UNITED NATIONS ENVIRONMENT PROGRAMME

MICROBIAL DEGRADATION OF XENOBIOTICS

Lecture Course

Pushchino 1990

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"Microbial Technologies for Degradation of Persistent Pollutants"

MICROBIAL DEGRADATION OF XENOBIOTICS

Lecture Course

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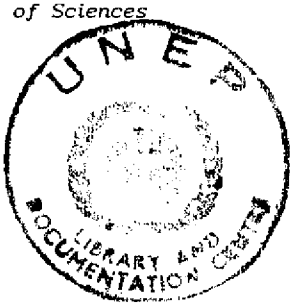

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FOREWORD

Interest in microbial technologies to overcome environmental problems resulting from persistent pollutants prompted the UN Environment Programme (UNEP) to organize an International workshop in Paris, July 1983 to review and assess activities in this area and to produce a state-of-the art report on the subject. An Expert Group meeting on Applied Microbiology organized by UNEP in 1985 stressed that training is a critical factor for the successful development and application of microbial technologies for degradation of persistent pollutants in developing countries.

Other international organizations such as the UN Food and Agriculture Organization (FAO) and the World Health Organization (WHO) concentrate on monitoring and assessment of the level and impact of pesticides and fertilizers on ecosystems and human health. In 1981 the United Nations Industrial Development Organization (UNIDO) launched an important project aimed at establishing an International Centre for Genetic Engineering and Biotechnology devoted to research, development and training in biotechnology directed specifically toward the needs of developing countries.

In June 1987 UNEP and the USSR Commission for UNEP in association with the USSR Academy of Sciences signed an agreement on launching a UNEP/USSR project on Microbial Technologies for Degradation of Persistent Pollutants. The project is concerned with practical implementation of the recommendations of the above UNEP-sponsored Workshop and Expert Group meeting.

The training course has been planned to impart theoretical background and practical training to participants interested in the application of microbial technologies for degradation of persistent xenobiotics. It is hoped that the course will contribute to promoting the use of environmentally sound biotechnologies in developing countries to overcome problems of persistent pollutants applied in agriculture and the environment. The lecture notes comprise relevant topics that focus on: methods of extraction of persistent pollutants from soil and their

qualitative and quantitative analyses; elucidation of the structure of xenobiotics and the products of their microbial bioconversion; description of techniques used for screening and isolation of active natural microbial strains, and genetic engineering of microorganisms with enhanced capabilities for degrading persistent pollutants; special attention is paid to the use of active strains for the cleanup of industrial sewage with due regard to the stability of such strains under selective and non-selective conditions.

This was the first attempt to plan and conduct a training course in line with the objectives set for the project. Therefore, the lecture notes and the practical manual have been revised and edited based on the experience gained and the comments received from the project Scientific Advisory Committee and the participants in the 1988 training course.

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MICROBIAL BIOCONVERSION OF XENOBIOTICS

L.A. Golovleva

Introduction

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Introduction

Microorganisms play an essential role in the bioconversion and total breakdown of pesticides and other xenobiotics in the environment. In recent years, however, we have encountered a number of problems related to the microbial degradation of pesticides. Some examples showing their specific characters follow.

In certain 'problem' soils, the efficiency of the pesticides applied is reduced due to their enhanced degradation, whereas environmental accumulation of persistent pesticides has become a problem.

Some pesticide residues are bound by soil but, under specific conditions, they may become subject to a large-scale release under the influence of microbial attack.

Despite the fact that most steps of the pesticide bioconversion are detoxification reactions, there have been reports on the formation of highly toxic pesticidal metabolites.

Certain pesticides are leached into deeper subsoil horizons or underground water systems but to date our understanding of the microbial processes occurring in these systems which undoubtedly affect the fate of pesticides, is far from adequate as well as our lack of knowledge on the possible conversion of pesticides in other anaerobic zones, e.g., water sediments.

A review of the relevant literature suggests that microorganisms possess a high potential in relation to bioconversion and the total degradation of xenobiotics by microorganisms, especially so since modern experimentation skills enable the successful manipulation of their specific characters.

The purpose of the present lecture is to present a critical review of the mechanisms of microbial bioconversion of xenobiotics indicative of the existing problems, in order to outline basic ways to address rationally these problems using recent developments in microbiology and biotechnology.

I. Basic Biochemical Processes in Bioconversion of Xenobiotics

A common distinctive feature of xenobiotics is the presence in the molecules of unusual elements, groupings, and bonds, as judged by biological standards. For example, many pesticides are presented as bulky complex molecules, while others display hydrophobic properties due to the presence of reduced hydrocarbon fragments. Therefore, the principal role in the bioconversion of xenobiotics is played by various lyases and oxyreductases, specifically hydrolases, oxygenases and various dehalogenation enzymes.

1. Hydrolytic Processes

Amidic and ester bonds are known to undergo hydrolytic cleavage in acyl anilides, phenyl ureas, esters of carbamino and thiocarbamino, phosphoric and thiophosphoric, and of other acids. Hydrolases responsible for the cleavage of pesticides are among the most studied groups of enzymes. Many of the hydrolases studied are extracellular enzymes, except for the cell wall-bound enzymes from the fungi of *Penicillium* and *Arthrobacter* sp. which hydrolyze barban and propham, respectively. Arylacylamidases, which hydrolyze the amidic bond in phenylamides, have been studied and described in detail (EC 3.5.1.13). As a rule, these enzymes are induced by a broad spectrum of substrates and, what is essential, they exhibit low substrate specificity (Engelhardt *et al.*, 1973; Tables 1, 2).

Münnecke and Hsich (1976) reported similar results: a culture capable of a rapid cleavage of the thioester bond (416 nmol/min per 1 mg protein) also brought about enzymic hydrolysis of another 8 of the 12 insecticides tested. This property of microbial hydrolases underlies the emergence of 'problem' soils , i.e., one of the problems we mentioned in the introduction.

TABLE 1. Induction of acylamidase in *Bacillus sphaericus* 12123 (Engelhardt *et al.*, 1973)

Inductor	Specific activity 10^{-3} units/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-Dimethylfurane--3-carboxyanilide	0.05	40

TABLE 2. Hydrolysis of phenylamides by a cell-free extract of *Bacillus sphaericus* induced by linuron (Engelhardt *et al.*, 1971)

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

2. Dehalogenation

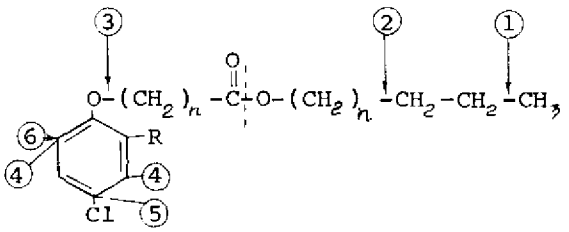
The molecules of most xenobiotics contain halogens bonded to aliphatic or aromatic carbon. This implies that dehalogenation reactions should be important initial steps in the degradation of xenobiotics. Though an adequate knowledge of molecular mechanisms of dehalogenation calls for further studies, yet certain reaction types are fairly well known, e.g., hydrolytic, reductive and oxidative dehalogenations. The latter reactions may be specified as including dehydrohalogenation, i.e. dehalogenation which involves molecular oxygen and results in the formation of a double bond. A number of enzymes have been isolated and characterized which effect dehalogenation. These are haloacetate halidohydrolase (EC 3.8.1.3) that splits-off a halogen off halide from

acetate to form glycolic acid; hydrolases 1 and 2 which effect dehalogenation of dichloroacetate; and 2-haloacid halidohydrolase (EC 3.8.1.2). The above enzymes are responsible for the hydrolytic release of a halide in position 2 of short-chain fatty acids. Enzymes which carry out reductive dehalogenation of aromatic carbon have not yet been characterized, though the literature provides several descriptions of the process proper comprising 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 details are available for the dioxygenase-mediated dechlorination of picloram and monooxygenase dechlorination of chlorophenoxyacids, chlorobenzoic acids, and chlorophenols.

3. Oxidation

Oxidative processes are very important in the bioconversion of xenobiotics, especially pesticides, in the steps of bioconversion which follow hydrolysis and dehalogenation of toxicants.

Here, the basic reactions involved are hydroxylation, cleavage of the aromatic ring, oxidative *O*- and *N*-dealkylation and epoxidation. Certain compounds,e.g. derivatives of aromatic acids, are totally degraded due to the involvement of oxidative enzymes (Scheme 1).



SCHEME 1. Susceptible positions for the oxidative attack by enzymes during degradation of alkyl esters of chlorophenoxyacids.
1 - hydroxylation of terminal methyl; 2 -β-oxidation; 3 - oxidative cleavage of ester bond; 4 - hydroxylation of the ring; 5 - oxidative dechlorination; 6 - cleavage of the ring; dashed line - possible hydrolytic fission by esterase, R = methyl or Cl.

It appears that there is no need to describe here in greater detail oxidative enzymes, which carry out the above bioconversions, since they are adequately treated in Hayaishi's monograph (Hayaishi, 1974).

4. Reduction

Generally, reductive processes take place in the early stages of degradation under anaerobic conditions. Above, we have already mentioned reductive dechlorination. Another no less important reaction is the reduction of nitro groups. Enzymes which bring about this process are called nitroreductases. They perform their functions in the presence of $NADH_2$, the reaction being stimulated by the reduced form of FAD, and Mn and Fe ions. A number of authors reported thatthese enzymes have a low specificity. Thus, the enzymic preparation of *Vellonella alkalescens* was found to catalyze the reduction of the nitro group of some 40 different compounds. The products of nitro group reduction may be recalcitrant and resist further transformation, whereas the nitroso-derivatives may exhibit potent mutagenic or carcinogenic properties thus creating the second problem we mentioned above, i.e., microbial bioconversion of xenobiotics into metabolites which are more toxic than the parent compounds.

II. Microorganisms Effecting Detoxification of Xenobiotics

Analysis of many years of research into the microbial degradation of xenobiotics reveals that bacteria are the leaders in the detoxication of xenobiotics, fungi being the next important, with yeast, microalgae and Protozoa being only rarely mentioned in this connection. Among bacteria, pseudomanads are reputed as the best degraders of xenobiotics. It is also noteworthy that if bacilli are responsible for pesticide hydrolysis, then pseudomonads can bring about hydrolysis as successfully as they do dehalogenation, hydroxylation, aromatic ring cleavage and nitro group reduction. Examples of bioconversion of various compounds effected by pseudomonads are listed below in Table 3.

Of the other bacteria, the genera *Acinetobacter, Arthrobacter, Rhodococcus, and Flavobacterium* deserve some mention, since they often

bring about various transformations of xenobiotics differing in composition.

Fungal cultures are often involved in the bioconversions of sym-triazines, the genera *Aspergillus and Penicillium* being the most important. Certain fungi have also been reported to bring about the methylation of oxy- and amino groups, and metals. Thus the fungus *Trichoderma virgatum* effected methylation of pentachlorophenol, whereas *Penicillium notatum, Aspergillus niger* and *Scopulariopsis brevicaulis* methylated arsenic derivatives, etc.

TABLE 3. Bioconversion of xenobiotics effected by pseudomonads.

Mode of action	Species	Reference
Hydrolysis of carbaryl, dichlophos, diazinon, parathion	*Pseudomonas melophthora*	Matsumura and Boush, 1968
Hydrolysis of parathion	*Pseudomonas stutzeri*	Münnecke and Hsich, 1976
Dehalogenation of halide acetate	*Pseudomonas* sp.	Goldman and Milne, 1966
Total dehalogenation of DDT, aromatic ring cleavage	*Pseudomonas aeruginosa*	Golovleva *et al.*, 1980, 1981
Total degradation of 3-chlorobenzoate	*Pseudomonas putida*	Golovleva *et al.*, 1984
Oxidative dehalogenation of lindane	*Pseudomonas putida*	Matsumura *et al.*, 1976
Reduction of nitro group in 4,6-dinitro-*o*-cresol	*Pseudomonas* sp.	Tewfik and Evans, 1966
Total degradation of 2,4,5-T	*Pseudomonas sepacia*	Chatterjee *et al.*, 1982
Degradation of toluene, xylenes, styrene, α-methylstyrene	*Pseudomonas putida*	Golovleva *et al.*, 1976, 1984
	Pseudomonas aeruginosa	Williams and Worsey, 1976

III. Role of Microorganisms in Detoxification of Pesticides

Currently, nobody will deny the important role played by the biotic factor in the degradation of pesticide residues. According to various authors, microorganisms degrade 25 to 70 % of xenobiotic residues. Literature cites microbial strains capable of degrading such

recalcitrant pesticides as DDT, polychlorodiphenyls, kelthane, 2,4,5-T and others (Golovleva and Skryabin, 1981; Chatterjee and Chakrabarty, 1983; Golovleva *et al.*, 1980).

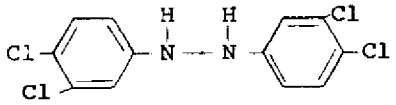
Unfortunately, very few xenobiotics can be utilized by pure cultures as the sole source of carbon. As a rule, they are fairly simple compounds such as dalapone, chlorobenzoates, 2,4-D. Of greater significance are bioconversions of pesticides effected by mixed cultures, when the initial degradation steps are brought about by one culture and the metabolites produced are further degraded other microbial strains. An example is the hydrolysis of parathion by *Pseudomonas stutzeri* which produces nitrophenol that in turn is degraded by another strain - *Pseudomonas aeruginosa* (Münnecke and Hsich, 1976). The exchange of genetic information by natural microbial populations also appears to be a potent factor that facilitates total degradation of xenobiotics. For example, there is an opinion that phenoxyalkane herbicides, such as 2,4-D and MCPA, do not accumulate in the environment because plasmids, which several years ago were found to determine their degradation, can be transferred under natural conditions from strain to strain (Don and Pemberton, 1981). More recent is the discovery of plasmids for the biodegradation of polychlorodiphenyls, chlorobenzoates, petroleum, surfactants and other compounds that apparently play an important part in degradation of numerous foreign compounds by the natural microflora.

IV. Microbial Bioconversion of Xenobiotics to More Persistent Compounds

Despite the fact that individual xenobiotics are readily degraded in natural ecosystems, processes involving partial bioconversion of complex alien molecules also occur widely. Assessment of the biodegradability of a specific toxicant based on its residence time in natural environments, provides only meagre information on the ecological safety of the compound in question. Many compounds are transformed into intermediates that are highly resistant to microbial attack and thus become a serious ecological nuisance.

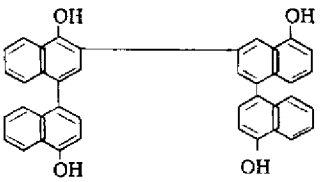
Hydrolysis of phenylamide herbicides, for instance, results in the

formation of chloroanilines which under the action of microbial enzymes are condensed to produce complex structures like tetrachlorobenzenes:

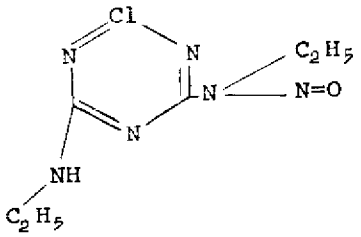


3,3',4,4'-tetrachloroazobenzene

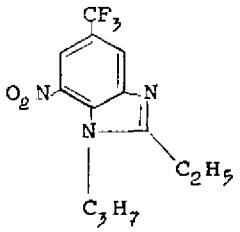
As a result, polychloroaromatic compounds appear in the biosphere and are much more resistant to degradation compared to the parent pesticides or chloroanilines. Thus Kearney and co-workers detected these compounds in the soil from rice fields 3 years after treatment with propanil. Peroxydase and, to a lesser extent, aniline oxydase are responsible for the production of chlorobenzenes. Similar processes also occur during degradation of carbaryl (1-naphthyl-*N*-methylcarbamate); the hydrolysis of carbaryl gives α-naphthol, which under the effect of phenol oxydase is condensed to form complex compounds that are highly toxic to aquatic organisms.

