of reversed-phase chromatography. In a more recent development, Cias and Shimoni (1981a,b) described a novel liquid/liquid partitioning system (Mixxor), which utilized a new concept in mass transfer, and applied the system to extractions and separations in immunoassays. In the current study, the Mixxor liquid/liquid partitioning device was applied to the extraction of endosulfan, aldrin, and their major metabolites from small samples of aqueous media, as is commonly required in studies of the microbial degradation of pesticides. The optimal gas chromatographic conditions for the determination of the metabolites of these cyclodiene pesticides using packed columns and an ECD are also described.

MATERIALS AND METHODS

Chemicals and Glassware. The parent compounds endosulfan I (99.8%) and endosulfan II (99.8%) and the metabolites, endosulfan diol (98%), endosulfan sulfate (98%), endosulfan ether (98%), endosulfan lactone (98%), and endosulfan hydroxy ether (98%), were kindly provided by Dr. Klaus Stumpf, Hoechst AG, Frankfurt am Main, FRG, and Hoechst Limited Australia. Aldrin metabolites were a gift from Dr. David H. Hutson, Shell Research, Sittingbourne, U.K., and Shell Chemicals Australia. A dechlorinated cyclodiene derivative, *syn*-monodechlorodieldrin, was kindly provided by Dr. Andrew Maule, Porton Down Laboratories, Salisbury, U.K. All parent compounds and metabolites were prepared as 1000 and 100 ppm stocks in methanol, which were used as spiking solutions. Dilutions were made into hexane to prepare gas chromatography standards. The standard solutions were stored at −10 °C. All solvents used in the extraction and analysis were of Nanograde (Mallinckrodt). Glassware used was unsilanized borosilicate glass, washed twice in concentrated Pyroneg detergent (Diversey Australia Pty. Ltd) and rinsed with distilled water. The glassware was rinsed once with AR grade acetone (Pronalysis) prior to use. Anhydrous sodium sulfate was of AR grade (BDH Chemicals). Boron trifluoride/methanol (12–14%) was purchased from BDH Chemicals. Acetic anhydride was of laboratory grade, purchased from May and Baker. Pyridine was of analytical grade, purchased from Univar. The pyridine was dried over KOH and redistilled. The distillation fraction of 114.5–115 °C was stored over KOH.

Extraction Procedure. Pesticides were added at 1–5 $\mu g\ mL^{-1}$ to 500–1000 μL of aqueous medium and this was extracted with a total solvent volume of 10 mL in hexane/acetone/methanol/medium (15:5:2:2) in the Mixxor reservoirs (Figure 1). The level of added pesticide exceeded the limits of the solid-phase solubility for these compounds in water. The piston of the Mixxor was moved 60 times in its reservoir to partition the pesticides into the solvent phase. After the phases were allowed to separate (approximately 1 min), the solvent layer was decanted off the aqueous phase directly from the Mixxors into volumetric flasks, and the total volumes were made to either 10.0 or 25.0 mL with hexane only. A subsample was dried with anhydrous sodium sulfate to remove all traces of water prior to gas chromatography. The liquid/liquid partitioning devices (tradename Mixxors, manufactured by Genex Corp., Gaithersburg MD 20877, and distributed by Alltech Australia Pty. Ltd.) of 10-mL capacity (cat. no. 69104) were used in the routine extractions, all carried out in duplicate.

