

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION
COMMISSION ON AGROCHEMICALS*

IUPAC Reports on Pesticides (27)

THE ROLE AND LIMITATIONS OF MICROORGANISMS IN THE CONVERSION OF XENOBIOTICS

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The role and limitations of microorganisms in the conversion of Xenobiotics

Abstract - The role of microorganisms in the detoxification of pesticides is described. Basic processes of microbial conversion of pesticides are considered with characterization of the enzymes that bring about these bioconversions. Special attention is paid to the processes of microbial transformation of pesticides that lead to more recalcitrant and toxic intermediates compared to their parent compounds. Microorganisms prove to be responsible for both enhanced degradation of pesticides and formation of soil-bound pesticide residues and, conversely, their release. A few examples of such transformations are considered and causes underlying them are discussed. A separate section is devoted to the use of biotechnology in agriculture and to prospects of practical application of microorganisms for degradation of pesticides.

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1 INTRODUCTION

Microorganisms play an essential role in the bioconversion and total breakdown of pesticides and other xenobiotics in the environment. In recent years, a number of problems related to microbial degradation of pesticides have been encountered. Some examples are given below.

In certain 'problem' soils, the efficacy of applied pesticides is reduced due to their enhanced rate of degradation. In other cases, environmental accumulation of persistent pesticides has become a problem. Some pesticide residues

are bound by the soil but under specific conditions may be released due to microbial attack.

Most bioconversions are detoxification reactions. However, there have been reports of the formation of more toxic pesticidal metabolites.

Certain pesticides are leached down to deeper subsoil horizons or underground water systems but, to date, our understanding of microbial processes occurring in these systems and undoubtedly affecting the fate of pesticides is far from adequate. In addition, there is limited knowledge of the conversion of pesticides in other anaerobic zones, e.g. water sediments.

The relevant literature shows that microorganisms possess high potential for bioconversion and total degradation of xenobiotics. This is especially true now that modern genetic engineering enables successful manipulation of specific abilities of microorganisms.

The present document presents a critical review of the mechanisms of microbial bioconversion of xenobiotics, indicates various problems and outlines a basic rational approach to these problems using recent developments in microbiology and biotechnology.

2 BASIC BIOCHEMICAL PROCESSES IN BIOCONVERSION OF XENOBIOTICS

Many xenobiotics contain functional groups and partial structures not usually found in nature. Many pesticides are bulky complex molecules, and others display hydrophobic properties due to the presence of reduced hydrocarbon fragments. The principal enzymes responsible for the bioconversion of xenobiotics are various lyases and oxydoreductases, specifically hydrolases, oxygenases and various enzymes capable of dehalogenation.

2.1 Hydrolysis

Amide and ester bonds undergo hydrolytic cleavage in anilides, phenylureas, esters of carbamic, thiocarbamic, phosphoric, thiophosphoric, and of other acids. Hydrolases responsible for the cleavage of pesticides are among the best studied groups of enzymes. Most of these hydrolases are extracellular enzymes, except for the cell wall-bound enzymes of *Penicillium* and *Arthrobacter* sp., which hydrolyse barban and propham, respectively. Aryl- and acylamidases, which hydrolyse the amide bond in phenylamides, have been studied and described in sufficient detail (EC 3.5.1.13). Usually, these enzymes are induced by a broad spectrum of substrates and exhibit low substrate specificity (ref. 1; Tables 1, 2).

TABLE 1. Induction of acylamidase in *Bacillus sphaericus* 12123 (ref. 1)

Inductor	Specific activity 10^{-3} unit*/mg protein	Induction rate, % of that of linuron
Linuron	0.13	100
Maloran	0.015	12
Monalide	0.029	22
Propanil	0.002	2
Propham	0.008	6
2-Chlorobenzanilide	0.03	23
2,5-Dimethylfuran-3-carboxanilide	0.05	40

* - micromoles of substrate degraded per minute

In 1976, Munnecke and Hsieh (ref. 2) reported similar results: a culture capable of the thioester bond cleavage (416 nmol/min per 1 mg protein) also brought about enzymic hydrolysis of another 8 of the 12 insecticides tested. This ability of microbial hydrolases underlies the emergence of 'problem' soils, which are capable of hydrolyzing a wide variety of pesticides.