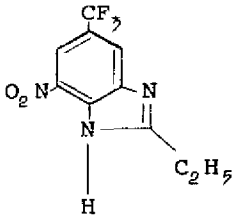


A particular threat to the environment are the metabolites of nitrogen-containing pesticides, since they are further degraded being readily transformed into highly mutagenic and carcinogenic compounds. For example, simazine is oxidized into a carcinogen with the following structure:



Two trifluralin degradation products are also hazardous as mutagenic factors:





The list of examples could be continued but the above illustrations leave little doubt as to the vital need of doing thorough studies of the properties of the intermediates of microbial pesticide bioconversion, since their effect on biocenoses and man may be more deleterious or even pernicious compared to that of the parent xenobiotics.

V. 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: accessibility of toxicants to microorganism's enzymes which is determined by the extent of the toxicant sorption by soil particles and colloids, availability of organic compounds which can be utilized by the microflora as energy substrates, moisture conditions, aeration level, temperature, pH and other factors.

Certain pesticides are known to degrade readily in liquid media, but in soil they become resistant to microbial attack. A classical example is the herbicide paraquat which is strongly sorbed by soil thus becoming inaccessible to microorganisms. A similar effect is observed with 4,6-dinitro-o-cresol, which is intensively sorbed by clays and, due to this fact, its degradation is highly inhibited. Quite often the primary transformation of a pesticide, e.g. hydrolysis, leads to phenols or anilines followed by their strong binding to soil and the resulting inaccessibility to microorganisms. The problem of pesticide sorption and desorption by soils will receive more complete coverage in the lecture

by V.P. Sukhoparova. Therefore, we will concentrate on examining other factors which affect biodegradation processes.

Data pertinent to the effects of organic compounds on microbial degradation of xenobiotics are rather conflicting. Some authors report an inhibiting effect with increasing organic matter on the rate of microbial decomposition of toxicants in soil, whereas others insist on a stimulating influence of organic matter on biodegradation. The cause of such controversy apparently may be explained by the dual role played by organic matter in water and soil, in relation to microorganisms, i.e., to be a sorbent of pesticides and their metabolites and a substrate for microbial cultures, source of carbon and providing energy for large populations of microorganisms, source of energy and factors required for the occurrence of the primary steps of xenobiotic degradation, and, moreover, specific components of organic matter may have a regulatory effect on microbial enzymes.

The stimulating action of organic matter on microbial degradation was given a tenable explanation by studies of the phenomenon called "co-metabolism".

Co-metabolism is defined as degradation or bioconversion of various organic compounds, mainly of xenobiotics, by microorganisms related to the transformation of other, more readily accessible substrates in a manner enabling the metabolism of cosubstrates to be stimulating for the degradation of xenobiotics. Co-metabolism in its biological sense consists in providing the processes of peripheral metabolism of xenobiotics with energy, cofactors, effectors and, possibly, oxidizers produced in the course of metabolism of additional substrates.

Co-metabolism of pesticides with various cosubstrates has been described by numerous authors for both laboratory and natural conditions. Thus the herbicide ordram was degraded by cultures of *Bacillus sp.* in the presence of ethanol; DDT was found to be co-metabolized by the strain *P. aeruginosa* 640x on the medium amended with glycerol and hexadecane, with alvison - during microbial growth on organic acids. Numerous experiments on the introduction of co-substrates into soils and water bodies showed a substantial increase of the soil microflora activity towards simazine, dexon, diazinon, pentachloronitro-

phenol, paraquat and other pesticides.

Hence, the organic matter of soil colloidal systems may impede microbial degradation of pesticides as the result of their sorption. In contrast, organic compounds of low molecular weight generally stimulate the biodegradation of xenobiotics.

Conditions of medium aeration under which degradation of xenobiotics occurs, call for a special discussion. Broad participation of oxidative enzymes in the conversion of foreign compounds favoured the view on the common necessity for accessible oxygen for effective degradation of pesticide residues. However, studies of microorganisms populating water and mud of flooded rice fields treated with pesticides have shed a new light on the role of anaerobic processes in the microbial degradation of xenobiotics.

It was found that many persistent pesticides, reputed as recalcitrant to microbial attack and therefore residing in soil for years, are degraded much faster in the tropical soils of flooded rice fields. In this respect, lindane is the best example. It was subject to anaerobic microbial degradation in mud and lake sediments. In addition to lindane, heptachlor, endrin, methoxychlor and kelthane underwent intensive degradation in flooded soils. The organophosphorus pesticide, diazinon was removed from the flooded soil in two months, whereas its residence time in a non-flooded soil was 6 months. Hence, it should be kept in mind that anaerobic conditions favour the accumulation of metabolites – the products of hydrolysis and dechlorination whose further decomposition requires a supply of air. Such a situation was observed during degradation of parathion, diazinon, the carbamate pesticide carbofuran and other pesticides in soil.

The effect of pH on the microbial degradation of xenobiotics is displayed not only in relation to properties of the microorganisms and/or enzymes but also by the fact that the acidity of the medium affects the rates of sorption of pesticides and their metabolites and influences the processes of decomposition or formation of conjugates of condensed compounds.

Anaerobic processes prove to be highly dependent on the Eh levels.

VI. Enhanced Degradation of Pesticides as Evidence for Microbial Activities

Repeated applications of the same biodegradable pesticides to specific crops often leads to the development in treated soils of microbial populations capable of rapid degradation of the applied pesticides thereby decreasing their efficiency in soil. This is the case with many carbamate, acetamide, acylanilide and organophosphate insecticides, herbicides and nematocides, which on application to soil undergo rapid degradation. The soils in which such preparations produce no positive effect and do not affect the target organisms are called the "tired", "problem" or "aggressive" (Kaufman, 1987). The VI International Congress on Chemistry of Pesticides held in Canada devoted much time to this topic (Filsot, 1986; Hamm and Thomas, 1986; Tam *et al.*, 1986). It was clearly demonstrated that frequent, unsystematic application of sym-triazines, carbofuran, chloroethiocarb and other toxicants led to a total loss of activity due to a drastically enhanced degradation by the soil microflora. Isolation and identification of microorganisms from such soils indicated the presence of strains capable of the rapid metabolism of the pesticides and their structural analogues. This fact essentially restricts the arsenal of pesticides used in farming practices. Many factors appear to contribute to the emergence of problem soils. The most common of these are the concentration of organic matter in soil, pH, moisture level, temperature, aeration conditions, soil capacitance, level and quantity of applied pesticides. No systematic studies have been conducted to establish the number of pesticide applications required before the phenomenon of accelerated degradation of specific pesticides becomes operative in soils. Laboratory kinetic studies of pesticide degradation 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 (Barik *et al.*, 1979). At the same time, degradation of carbofuran showed drastic acceleration even after its first application to sandy soils. In practice, certain toxicants, e.g. herbicides, are usually used once a season on the farm, whereas specific insecticides are applied more

frequently (2–4 times a season in rice fields and over 10 times in cotton plantations). For various pesticide preparations, the threshold levels that induce enhanced degradation, and consequently activate the adaptive microflora, have different values, while in the laboratory, experiments are generally conducted at higher concentrations of pesticides compared to those used in real farming practice. These points should be borne in mind while elaborating practical recommendations for averting the appearance of problem soils and attempting to extrapolate laboratory results to field conditions. Dr. Kaufman, an expert in this field, proposed the following techniques as preventive measures against the development of problem soils: crop rotation, alternation of pesticides, application of microbial inhibitors, introduction of new preparations into the arsenal of applied pesticides, new formulations with inhibited action of pesticides, etc. (Kaufman, 1987). Of course, any of the proposed techniques has its own advantages, restrictions and drawbacks. The ideal case will be to bring about a more effective control of pesticide residues in real environmental conditions in order to minimize the level of pesticide use and thus reduce the probability of the development of problem soils.

VII. Role of Microorganisms in Formation and Release of Soil-Bound Pesticides

In the course of a pesticide applied to a soil, certain portions of the toxicant or its metabolites may after a time be bound to specific soil components and thus become unextractable by conventional analytical techniques. The binding of pesticides by soil is of great ecological and toxicological significance. When bound by soil, a pesticide is detoxified and loses its activity. However, until now it is not quite clear whether such binding is irreversible or only temporary. Many researchers have reported data supporting the release of soil-bound pesticides and their possible accumulation by plants or leaching into underground waters (Fuhr *et al.*, 1985; Aharanson *et al.*, 1987). Moreover, the incorporation of foreign chemicals may essentially affect the soil structure and various microbial and biochemical processes

occurring therein. The rate of pesticide inclusion into organic matter of the soil depends on the type of the pesticide applied and its capacity to undergo biotic and abiotic transformations. The proportion of incorporated pesticides may vary from 50% after a few weeks to 1-2% after a year.

The modes of microorganisms' involvement into the formation of soil-bound residues of pesticides may differ. They may degrade pesticides to highly reactive products forming complexes with the soil humus. Most phenylureas, phenylcarbamates, acylanilides as well as specific fungicides contain halogens or alkyl-substituted aniline moieties. In the course of microbial conversion these compounds are released and can also interact with organic matter of the soil (Bartha, 1968; Bollag *et al.*, 1978; Hsu and Bartha, 1974).

1. Formation of Soil-Bound Residues by Pure Cultures and Enzymes

Many microorganisms synthesize the enzymes called phenol oxydases capable of bringing about the formation of oligomers and polymers. Polymerization of humus monomers often involves chlorophenols that are produced during the bioconversion of a number of pesticides: 2.4-D, MCPA, 2,4,5-T and many others. Thus laccase of the fungus *Phizoctonia praticola* brings about the coupling of dichlorophenyl with syringic and vanillic acids, vanillin, and orcin which are components of humus. A number of fungal enzymes effected the conjugation of pentachlorophenol with syringic acid to form various oligomers (Bollag *et al.*, 1977). It was found that laccase of the fungus *Trametes versicolor* formed a conjugation with xylenol and syringic acid and polymerized various substituted anilines (Bollag and Liu, 1985; Hoff *et al.*, 1985). The fungus *Aspergillus versicolor* effected the incorporation into a humic polymer even of such a complex molecule as methoxychlor. It should be noted that methoxychlor does not contain free phenyl groups (Mathur and Morley, 1978). The bound materials are more resistant to microbial degradation as compared to unbound ones (Haider, 1976).

It is also worthy of note that the binding of pesticides to soil is enhanced by application of mineral fertilizers, manure and organic additifs (Lichtenstein *et al.*, 1982).

2. Release of Soil-Bound Pesticide Residues by Microorganisms

As mentioned above, the pesticide residues bound to soil are rather persistent. Thus, two years after application, 46% of 3,4-dichloroaniline applied was still bound in the soil as a pesticide residue. Nonetheless, certain reports indicate that bound pesticides and their metabolites may be released faster under the impact of various microorganisms. These data are, however, of an indirect character: such as increased emission of $^{14}CO_2$ in experiments where glucose had been added to the soil, asparagine or activated sludge waste system with bound pesticides labelled with radioactive carbon or increasing uptake by plants of bound pesticide residues as the result of a presumably high activity of foreign microflora. There exist some direct evidences but these are not examples. The above process of methoxychlor coupling with the soil humus effected by *Aspergillus versicolor*, became reversible under the action of another fungus *Marasmius oreades* and resulted in the release of methoxychlor from the complex (Mathur and Morley, 1978).

The fungi *Penicillium frequentans* and *Aspergillus versicolor* were found to release (^{14}C-3,4-dichloroaniline from the complex with the humic substance of soil (Hsu and Bartha, 1974). Incubation of the (^{14}C)-prometrin complex bound to soil with 4 different microbial mixtures also has led to the release of some 25-30% of the radioactivity after 28 days (Khan and Ivarson, 1982). But it should be stressed once again that the available information is too limited to assess the contribution of microorganisms to the release of bound pesticide residues and their subsequent degradation.

VIII. Potential of Biotechnology for Degradation of Xenobiotics

Recent developments in the genetic engineering of microorganisms have opened many possibilities for the use of microbial strains with enhanced degradative activity to persistent pollutants, for designing, using gene engineering techniques, crops resistant to herbicides or capable of degrading herbicide residues accumulated by plants and also for constructing bacteria capable of producing enzymes for the pesticide detoxification (Kearney *et al.*, 1987; Chaleff, 1986; Stotzky and Babich,

1986).

At the present time, a number of laboratories have produced strains capable of degrading such persistent pollutants as mono- and dichlorophenols, mono- and dichlorobenzoates, DDT and kelthane, 2,4,5-T, etc. (Chatterjee *et al.*, 1981; Pemberton and Don, 1981; Golovleva *et al.*, 1980, 1981, 1986, 1987). These strains were formed by strategies based on both engineering in vivo, i.e. direct strain-to-strain transfer of an entire plasmid or of its fragments with subsequent selection of transconjugants, and using in vitro techniques, i.e. the gene cloning. The first strategy proved to be successful in producing the strain *Pseudomonas aeruginosa* BS827 with enhanced capability for degrading DDT and kelthane (Golovleva *et al.*, 1982). The same approach was successful in engineering bacterial strains capable of degrading mono- and dichlorobenzoates (Chatterjee and Chakrabarty, 1982), and 2,4,5-T. In fact, this method has opened up new avenues for constructing novel strains. However, the transconjugants produced are not merely strains with an increased number of plasmids, but as a rule, they harbour complex genetic events which often lead to the establishment of new biochemical pathways that prove difficult to predict.

Gene cloning presents a number of advantages, specifically it allows selection of those genes which are indispensable for a specific hybrid pathway while excluding those genes which encode unproductive biodegradation steps. However, the method suffers a substantial limitation - in order to successfully manipulate with DNA enzymes, it requires a thorough knowledge of modern biochemistry and the genetic organization of initial metabolic pathways. The lack of such information of degradative processes of most xenobiotics greatly impedes a wider use of this method. One example of a successful use of this strategy is the construction of a microorganism based on the strain *Pseudomonas sp.* B13 that is capable of utilizing 3,4-,3,5-dichloro- benzoates (Lehrbach *et al.*, 1984). Fragments of the TOL plasmid containing genes xylD and xylL and DNA of the plasmid NAH7 harbouring gene nahG have 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 resulting

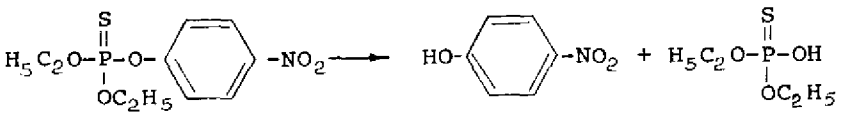
in a drastic increase of the metabolic potential of the strain enabling it to utilize 3,4-, 3,5- dichlorobenzoates and 3-, 4-, 5-chlorosalicylates (Lehrbach *et al.*, 1984).

The successful development of strains able to degrade persistent pollutants brought to the forefront the problem of stability and resistance of such strains. At present, one of the most urgent problems in this field is the establishment of regulations that control the stability of a plasmid-carrying microbial population and investigation of the possibilities of effective use of the constructed strains under natural conditions. Of special importance are studies on the behaviour of microorganisms, produced by genetic manipulations in model ecosystems in order that their behavioural characters can be extrapolated to natural ecosystems. In addition to their viability and genetic stability, on releasing such microorganisms into soil or water other aspects acquire special significance. These embrace the impact on natural water and soil ecosystems, ability to exchange genetic material with the autochthonous microflora, the capacity for distribution over substantial distances and to occupy new ecological niches (Stotzky and Babich, 1984; Colwell, 1986). Studies of the viability and stability of a number of strains are interesting as potential subjects for subsequent genetic manipulations and reveal that individual strains are rather tolerant to abiotic stresses, remain viable under conditions of nutrient deficiency and can exist in the presence of antagonists.