Gas Chromatographic Analysis. Extracts were analyzed

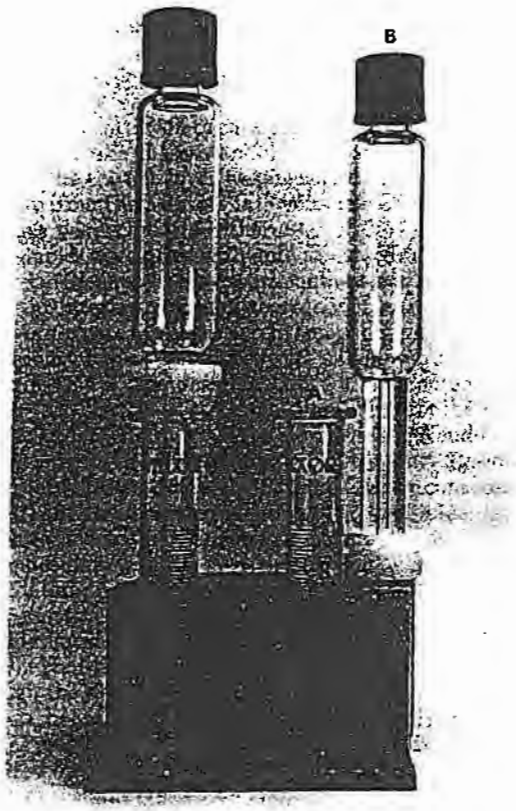

Figure 1. Mixxor liquid/liquid partitioning device. Sample to be extracted and solvent are added to the glass mixing reservoir (A). The mixer–separator (B) is inserted into the mixing reservoir and moved up and down up in a piston-like fashion to extract the pesticides.

Gaschrom Q, 80/100 mesh, packed into 3 m × 2.0 mm custom-made unsilanized borosilicate glass columns. The N_2 gas flow rate on all columns was adjusted to 25 mL min^{-1}. The column and injector/detector temperatures were set at 210 and 290 °C, respectively. The columns were prepared for routine use by standard procedures followed by conditioning in the gas chromatograph at 250 °C for 48–60 h (Zweig and Sherma, 1972). Before routine use, the liquid phases were equilibrated by injecting four or five times 1–5 μL of 1–10 ppm of pesticides and metabolites in hexane, with the end of the column disconnected from the detector. When the parent compounds and metabolite standards showed consistent sensitivity and retention time behavior, the column was considered ready for use. Columns were maintained by using extra glass wool packing as a pseudo-precolumn and replacing it when it became discolored after many injections. Peak area integration was performed using a Shimadzu Chromatopac CR-3A integrator set at 32 mV full-scale deflection (FSD) sensitivity. The external standard method was used for calculating residue values.

Determination of Limits of Detection. These were determined for each of the compounds analyzed by calculating the size of the smallest peak able to be distinguished from baseline noise under the conditions described under Gas Chromatographic Analysis. This was defined as being a peak 3 times the mean baseline noise at 32-mV FSD.

Derivatization Procedure. Derivatization of endosulfan diol was achieved by mixing 1 mL of extract, 2 mL of pyridine (treated as described), and 1 mL of acetic anhydride and incubating for 15 min at 37 °C. After 15 min, 5 mL of water was added and the solution was extracted with 5 mL of hexane. The hexane layer was washed in sequence with 5 mL of H_2SO_4 (1 M), 5 mL of $NaHCO_3$ (1 M), and 5 mL of water. The sample was dried with anhydrous Na_2SO_4 and made to volume for gas chromatographic analysis.

RESULTS AND DISCUSSION

Comparison of the Efficiency of Extraction. Extraction efficiencies of aldrin, dieldrin, and endosulfans I and II from growth medium and pure water were equal to, or above, 83% when the Mixxor was used without subsampling, i.e., extracting the entire vessel and its contents (Table I). The average coefficient of variation for all of the compounds using the Mixxor without subsampling was 5% at 1.0 ppm level of spiking. The value for endosulfan sulfate over all of the treatments was slightly higher than this. The liquid/liquid partitioners gave comparable recoveries to the separating funnel for equal amounts of time spent partitioning. However, use of the Mixxors was more convenient, allowing multiple samples (three or four) to be processed at once. Another benefit of the Mixxor was that consistent phase separation was achieved. An alternative method for liquid extraction method was used preliminary in this study. This approach involved extraction of the media directly from vials by adding the extraction solvent and vortexing for 30 s. The extraction efficiency was relatively low (70%) and variable. In addition, phase separation was erratic and low-speed centrifugation was often required to bring this about.