TABLE 2. Hydrolysis of phenylamides by a cell-free extract of *Bacillus sphaericus* induced by linuron (ref. 3)

Phenylamide	Specific activity, 10^{-3} unit*/mg protein
Linuron	0.20
Monolinuron	0.20
Maloran	0.11
Carboxin	2.52
2-Chlorobenzanilide	18.7
Glycine 4-nitroanilide	106.0
L-Alanine 4-nitroanilide	309.0
L-Leucine 4-nitroanilide	55.8
L-Phenylalanine 4-nitroanilide	7.63

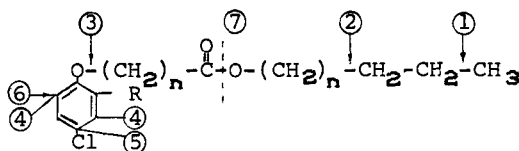
* - micromoles of substrate degraded per minute

2.2 Dehalogenation

Many xenobiotics contain halide atoms bonded to aliphatic or aromatic carbon. Therefore, dehalogenation reactions are important initial steps in the degradation of such xenobiotics. Although molecular mechanisms of dehalogenation are not fully understood and require further studies, certain reaction types are fairly well known: hydrolytic, reductive and oxidative dehalogenations. The latter type includes dehydrohalogenation, i.e. dehalogenation which involves molecular oxygen, and results in the formation of a double bond. A number of enzymes have been isolated, described and characterized as effecting dehalogenation (ref. 4). These are haloacetate-halohydrolyase (EC 3.8.1.3) which splits off a halogen from halide acetate to form glycolic acid; hydrolases effecting dehalogenation of dichloroacetate; 2-haloacid halohydrolyase (EC 3.8.1.2). The above enzymes are responsible for the hydrolytic release of a halide from the 2 position of short-chain fatty acids. The enzymes which carry out reductive dehalogenation of aromatic carbon have not yet been characterized, although literature provides a number of examples of the process including with DDT, 2,4-D and polychlorophenols. As far as oxidative dehalogenation is concerned, the enzyme - DDT-dehydrochlorinase (EC 4.5.1.1.) has been characterized and descriptions of the dioxygenase-mediated dechlorination of picloram and monooxygenase dechlorination of chlorophenoxyacids, chlorobenzoic acids, and chlorophenols are available.

2.3 Oxidation

Oxidative processes are very important in the bioconversion of pesticides and other xenobiotics, especially bioconversions which follow hydrolysis and dehalogenation of the parent. In this case, the basic reactions are hydroxylation, followed by cleavage of the aromatic ring, oxidative O- and N-dealkylation, and epoxidation. Certain compounds, e.g. derivatives of aromatic acids, are completely degraded due to the involvement of oxidative enzymes (Scheme 1).



Scheme 1 Sites of oxidative attack by enzymes during degradation of alkyl esters of chlorophenoxyacids

- R - Methyl or Cl;
- 1 - hydroxylation of terminal methyl;
- 2 - ω -oxidation;
- 3 - oxidative cleavage of ether bond;
- 4 - hydroxylation of the ring;
- 5 - oxidative dechlorination;
- 6 - cleavage of the ring;
- 7 - dashed line - possible hydrolytic fission by esterase.

The above bioconversions are adequately described in Hayaishi's monograph (ref. 5).

2.4 Reduction

In general, reductive processes take place under anaerobic conditions in the early stages of degradation. Mention has already been made of reductive dechlorination. Another no less important process is reduction of nitro groups. Enzymes which bring about this process are called nitroreductases. They perform their functions in the presence of NADH, the reaction being stimulated by the reduced form of FAD, as well as by Mn and Fe ions. A number of authors reported that these enzymes have low substrate specificity. Thus, the enzymic preparation from *Vellonella alkalescens* was found to catalyze the reduction of nitro groups of some 40 different compounds. The amino products of nitro group reduction may resist further transformation due to the formation of bound residues (see Section VIII.1).

3 MICROORGANISMS EFFECTING DETOXIFICATION OF XENOBIOTICS

A review of the research on microbial degradation of xenobiotics reveals that bacteria are chiefly responsible for the detoxification of xenobiotics. Fungi and yeasts are less important, and microalgae and Protozoa appear to be only rarely involved in the degradation of xenobiotics. Among the bacteria, pseudomonads are considered to be the most efficient group involved in the degradation of xenobiotics. Bacilli are quite often responsible for processes of pesticide hydrolysis. However, pseudomonads can bring about hydrolysis as successfully as they do dehalogenation, hydroxylation, aromatic ring cleavage and nitro group reduction. Some examples of bioconversion of various compounds effected by pseudomonads are listed in Table 3.

The bacterial genera *Acinetobacter*, *Arthrobacter*, *Rhodococcus* and *Flavobacterium* also deserve mention, since they often bring about various bioconversions of different xenobiotics.