Constructed strains also proved to be fairly viable when released into soil. In our experiments, the strain *P. aeruginosa* BS827 was active in degradation of kelthane in soil and persisted for several field seasons, whereas the plasmid introduced in the strain remained rather stable under selective conditions in the presence of the xenobiotic (Golovleva *et al.*, 1987). However, the lack of adequate genetic characteristics of the initial and constructed strains, scanty data on genetic interactions of microorganisms, and in particular on those engineered by genetic methods, under natural conditions, as well as 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 in general. Some scientists are of the opinion

that such strains are not advisable for practical use due to potential hazards to the biosphere. Genetically constructed strains appear to show promise for the cleanup of industrial sewage which is a step required in order to avert environmental pollution. Experiments on the purification of industrial effluents from residual toxicants using the strain *P. aeruginosa* DC13 carrying the plasmid for the biodegradation of α-methylstyrene, toluene and diphenyl, showed that both the strain and plasmid remained stable and active during the entire 4-month experiment. In these experiments it was also found that the amount of toxicants removed exceeded 99%.

Another direction where biotechnology may have a role in the future is the development of higher yields of microbial enzymes which carry out the degradation of xenobiotics. To date, success is still modest but the work carried out at the Agricultural Environmental Quality Institute in Beltsville (USA) shows the potential (Kearney *et al.*, 1987). The enzyme parathion hydrolase (E.C.3.1.3.) carries out hydrolysis of parathion:



The enzyme displays a broad substrate specificity in relation to *O,O*-diethylphosphorothioate, and it was isolated from *Ps. diminuta* and *Flavobacterium sp.* Both strains carry plasmids responsible for biosynthesis of this esterase. In *Ps. diminuta*, the plasmid pCMS1 has molecular weight of 66 kb and 43 kb in *Flavobacterium*. 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 being continued.

IX. Potential of the Practical Utilization of Microorganisms

The practical application of microorganisms for the degradation of

xenobiotics is presently brought about almost exclusively in the treatment of sewage from various industries.

Experience gained from laboratory assays and industrial uses indicates that pure and mixed cultures of microorganisms are capable of degrading individual pesticides rapidly. Such evidence was obtained for the degradation of 2,4-D, 2,4-dichlorophenol and parathion using pseudomonads (Münnecke and Hsich, 1974). Immobilization of cells and enzymes opens up broader prospects for purification of industrial effluents from toxicants. Thus an enzyme preparation capable of hydrolyzing nine organophosphate pesticides was immobilized on fragmented glass, and a column packed with the immobilized enzyme had a half-residence time of 280 days while maintaining the hydrolase specific activity level of 0.035 to 0.5 μmol/mg glass. The cells of pseudomonads immobilized on glass wool were of total purification of industrial effluents from such toxic and volatile compounds as α-methylstyrene, toluene and styrene. The purification efficiency was over 99%, and the cells still displayed high activity after several months (Golovleva *et al.*, 1987).

Efforts to use active microbial strains for the degradation of pesticides and other xenobiotics under natural conditions follow two principal directions. The first is the release of active microorganisms into soil and water bodies which are studied in model ecosystems as well as real natural conditions. In some instances the desired results were obtained, especially with genetically constructed strains, whereas most experiments did not provide the expected results and the strains released into ecosystems were either rapidly eliminated or did not display degradative characters (Anderson and Lichtenstein, 1972; Golovleva *et al.*, 1977). Such an approach calls obviously for more adequate investigations, especially when using genetically constructed strains to eliminate or at least reduce the hazard to the minimum with respect to their control distribution.

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

The Sun Oil company has gained a reputation based on their cleanup of water bodies, underground waters and limestones polluted with petroleum products by the introduction of dissolved nitrogen and phosphorus salts (US patent, 1974). Finnish researchers obtained positive results in the purification of soils polluted with petroleum waste by the application of effluents from industries manufacturing yeast. Earlier, work was mentioned aimed at enhancing the microbial degradation of lindane, DDT and other polychloroaromatic pesticides by ploughing alfalfa, straw and green pea mass into fields. Consequently, the problem of the cleanup of pesticide residues from natural habitats by the activation of the natural microflora calls for a systematic work in this direction.

Conclusion

1. A review of the state of the art in microbial degradation of xenobiotics makes it possible to indicate some basic practical tasks and identify priority areas for further research.
2. First and most important is the development of the principles of synthesis of xenobiotics (pesticides in particular) that will present no hazards to the biosphere both in the initial state and in the course of their transformations in the environment.
3. Gradual substitution of chemical means of protection of plants and animals by the biological ones.
4. Improvement of the biodegradability control system and investigation of metabolic pathways in different natural ecosystems for all synthetic chemicals intended for release into the biosphere in order to avert the risk of the formation of intermediates which are deleterious to the biota.
5. Strict monitoring of residues of pollutants in natural environments. The elaboration of agrotechnical methods preventing formation of problem soils by crop rotations, alternation of applied chemicals, microbiological control, use of inhibitors etc.
6. Development of the fundamental principles for the establishment and control of integrated sewage purification systems extended from

activated sludge and biological ponds to the use of immobilized cells and enzymes.

7. Feasibility studies on the control and potential monitoring of the activity of microflora in natural habitats: soils, water bodies, etc. by the release of microorganisms and/or chemicals. Investigation of the possibility of release of microorganisms, including those genetically constructed, into natural ecobiocenoses with the view to detoxifying residues of persistent pollutants. The latter task calls for special attention to the problems of biotechnology, ecology, and biocenology of microorganisms.

REFERENCES

Aharanson N., S. Cohen, N. Drescher, I. Gich, S. Gorbach, P. Kearney, S. Otto, T. Roberts. 1987. *Potential contamination of ground water by pesticides.* Project IUPAC PAC (in press).

Anderson J.P., E. Lichtenstein. 1972. Effect of various fungi and insecticides on the capacity of *Mucor alternants* to degrade DDT. *Can. J. Microbiol.* 18:553–560.

Barik S., P. Wakid, C. Ramakrishna, N. Sethunathan. 1979. A change in the degradation pathway of parathion after repeated applications to flooded soil. *J. Agr. Food Chem.* 27:1391–1392.

Bartha R. 1968. Biochemical transformations of anilide herbicides in soils. *J. Agr. Food Chem.*16:602–604.

Bollag J.M., P. Blattmann, T. Laanio. 1978. Adsorption and transformation of four anilines in soil. *J. Agr. Food Chem.* 26: 1302–1306.

Bordeleau L. and R. Bartha. 1972. Biochemical fransformations of herbicide derived anilines in culture medium and in soil. *J. Can. Microbiol.* 18:1857–1864.

Burge W.D. 1972. Microbial populations hydrolyzing propanil and accumulation of 3,4-dichloroaniline and 3,3',4,4'-tetrachloroazobenzene in soil. *Soil Biol. Biochem.* 4:379–386.

Bollag J., S. Liu, R. Minard. 1982. Enzymatic oligomerisation of vanillic acid. *Soil Biol. Biochem.* 14:157–163.

Bollag J.M. and S.-J. Liu. 1985. Copolymerisation of halogenated phenols and syringic acid. *Pesticide Biochem. Physiol.* 23:261–272.

Chaleff R.S. 1986. Engineering resistance in crops. *Abstracts of the Sixth Congress of Pesticide Chemistry.* Ottawa, SS 2–02.

Chatterjee D.K., S.T. Kellogg, K. Furukawa, J.Y. Kilbane, A.M. Chakrabarty. 1981. Genetic approaches to the problems of toxic chemical pollution. In: *Recombinant DNA* (A.C.Walton ed.). Elsevier, Amsterdam. p.199–212.

Chatterjee D.K., J.J. Kilbane, A.M. Chakrabarty. 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil by a pure culture of *Pseudomonas cepacia. Appl. Environ. Microbiol.* 45:1697–1700.

Chatterjee D.K., A.M.Chakrabarty. 1983. Genetic homology between

indepedently isolated chlorobenzoate-degradative plasmids. *J. Bacteriol.* 153:532-534.
Colwell R. 1986. The potential of biotechnology for developed and developing countries. *Mircen Journal of Applied Microbiol. Biotechn.* 2:5-12.
Don R.H., J.M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes eutrophus* and *Alcaligenes paradoxus*. *J. Bacteriol.* 145:681-686.
Engelhardt G., P.R. Wallenofer, R. Plapp. 1971. Degradation of linuron and some other herbicides and fungicides by a linuron-inducible enzyme obtained from *Bacillus sphaericus*. *Appl. Microbiol.* 22:284-288.
Engelhardt G., P.R. Wallenofer, R. Plapp. 1973. Purification and properties of an aryl acylamidase of *Bacillus sphaericus*, catalizing the hydrolysis of various phenylamide herbicides and fun gicides. *Appl. Microbiol.* 26:709-718.
Felsot A.S. 1986. Effect of conditioning soil for enhanced biodegradation of cabofuran and dissipation of other methyl carbamate insecticides. In: *Abstracts of the Sixth Internat. Congress of Pesticide Chemistry*. Ottawa, IUPAC, 6B-OB.
Fuhr F. 1986. Nonextractable pesticide residues in soil. In: *Abstracts of the Sixth Internat. Congress of Pesticide Chemistry*. Ottawa, IUPAC, 6S-02.
Goldman P., W.A. Milne. 1966. Carbon-fluorine bond cleavage. II Studies on the mechanisms of the defluorination of fluoroacetate. *J. Biol. Chem.* 241:5557-5559.
Goldman P., G.W. Milne, D.B. Keister. 1968. Carbon-halogen bond cleavage. III Studies on bacteria halidohydrolase. *J. Biol. Chem.* 243:428-434.
Golovleva, L.A., Z.I. Finkelshtein, N.A. Popovich, G.K. Skryabin. 1981. Transformation of ordram by microorganisms. *Izvestiya AN SSSR, ser.biol.* 3:348-357 (in Russian).
Golovleva, L.A., E.L. Golovlev, Kh.G. Ganbarov, G.K. Skryabin. 1976. Role of cosubstrates in microbial oxidation of isomeric xylenes by *Pseudomonas aeruginosa*. *Mikrobiologiya* 46:5-10 (in Russian).
Golovleva, L.A., A.M. Ilyaletdinov, R.M. Alieva, and D.B.Dzhusupova. 1984. Strain *Pseudomonas aeruginosa* 8 capable of degrading α-methylstyrene. Author's certificate No.1117316, BI No.37 (in Russian).
Golovleva, L.A., R.N. Pertsova, A.M. Boronin, B.G. Grishchenkov, B.P. Baskunov, A.V. Polyakova. 1982. Degradation of polychloroaromatic insecticides by *Pseudomonas aeruginosa* carrying biodegradation plasmids. *Mikrobioloiya* 51:973-978 (in Russian).
Golovleva L.A., R.N. Pertsova, V.M. Travkin. 1987. Degradation of kelthane in soil by *P. aeruginosa* BS827 carrying plasmid pBS3. In: *Pesticide Science and Biotechnology* (Greenhalgh R., Roberts T., eds). Blackwell Scientific Publications. Ottawa, P.421-424.
Golovleva L.A. and G.K. Skryabin. 1981. Microbial degradation of DDT. In: *Microbial Degradation of Xenobiotics and Recalcitrant Compounds* (Leisinger T., Cook A., Hutter R., eds). London, N.Y. P.291-298.
Golovleva L.A., A.M. Zyakun, B.P. Baskunov, R.N. Pertsova, G.K. Skryabin. 1980. Conditions of degradation of DDT and its analogues by *Pseudomonas aeruginosa* 640x. *Izvestiya AN SSSR,*

ser.biol. 2:243-253 (in Russian).
Haider K. 1976. In: *Bound and Conjugated Pesticide Residues* (D. Kaufman, G. Still, G. Paulson, S. Bandal, eds). ACS Symposium, series 29. Washington D.C. 244 p.
Haider K., G. Jagnow, K. Rohr. 1976. Anaerober Abbau von Hexachlorocyclohexan durch eine bakterielle Mischflora des Bodens und des Kuhpansens. *Landwirt. Forsch. Souderh.*, v.29, No.32/2, p.147-152.
Hamm R.T. and J.H. Thomas 1986. Microbial degradation of chloethocarb and carbofuran in two different soils. In: *Abstracts of the Sixth Interanat. Congress of Pesticide Chemistry.* Ottawa, IUPAC, 6C-14.
Hayaishi O. 1974. *Molecular Mechanisms of Oxygen Activations.* A,.P., N.Y., London. 687 p.
Hoff T., S. Liu, J.M. Bollag. 1985. Transformation of halogen-, alkyl-, and alkoxy-substituted anilines by a laccase of *Frametes versicolor. Appl. Environ. Microbiol.* 49:1040-1045.
Hsu T.S. and R. Bartha. 1974. Biodegradation of chloroaniline-humus complexes in soil and in culture solution. *Soil Sciences* 118:213-220.
Kaufman D., N. Aharanson, L.C. Horng. 1986. Accelerated biodegradation of pesticides: problems, implications and progress. In: *Abstracts of the Sixth Internat. Congress of Pesticide Chemistry.* Ottawa, IUPAC, 6S-04.
Kearney P., J.S. Karns, W. Milbry. 1987. Engineering soil microorganisms for pesticide degradation. In: *Biotechnology and Pesticide Science* (Greenhalgh R., Roberts T., eds). Blackwell Scientific Press), p.591-596.
Kilbane J.J., D.K. Chatterjee, J.S. Karns, S.T. Kellogg, A.M.Chakrabarty. 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Ps. cepacia. Appl. Environ. Microbiol.* 44:72-78.
Khan S.U. and K.S. Ivarson. 1982. Microbial release of unextracted bound residues from an organic soil treated with prometryne. In: *Environmental Science and Health*, Part B, B 17, p.737-749.
Lehrbach P., J. Zeyer, W. Reineke, H. Knackmuss, K. Timmis. 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas sp.* strain B13. *J. Bacteriol.* 158:1025-1032.
Lichtenstein E., P. Linag, M. Koeppe. 1982. Effect of fertilizers, captafol, and atrazine on the fate translocation of (^{14}C) fonofos and (^{14}C) parathion in a soil-plant microcosm. *J. Agr. Food Chem.* 30:871-878.
Mathur S.P. and H.V. Morley. 1978. Incorporation of methoxychlor-^{14}C in model humic acids prepared from hydroquinone. *Bull. Environ. Contam. Toxicol.* 20:268-274.
Matsumura F., H. Benezet, K.C. Patil. 1976. Factors affecting microbial metabolism of BHC. *J. Pesticide Sci.* 1:3-8.
Matsumura F. and M. Boush. 1968. Degradation of pesticides by a soil fungus *Trichoderma viride. J. Econom. Entomol.* 61:610- 612.
Münneke D and D.Hsich. 1975. Microbial metabolism of parathion-xylene pesticide formulation. *Appl. Microbiol.* 30:575-580.
Münneke D and D Hsich. 1976. Pathways of microbial metabolism in parathion. *Appl. Environ. Microbiol.* 31:63-69.
Pertsova R.N., F. Kunc, L.A. Golovleva. 1984. Degradation of 3-chlororobenzoate in soil by pseudomonads carrying biodegradative plasmids.