Subsampling from the total bulk of the growth medium, at the 1.0 ppm spiking level, had the effect of reducing the extraction efficiency to approximately a third of that obtained without subsampling (Table I). The average coefficient of variation when the extract was subsampled increased to 8.4%. This indicates that if cyclodiene residues are to be determined in an aqueous sample, and their residues are likely to be above the limits of their solubility, it is necessary that the entire vessel be extracted to ensure that material absorbed at the interfaces is recovered. For example, if a water sample is collected in a glass vessel, it is recommended that the vessel should be rinsed with the extracting solvent rather than subsampling from the total volume and extracting this subsample. This is also important where metabolites are sought for, for example, endosulfan sulfate and endosulfan diol, even though these compounds are slightly more water soluble than either of the parent isomers.

Effect of Media Composition and Level of Spiking. There was little difference between recoveries from different media types, but there were significantly higher recoveries of all compounds from pure water as compared with that from microbial growth medium (Table II). The mean coefficients of variation for each of the media types were close to 5%. Optimal extraction efficiency from the phosphate-buffered soil extract yeast mannitol broth (SEYMB) was achieved when the compounds were present at between 0.1 and 1.0 μg mL^{-1} (Table II). Recoveries significantly decreased at 10 μg mL^{-1}, although the coefficient of variation was considerably reduced.

Optimization of Extraction Conditions. An optimum extraction was considered to be reached after 60 strokes of the partitioner piston in its reservoir. Where further extractions of a sample are desired, the Mixxor reservoir may be emptied into a quick-fit flask for rotary evaporation and then refilled with the extraction solvent and the sample re-extracted. There was no significant improvement in extraction efficiency when the duration of extraction was increased to 120 or 180 strokes; therefore, 60 strokes was used routinely throughout the study to minimize unnecessary wear of the glass surfaces.

Optimization of Gas Chromatographic Conditions. The analysis of a wide range of chlorinated cyclodiene compounds using gas chromatography is common practice. There are, however, no descriptions in the literature of

Table I. Comparison of Efficiency of Extraction of Pesticides from Various Sterile Aqueous Media Using a Separating Funnel and a Mixxor Extractor[a]

extraction method and media	extraction efficiency, %					CV,[d] %
	aldrin	dieldrin	endosulfan I	endosulfan II	endosulfan sulfate	
separating funnel (250 mL)[c]	84.5 ± 5.9	87.5 ± 4.5	83.5 ± 3.5	87.0 ± 5.4	95.6 ± 7.3	6.1
Mixxor[c]	85.4 ± 2.9	88.5 ± 4.1	89.4 ± 4.1	82.7 ± 3.4	83.9 ± 6.6	4.9
Mixxor subsampling[c]	29.8 ± 2.2	27.8 ± 2.5	20.0 ± 1.4	27.1 ± 2.5	35.4 ± 3.2	8.4
Mixxor anaerobic medium[d]	84.3 ± 3.2	85.0 ± 5.0	88.6 ± 3.5	83.1 ± 3.7	82.5 ± 6.5	4.4
Mixxor pure water	92.7 ± 2.9	91.5 ± 2.6	91.3 ± 2.6	82.7 ± 1.8	72.6 ± 3.0	3.0

[a] Pesticide concentration in the medium was 1 ppm for aldrin, dieldrin, endosulfan I, and endsofulan II and 5 ppm for endosulfan sulfate. There was no subsampling from larger total volumes unless indicated. [b] CV, coefficient of variation across each treatment. [c] Soil extract yeast mannitol medium (SEYMB) contained soil extract (10%), yeast extract (2.0 g/L), mannitol (5.0 g/L), sucrose (2.0 g/L), and trace elements in 100 mM phosphate buffer (Allen, 1957). [d] Anaerobic medium was described by Balch et al. (1982) and modified by Maule et al. (1987) with the addition of formate (2.0 g/L).