Fungal cultures, especially the genera *Aspergillus* and *Penicillium*, are often involved in bioconversions of *s*-triazines. Certain fungi have also been reported to bring about methylation of hydroxy- and amino groups, and metals. Thus the fungus *Trichoderma virgatum* effected the methylation of pentachlorophenol, whereas *Penicillium notatum*, *Aspergillus niger* and *Scopulariopsis brevicaulis* methylated arsenic derivatives (ref. 6).

TABLE 3. Bioconversion of xenobiotics effected by pseudomonads

Mode of action	Species	Reference
Hydrolysis of carbaryl, dichlorophos, diazinon, parathion	<i>Pseudomonas melophthora</i>	(ref. 7)
Hydrolysis of parathion	<i>Pseudomonas stutzeri</i>	(ref. 2)
Dehalogenation of halide acetate	<i>Pseudomonas</i> sp.	(ref. 8)
Total dehalogenation of DDT, aromatic ring cleavage	<i>Pseudomonas aeruginosa</i>	(ref. 9, 10)
Total degradation of 3-chlorobenzoate	<i>Pseudomonas putida</i>	(ref. 11)
Oxidative dehalogenation of lindane	<i>Pseudomonas putida</i>	(ref. 12)
Reduction of nitro group in 4,6-dinitro- <i>o</i> -cresol	<i>Pseudomonas</i> sp.	(ref. 13)
Total degradation of 2,4,5-T	<i>Pseudomonas sepacia</i>	(ref. 14)
Degradation of toluene, xylenes, styrene, α -methylstyrene	<i>Pseudomonas putida</i>	(ref. 15, 11)
	<i>Pseudomonas aeruginosa</i>	(ref. 16)

4 ROLE OF MICROORGANISMS IN DETOXIFICATION OF PESTICIDES

The important role played by microorganisms in the degradation of pesticide residues is well recognized. According to various authors, microorganisms degrade 25 to 70% of the xenobiotic residues. Literature carries reports on microbial strains capable of degrading such recalcitrant pesticides as DDT,

polychlorobiphenyls, dicofol, 2,4,5-T and others (ref. 17, 18, 9).

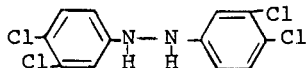
Unfortunately, very few xenobiotics can be utilized by pure cultures as the sole source of carbon. Those, which can, are fairly simple compounds such as dalapon, chlorobenzoates, 2,4-D. Of greater significance are bioconversions of pesticides effected by mixed cultures when the initial degradation steps are brought about by one culture, and metabolites produced are further degraded by other microbial strains. One example is the hydrolysis of parathion by *Pseudomonas stutzeri* to produce nitrophenol which is further degraded by another strain - *Pseudomonas aeruginosa* (ref. 2).

Exchange of genetic information by natural microbial populations also appears to be a potent factor facilitating total degradation of xenobiotics. For example, one hypothesis states that phenoxyalkane carboxyherbicides, such as 2,4-D and MCPA, do not accumulate in the environment exactly because plasmids, which several years ago were found to encode their degradation, can be transferred under natural conditions from strain to strain (ref. 19). Recently, plasmids responsible for biodegradation of polychlorobiphenyls, chlorobenzoates, petroleum, surfactants and other compounds were discovered. They apparently play an important role in degradation of numerous foreign compounds by the natural microflora.

5 MICROBIAL BIOCONVERSION OF XENOBIOTICS TO MORE PERSISTENT COMPOUNDS

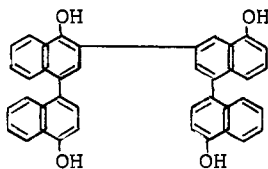
Most xenobiotics are readily degraded in natural ecosystems. However, some compounds are transformed into intermediates which are highly resistant to microbial attack or are more toxic than the parent compound.

The hydrolysis of phenylamide herbicides, for instance, results in the formation of chloroanilines, which under the action of microbial enzymes are condensed to form tetrachlorohydrazobenzenes: e.g.

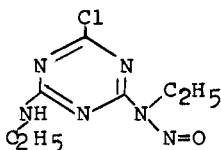


3,3',4,4'-tetrachlorohydrazobenzene

As a result, polychloroaromatic compounds appear in the biosphere. These are much more resistant to degradation and more toxic than the parent pesticides and chloroanilines. Thus Kearney and co-workers (ref. 20) have detected 3,3',4,4'-tetrachlorohydrazobenzene in soil of rice fields some three years after their treatment with propanil. It should be stated that peroxidase and, to a lesser extent, aniline oxidase are responsible for the production of chlorohydrazobenzenes. Similar processes also occur during the degradation of carbaryl (1-naphthyl N-methylcarbamate), where the hydrolysis of carbaryl leads to α -naphthol which under the effect of phenol oxydase is condensed to give complex compounds highly toxic for aquatic organisms.