Folia microbiologica 29:242-247.
Stotzky G. and H. Babich. 1984. Fate of genetically engineered microbes in natural environments. *Advances in Applied Microbiology* 31:93-138.
Tam A.C., R.M. Behki, S.U. Khan. 1986. Isolation and characterization of an *Arthrobacter spp.* from EPTC exposed soil: plasmid-mediated degradation of the herbicide. In: *Abstracts of the Sixth Internat. Congress of Pesticide Chemistry.* Ottawa, IUPAC, 6C-08.
Tewfik M.S., W.S. Evans. 1966. The metabolism of 3,5-dinitro-*o*-cresol (DNOC) by soil microorganisms. Proceedings of the Biocheical Society. *Biochem. J.* 99:31-32.
Williams P. and M.J. Worsey. 1976. Ubiquity of plasmids encoding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL-plasmids. *J. Bacteriol.* 125:818-828.

MICROBIAL INTERACTIONS WITH HALOGENATED ALIPHATIC HYDROCARBONS UNDER AEROBIC AND ANAEROBIC CONDITIONS

Thomas Leisinger

Ten years ago virtually nothing was known about the interactions of microorganisms with halogenated aliphatic hydrocarbons. Today one can state that all the relevant chlorinated, brominated and iodinated aliphatic hydrocarbons are, under some conditions, subject to microbial attack. This statement is not valid for the fluorinated aliphatic hydrocarbons which either have yielded negative results or have not been tested Microbial interactions with halogenated aliphatic hydrocarbons have been reviewed by Janssen and Withold (1985), Leisinger (1983), Müller and Lingens (1986) and by Vogel *et al.* (1987). The interest in microbial interactions with halogenated aliphatic hydrocarbons is evidently due to the fact that these compounds have proven to be bothersome environmental pollutants that are resistant to microbial degradation by many ecosystems. Their significance as pollutants relates the large quantities of these compounds that are produced worldwide (approx. 30 million tons per year according to Pearson (1982) and with their widespread use as solvents or intermediates in chemical syntheses. The most important representatives, which are also the most significant pollutants, are trichloromethane, dichloromethane, tetrachloroethene, trichloroethene, chloroethene, trichloroethane and 1,2-dichloroethane.

Halogenated aliphatic hydrocarbons enter the environment not exclusively as a result of industrial activities. Many of these compounds have been shown to occur naturally. They are synthesized by fungi, formed in forest fires and, most importantly, continuously released by marine macroalgae (Gschwend *et al.*, 1985).

The three basic types of interactions of microorganisms with halogenated aliphatic hydrocarbons are listed in Table 1.

TABLE 1. Bacterial systems for the degradation of aliphatic hydrocarbons.

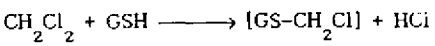
Function of halohydrocarbon	Dehalogenation mechanism
Carbon/energy source	Hydrolytic (thiolytic)
Cometabolic substrate	Hydrolytic
	Oxidative
Electron acceptor	Reductive

These compounds may serve as the only carbon and energy sources for aerobic chemoheterotrophic bacteria, and the dehalogenation mechanisms involved are either hydrolytic (thiolytic in some cases) or oxidative. Alternatively, halogenated aliphatic hydrocarbons serve as cometabolic substrates for aerobic chemotrophs. In this case the organisms grow on another compound and fortuitously transform the halogenated compound into less halogenated or non-halogenated products by the enzymes induced under the particular growth conditions. The dehalogenation mechanisms that have been observed under these conditions again are either hydrolytic or oxidative.

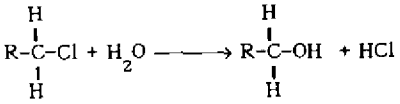
As a third possibility, halogenated aliphatic hydrocarbons may function as electron acceptors in anaerobic systems thereby being reductively dehalogenated. This type of reaction has been observed in methanogenic mixed cultures grown on acetate as well as in mixed cultures grown under denitrification conditions (Vogel *et al.*, 1987). The dehalogenation reactions operating in bacterial systems are summarized in Table 2.

TABLE 2. Dehalogenation reactions in bacteria.

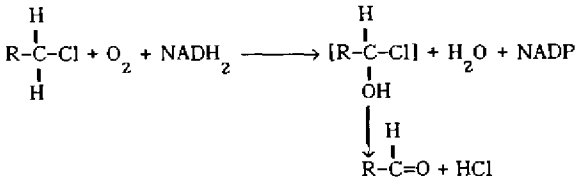
Thiolysis: Dichloromethane dehalogenase



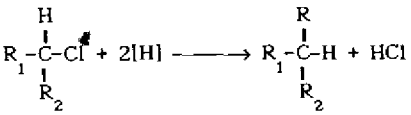
Hydrolysis: Haloalkane dehalogenases



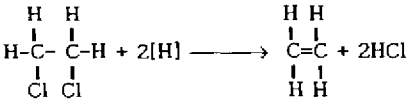
Oxidation: e.g. Alkane-monooxygenases



Reduction (Hydrogenolysis)



Reduction (Dihalo-elimination)



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1. Hydrolytic (Thiolytic) Dehalogenation

Several haloalkane dehalogenases from bacteria have been purified and characterized. The most specific one is dichloromethane dehalogenase, an enzyme found in facultatively methylotrophic bacteria of the genera *Methylobacterium, Hyphomicrobium and Pseudomonas.* All organisms growing on dichloromethane as the only carbon and energy source possess this enzyme. Using reduced glutathione as a cofactor, it converts one molecule of dichloromethane into one molecule of formaldehyde and two molecules of hydrochloric acid. The formation of formaldehyde is thought to proceed through the following intermediates:

$$CH_2Cl_2 + GSH \longrightarrow GS\text{-}H_2CCl + HCl$$
$$GS\text{-}CH_2Cl + H_2O \longrightarrow GS\text{-}CH_2OH + HCl$$
$$GS\text{-}CH_2OH \longrightarrow GSH + CH_2O$$

Dichloromethane dehalogenase, a hexamer with a subunit molecular weight of 33 kDa, is highly inducible by its substrate and constitutes about 16% of the total soluble protein in induced cells. The enzyme utilizes CH_2Cl_2, CH_2BrCl, CH_2Br_2 and CH_2I_2 as substrates but is inactive with a number of other aliphatic hydrocarbons that were tested. It has an extremely low turnover number of 33 mol of dichloromethane per mol of enzyme subunit per minute, which has given rise to the speculation that dichloromethane dehalogenase represents an enzyme at an early stage in its evolution, whose catalytic activity is still in the process of being improved (Kohler-Staub *et al.*, 1986). The dichloromethane utilization genes of *Methanobacterium sp.* DM4 have been cloned (Galli and Leisinger, 1988). A fluidized bed reactor with sand particles as a carrier for immobilization of dichloromethane-degrading bacteria was operated under non-sterile conditions. It exhibited a maximum degradation rate of 1.6 g of dichloromethane per hour and was stable during long-time operation (Galli and Leisinger, 1985).

Another group of well characterized enzymes specifically involved in the degradation of halogenated aliphatic hydrocarbons are bacterial haloalkane dehydrogenases. As shown in Table 3, four enzymes of this type have recently been purified and characterized from Gram-negative as well as from Gram-positive bacteria. All four enzymes are composed of a single subunit of roughly similar size. They have no requirement for an added cofactor but the catalytic mechanism is thought to be broadly similar to the one described for dichloromethane dehalogenase in requiring a sulfhydryl group at the active site. The enzymes differ with respect to their inducibility, the *Xanthobacter* enzyme being formed constitutively, whereas the other three enzymes are induced by haloalkanes. They also differ with respect to their substrate range. The preferred substrates are mono- or biterminally chlorinated, brominated or iodinated alkanes of varying chain length. The enzymes dehalogenate

also the haloalcohols but very weakly or not at all the corresponding haloacids.

TABLE 3. Bacterial haloalkane dehalogenases.

Organism	Sub-unit	MW (kDa)	Induction	Substrate range	Reference
Xanthobacter autotrophicus	1	36	-	C_1–C_4	Keuning *et al.*, 1985
Acinebacter sp.	1	28	+	C_2–C_9	Janssen *et al.*, 1986
Corynebacter sp.	1	36	+	C_2–C_{12}	Yokota *et al.*, 1987
Arthrobacter sp.	1	37	+	C_1–C_{10}	Scholtz *et al.*, 1987

2. Oxidative Dehalogenation

The hydrolytic dehalogenations discussed so far provide the means for the mineralization of the important pollutants dichloromethane and 1,2-dichloroethane. Hydrolytic enzymes attacking chlorinated ethenes like chloroethene, trichloroethene and tetrachloroethene or polyhalogenated methanes like trichloromethane or tetrachloromethane have not been observed. To deal with these important environmental pollutants one has to rely on oxidative or reductive mechanisms. The biochemistry of these reactions is not understood at present. Industrially important chlorinated aliphatic hydrocarbons which are utilized via oxidative dehalogenation reactions as growth substrates for bacterial pure cultures include chloromethane (Hartmans *et al.*, 1986), 1,2-dichloroethane (Stucki *et al.*, 1983) and chloroethene (Hartmans *et al.*, 1985). However, many oxidative and all reductive dehalogenations of C_1 and C_2 halohydrocarbons are transformation reactions which do not provide the dehalogenating organisms with utilizable carbon sources.

There exists indirect evidence that some long chain mono-haloalkanes are oxidatively dehalogenated by non-specific alkane mono-oxygenases. The corresponding dehalogenation reactions, however, have not been measured in cell-free systems (Yokota *et al.*, 1986). Recently it was observed that trichloroethene is completely

dehalogenated by an inducible component of a bacterial aromatic degradative pathway (Nelson *et al.*, 1987). Several important halogenated aliphatic hydrocarbons are co-oxidized by methane mono-oxygenase, an enzyme which is notorious for its wide substrate specificity. The evidence for this co-oxidative transformations rests on experiments, in which ^{14}C-labeled trichloromethane, trichloroethane, dichloroethene or chloroethene was added at low concentration to growing methanotrophic mixed cultures. The labeled halogenated compounds disappeared, and most of the label was recovered in CO_2. The reactions and the intermediates in these transformations, which ultimately lead to CO_2, are not known. The system is, however, of considerable interest for the removal of chlorinated ethenes from contaminated groundwater (Wilson *et al.*, 1987).

3. Reductive Dehalogenation

The highly chlorinated compounds tetrachloromethane and tetrachloroethene are not attacked by methane mono-oxygenase. The high extent of halogenation of these compounds increases their electrophilicity and their oxidation state and makes them more susceptible to reduction and less susceptible to oxidation. The effect of the extent of halogenation on the relative rates of oxidation and reduction of chlorinated methanes, ethanes and ethenes was discussed by Vogel *et al.* (1987). From their considerations it is evident that an initial reductive dehalogenation may represent the only possibility for the microbial degradation of the highly chlorinated solvents. A major problem in studying anaerobic dehalogenations was the fact that these reactions had only been observed in undefined mixed cultures and that the organisms involved were unknown. Recently reductive dehalogenations have been observed with pure cultures of strict anaerobes, namely with methanogens and with sulfate reducing bacteria (Table 4) as well as with acetogens (Egli *et al.*, unpublished).

This should allow the mechanism to be explored. Since we observe reductive dehalogenation of chlorinated aliphatic hydrocarbons only in anaerobic bacteria possessing the acetyl-CoA pathway, but not in organisms using the citric acid cycle for acetyl-CoA oxidation, we

speculate that a component of the acetyl-CoA pathway, possibly a corrinoid protein, is involved in anaerobic dehalogenation.

TABLE 4. Anaerobic dehalogenation of halogenated aliphatic hydrocarbons by bacterial pure cultures.

Halogenated compound	Product formed	Organism*	Reference
CCl_4	$CHCl_3$	M, S	Egli *et al.*, 1987
$CHCl_3$	CH_2Cl_2	M, S	Egli *et al.*, 1987
CH_2Cl-CH_2Cl	$CH_2=CH_2$	M	Egli *et al.*, 1987; Belay and Daniels, 1987
CH_3-CCl_3	CH_3-CHCl_2	M, S	Egli *et al.*, 1987;
$CCl_2=CCl_2$	$CCl_2=CHCl$	M, DCB-1	Egli *et al.*, 1987; Fathepure *et al.*, 1987
CH_2Br-CH_3	CH_3-CH_3	M	Belay and Daniels, 1987
CH_2Br-CH_2Br	$CH_2=CH_2$	M	Belay and Daniels, 1987
$CHBr=CHBr$	$CH\equiv CH$	M	Belay and Daniels, 1987

* M - methanogenic bacteria, S - *Desulfobacterium autotrophicus*, DCB-1 - dechlorinating bacterium DCB-1.

TABLE 5. Approximate degradation rates of bacterial cultures for chlorinated hydrocarbons.

System	Compound	Rate (mg/g protein h)	Reference
Aerobic mineralization	CH_2Cl_2	540	Galli and Leisinger, 1985
	$CH_2=CHCl$	60	Hartmans *et al.*, 1985
	CH_2Cl-CH_2Cl	450	Stucki *et al.*, 1983
Co-oxidation by methanotrophs	$CHCl=CCl_2$	0.33	Fogel *et al.*, 1986
	$CHCl=CHCl$	1.00	Fogel et al., 1986
Anaerobic transformation	CCl_4	54	Scholtz *et al.*, unpubl.
	$CHCl_3$	0.70	Scholtz *et al.*, unpubl.
	CH_3-CCl_3	0.22	Egli *et al.*, 1987
	$CCl_2=CCl_2$	0.016	Fathepure *et al.*, 1987

Conclusions

Bacteria for the partial and in many cases for the complete microbial degradation of the important chlorinated aliphatic hydrocarbons are

available. The most efficient systems are those, in which the chlorinated compounds are utilized as carbon sources via hydrolytic or oxidative dehalogenation mechanisms. Halogenated compounds not serving as carbon sources are either co-oxidatively transformed by methanotrophic mixed cultures or reductively dehalogenated by methanogens, certain sulfate reducers and acetogens. Some compounds are subject to both types of transformations. The total degradation of highly chlorinated compounds like tetrachloroethene is feasible by combining anaerobic dehalogenation with subsequent co-oxidation in a methanotrophic system. As shown in Table 5, the degradation rates observed in co-oxidative transformation systems with methanotrophs are 2 to 3 orders of magnitude lower than the degradation rates of systems permitting growth on chlorinated aliphatic hydrocarbons. With the exception of the transformation of tetrachloromethane, the same holds true for the anaerobic dehalogenation systems. It is now important to understand the biochemistry of co-oxidation and of anaerobic dehalogenation. Insight into the mechanisms of these processes may lead to possibilities for their optimization and their application in the solution of environmental problems.

REFERENCES

Belay N. and Daniels L. 1987. *Appl. Environ. Microbiol.* 53:1604-1610.

Egli C., Scholtz R., Cook A.M., Leisinger T. 1987. *FEMS Microbiol. Lett.* 43:257-261.

Fathepure B.Z., Nengu J,P., Boyd S.A. 1987. *Appl. Environ. Microbiol.* 53:2671-2674.

Fogel M.M., Taddeo A.R., Fogel S. 1986. *Appl. Environ. Microbiol.* 51:720-724.

Galli R. and Leisinger T. 1985. *Conserv. Recycl.* 8:91-100.

Galli R. and Leisinger T. 1988. *J. Gen. Microbiol.* (in press).

Gschwend P.M., MacFarlane J.K., Newman K.A. 1985. *Science* 227 1033-1035.

Hartmans S., de Bont J.A.M., Tramper J., Luyben K.C.A.M. 1985. *Biotechnol. Lett.* 7:383-388.