Table II. Effect of Level of Spiking on the Efficiency of Pesticide Extraction from Growth Medium Using the Mixxor Liquid/Liquid Partitioning Devices

spike,[a] ppm	extraction efficiency, % ± SD					CV,[b] %
	aldrin	dieldrin	endosulfan I	endosulfan II	endosulfate	
0.1	88.5 ± 1.3	83.4 ± 2.0	91.9 ± 2.5	78.0 ± 4.0	88.7 ± 7.6	4.0
1.0	86.8 ± 3.2	86.3 ± 3.7	83.7 ± 4.5	79.1 ± 3.5	86.0 ± 7.1	5.1
10.0	62.7 ± 1.4	61.2 ± 1.6	72.1 ± 1.9	72.1 ± 1.8	65.1 ± 3.4	3.0

[a] Endosulfan sulfate was spiked at 5 times the amount of the other cyclodienes, i.e., 0.5, 5.0, and 50.0 ppm into SEYMB (described in Table I). There was no subsampling in this experiment. [b] CV, coefficient of variation across each spiking treatment.

the relative sensitivity and linearity of the electron capture detector toward the various cyclodiene metabolites in their underivatized forms or on their limits of detection using the gas chromatographic technique. An attempt was made to characterize optimal conditions for the analysis of cyclodiene metabolites using ECD gas chromatography with packed columns.

It was apparent that the chromatography columns required a period of equilibrating with the compounds to be analyzed prior to use. This was demonstrated by the very low relative sensitivities of the compounds, in particular the diols, prior to equilibrium. In fact, the sensitivity of the detection of endosulfan diol, *cis*- and *trans*-aldrin diol, increased 3-fold after the equilibration period. This period was kept to a minimum when 1–10 ppm solutions in hexane were injected with the detector end of the column disconnected. This effect is likely to be due to strong interactions of the analytes at sites within the packed column.

Although both columns tested were able to chromatograph the parent compounds and metabolites in the study, only one of the columns successfully separated all of the endosulfan compounds when they were present in a mixture (Figure 2). This was the column containing a mixture of the liquid phases SE-30 (5%):DC-200 (5%) in a 3-to-1 ratio.

In studies where XE-60, QF-1, DC-200, SE-52, OV-225, and XE-60:SE-52 were used as liquid phases at 2% in packed columns, useful separation was only achieved with XE-60 (Goebel et al., 1982, and references cited therein). The major difficulty in separating the endosulfan metabolites, on the column configurations previously reported, arisea from the similarity of retention times of endosulfan diol and endosulfan hydroxy ether. When both are present in a mixture, they tend to elute with the same retention time, although when analyzed individually they have slightly different retention times. Therefore, to be able to quantify all of the endosulfan compounds in a study, it has been necessary to use two columns routinely. The choice of the SE-30:DC-200 liquid-phase mix in the current study has overcome this inconvenience, so that two columns need only be used for metabolite confirmation

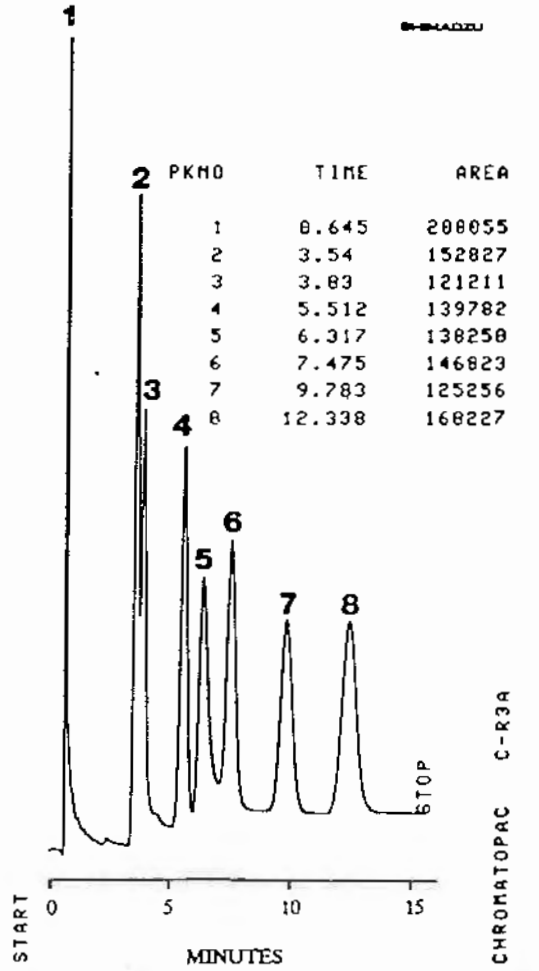

Figure 2. Typical chromatogram showing the elution of endosulfan compounds on the packed column containing a 3:1 mixture of SE-30 (5%):DC-200 (5%) liquid phases on Gaschrom Q 80–100 mesh. Injection, column, and detector temperatures were 290, 210, and 290 °C, respectively. The compounds in order of elution are solvent (1), endosulfan ether (2), endosulfan hydroxy ether (3), endosulfan lactone (4), endosulfan diol (5), endosulfan I (6), endosulfan II (7), and endosulfan sulfate (8).