A particular threat for the environment are the metabolites of nitrogen-containing pesticides, which on further degradation are readily transformed in highly mutagenic and carcinogenic compounds. For example, simazine is oxidized into a carcinogen of the following structure (ref. 21):



Two of the trifluralin degradation products are also hazardous as mutagenic factors:



These examples show the need for the thorough studies of the properties of the intermediate products from the microbial bioconversion of pesticides. Their effect on biocenoses and man may be more deleterious than that of parent xenobiotics.

6 CONDITIONS FOR MICROBIAL DEGRADATION OF XENOBIOTICS

The principal cause of pesticide persistence in soil is commonly the lack of conditions favouring microbial degradation. Of these, the most important are: low accessibility of toxicants to enzymes of microorganisms (determined by the extent of the sorption of toxicant by soil particles and colloids), availability of organic compounds utilized by the microflora as energy substrates, moisture conditions, aeration level, temperature, pH etc. Certain pesticides are readily degradable in liquid media but, in soil, become resistant to microbial attack. A classical example is the herbicide paraquat which is strongly sorbed by soil thus becoming inaccessible to microorganisms. Quite often the primary transformation of a pesticide, e.g. hydrolysis, leads to phenols or anilines followed by their strong binding to soil. This results in their inaccessibility to microorganisms. The data concerning the effect of organic matter in soil on microbial degradation of xenobiotics are rather conflicting. Some authors report an inhibiting effect of increasing organic matter on the rate of microbial decomposition of toxicants in soil, whereas others report that organic matter stimulates biodegradation. The contradictory effect may be explained by the dual role played by organic matter in water and soil in relation to microorganisms. In the latter case, it may provide a source of energy and factors required for the occurrence of primary steps of xenobiotic degradation. Finally, specific components of organic matter may affect the regulation of microbial enzymes.

An explanation of the stimulating action of organic matter on microbial degradation was provided by studies of cometabolism. This is defined as the degradation or bioconversion of organic compounds, mainly of xenobiotics, by microorganisms in the presence of more readily accessible substrates. Biologically, cometabolism provides the processes of xenobiotic metabolism with energy, cofactors, effectors and, possibly, oxidants produced in the course of the cosubstrate metabolism. Cometabolism of pesticides with various cosubstrates has been described for both laboratory and natural conditions. Thus, the herbicide molinate was degraded by cultures of *Bacillus* sp. in the presence of ethanol; DDT was found to be cometabolized by the strain *P. aeruginosa* 640x faster on a medium with glycerol and hexadecane, and alvison during microbial growth on organic acids. Numerous experiments, on the introduction of cosubstrates into soils and aquatic systems, showed a substantial increase of the soil microflora activity towards simazine, dexton, diazinon, pentachloronitrophenol, paraquat and other pesticides. Hence, the polymeric organic matter of soil colloidal systems may impede microbial degradation of pesticides as a result of their adsorption. In contrast, low molecular weight compounds generally stimulate biodegradation of xenobiotics. The role of oxygen in the degradation of xenobiotics merits special discussion. General participation of oxidative enzymes in the conversion of foreign compounds demonstrates the necessity for accessible oxygen in order to effect degradation of pesticide residues. However, studies of the degradation of applied pesticides in water and the soil of flooded rice fields have shed new light on the role of anaerobic processes in microbial degradation of xenobiotics. Many persistent pesticides, reputed to be recalcitrant to microbial attack and thereby residing in soil for many years, are degraded much faster in tropical soils from flooded rice fields. Lindane was subject to anaerobic microbial degradation in muds and lake sediments (ref. 22). In addition, heptachlor, endrin, methoxychlor and dicofol also underwent intensive degradation in flooded soils. The organophosphorus pesticide diazinon was eliminated from the flooded soil in two months, whereas its residence time in a non-flooded soil was six months. It should be noted that anaerobic conditions favour the accumulation of metabolites whose further decomposition requires the presence of oxygen. This phenomenon was observed during degradation of parathion, diazinon, carbofuran and other

pesticides in soil. Anaerobic processes are highly dependent on the redox potential. Microbial degradation of xenobiotics is also related to acidity of the medium. This affects the rates of sorption of pesticides and their metabolites and also influences the processes of decomposition or the formation of conjugants.