Hartmans S., Schmuckle A., Cook A.M., Leisinger T. 1986. *J. Gen. Microbiol.* 132:1139-1142.

Janssen D.B. and Withold B. 1985. *The Science of the Total Environment* 47:121-135.

Janssen D.B., Jager D., Withold B. 1986. Abstr. *14th Int. Congr. Microbiol.* P.B. 13-10, p.82.

Keuning S., Janssen D.B., Withold B. 1985. *J. Bacteriol.* 163:635-639.

Kohler-Staub D., Hartmans S., Galli R., Suter F., Leisinger T. 1986. *J. Gen. Microbiol.* 132:2837-2843.
Leisinger T. 1983. *Experientia* 39:1183-1191.
Müller R. und Lingens F. 1986. *Angew. Chem.* 98:778-788.
Nelson M. J.K., Montgomery S.O., Mahaffey W.R., Pritchard P.H. 1987. *Appl. Environ. Microbiol.* 53:949-954.
Pearson C.R. 1982. In: *The Handbook of Environmental Chemistry* 3B:69; (O. Hutzinger, ed.) Springer Verlag, Berlin.
Scholtz R., Leisinger T., Suter F., Cook A.M. 1987. *J. Bacteriol.* 169:5016-5021.
Stucki G., Krebser U., Leisinger T. 1983. *Experientia* 39:1271-1273.
Vogel T.M., Criddle C.S., McCarty P.L. 1987. *Environ. Sci. Technol.* 21:722-736.
Wilson J.T., Fogel S., Roberts P.V. 1987. In: *Detection, Control and Renovation of Contaminated Ground Water.* (N.Dee *et al.*, eds.) Am. Soc. Civil Engineers, New York.
Yokota T., Fuse H., Omori T., Minoda Y. 1986. *Agric. Biol. Chem.* 50:453-460.
Yokota T., Omori T., Kodama T. 1987. *J. Bacteriol.* 169:4049-4054.

GENETICS OF BIODEGRADATION

A.M. Boronin

Introduction

The last several decades have been characterized by broad industrial and agricultural applications of numerous synthetic organic chemicals as solvents, refrigerants, explosives, dielectrics, plasticizers, fire retardants, pesticides, herbicides, etc. Most of these compounds are referred to as xenobiotics, i.e. substances alien to the environment. Many of them are halogenated compounds which exhibit highly toxic characters. Natural compounds very rarely contain halogen–carbon bonds. Therefore the presence of such groups in xenobiotic molecules creates problems with regard to their microbial degradation. Consequently, accumulation of halogenated organic compounds occurs in water and soil (Schneider, 1979). This in turn leads to the concentration of such compounds in plants, animals, and even humans (Ramel, 1978). Since many of these compounds are potential carcinogens and, therefore, are hazardous to man, their accumulation in the biosphere arouses a growing concern on the part of scientists and the public in general. Evident economic benefits derived from the application of chlorinated compounds, for example pesticides and herbicides in agriculture, become conflict with the potential biological hazards due to their presence in the biosphere.

In order to resolve this problem there is a need, in the first place, for adequate information on the capabilities of microorganisms to degrade toxic organics released to the biosphere. Studies of the degradation of xenobiotics by the soil microflora revealed that certain chlorinated compounds, e.g. the widely used herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), are effectively degraded by soil microorganisms. However, most chlorinated organic compounds prove to be fairly persistent and undergo very slow and only partial degradation by the soil microflora as a result of a process known as co-oxidation (Alexander, 1981).

In recent years intensive studies have been carried out on the analysis of genetic systems which control degradation of organic compounds by bacteria. Such investigations were aimed at elucidating the evolutionary aspects of the capacity of bacteria to degrade new synthetic chemicals, studies of the mechanisms of distribution of this character among bacteria, which are referred to different taxonomic groups, and, finally, assessment of the possibility of practical uses of bacteria posessing such characters for the cleanup of the environment from toxic pollutants.

1. Plasmids of Biodegradation

The term "biodegradation" is used to describe processes of partial or total decomposition of organic compounds by microorganisms with the possible utilization of decomposition products as sources of carbon and energy for microbial populations which bring about this process. In general, they are mostly organic compounds which are not utilized as common growth substrates by the majority of microorganisms.

To date, the degradation of organic compounds by bacteria has been shown to be controlled in many cases by extrachromosomal elements capable of stable autonomous replication. These elements have been named plasmids (Novick *et al.*, 1976). Plasmids which determine reactions of degradation of organic compounds were discovered in the early 1970s in bacteria of the genus *Pseudomonas*. Chakrabarty (1973) proposed to term them "biodegradation "plasmids, whereas Broda was the first to use for

their designation the abbreviation "D-plasmids" (Bayley *et al.*, 1979).

At present, a number of biodegradation plasmids have been identified. Most of them were found in the soil strains of *Pseudomonas* genus, most often in strains of *Pseudomonas putida* (Table 1). These plasmids control the bacterial catabolism of such compounds as salicylate, naphthalene, camphor, octane, xylene, toluene, nicotine, biphenyl. The D-plasmids were also found in bacteria of other taxonomic groups. Thus, the D-plasmid controlling the phenanthrene metabolism was found in cells of the strain *Alcaligenes faecalis,* whereas cells of a strain of *Flavobacterium sp.* were found to harbour the plasmid of degradation of cyclic dimers of 6-aminohexanoic acid.

Degradative plasmids can control either the primary steps of organic compound degradation or its complete decomposition: this problem will be treated in greater detail in subsequent sections.

Of special interest are the D-plasmids involved in the control of degradation or cometabolism of chlorinated compounds such as 3-chlorobenzoate, 2,4-dichlorophenoxyacetate, chlorinated biphenyls (Table 1).

It is worthy of note that to identify D-plasmids, extensive use is made of the method of conjugational transfer of the character pointing to capacity for degradation of individual compounds. Therefore, one should not wonder when seeing that all plasmids presented in Table 1 are transmissible ones. Plasmids are presently classified by their incompatibility properties. In the case of bacteria of *Pseudomonas* genus, such groups were found to number 13 of which four - IncP-1, P -2, P-7 and P-9 - include biodegradation plasmids in addition to other ones (Table 1).

Localization of genetic systems, which specifically degrade organic compounds, on transmissible genetic elements enables one to use various strains of bacteria whose chromosomes are free of or contain genes or their mutant alleles required for conducting specific experiments. The availability of plasmids containing biodegradation genes facilitates molecular cloning of degradation genes in vitro and construction of hybrid plasmids using in vivo genetic techniques. All this makes it possible to attempt utilizing biodegradation plasmids to obtain hybrid

bacterial strains with enhanced capabilities to degrade organic toxicants or able to bring about the degradation of compounds resistant to attack by natural microorganisms.

TABLE 1. Plasmids controlling catabolic functions (according to Harayama and Don, 1985; Boronin, 1987).

Plasmid	Host	Characterization
SAL	*Pseudomonas putida*	degradation of salicylate IncP-9
CAM	*P. putida*	degradation of camphor IncP-2
OCT	*P. devorans*	degradation of n-alkanes IncP-2
pBS250	*P. aeruginosa*	degradation of n-alkanes IncP-2
NAH	*P. putida*	degradation of naphthalene IncP-9
NPL-1	*P. putida*	degradation of naphthalene to salicylate, IncP-9
pBS2	*P. putida*	degradation of naphthalene IncP-7
TOL	*P. putida*	degradation of toluene, *p*- and *m*-xylene
NIC	*P. conveza*	degradation of nicotine and nicotinate, IncC
pBS271	*P. ellipsoidea*	degradation of ε-caprolactam
pJp1	*Alcaligenes paradoxus*	degradation of 2,4-dichlorophenoxy-acetate and phenoxyacetate, IncC
pJp4	*Alcaligenes eutrophus*	degradation of 2,4-dichlorophen-oxyacetate and 3-chlorobenzoate, IncP-1
pOAD1	*Flavobacterium sp.*	degradation of cyclic dimer of 6-amino-hexane
pUO1	*Moraxella sp.*	degradation of haloacetate
pAC25	*Pseudomonas sp.*	degradation of 3-chlorobenzoate, IncP-9
pAC21	*Pseudomonas sp.*	degradation of p-chlorobiphenyl
pRE1	*P. putida*	degradation of isopropyl benzene
pCIT1	*Pseudomonas sp.*	degradation of aniline
pBS241	*P. putida*	degradation of biphenyl
DBT2	*P. alcaligenes*	degradation of dibenzothiophene
pST	*P. fluorescens*	degradation of styrene
pWW17	*Pseudomonas sp.*	degradation of phenylacetate
pBS290	*P. acidovorans*	degradation of α-methylstyrene

2. Genetic Systems of Biodegradation

Few plasmid systems for the biodegradation of organic compounds have been studied to date, and of these, a substantiated characterization is available for the systems that control degradation of aromatic compounds and their derivatives - toluene, xylenes, naphthalene, 3-chlorobenzoate, 2,4-dichlorophenoxyacetate. Detailed information can be found for the

alk-system of the OCT plasmid (Table 1) that specifies the oxidation of linear hydrocarbons - *n*-alkanes.

It should be noted, however, that despite the ability of pseudomonads to degrade a broad spectrum of organic substrates (Clarke and Ornston, 1975) individual strains normally oxidize only one or several compounds. The essence of a general concept on the development of catabolic pathways in pseudomonads is that, although pseudomonads possess a large set of specific enzymes that specify primary oxidation of various substrates, the catabolic pathways generally converge to a small number of central metabolites such as catechols, protocatechoates, and β-ketoadipates which are further metabolized via the central routes (Ribbons and Eaton, 1982).

In this context, it should be stressed that progress in studies on the structural and functional organization of plasmid systems of biodegradation are related to the development of modern molecular genetic techniques such as gene cloning, restriction and hybridization analysis of nucleic acids, etc. Of special importance are the methods of gene analysis and methodology of gene engineering in their application to bacteria differing from the classical *Escherichia coli*. In the first place, these are techniques of effective transposon mutagenesis of DNAs and construction of vector systems for gene cloning in a broad range of Gram-negative bacteria.

2.1. TOL Plasmid pWWO

This plasmid (Table 1) is nowadays considered as an archetype for the whole group of plasmids that control oxidation of aromatic compounds, such as *m*- and *p*-xylenes, 1,2,4-trimethylbenzene, 3-ethyltoluene and their corresponding alcohols and carboxylic acids, via the meta-pathway cleavage in the central metabolism of catechol (Harayama and Donn, 1985). Using methods of transposon mutagenesis, induced mutagenesis and molecular cloning, several teams of researchers, and in the first place, those headed by K. Timmis (Switzerland) and T. Nakazawa (Japan) showed by independent studies that the genes encoding catabolic pWWO genes are organized in two pWWO clusters separated by the 7 kb spacer of of pWWO on the map (Figs. 1, 2).

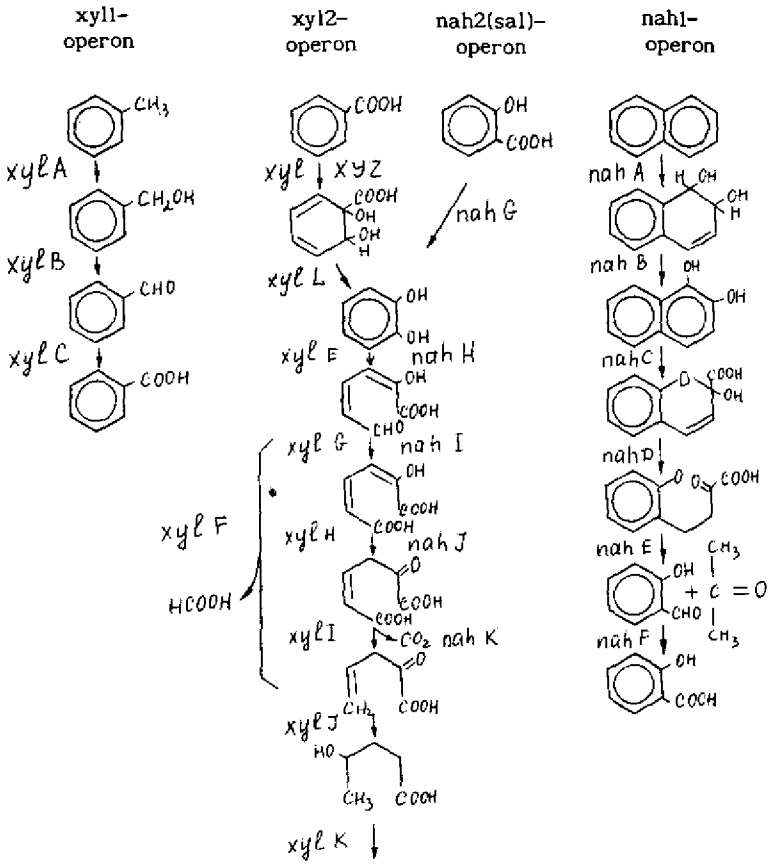


FIGURE 1. Catabolic pathways controlled by TOL and NAH7 plasmids. A, B, C, D etc. denote structural genes. Convergence of both pathways occurs at the level of catechol cleavage.

Xyl-operons: A - xylene oxydase, B - benzylalcohol dehydrogenase, XYZ(D) - toluate 1,2-dioxygenase, L - dihydrocyclohexadiene carboxylate dehydrogenase, E - catechol 2,3-dioxygenase, F - hydroxymuconic semialdehyde hydrolase, H - 4-oxalocrotonate tautomerase, I - 4-oxalocrotonate decarboxylase, J - 2-oxopent-4-enoate hydratase, K - 2-oxo-4-hydroxypentenoate aldolase.

Nah-operons: A - naphthalene 1,2-dioxygenase, B - cis-dihydrodiol naphthalene dehydrogenase, C - 1,2-dihydroxynaphthalene dioxygenase, D - 2-hydroxychromene-2-carboxylate isomerase, E - 2- hydroxybenzylpyruvate aldolase, F - salicylaldehyde dehydrogenase, G - salicylate hydroxylase, H - catechol 2,3-dioxygenase, I - 2-hydroxymuconate semialdehyde dehydrogenase, J - 2-hydroxymuconate tautomerase, K - 4-oxalocrotonate decarboxylase.

The first cluster embraces the genes encoding enzymes of the "upper" pathway, i.e. oxidation of hydrocarbons to corresponding carboxylic acids, whereas the second cluster includes the genes encoding enzymes of the "lower" or meta-pathway along which carboxylic acids are oxidized to catechols with subsequent meta-cleavage of the aromatic ring and conversion into short-chain carboxylic acids, pyruvate and aldehydes (Nakazawa *et al.*, 1985). Each gene cluster represents an operon, i.e. a jointly regulated block of genes (Worsey *et al.*, 1978).

The gene order (Fig. 2) is the following: xylC- A- B (xyl1 operon) and xylXYZ- L- E- G- F- J- I- H (xyl2-operon) (Harayama *et al.*, 1984).

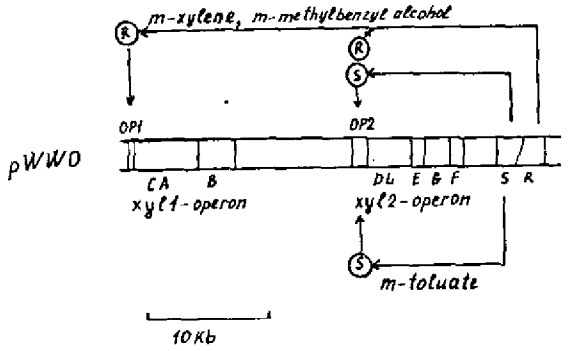


FIGURE 2. Model of positive regulation of xyl-operons of the TOL plasmid pWW0 (according to Nakazawa *et al.*,1985).