Table III. Analysis of Endosulfan and Its Metabolites Using Two Chromatographic Columns on an Electron Capture Gas Chromatograph[a]

parent compound or metabolite	column I[b] retention time, min	column I[b] relative sensitivity[d]	column II[c] retention time, min	column II[c] relative sensitivity
endosulfan I	7.88	1.00	8.67	1.00
endosulfan II	10.43	0.79	11.34	1.11
endosulfan sulfate	13.26	0.08	21.87	0.67
endosulfan diol[e]	6.67	0.01	4.73	0.01
endosulfan ether	3.62	1.28	3.35	1.04
endosulfan lactone	5.78	0.98	7.23	0.98
endosulfan hydroxy ether	3.95	1.25	5.08	1.17

[a] Conditions described under Materials and Methods. [b] SE-30 (5%):DC-200 (5%) (3:1) liquid phase mix. [c] OV-210 (5%):SE-30 (5%) (7:3) liquid phase mix. [d] Relative to endosulfan I. [e] The acetylated derivative of the diol eluted at 11.6 min and showed a 60-fold increase in sensitivity compared to endosulfan I.

Table IV. Chromatographic Characteristics of Aldrin and Related Compounds Using Two Chromatographic Columns on an Electron Capture Gas Chromatograph[a]

parent compound or metabolite	column I[b] retention time, min	column I[b] relative sensitivity[d]	column II[c] retention time, min	column II[c] relative sensitivity
aldrin	5.23	1.00	4.08	1.00
dieldrin	8.82	1.08	8.56	0.85
syn-dechlorodieldrin	7.63	0.54	9.05	0.42
pentachloroketo-dieldrin	11.18	0.80	19.28	0.23
dieldrin ketone	8.48	0.16	10.43	0.30
dihydrochlordene-dicarboxylic acid dimethyl ester	15.12	0.78	15.09	0.26
photoaldrin	11.09	0.04	11.95	0.14
photodieldrin	19.69	0.05	28.10	0.02
cis-aldrin diol	16.37	0.02	12.05	0.02
trans-aldrin diol	12.53	0.004	15.0	0.001

[a] Conditions described under Materials and Methods. [b] SE-30 (5%):DC-200 (5%) (3:1). [c] SE-30 (50%):OV-210 (5%) (1:1) liquid phase mix. [d] Relative to aldrin.

endosulfan compounds, except endosulfan diol and endosulfan hydroxy ether. The second column used in the study, containing the OV-210:SE-30 liquid-phase mix, gave good separation of all the endosulfan compounds except for endosulfan diol and endosulfan hydroxy ether and therefore was not used routinely in the analysis of endosulfan (Table III).

Only one potential metabolite of aldrin, dihydrochlordenedicarboxylic acid, could not be chromatographed without derivatization, even when injecting concentrations of up to 10 ppm were injected. The dimethyl ester standard (provided by Shell Chemicals), however, could be detected under these chromatographic conditions. For analyzing this compound in environmental samples it is therefore necessary to form the dimethyl ester derivative prior to its analysis. A simple and efficient method using BF_3/methanol, modified from that of Hallas (1965), has been developed by N. Ahmad (1989, Fox Anamet Laboratory, Sydney, personal communication). The methyl ester derivative is considerably more volatile and less water soluble than the diacid, and this has allowed it to be detected in the gas chromatograph under the conditions described. All other potential metabolites of aldrin were successfully chromatographed on both of the columns used (Table IV).