7 ENHANCED DEGRADATION OF PESTICIDES AS AN EVIDENCE OF MICROBIAL ACTIVITIES

Repeated applications of the same biodegradable pesticides to soils can lead to a build-up of microbial populations capable of more rapid degradation of the applied pesticides thereby decreasing their efficacy in soil (ref. 23). This is the case with many carbamates, acetamides, acylanilides and organophosphorus insecticides, herbicides and nematocides which upon application to soil undergo rapid degradation. The soils in which such pesticides produce no control of the target organisms are called the 'tired', 'problem' or 'aggressive' soils (ref. 24, 25, 26, 27). It was clearly demonstrated that frequent applications of \bar{s} -triazines, carbofuran, chloroethiocarb or other toxicants led to the complete and rapid loss of their activity due to their drastically enhanced degradation by the soil microflora. The isolation and identification of microorganisms from such soils identified the presence of strains capable of rapid metabolism of pesticides and their structural analogues. This fact essentially restricts the efficient use of some pesticides in agriculture. Many factors appear to contribute to the emergence of problem soils. The most common are the concentration of organic matter in soil, pH, moisture level, temperature, aeration conditions, soil adsorption capacity, application rate and quantity of applied pesticides. To date, no systematic studies have been conducted to establish the number of pesticide applications after which the phenomenon of accelerated degradation of specific pesticides becomes operative in soils. Laboratory studies of pesticide degradation kinetics in problem soils with parathion, pointed to a distinct enhancement of the degradation rate after the second or third application of the toxicant to a flooded soil (ref. 28). In the field, the degradation of carbofuran can show a drastic enhancement even after one application to sandy soils. In practice, certain pesticides, for example herbicides, are usually applied only once a season, whereas certain insecticides are applied more frequently (2-4 times a season in rice fields and 10 or more applications to cotton).

For various pesticide preparations, the threshold levels, that induce enhanced degradation and consequently activate the adaptive microflora, have different values. Repeated applications of the same pesticide may, in some cases, slow down the rate of its degradation, e.g. alachlor and terbutryn (ref. 29, 30). Certain treatments such as soil fumigation or solarization or treatment with various fungicides and bactericides have also been shown to affect microbial degradations and to slow down the degradation (ref. 31). In the laboratory, experiments are generally conducted with higher concentrations of pesticides than those used in the field. This fact should be borne in mind when making recommendations for averting the development of problem soils and attempting to extrapolate laboratory results to field conditions. The following techniques have been proposed as preventive measures against the development of problem soils: crop rotation, alternating pesticides, application of microbial inhibitors, new formulations which inhibit the degradation of pesticides, etc. (ref. 24). Of course, any of the proposed techniques will have some advantages and disadvantages. The ideal case will be to achieve a more effective control of pesticide residues in real environmental conditions in order to minimize the level of pesticide use and thus bring down the probability of development of 'problem' soils.

8 THE ROLE OF MICROORGANISMS IN THE FORMATION AND RELEASE OF SOIL-BOUND RESIDUES OF PESTICIDES

When a pesticide is in contact with soil for some time, a proportion of the compound or of its conversion products can become bound to the soil organic matter and cannot be extracted by normal analytical methods. The IUPAC Commission on Agrochemicals has defined soil-bound residues as: "chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products" (ref. 32). The binding of pesticides to soil is a matter of

some ecological and toxicological importance. Once bound to soil, it appears that pesticides are detoxified and lose their activity. However, it is not known if detoxification is permanent or temporary. Some studies indicate that certain bound residues can be released and subsequently perhaps taken up by plants (ref. 33) or leached into the groundwater. The limited information available on the role of microorganisms in the formation and degradation of soil-bound residues of pesticides is critically reviewed below.

8.1 Formation of soil-bound residues

Pesticide residues are often bound to soil organic matter. The rate of incorporation of pesticides into soil organic matter depends upon the type of pesticide and its subsequent transformation by microorganisms and abiotic factors. The extent of binding varies from 50% within a few weeks to only a few percent after one year. It is difficult to predict from the structure of a pesticide its potential to form non-extractable residues. Microorganisms are involved in several ways in the formation of soil-bound residues of pesticides. For instance, they may degrade pesticides into conversion products that are reactive towards humus. A large number of pesticides such as phenylureas, phenylcarbamates, anilides, dinitroherbicides and certain fungicides are converted into halogenated and alkylsubstituted anilines which are bound to organic matter (ref. 34, 35, 36). Little is known about the mechanisms of formation of soil-bound residues of pesticides. Many soil microorganisms have polyphenol oxidases which catalyse oxidative couplings (ref. 37). These organisms have been shown to form oligomers from typical humus monomers. Bollag and Liu (ref. 38) studied the enzymic polymerization of humic monomers with 2,4-dichlorophenol, a breakdown product of 2,4-dichlorophenoxyacetic acid. They found that an extra-cellular laccase from the fungus *Rhizoctonia praticola* coupled dichlorophenol with several phenolic compounds, all of which were precursors of humus. Oligomeric mixtures (up to pentamers) of 2,4-dichlorophenol and the humic monomers were also obtained. The same fungal enzyme also coupled pentachlorophenol and syringic acid to form several oligomers (ref. 39). 2- and 4-chlorophenol, 4-chloro-2-methylphenol, and 4-bromo-2-chlorophenol, conversion products of 2- and 4-chlorophenoxyacetic acid and 4-bromo-2-chlorophenoxyacetic acid respectively, were polymerized by the enzyme (ref. 40). Substituted anilines but not nitrophenols were also coupled.