The organization of xyl-genes in operons of xyl1 (CAB) and xyl2 (XYZEGF) is schematized above. The protein xylR is linked with OP1 in the presence of *m*-xylene or *m*-methylbenzyl alcohol and activates operon xyl1. The protein xylS is linked to OP2 in the presence of *m*-toluate and activates operon xyl2. Both proteins xylR and xylS are required for activation of xyl2-operon in the presence of m-xylene or *m*-methylbenzyl alcohol.

Previously, xylXYZ was designated as the gene xylD (Franklin *et al.*, 1983; Inouye *et al.*, 1983) encoding toluate 1,2-dioxygenase - the first enzyme of meta-pathway. The toluate 1,2-dioxygenase is referred to

the family of multicomponent enzymes - dioxygenases which play the key role in the biological attack of aromatic rings by their double hydroxylation with production of *cis*-dihydrodiols (Gibson and Subramanian, 1984) that are further readily attacked by subsequent enzymes of catabolic pathways - dihydrodiol dehydrogenases. Several dioxygenases of the aromatic rings were purified and characterized. Nearly all of them, except for benzoate 1,2-dioxygenase of *P. arvilla* (Yamaguchi and Fujisawa, 1978), are composed of four subunits: ferredoxin, flavin-containing NADH-oxydase and the terminal oxydase composed of two different subunits (Harayama *et al.*, 1986).

Little is known, however, about detailed mechanisms of the dihydroxylation of aromatic rings by dioxygenases, this situation being due to the complexity and instability of such multicomponent systems.

Toluate 1,2-dioxygenase as well as the next enzyme of meta-pathway dihydroxycyclohexadiene carboxylate dehydrogenase (xylL) transform benzoate and *p*- and *m*-toluates to corresponding catechols (Harayama *et al.*, 1984; Lehrbach *et al.*, 1984), substrates for the aromatic ring cleavage. The latter reaction is catalyzed by the most important enzymes of the meta-pathway of aromatic ring cleavage - catechol 2,3-dioxygenase (C230, gene xylE) which transforms catechols to 2-hydroxymuconic semialdehydes This enzyme is the most studied of all other enzymes of catabolic pathways. It was isolated and characterized (Nakai *et al.*, 1983), cloned, and its nucleotide and amino acid sequences were determined (Nakai *et al.*, 1983; Zukowski *et al.*, 1983). The enzyme is composed of 4 identical subunits, each having the MW of 35 kDa. The reaction of catechol transformation to 2-hydroxymuconic semialdehyde may be used as colour test, since the latter has a bright yellow colour which is readily identified visually after spraying clones, growing on solid media and containing xylE gene, with millimolar solution of catechol. All this made xylE a useful genetic marker for detection and quantitative determination of promoters as well as for construction of vectors allowing direct selection of cloned DNA within a wide range of organisms, from Gram-negative bacteria to Gram-positive ones and yeast (Ebina *et al.*, 1983; Zukowski and Miller, 1985; Warcoin, 1984; Patent of the Transgene company, 1984).

Compared to the meta-pathway (xyl2-operon), the upper pathway (xyl1-operon) has been investigated less. It comprises three enzymes - xylene oxygenase (xylA), benzyl alcohol dehydrogenase (xylB) and benzaldehyde dehydrogenase (xylC) (Fig.1). Analysis of the expression of cloned genes of xyl1-operon showed that benzyl alcohol is attacked by two different enzymes: xylene oxygenase and benzyl alcohol dehydrogenase. Both enzymes display additive catalytic activities to this substrate. The fact that benzyl alcohol is both product and subproduct for xylene oxygenase suggests that this enzyme has a very poor substrate affinity. Low substrate affinity of xylene oxygenase (xylA) pWWO leads to the synthesis of indigo dye, though at a very low level (about 1.5 mg/l culture) (Mermod *et al.*, 1986), during cloning in *E. coli* cells on a multicopy vector.

Indole was found to be a precursor of the indigo biosynthesis. The aromatic derivatives of toluene, indole and benzyl alcohol are all subject to oxidation by xylene oxygenase, but differ essentially from each other. This is suggestive of xylene oxygenase to be an example of degradative enzymes with an unusually broad substrate affinity (Kunz and Chapman, 1981; Reineke and Knackmuss, 1978; Lehrbach *et al.*, 1984).

As mentioned above, genes encoding the toluene/xylene catabolism are grouped into two operons, the "upper" and the "lower" (meta-) pathways. Two regulatory loci xylR and xylS have been localized on the pWWO map immediately after xyl2-operon (Fig.2). Herewith, xylR takes part in the induction of enzymes of both operons by primary pathway substrates and their alcoholic derivatives, whereas xylS is required for inducing the enzyme synthesis solely of the "lower" pathway (xyl2-operon) by carboxylic acid - the first substrate of the operon. Both loci, xylR and xylS, are necessary for inducing the synthesis of xyl2-operon enzymes only when the primary substrates of the complete pathway (*m*-xylene) or their corresponding alcohols (*m*-methylbenzyl alcohol) act as inducers (Nakazawa *et al.*,1985). Inouye *et al.* (1981) cloned the lower operon xyl DEGF and showed that enzymes encoded by genes of this operon were not inducible until the cell received gene xylS in the trans-position. This positively acting gene was activated in the presence of *m*-toluate or benzoate, whereas *m*-xylene or its

derivative - *m*-methylbenzyl alcohol effected no activation. Similar role of xylS was demonstrated in an independent study by Franklin *et al.* (1981).

Inouye *et al.*(1983) cloned another regulatory gene - xylR. They showed that, when in trans-position, this gene participated in the induction of the synthesis of catechol 2,3-oxygenase, if its encoding gene xylE was arranged to be controlled by the regulatory site of the "upper" operon xyl BAC, in the presence of *m*-xylene or *m*-methylbenzyl alcohol. However, the regulatory gene xylR was not effective towards the "lower" operon xyl XYZEGF until the cell received the gene xylS with inducers. In other terms, induction of the "lower" operon by *m*-xylene or *m*-methylbenzyl alcohol requires products of both regulatory genes - xylR and xylS.

In conclusion it should be noted that a thorough molecular genetic analysis of the regulation of xyl-genes expression enabled researchers to derive results that are in full agreement with the model of positive regulation of operons (Raibaum and Schwartz, 1984).

2.2. Degradation Pathways of Naphthalene and Salicylic Acid

To date, the degradative routes of naphthalene and salicylic acid are known in sufficient detail for the bacteria of the genus *Pseudomonas* (Fig. 1). The first six consecutive enzymatic reactions of naphthalene oxidation lead to the formation of salicylic acid. Even when further oxidation of salicylic acid does not occur, the growth of bacterial cells that effect such a transformation is possible at the expense of pyruvate released during formation of salicylic aldehyde, which in turn is oxidized to salicylic acid (Fig. 1) (Boronin, 1987; Yen and Gunsalus, 1982). Further, salicylic acid is usually oxidized via catechol or, in rarer cases, via gentisic acid. Cleavage of catechol proceeds following the meta- or ortho-pathway (Feist and Hegeman, 1969).

At present, of all biodegradative plasmids of naphthalene the best known is NAH (NAH7) (Dunn and Gunsalus, 1973). This plasmid specifies the oxidative pathway of naphthalene via the intermediate salicylate which is converted into catechol followed by its cleavage via the meta-pathway (Fig. 1).

Connors and Barnsley (1980) studied the characteristics of the induction of enzymes involved in the metabolism of naphthalene in various strains of pseudomonads and showed that the induction characters varied from strain to strain, but in all cases salicylic aldehyde and salicylate proved to be efficient inducers of the entire pathway.

The genetic system of NAH7, which controls the complete oxidation of naphthalene (Fig. 1) comprises no less than 11 structural genes determining synthesis of enzymes, that specify oxidative reactions, and organized into two clusters, as shown by the transposon mutagenesis (Yen and Gunsalus, 1982). Like the pWWO operons, the two clusters contain genes of the "upper" and "lower" (meta-) oxidative pathway that are separated on the NAH map by the DNA fragment of about 7 kb. The transposon mutagenesis as well as nah-genes cloning showed that the gene order in the nah1-operon ("upper" pathway), which controls naphthalene oxidation to salicylate, is as follows: nahA⇒nahB⇒nahC⇒nahD⇒nahE⇒nahF (Yen and Gunsalus, 1982, 1985). The operon nah2 (sal) ("lower" or mata-pathway) of NAH7 includes the genes nahG⇒nahH⇒⇒nahK, which specify the salicylate oxidation to catechol with the subsequent meta-cleavage of the latter to form 2-oxo-4-pentenoic acid (Fig. 1) This is analogous to the operon xyl2 of pWWO plasmid. An exception is made by the first gene nahG of nah2(sal)-operon encoding salicylate hydroxylase, whereas the first enzyme of the "lower" pathway of pWWO is toluate 1,2-dioxygenase (xyl XYZ). Salicylate hydroxylase (nahG) is an enzyme with a broad substrate affinity, and due to this property, it can effectively utilize not only salicylate but also its various derivatives including halogenated ones (Lehrbach *et al.*, 1984).

Of all genes of the nah1-operon, the interesting are apparently two genes which encode the key enzymes of naphthalene degradation: nahA - encoding naphthalene dioxygenase and nahC encoding 1,2-dihydroxy-naphthalene dioxygenase. Ensley *et al.* (1983) showed that the expression of nahA gene in *E. coli* cells results in accumulation of indigo dye at the expense of indole transformation. Under conditions of normal metabolism in *E. coli* cells tryptophanase induces formation of tryptophan from indole which is further oxidized by naphthalene dioxygenase to *cis*-indole-2,3-dihydrodiol. Thereafter, *cis*-dihydrodiol

undergoes spontaneous conversion into indigo as a result of dehydration with subsequent oxidation.

In one of his earlier works Barnsley reported the isolation and partial purification of 1,2-dihydroxynaphthalene dioxygenase - the product encoded by the nahC gene of NAH2 plasmid. The enzyme preparation displayed affinity not only to its substrate - 1,2-dihydroxynaphthalene but also to methyl-substituted catechols, 3- and 4-methylcatechols. Analysis of the nahC gene of nahl-operon of the plasmid pBS286 NPL-41:TnA) cloned by Tsoy *et al.*, (1988) was found to encode a polypeptide with a molecular weight of 32.5 kDa and showed that 1,2-dihydroxynaphthalene dioxygenase exhibited affinity not only to 3- and 4-methylcatechols but also to catechol, although its activity to the latter substrate was less.

Since 1983, successful developments in cloning and analysis of structural genes of the naphthalene degradation pathway have stimulated thorough investigations of the regulatory mechanism of nah-genes, and the basic findings were described by Schell (Schell, 1985, 1986; Schell and Wender, 1986).

On the basis of results of cloning and analysis of the nahR locus, Schell (1985) put forward the following model of coordinating regulation of naphthalene degradative genes. Cells of *P. putida* (NAH7) capable of growth in the absence of salicylate are characterized by low levels of transcription and translation of nah-, sal- and nahR-regions. Addition to the growth medium of salicylate (or naphthalene that can be gradually metabolized to salicylate) results in activation of the transcription of nah- and sal-operons mediated by the preceding product of gene nahR. Assumedly, nahR protein is linked to salicylate and transformed, in conformity with the concepts of the operon positive regulation model (Raibaum and Schwartz, 1984), to an active form of activator of the nah- and sal-operons transcription. Comparison of promoter nucleotide sequences of nah- and sal-operons with similar sites of pWWO xyl-operons failed to reveal substantial homology. In addition, the authors succeeded in localizing the site of initiation of the gene nahR transcription and determining the nucleotide sequence of its promoter. It should be noted that the promoters for nahR and sal-operon overlap in

position -35, the gene nahR being expressed in reverse direction. As the gene nahR is expressed constitutively, of no surprise is that the boxes -35 and -10 close to those for *E. coli* promoter were found in P_{nahR} in the corresponding positions. However, based on the positive regulation model assuming the absence of typical -35 and -10 sites in positively regulated promoters (Raibaum and Schwartz, 1984), it appears surprising that such sites in the corresponding positions were also found in promoters of nah- and sal-operons (Schell, 1986).

According to the data from gel-filtration analysis of *E. coli* maxi-cells, the product of nahR gene has a molecular weight of about 36 kDa and forms a stable complex with DNA irrespectively of the presence or absence of a salicylate inducer. Synthesis of an additional polypeptide of 14 kDa was also observed in maxi-cells.

A more detailed analysis of the nahR loci appears to be required, in particular a more thorough purification of the product, as well as the studies on the nah- and sal-operons in vitro transcription intended at elucidating details of specific interaction of the regulatory protein with the regulatory sites of operons. However, if further evidence is adduced for the idea of formation of stable nahR protein (or one of the proteins) complex with the DNA matrix in the absence of salicylate, the model of positive regulation of nah- and sal-operons will be called in question, at least in its pure form.

Thus, only essentially external similarity appears to exist between the regulatory systems of pWWO and NAH7, and if in the case of pWWO we deal apparently with the system of positive regulation in its "pure" form, the situation with NAH7 may be complicated by the presence of elements of negative control. This assumption is supported by the findings revealing that in the case of NPL-1 plasmid with inducible synthesis of nah-operon enzymes, the transition to constitutiveness (NPL-41) is accompanied by an inversion of segment X localized upstream the structural part of the operon (Boronin, 1987) - a fact which cannot be interpreted within the frames of "pure" model of positive regulation of nah-genes transcription.

2.3. Plasmids Involved in the Degradation of Chlorinated Organic Compounds

To date, numerous strains of Gram-negative microorganisms have been isolated (including those of *Alcaligenes*, *Moraxella* and others) and found to carry plasmids which participate in degradation of chloroorganic compounds (Table 1). Only two of such plasmids pAC27 (a deletion derivative of pAC25 isolated from a strain of *P. putida*) and pJP4, found in the strain *Alcaligenes eutrophus* JMP134 (Don and Pemberton, 1981) have been studied in sufficient detail.

The plasmid pJP4 having a size of 80 kDa and referred to the group IncP-1 (Table 1) specifies the degradation of 3-chlorobenzoate (3-CB) and that of 2,4-dichlorophenoxyacetate (2,4-D) both brought about by the strain *A. eutrophus* JMP134 (Fig. 3). The transposon mutagenesis Tn5 and Tni771 (Don *et al.*, 1985) was effected for analysis of the organization of plasmid genes which control specific degradation pathways. Two classes of mutants have been obtained, $2,4\text{-}D^{-}3\text{-}CB^{+}$ (I) and $2,4\text{-}D^{-}3\text{-}CB^{-}$ (II). In all mutants of class I the insertion of transposons was mapped in the gene encoding 2,4-dichlorophenol hydroxylase (gene tfdB). An attempt to isolate mutants for the gene tfdA (2,4-monooxygenase) failed, though this gene must be localized on the plasmid, since plasmid-free derivatives of the strain jMP134 do not exhibit activity of this enzyme (Don and Pemberton, 1985).

Mutants of class II ($2,4\text{-}D^{-}$, $3\text{-}CB^{-}$) were deficient in enzymes of the ortho-pathway of chlorocatechol cleavage, thereby pointing to the fact that the oxidation routes of 2,4-D and 3-CB converge at the level of chlorocatechol, which is the first common metabolite (Don *et al.*, 1985). The ortho-pathway of chlorocatechols cleavage is by its origin, also common to plasmid pAC27 (Frantz and Chakrabarty, 1986). The products of the genes tfdC, D and E have been characterized similarly to earlier identifications in variations of the ortho-pathway for chlorocatechols (Dorn and Knackmuss, 1978) and called, respectively, dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and chlorodienelactone hydrolase. Based on the analysis of transposon mutants, the authors inferred that the genes controlling degradation of 2,4-D are organized in two operons, the "upper" one comprising the genes

tfdB (2,4-dichlorophenol hydroxylase) and tfDA (2,4-D monooxygenase), the latter not having been localized so far. The "lower" pathway includes the genes tfdC, D and E specifying 3-chlorocatechol metabolism to chloromaleylacetate via the ortho-pathway.