Linearity of Detector Response. The linearity of the response of the ECD to the endosulfan compounds was determined. Both parent compounds and metabolites

Table V. Detection Parameters for Endosulfan and Its Metabolites (Underivatized) on the Electron Capture Gas Chromatograph[a]

compound	LOD,[b] pg	mass range analyzed, pg	linearity of detector response[c]
endosulfan I	1.01	0–1600	0.997
endosulfan II	1.36	0–1600	0.992
endosulfan sulfate	7.41	0–1000	0.965
endosulfan diol	42.0	0–1000	0.989
endosulfan ether	1.1	0–200	0.974
endosulfan lactone	1.20	0–200	0.974
endosulfan hydroxy ether	1.23	0–200	0.995

[a] Using SE-30:DC-200 column under the conditions described under Materials and Methods. [b] Limit of detection is defined as being the quantity of compound which gives a peak 3 times the baseline noise at 32 mV FSD. [c] Regression coefficients determined from linear plot of mass of compound analyzed vs peak area.

Table VI. Detection Parameters for Aldrin and Its Metabolites on the Electron Capture Gas Chromatograph[a]

compound	LOD,[b] pg	concn range, pg	linearity of detector response[c]
aldrin	1.03	0–4000	1.000
dieldrin	1.11	0–4000	0.998
syn-dechlorodieldrin	1.79	0–4000	1.000
pentachloroketodieldrin	1.22	0–4000	0.999
dieldrin ketone	167	0–4000	0.999
dihydrochlordenedicarboxylic acid dimethyl ester	1.24	0–4000	0.998
photealdrin	23.8	0–1500	0.990
photodieldrin	18.2	0–1500	0.996
cis-aldrin diol	66.2	0–5000	0.985
trans-aldrin diol	250	0–5000	0.956

[a] Using SE-30 (5%):DC-200 (5%) (3:1) column under the conditions described under Materials and Methods. [b] Limit of detection is defined as being the quantity of compound which gives a peak 3 times the baseline noise at 32 mV FSD. [c] Regression coefficients determined from linear plot of mass of compound analyzed vs peak area.

showed good linearity over the working concentration range. The indicator of linearity used was the regression coefficient from the mass of compound injected vs peak area plots (Table V). Endosulfans I and II and hydroxy ether data points showed the closest fits on the linearity plots. Endosulfan sulfate, although linear over the entire concentration range examined, gave the lowest regression coefficient of all the endosulfan compounds, i.e., compared with that of endosulfan I. Similarly, the ECD response for aldrin and related compounds was determined. All of the aldrin-related compounds gave very good linearity response curves over the concentration ranges analyzed, except for *trans*-aldrin diol (Table VI). This response for the *trans*-aldrin diol is correlated with a low sensitivity and therefore a high limit of detection. The response curves demonstrate that after the column is initially equilibrated with parent compounds and metabolites, there is a proportional response with amount of pesticide injected (Figures 3 and 4). Since the response curves for all of the compounds, including the diols, pass through the origin, any losses of compounds occurring in the chromatography system are proportional to the amount injected.

Sensitivity of Detector toward Metabolites. After many hours of routine use of the OV-210:SE-30 column, the apparent sensitivity of the ECD toward endosulfan hydroxy ether decreased. Full sensitivity was restored by replacing the glass wool packing located at the injection zone, which had become discolored with use. This effect was not observed with any of the other endosulfan or aldrin