Mathur and Morley (ref. 41) observed incorporation of methoxychlor into a humic acid-like polymer by *Aspergillus versicolor*. Pure culture studies with *Hendersonula toruloidea* have indicated that substantial percentages of the ring carbons from 2,4-D are linked into humus-like polymers. The linked material is more resistant to decomposition in soil than the unbound compound (ref. 42).

There is evidence that for certain compounds like phenols, microbial activity of soil enzymes plays an important role in the formation of pesticide-bound residues. This conclusion is supported by the fact that soil conditions which are optimal for microbial activity, such as addition of manure and higher temperature increase, the incorporation of pesticides in the soil matrix (ref. 43).

Soil microorganisms may degrade pesticides into small fragments which are transformed into natural products. Such fragments are, by definition, not regarded as pesticide-bound residues. However, it may be very difficult to distinguish between these natural breakdown products and relevant bound pesticide residues when C is found in exhaustively extracted soil incubated with a C-labelled pesticide.

8.2 Release of soil-bound residues by microorganisms

Once incorporated into soil organic matter, bound residues are generally rather stable. When 3,4-dichloroaniline was applied outdoors to soil in a plant-soil system, 46% was still present, in the form of bound residue, two years after treatment (ref. 44). The turnover time for soil organic matter is quite slow and release of bound residues is probably gradual (ref. 45). However, several authors have obtained evidence that bound pesticides or their metabolites are released faster by the action of some species of microorganisms. For instance, Hsu and Bartha (ref. 36) found that small amounts of soil-bound residues of 3,4-dichloroaniline were mineralized to CO₂ upon incubation of soil containing bound residues with fresh soil. This mineralization decreased with sterilization or anaerobic incubation. Roberts and Standen (ref. 46) reported that between 25% and 40% of soil-bound cypermethrin residues were mineralized to CO₂ after 26 weeks of incubation of soil containing bound residues to which fresh soil had been added. Khan and Ivarson (ref. 47) investigated microbial release of [¹⁴C] prometryn from an organic soil. Ext-

racted soil samples were incubated with a liquid soil inoculum. After 32 days, 27% of ^{14}C which has been initially unextractable could be extracted. No ^{14}C was released when soil was incubated with sterilized distilled water.

Many workers observed the uptake by plants of soil-bound residues (ref. 48, 32). It is possible that microorganisms which are active in the rhizosphere of the plant roots play a role in the release of bound residues from the soil. Also, increased microbial activity enhanced the release of CO_2 from soil-bound residues. This latter approach may be used to estimate the rate of mineralization and persistence of soil-bound residues.

Several examples of the release and breakdown of pesticide soil-bound residues by pure or mixed cultures of microorganisms have been reported. Mathur and Morley (ref. 41) found that methoxychlor, incorporated into a humic material produced by the fungus *Aspergillus versicolor*, was subsequently released by the action of another soil fungus. Khan and Ivarson (ref. 49) incubated soil, containing bound residue of [^{14}C] prometryn, with different types of microbial populations. They found release of bound ^{14}C -labelled compounds and $^{14}\text{CO}_2$. Hsu and Bartha (ref. 50) studied the biodegradation of a humic- ^{14}C -3,4-dichloroaniline complex isolated from soil, by the soil fungi *Penicillium frequentans* and *Aspergillus versicolor*. The complex served as the only organic substrate for the fungi. Both fungi released $^{14}\text{CO}_2$ from the humic-dichloroaniline complex. As yet, it is not known whether studies with pure cultures may represent the actual situation in soils.

It may be concluded that microorganisms are involved in the formation of soil-bound residues of pesticides either by incorporating intact compounds of metabolites into soil organic matter, or by generating breakdown products which can react with soil components. Indirect evidence seems to indicate that under certain conditions, microbial activity can release and mineralize soil-bound residues. From the limited literature data available, no conclusion can be drawn about the potential environmental significance of the microbial release of bound residues of pesticides.