The primary steps of 3-CB degradation appear to be controlled by chromosomal genes. In any case, the plasmid-free derivatives of the strain JMP134 are capable of cometabolizing 3-CB to chlorocatechol. Moreover, the Tn5 mutagenesis of chromosomal genes of the strain JMP134 (pJP4) revealed mutants capable of growth in the medium with 2,4-D as sole source of carbon and energy, but unable to degrade 3-CB (Don *et al.*, 1985). The regulation of tfd-operons is still poorly studied, but is supposed to be positive.

The cloning of genes which specifically degrade chloro- and dichlorocatechols appears very promising for their prospective application in engineering bacterial strains capable of degrading a broad spectrum of organochlorine compounds. Although most natural strains are unable to degrade chloroaromatic compounds, they are, on the other hand, known to often cometabolize such compounds to cell-toxic chlorocatechols due to broad substrate affinity of the degradative enzymes of unsubstituted aromatics (Dorn and Knackmuss, 1978).

2.4. Plasmids CAM and OCT

The plasmid CAM (Table 1) comprises a set of genes organized in concertedly regulated clusters which control conversion of camphor down to isobutyrate. Some genes responsible for further oxidation of isobutyrate are also localized on the plasmid; however, the total set of genes specifying utilization of isobutyrate is localized in a *P. putida* chromosome.

Though the large size of the plasmid (500 Kb) makes its analysis difficult, sufficient information is already available on the enzymology and biochemistry of the process of camphor degradation (Unger *et al.*, 1986). The primary reaction of 5-exo-hydroxylation of camphor requires the participation of a three-component monooxygenase (similar to xylXYZ of TOL plasmid) encoded by the genes camA, B and C. The next reaction, oxidation of 5-exo-alcohol, is catalyzed by the product of camD gene -

F-dehydrogenase. The gene camA encodes flavoprotein, whereas camB - FeS-containing protein and camC - the terminal hydroxylase (P-450). These genes are organized into an operon camR⇒D⇒C⇒A⇒B negatively regulated by the locus camR (Koga *et al.*, 1985).

The plasmid OCT which specifies oxidation of *n*-alkanes (octane, hexane, decane) resembles CAM in many respects. The oxidation pathway of *n*-alkanes in the studied bacterial strains of the *Pseudomonas* genus studied may be schematized as follows: $R-CH_3 \Rightarrow R-CH_2OH \Rightarrow R-CHO \Rightarrow R-COOH$.

Genetic systems of the catabolism of *n*-alkanes were studied by methods of genetic analysis based on the induction and transposon mutagenesis, transduction analysis with the phage F116, and cloning of alk-genes. The OCT plasmid was shown to control the oxidation of *n*-alkanes only to corresponding aldehydes, subsequent reactions being controlled by chromosomal genes. Plasmid genes are organized into the operon alkB- A- C. The gene alkB encodes the membrane component alkane hydroxylase, alkA - the soluble component of alkane hydroxylase, whereas alk-operon is space-regulated in the OCT-plasmid map by the remote locus alkR encoding the synthesis of at least two regulatory enzymes. According to recent findings, these regulatory proteins may be acting negatively and not positively (Shapiro *et al.*, 1984).

3. Construction of Active Strains Capable of Degrading Xenobiotics

Genetic engineering of highly efficient degradative strains is an urgent problem today that may be tackled by various means, the choice and realization being directly dependent on the level of genetic and biochemical knowledge on the biodegradation processes and techniques of gene manipulations.

There is another aspect which adds to the urgency of the above problem. While certain compounds (mainly of natural origin) are readily mineralized by microorganisms and therefore do not accumulate in the biosphere, other compounds (mostly of anthropogenic origin) prove to be recalcitrant, i.e. they are either slowly or not degraded by the natural microflora. As a result, they accumulate in the biosphere and become a real threat as ecological pollution. The bulk of recalcitrant

pollutants, consequently, include compounds with structural components that are either highly stable chemically or have a structure generally not found in molecules of biological origin, are toxic to microorganisms or inhibit degradative enzymatic attack. It is precisely the problem of degradability of such compounds that preoccupies most microbiologists, biochemists and geneticists.

It should be kept in mind that though the soil and water microorganisms are capable of degrading a great diversity of organic compounds, their metabolic potential is the sum total of capabilities of individual strains to degrade or transform only one or several compounds. Apparently, under natural conditions stable microbial communities develop that subsist on a common nutrient source. Certain members of the community are only able to cometabolize such nutrient source, i.e. transform the compound to non-growth substrate while using other compounds as a source of energy.

At present, it has become obvious that continued production of toxic and recalcitrant compounds is fraught with hazards to the environment and therefore should be substituted by alternative technologies yielding innocuous and readily metabolizable compounds. However, since it is hardly possible to predict how many years will it take before this idea becomes a reality, we should start to look for other approaches capable of a more rapid and practical contribution. In this context, one way that appears most promising is optimization of the biodegradative capabilities of microorganisms, improving the evolution of novel microbial activities and their use for the elimination of the most difficult pollutants.

Yet early experiments pointed to the capabilities of microorganisms to evolve new degradative routes within rather short time limits in the course of selection for large concentrations of xenobiotics. For example, intensive treatments of cultivated soils with 2,4-D (2,4-dichlorophenoxyacetate) or delapon (2,2-dichloropropionate) resulted in the emergence of microorganisms capable of mineralizing these compounds (Senior *et al.*, 1976; Don *et al.*, 1985), whereas application of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane provoked evolution of a whole consortium capable of cometabolizing this

pesticide (Subba-Rao and Alexander, 1985). Genetic analysis of biodegradation processes conducted during the last decade suggests that such a rapid evolution of new pathways, which generally included many biochemical steps, is not as surprising as it might seem at the first glance. In our opinion, the background of the evolutionary variation of degradative characters of microorganisms is formed by the following genetic peculiarities of the structural and functional organization of biodegradation systems:

- total or partial localization of biodegradation genes on transmissible replicons, thus making possible effective horizontal transfer inside and among microbial populations;
- transposon-type organization of biodegradation regions providing for efficient recombination and insertion into plasmid replicons thus favouring further transfer of genetic material;
- transposon-type or IS-like organization of specific blocks of structural genes of biodegradation pathways providing under natural conditions for manipulations at the level of individual blocks of encoding sequences. This also presupposes a possibility of submission of structural genes to the control of new regulatory mechanisms;
- availability inside biodegradation regions of repeated DNA sequences, possibly very short ones, providing for short duplications at the level of one gene or even less than that. This may underlie a rapid evolution of novel enzymatic activities by gene duplications.

The above statements are suggestive of certain experimental approaches to a laboratory evolution of metabolic pathways, of which some have already been successfully tested while others are in order:

- enrichment: selection conceived as selective stress with appropriate compounds for bringing about evolution of microorganisms capable of utilizing new substrate. Herein, use is often made of a progressive substitution of mineralizable substrate by its recalcitrant analogue;
- in vivo genetic transfer when genes of critical enzymes are transferred from strain to strain for their incorporation into the pathway of the recipient strain in experiments of the type of conjugation, transduction, transformation. Such experiments are mostly feasible precisely owing to plasmid localization or transposon organization

of biodegradation systems;

- in vitro evolution of novel metabolic pathways when the cloned and well studied genes are selectively introduced into a microbial strain for developing new pathway;
- protein engineering under which the cloned and well studied genes are used for target-directed changes in the structure of encoded enzyme for generating new substrate affinities or, vice versa, restricting the range of utilized substrates.

4. Examples of Experimental Evolution of Biodegradation Pathways

(1) Localization of biodegradative genes on plasmids enables assembling within one bacterial cell of a genetic material which controls degradation of various organic compounds. Dr. A. Chakrabarty, who one time collaborated with the industry in the USA, showed the possibility of constructing pseudomonads strains carrying a few plasmids specifying degradation of various petroleum hydrocarbons: aliphatic (OCT plasmid), cyclic (CAM), polycyclic aromatics (NAH). In the author's opinion, such strains have a number of advantages compared to mixed cultures (which can, e.g., exhibit antagonistic interrelations) They were proposed for abating the environmental pollution caused by crude petroleum and its products. Similar strains were also constructed a few years ago by Boronin *et al.* (1987) (USSR Academy of Sciences, Institute of Biochemistry and Physiology of Microorganisms). Polyplasmid strains proved to be capable of effective utilization of crude petroleum, gas oil and lubricants.

(2) Plasmids were found to be a suitable material for constructing bacterial strains with an enlarged degradation spectrum of chlorinated organics. The strain *Pseudomonas sp.* B13 (pWR1) displays a remarkable ability to utilize chlorinated aromatic compounds, e.g. 3-chlorobenzoate or 4-chlorophenol as sole source of carbon. Nonetheless, it has been shown that narrow specificity of the enzyme carrying out the primary attack on chloroaromatic compounds prevents utilization of 2- or -4-chlorobenzoate. At the same time, the plasmid TOL controls the synthesis of dioxygenase which is much less specific and readily

transforms 2- or 4-chlorobenzoates into intermediate oxidation products. The latter, however, cannot be utilized by the strain carrying the TOL plasmid because of its inability to oxidize chlorinated metabolites of the "meta"-pathway of catechol oxidation. Transfer of the TOL plasmid into the strain *Pseudomonas sp.* B13 enabled Reineke and Knackmuss (1979) to obtain a new strain capable of degrading 4-chlorobenzoate or 3,5-dichlorobenzoate.

We obtained similar results by incorporating the biodegradative plasmid of naphthalene into the strain *P. aeruginosa* 640x which was able to degrade DDT. The constructed strains were not only more efficient in DDT degradation but also acquired the capability to degrade another pesticide, kelthane.

(3) Methodology of genetic engineering as applied to horizontal evolution was demonstrated in recent studies (Lehrbach *et al.*, 1984; Ramos *et al.*, 1987). In experiments on the introduction of plasmid TOL into the strain B13 (pWR1), the transconjugants previously obtained showed no growth on 4-CB, and the mutants degrading 4-CB were found only by subsequent selection. These mutants proved to be deficient in the catechol 2,3-oxygenase of TOL plasmid. It was inferred that B13 (pWR1; TOL) processed 4-CB to 4 -chlorocatechol using the TOL enzymes, whereas 4-chlorocatechol was subjected preferentially to the meta-cleavage by the TOL 2,3-oxygenase but did not follow the ortho-cleavage by catechase II of a modified pWR1 ortho-pathway. This resulted in the inhibition of pyrocatechase and lethal conditions for cells. Mutational inactivation of the TOL catechol 2,3-oxygenase prevented 4-chlorocatechol from following meta-cleavage route thus imparting to B13 cells (pWR1; TOL-C230) the ability to utilize 4-CB as a growth substrate. To check experimentally this conclusion, Lehrbach *et al.* (1984) cloned genes of the multicomponent enzyme toluate 1,2-dioxygenase (xylXYZ) and the next enzyme of TOL plasmid xyl-route dihydrocyclohexadiene carbo..ylate dehydrogenase (xylL) as well as their promoter p_m and the regulatory locus xylS of xyl2-operon on a broad host range vector (derivative RSF1000, IncP-4). The hybrid plasmid was introduced into B13 (pWR1) (Fig. 3) which developed a degradative and growth capability on 4-CB. This experiment showed unequivocally that to utilize 4-CB, the strain

B13 (pWR1) requires additionally only the enzymes of the initial reactions of the TOL benzoate oxidation route with a broad substrate affinity.

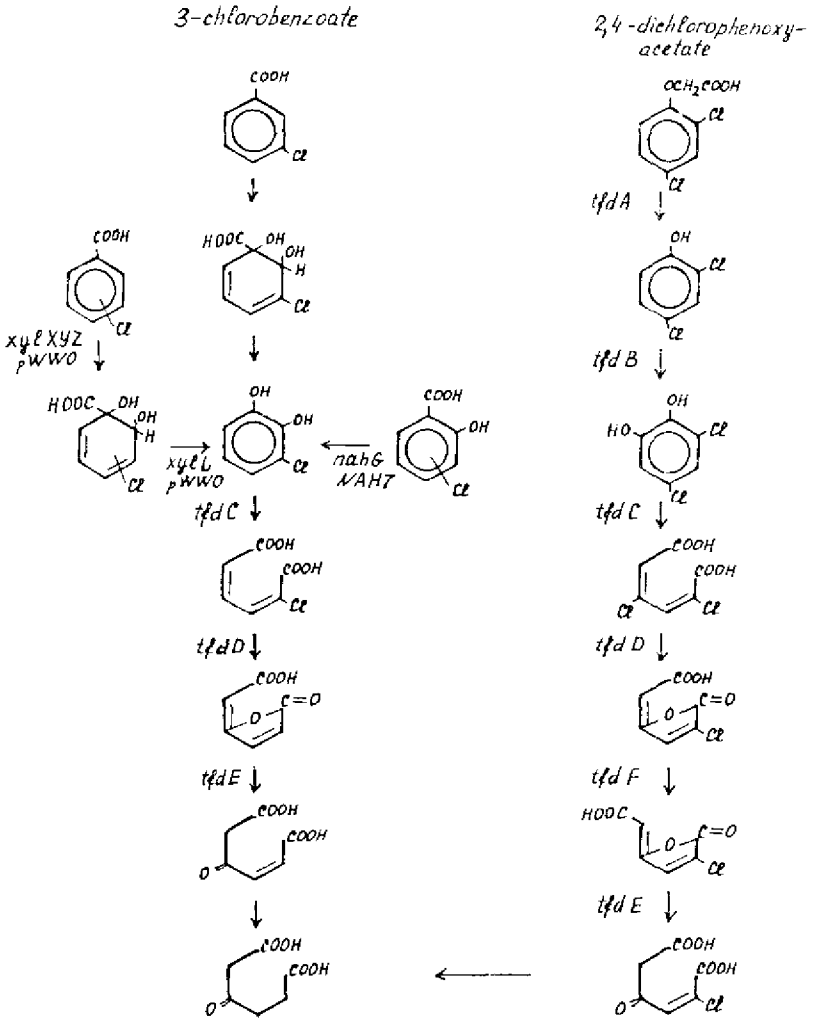


FIGURE 3. Degradation pathways of 3-chlorobenzoate and 2,4-dichlorophenoxyacetate controlled by the the strains *A. eutrophus* JMP134, *Pseudomonas sp.* b13 (pWR1), *P. putida* (pAC27). A, B, C, D, E, F denote genes localized on plasmids.

Tfd1-operon: A - 2,4-dichlorophenoxyacetate monooxygenase, B - 2,4-dichlorophenol hydroxylase.
Tfd2-operon: C - chlorocatechol 1,2-dioxygenase, D - chloromuconate cycloisomerase, E - dienelactone hydrolase, F - *trans-cis*-chlorodiene-lactone isomerase.
A strategy is schematized for constructing hybrid pathways: horizontal (left) and vertical (right) broadened ranges of substrates (Lehrbach *et al.*, 1984).