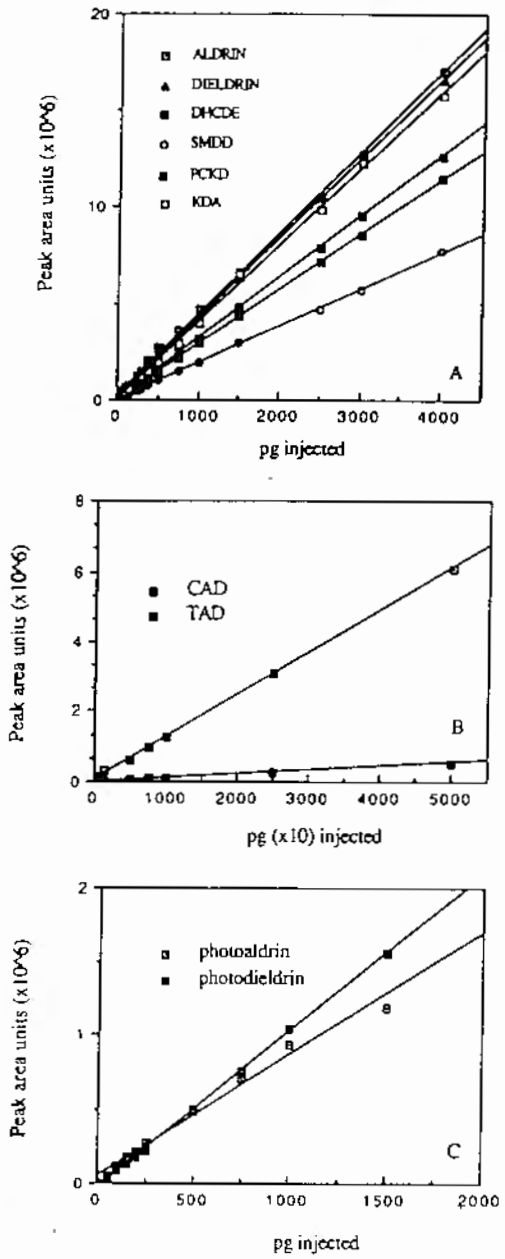

Figure 3. Comparison of the ECD response toward (A) aldrin, dieldrin, their pentachloro, ketone, and methylated acid derivatives, (B) aldrin diols, and (C) photoisomers of aldrin and dieldrin.

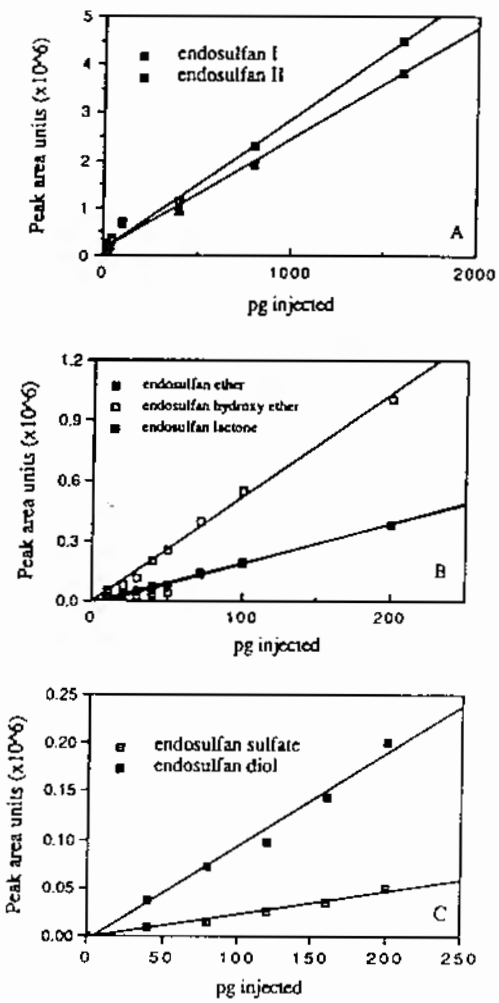

Figure 4. Comparison of the ECD response toward (A) the endosulfan isomers, (B) endosulfan hydroxy ether, ether, and lactone, and (C) endosulfan diol and sulfate.

compounds. This observation indicates that endosulfan hydroxy ether is particularly sensitive to degradation in the injection zone once the glass wool packing has aged. This problem will be avoided in routine analysis if the glass wool packing is replaced frequently, i.e., after approximately 200 injections.

The mean coefficients of variation from each of the linearity plots were determined as follows: endosulfan I, 5.1%; endosulfan II, 6.6%; endosulfan sulfate, 14%; endosulfan diol, 7%; endosulfan ether, 6.4%; endosulfan hydroxy ether, 6.9%; and endosulfan lactone, 3.5%. The greater variability in the detection of endosulfan sulfate means that when the sulfate is being analyzed, at least duplicate or preferably triplicate injections of the same sample should be made. The variation does indicate that during analysis endosulfan sulfate may show erratic stability. The limits of detection of all compounds studied demonstrated relatively high limits of detection, indicating a possible need to concentrate the solvent phase of extracts of these samples when these degradation products are analyzed in environmental samples.