9 POTENTIAL OF BIOTECHNOLOGY FOR THE DEGRADATION OF XENOBIOTICS

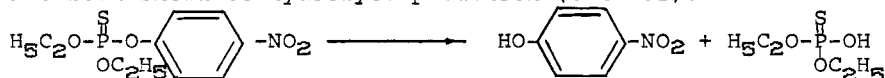
Recent developments in genetic engineering of microorganisms have opened possibilities for the use of new microbial strains having enhanced degradative activity towards persistent pollutants. Genetic engineering techniques can be used to construct bacteria, capable of producing enzymes for pesticide detoxification (ref. 51, 52, 53). At the present time, strains capable of degrading such persistent pollutants as mono- and di-chlorophenols, mono and dichlorobenzoates, DDT and dicofol, 2,4,5-T, etc. have been constructed (ref. 54, 19, 9, 10, 55, 56). These strains were constructed using strategies based both on engineering 'in vivo' (i.e. direct strain-to-strain transfer of an entire plasmid or of its fragments with subsequent selection of transconjugants) and 'in vitro' techniques, i.e. gene cloning. The in vivo strategy proved to be successful for constructing the strain *Pseudomonas aeruginosa* BS827 with enhanced capability for degrading both DDT and dicofol (ref. 55). The same approach was used successfully in engineering bacterial strains capable of degrading mono- and dichlorobenzoates and 2,4,5-T (ref. 54, 14, 18). In fact, this strategy has opened up broad vistas for the construction of novel strains. However, the transconjugants produced are not merely strains with an increased number of plasmids but, as a rule, they contain complex genetic elements which lead to the establishment of new biochemical pathways that are difficult to predict.

The in vitro approach presents a number of advantages. It allows the selection of those genes which are indispensable for specific hybrid pathways while excluding the genes encoding unproductive steps of biodegradation. However, this method also suffers a substantial limitation. To successfully manipulate DNA enzymes, a complete knowledge of modern biochemistry and genetic organization of the initial metabolic pathways are required. The lack of such information on the degradative processes of most xenobiotics impedes greatly a wider use of this method. One successful use of the strategy is the construction of a microorganism, based on the strain *Pseudomonas* sp. B13 which is capable of utilizing 3,4- and 3,5-dichlorobenzoates (ref. 57). Fragments of the TOL plasmid containing the genes *xy1D* and *xy1L* and DNA of NAH7 plasmid harbouring the *nahG* gene had also been cloned in the vector pBR322, which was subsequently ligated with the vector pKT231 for a wide range of Gram-negative bacterial hosts. The hybrid plasmids were introduced into the strain *Pseudomonas* sp. B13 to produce a dramatic increase in the me-

tabolic potential of the strain enabling it to utilize 3,4- and 3,5-dichlorobenzoates and 3-, 4-, 5-chlorosalicylates (ref. 57).

Successful developments in constructing strains able to degrade persistent pollutants have brought to the forefront the problems of stability and resistance of such strains. Therefore, at present, one of the most urgent problems in this field, is the establishment of the factors which control the stability of a plasmid-carrying microbial population and the investigation of possibilities of effective use of constructed strains under natural conditions. Studies of the fate of genetically manipulated microorganisms in model ecosystems are specially important to predict their behaviour in natural ecosystems. The release of such microorganisms into soil or water systems, requires special consideration in addition to their viability and genetic stability. These embrace their impact on natural water and soil ecosystems, ability to exchange genetic material with the autochthonous microflora and the capacity for distribution over substantial distances and to occupy new ecological niches (ref. 53, 58). Studies of the viability and stability of a number of strains which are interesting as potential subjects for subsequent genetic manipulations, revealed that individual strains were rather tolerant to abiotic stresses, remained viable under nutrient deficit conditions and could exist in the presence of antagonists. Constructed strains also proved to be fairly viable when released into soil. The strain *P.aeruginosa* BS827 was active in the degradation of kethane in soil and was persistent for several field seasons, whereas the plasmid introduced in the strain remained rather stable under selective conditions in the presence of the xenobiotic (ref. 56). However, the lack of adequate genetic characteristics of the initial and constructed strains, scanty data on genetic interactions of microorganisms, especially on those engineered by genetic methods, under natural conditions, and imperfection of the monitoring of constructed strains released in the environment are becoming the cause of growing concern on the part of scientists and the public. Some scientists are of the opinion that the use of such strains is not advisable in practice due to potential hazards to the biosphere.