When endosulfan diol was derivatized with acetic anhydride, the sensitivity of the detector response was increased. On column I (Table III), the acetylated diol derivative eluted at 11.6 min, which was equivalent to a relative retention time of 1.5 when compared with endosulfan I. This increase in sensitivity was 60-fold over that of the underivatized diol. Although it is not possible to claim this result alone means there are on-column losses of the underivatized form, we have found using the mass selective detector (Hewlett-Packard HP 5971 series) that a similar loss also occurs, indicating that the loss of diol occurs before detection (unpublished data).

Given that all of the cyclodiene residues could be detected with the chromatographic conditions described, except for the very polar diacid derivative of aldrin, it is apparent that lengthy derivatization procedures can be kept to a minimum in analyzing for environmental residues of the metabolites of aldrin and endosulfan. The limitation, however, of not derivatizing the diol compounds is that their limits of detection are greatly increased using the detection system described. The low sensitivity of the ECD detector to the other oxygenated aldrin com-

Relevance to Microbiological and Aqueous Residue Analysis Studies. A consequence of the higher water solubility of many of the cyclodiene metabolites is that they can be more difficult to detect, in their underivatized forms, by the gas chromatographic technique. These metabolites generally have additional oxygen atoms, increasing their polarity and solubility in water and so reducing their volatility in the gas chromatograph and increasing interaction within the column system. These studies demonstrated that the liquid phase of a column requires an equilibration period with the metabolite to achieve consistent chromatography. Therefore, in microbial degradation studies where the parent cyclodienes are being detected, potential metabolites in their underivatized form may escape detection. Although likely to be detected after several injections, the metabolite would probably pass undetected if the column were not previously equilibrated with the compound. The appropriate standards therefore are necessary prior to any degradation study so that the chromatographic system can be properly equilibrated prior to any routine analyses.

CONCLUSION

The current study describes an efficient one-step method for extracting organochlorine compounds and their metabolites from small volumes of aqueous media. Although the extraction procedure described has been applied to the removal of cyclodiene organochlorines from aqueous phases, it may be equally applied to the extraction of other water-insoluble compounds. The extraction procedure may be coupled to a Pasteur pipet cleanup column method (Ahmad et al., 1988) to provide a simple and efficient system for the extraction of pesticides and their cleanup from relatively small aqueous samples prior to analysis.

The gas chromatographic configuration described allows the routine detection of endosulfan, aldrin, and their metabolites. Electron capture detection was linear over the working ranges of the dimethyl ester derivative of dihydrochlordenedicarboxylic acid, as well as all of the other underivatized compounds, indicating that they can be routinely analyzed, on a single packed column, without the need for capillary column technology, with minimal derivatization procedures.

A range of aqueous extraction procedures for removing chlorinated pesticides from microbial growth media have been developed for aldrin and endosulfan (Goebel et al., 1982; Schuenert, 1989, and references cited therein). These procedures, while effective, are time-consuming, often involving the use of a separating funnel with a range of different solvents with a series of evaporation and redissolving steps. The method described in the current study provides an attractive alternative in routine laboratory studies where small volumes of media are to be analyzed, as the recoveries are relatively high and the time required for each extraction is minimal (approximately 2 min). Solvent use was also kept to a minimum. Under the conditions described, the Mixxor was shown to be more reproducible, more convenient for multiple extractions, and therefore more suitable for routine use.

An advantage of the Mixxor was that effective phase separation was rapidly achieved for all of the extractions performed. This is a major improvement over the direct extraction of pesticides in the incubation vessels using a vortex device, where both phases may be difficult to separate when using a close ratio of solvent-to-extract volume. Such direct extraction methods, used in preliminary trials in the current study, did not warrant further investigation because of the relatively low extraction efficiency for the compounds tested and the erratic phase separation demonstrated after extraction.

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