Genetically constructed strains appear to have some promise for the cleanup of industrial sewage, a step required for averting environmental pollution. Experiments on purification of industrial effluents from residual toxicants using the strain *P.aeruginosa* DC13 carrying the plasmid of biodegradation of α -methylstyrene, toluene and diphenyl showed that both the strain and the plasmid were stable and active during the entire 4-month experiment. In these experiments, it was also found that more than 99% of the toxicant could be removed. Another direction where biotechnology may have a positive impact is the development of higher yields of microbial enzymes which degrade xenobiotics. To date, success has been modest. The enzyme parathion hydrolase (E.C. 3.1.3.) has been shown to hydrolyse parathion (ref. 51):



This enzyme, isolated from *Ps.diminuta* and *Flavobacterium* sp., displays a broad substrate specificity in relation to O,O-diethylphosphorothioates. Both strains carry plasmids responsible for the biosynthesis of this esterase. In *Ps.diminuta*, the plasmid pCMS1 has a size of 66 kb and in *Flavobacterium* 43 kb. The experiments on DNA hybridization showed a significant homology of the two genes responsible for the enzyme biosynthesis. Restriction analysis supported this homology. The work aimed at obtaining strains with enhanced capabilities for the enzyme biosynthesis is in progress.

10 PERSPECTIVES ON THE PRACTICAL UTILIZATION OF MICROORGANISMS

Practical application of microorganisms for the degradation of xenobiotics is presently used almost exclusively for treatment of industrial sewage. Experience gained from laboratory assays and industrial uses indicates that both pure and mixed cultures of microorganisms are capable of degrading individual pesticides. This has been demonstrated for the degradation of 2,4-D, 2,4-dichlorophenol and parathion using pseudomonads (ref. 2). Immobilization of cells and enzymes opens interesting prospects for purification of industrial effluents from toxicants. Thus, an enzyme preparation capable of hydrolysing nine organophosphorus pesticides was immobilized on fragmented glass which was packed in a column. The immobilized enzyme had a half-residence time of 280 days maintaining the specific hydrolase activity at the level of 0.035 to 0.5 $\mu\text{mol per mg glass}$. The pseudomonad cells immobilized on glass wool were

used to completely purify industrial effluents of such toxic volatile compounds as α -methylstyrene, toluene and styrene. The purification efficiency was over 99% , and the cells displayed high activity during several months (ref. 11).

Efforts to use active microbial strains for the degradation of pesticides and other xenobiotics under natural conditions have concentrated on two principal approaches. The first one is the release of the active microorganisms into the soil and water systems which have been studied in model and natural ecosystems. In some instances, the desired results were obtained. However, the majority of experiments with genetically constructed strains were not successful, and the strains released into the ecosystems were either rapidly eliminated or did not exhibit any degradative characters (ref. 59, 10). This approach calls for a more adequate investigation to eliminate or at least minimize the hazards associated with the uncontrolled distribution of genetically constructed strains.

The second approach, which also appears to be promising, attempts to activate the microflora of natural habitats by the introduction of appropriate inductive substrates, sources of nitrogen and phosphorus, and various cosubstrates.

The US company "Sun Oil" has developed much experience in the cleanup of water systems, underground waters and limestones polluted with petroleum products by the introduction of dissolved nitrogen and phosphorus salts (US patent, 1974). Finnish researchers have obtained positive effects in the purification of soils polluted with petroleum waste by the application of effluents from industries manufacturing yeast. Earlier, some work was mentioned that was aimed at enhancing the microbial degradation of lindane, DDT and other polychloroaromatic pesticides by ploughing alfalfa, straw and green pea mass into fields. The cleanup of pesticides in natural habitats by activation of their natural microflora calls for a more systematic effort.

11 CONCLUSIONS

1. Microorganisms play a vital role in the environmental fate of pesticides and other xenobiotics. These can be completely detoxified and mineralized. In addition, microorganisms are associated with enhanced degradation, the emergence of toxic and persistent intermediates and the formation and release of soil-bound residues.
2. The undesirable role of microorganisms can be controlled by two different approaches: (i) by using alternative pesticides and (ii) by manipulation of the properties of the microorganisms.
3. The mechanism by which microorganisms degrade pesticides in vitro are in general well understood. However, the environmental matrices in which pesticides and microorganisms interact are very complex. This makes prediction of the outcome difficult. More understanding is required to improve the accurate prediction of the environmental fate of pesticides.
4. In order to reduce the problem of enhanced degradation of pesticides in soil, the rotation of crops and of pesticides is recommended. These approaches may lead to the reduced use of pesticides.
5. Selected microbial cultures are now available to set up industrial processes for decontamination of effluents, agricultural wastes and dump sites. Such an approach is likely to be efficient and cost-effective for many problems.
6. Genetically modified microorganisms can provide improved activity which should prove useful in large-scale applications of microbial degradation to environmental problems. However, it is essential that such microorganisms are thoroughly evaluated for safety before release into the environment.

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