It was further found that though B13 (pWR1; xyl XYZLS) could grow on 3-CB and 4-CB, it was unable to utilize 3,5-dichlorobenzoate (3,5-DCB) because of low effector affinity of xylS regulatory protein to 3,5-DCB (Ramos *et al.*, 1986). To overcome this block, a mutant gene xylS was obtained whose product. xylS protein, was effectively activated by 3,5-DCB, and in this form it activated well the transcription of the xylXYZ gene. A strain capable of growth on 3,5-DCB was obtained by the introduction of a recombinant plasmid carrying the mutant gene xylS into the strain B13 (Ramos *et al.*, 1986). It is worthy of note that the transdominance of a positively acting regulatory protein provides an opportunity of the direct use of mutant regulatory genes for efficient changes in metabolic activities of cells, without the necessity of preliminary elimination of a wild type regulatory gene.

Ramos *et al.* (1987) used a similar approach with application of mutant regulatory gene xylS for enlarging the number of metabolizable substrates of the TOL meta-cleavage route in metabolism of alkylbenzoates. The TOL plasmid is known to specify the catabolic pathway of benzoate, 3- and 4-methylbenzoates (3-MB, 4-MB), 3,4-dimethylbenzoate (3,4-DMB) and 3-ethylbenzoate (3-CB). This pathway is controlled by genes of xyl2-operon (Fig. 3) whose transcription is regulated positively by the regulatory gene xylS. But the ability to grow on methylbenzoates and 3-EB does not embrace 4-EB, and this peculiarity proved to be due to the low effector affinity of xylS protein to 4-EB as an activator. Therefore, having obtained, via ingenious selection, the mutant gene xylS whose xylS protein was effectively activated by 4-EB and by introducing the recombinant plasmid carrying the mutant gene into the strain *P. putida* (TOL) Ramos *et al.* (1987) succeeded in obtaining a strain capable of growth on 4-EB.

(4) A vertical evolution of new degradative pathways was demonstrated by Lehrbach *et al.* (1984) pointing to experimental feasibility of the central pathway of chlorocatechols cleavage aimed at its vertical broadening and realizing a degradative route for chlorosalicylates. Numerous attempts to isolate from soil bacteria capable of catabolizing chlorosalicylate have been unsuccessful. The above-described plasmid NAH7 encodes, among others, the enzyme salicylate hydroxylase (nahG) (Fig. 3) which exhibits broad substrate affinity and oxidizes not only salicylate but also methyl and chlorosalicylates to corresponding catechols. The gene nahG occupies the first position in the operon nah2 (sal) and overlaps at the level of promoter sequences with the gene nahR encoding regulatory protein (Schell, 1985). A DNA fragment of NAH7 plasmid containing the genes nahG and nahR was cloned on a vector with a broad host range, and the recombinant plasmid was introduced into the strain B13 (pWR1). The resultant recombinant strain developed a capacity for growth on 3-, 4- and 5- chlorosalicylates (Fig. 3).

Hence, mobilization of the NAH7 salicylate hydroxylase into a bacteria capable of degrading chlorocatechols broadens vertically the degradation pathway resulting in construction of an organism capable of catabolizing compounds resistant to attack by soil microorganisms.

Conclusion

As seen from the above examples, experimental possibilities of a horizontal broadening of catabolic pathways are based on the availability of isofunctional degradation cycles of the structurally similar organic compounds and the existence in certain cycles of enzymes with broad substrate specificity, (this refers first to key the enzymes of degradative pathways - mono- and dioxygenases). In this context, of special significance is the possibility to change the affinity of proteins to their substrate effectors using mutagenesis. For attaining such an understanding of the experimental possibilities we are indebted to the pioneer works carried out by Clarke and co-workers (Clarke and Ornston, 1985), whose elegant studies clearly demonstrated that the substrate specificity of the amidase of pseudomonads, for which

acetamide is common natural substrate, and the effector affinity of the regulatory protein of amidase gene may be consistently broadened.

Experimental possibilities of a laboratory vertical evolution depend on a convergence of biodegradative pathways of various organic compounds to a small number of central routes. For example, the pathways of cleavage of aromatics converge to a limited number of dihydroxy-aromatic intermediate metabolites such as catechol, gentisate, protocatechoate, which are further subjected to cleavage of the aromatic ring to produce substrates of the Krebs cycle. This makes possible a vertical buildup of the central pathways, e.g. the ortho-route of chlorocatechol cleavage, by addition of supplementary enzymatic steps so that the constructed pathway acquire the capability to utilize more complex compounds, as it was shown by studies on genetic construction of a strain capable of degrading chlorosalicylate (Lehrbach *et al.*, 1984).

Developments in genetic construction of hybrid catabolic pathways are promising in the sense of their use for the cleanup of the environment. One should, however, be aware that the use of the approaches described provides no guarantee of success in an endeavour to solve certain biotechnological problems. The solution of certain problems will apparently call for the construction of entirely new catabolic pathways, as, for example, may be the case of the degradation of such compounds as cresols in combination with chlorophenols. This problem stems from the fact that industrial effluents commonly contain mixtures of chloro- and methyl-phenols which are not only a difficult subject for degradation but also destabilize phenol-degrading microbial communities. The difficulty is that catechols and chlorocatechols undergo the cleavage via ortho-routes, whereas methylcatechols follow the meta-fission. As a rule, in an individual microorganism only one pathway is functional, though both routes may be present. However, in the case of a mixture of chloro- and methylcatechols both pathways operate, and one organism becomes recipient of the products of cleavage of both types of catechols, the situation thus becoming lethal to cells. Theoretically, the problem may be solved by constructing catabolic cycles effecting only one mode of the aromatic ring fission, either meta or ortho (Ramos and Timmis, 1987).

Another problem is the stability of recombinants. Until now the problem was viewed as No.1 in biotechnology, and it appears that the stability of recombinants may be raised by introduction of cloned genes into chromosomes of the host strain. However, the maintenance of sufficient efficiency of substrate utilization may require amplification of key genes. This in turn brings to the forefront the problem of constructing transposon vectors of cloning that would permit multiple transposition of a desired gene into a chromosome.

The first successes realized in the construction of hybrid pathways for degradation of xenobiotics showed that complex problems related to biotechnological processes of the environment cleanup may be tackled solely by thorough genetic and biochemical investigations of the catabolic pathways. The prospective tasks of experimental strategy that await a solution are also becoming clearer. Of these, the most pressing are the development of cloning methodology, techniques of effective gene expression and stabilization of recombinants, and methods of protein engineering. Finally, if today one could see an efficient synergy of genetics and biochemistry for investigating biodegradation problems, tomorrow will require a similar use of genetics and physiology. On this agenda are also prospects of improvement and stabilization of recombinant strains, genetic manipulation of physiological barriers, and genetic analysis of physiological parameters. It is quite obvious that only a comprehensive approach embracing the entire potential of genetics, gene engineering, biochemistry and physiology will be instrumental in tackling the most complex problems of the cleanup and protection of the environment from deleterious anthropogenic products.

REFERENCES

Alexander M. 1981. Biodegradation of chemicals of environmental concern. *Science* 221:132-138.

Bayley B.A., Morris D.W., Broda P. 1979. The relationship of degradative and resistant plasmid of *Pseudomonas* belonging to the same incompatibility group. *Nature* 280:338-339.

Boronin A.M. 1987. *Plasmids of Resistance and Biodegradation of Bacteria.* Post-doctoral dissertation. 500 p. (in Russian).

Chakrabarty A.M. 1972. Genetic basis of the biodegradation of salicylate in Pseudomonas. *J. Bacteriol.* 112:815-823.

Chakrabarty A.M. 1973. Genetic fusion of incompatible plasmids in *Pseudomonas*. *Proc. Natl. Acad. Sci. USA* 70:1641-1644.
Chatterjee D.K. 1983. Genetic homology between independently isolated chlorobenzoate-degradative plasmids. *J. Bacteriol.* 153:532-534.
Clarke P.H., Ornston L.N. 1975. Metabolic pathways and regulation. In: *Genetics and Biochemistry of Pseudomonas* (Clarke P.H., Richmond M.H., Eds. London. J.Wiley and Sons. P.191-340.
Connors M.A., Barnsley E.A. 1980. Metabolism of naphthalene by *Pseudomonas*: salicylate as the first possible inducer in the metabolic pathway. *J. Bacteriol.* 141:1052-1054.
Don R.H., Pemberton J.M. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J. Bacteriol.* 145:681-686.
Don R.H., Pemberton J.M. 1985. Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pp4. *J. Bacteriol.* 161:466-468.
Don R.., Weightman A.J., Knackmuss H.J., Timmis K.N. 1985. Transposon mutagenesis and cloning analysis of the pathway for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134 (pJP4). *J. Bacteriol.* 161:85-90.
Dorn E., Knackmuss H.-J. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 2,3-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochem. J.* 174:73-84.
Dunn N.W., Gunsalus I.C. 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. *J. Bacteriol.* 114:974-979.
Ebina Y., Takohara Y., Shirake K., Yamada M., Nakazawa T., Nakazawa A. 1983. Plasmid encoded regulation of colicin E1 gene expression. *J. Bacteriol.* 156:487-492.
Ensley B.D., Ratzkin B.J., Osslund T.D., Simon H.J. Wackett L.P., Gibson D.T. 1983. Expression of naphthalene oxidation genes in *Eschirichia coli* results in the biosynthesis of indigo. *Science* 222: 167-169.
Feist C.P., Hageman G.D. 1969. Regulation of the meta cleavage pathway for benzoate oxidation by *Pseudomonas putida*. *J. Bacteriol.* 100: 1121-1123.
Franklin F.C.M., Bagdasarian M., Bagdasarian M.M., Timmis K.N. 1981. Molecular and function analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proc. Nat. Acad. Sci. USA* 78: 7458-7462.
Franklin F.C.H., Lehrbach P.R., Lurz R., Rueckert B., Bagdasarian M., Timmis K.N. 1983. Localization and functional analysis of transposon mutagenesis in regulatory genes of the TOL catabolic pathway. *J. Bacteriol.* 154:676-685.
Frantz B., Chakrabarty A.M. 1986. Degradative plasmids in *Pseudomonas*. In: *The Bacteria* (Sokatch J.R., ed.). N.-Y.-Lond. Acad. Press Inc. 10: 295-323.
Gibson D.T., Subramanian V. 1984. Microbial degradation of aromatic hydrocarbons. In: *Microbial Degradation of Organic Compounds* (Gibson D.T., ed.). N.-Y. Marcel Dekker. P. 181-252.
Harayama S., Don R.H. 1985. Catabolic plasmids: their analysis and

utilization in the manipulation of bacterial metabolic activities. In: *Genetic Engineering: Principles and Methods* (Setlow J.K., Hollaender A. eds.). N.-Y. Plenum Press. 7:283-307.
Harayama S., Lehrbach P.R., Timmis K.N. 1984. Transposon mutagenesis analysis of meta-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. *J. Bacteriol.* 160:251- 255.
Harayama S., Rekik M., Timmis K.N. 1986. Genetic analysis of a relaxed substrate specificity aromatic ring dioxygenase, toluate 1,2-dioxygenase encoded by TOL plasmid pWWO of *Pseudomonas putida*. *Mol. Gen. Genet.* 202:226-234.
Inouye S., Nakazawa A., Nakazawa T. 1981. Molecular cloning of gene xylS of the TOL plasmid: evidence for positive regulation of the xylDEGP operon by xylS. *J. Bacteriol.* 148:413-418.
Inouye S., Nakazawa A., Nakazawa T. 1983. Molecular cloning of regulatory gene xylR and operator-promotor regions of the xylABC and xylDEGF operons of the L plasmid. *J. Bacteriol.* 155:1192-1199.
Koga H., Rauchfuss B., Gunsalus I.C. 1985. P450cam gene cloning and expression in *Pseudomonas putida* and *Escherichia coli*. *Biophys. Biochem. Res. Commun.* 130:412-417.
Kunz D.A., Chapman P.J. 1981. Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida (arvilla)* mt-2: evidence for new functions of the TOL (pWWO) plasmid. *J. Bacteriol.* 146:179-191.
Lehrbach P.R., Zeyer J., Reineke W., Knackmuss W., Timmis K.N. 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas sp.* B13. *J. Bacteriol.* 158: 1025-1032.
Mermod N., Harayama S., Timmis K.N. 1986. New route to bacterial production of indigo. *Bio/Technology* 4:321-324.
Nakai C., Kagamiyama H., Nozaki ., Nakazawa T., Inouye S., Ebina Y., Nakazawa A. 1983. Complete nucleotide sequence of the metapyrocatechase gene of the TOL plasmid of *Pseudomonas putida* mt-2. *J. Biol. Chem.* 256:2923-2928.
Nakazawa T., Inouye S., Nakazawa A. 1985. Positive regulation and transcription initiation of xyl operons on TOL plasmid. In: *Plasmids in Bacteria* (Helsinki D.R., Cohen S.N., Clewell D.B. *et al.*, eds.). N.Y.-Lond. Plenum Press. P. 415-429.
Novick R.P., Clowes R.C., Cohen S.N., Curtiss R., Datta N., Falkow S. 1976. Uniform nomenclature for bacterial plasmids: a proposal. *Bacter. Rev.* 40:168-189.
Patel T.R., Barnsley E.A. 1980. Naphthalene metabolism by *Pseudomonas*: purification and properties of 1,2-dihydroxynaphthalene oxygenase. *J. Bacteriol.* 143:668-678.
Raibaum O., Schwartz M. 1984. Positive control of transcription initiation in bacteria. *Annu. Rev. Genet.* 18:173-206.
Ramel C. 1978. *Chlorinated Phenoxyacids and Their Dioxins*. Swedish Nat. Sci. Res. Council. Stockholm.
Ramos J.L., Stolz A., Reineke W., Timmis K.N. 1986. Broader effector specificities in regulators of gene expression: TOL plasmid xylS mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. *Proc. Nat. Acad. Sci. USA* 83:8467-8470.
Ramos J.L., Timmis K.N. 1987. Experimental evolution of catabolic pathways of bacteria. *Microbiol. Sci.* 4:228-237.

Ramos J.L., Wasserfallen A., Rose K., Timmis K.N. 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science* 235:593- 596.
Reineke W., Knachmuss H.-J. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biophys. Biochim. Acta* 542:412-423.
Reineke W., Knackmuss H.J. 1979. Construction of haloaromatics utilizing bacteria. *Nature* 277:385-386.
Ribbons D.W., Eaton R.W. 1982. Chemical transformation of aromatic hydrocarbons that support the growth of microorganisms. In: *Biodegradation and Detoxification of Environmental Pollutants* (Chakrabarty A.M., ed.). Boca Raton. Fla. CRC Press. P. 59-84.
Schell M. 1985. Transcriptional control of the nah and sal hydrocarbon degradation operons by the nahR gene product. *Gene* 36:301-309.
Schell M. 1986. Homology between nucleotide sequence of promotor regions of nah and sal operons of NAH7 plasmid of *Pseudomonas putida. Proc. Nat. Acad. Sci. USA* 83:369-373.
Schell M., Wender P. 1986. Identification of nahR gene product and nucleotide sequence required for its activation of the sal operon. *J. Bacteriol.* 166:9-14.
Schneider M.J. 1979. *Persistent Poisons: Chemical Pollutants in the Environment.* N.Y. - The N.-Y. Acad.Sci.
Senior E., Bull. .T., Salter J.M. 1976. Enzyme evolution in a microbial community growing on the herbicide dalapon. *Nature* 263:476-479.
Shapiro J.A., Owen D.J., Kok M., Eggink G. 1984. *Pseudomonas* hydrocarbon oxidation. In: *Genetic Control of Environmental Pollutants* (Omenn G.., Hollaender A., eds.). N.-Y. Plenum Press. P. 229-238.
Subba-Rao R.V., Alexander M. 1985. Bacterial and fungal cometabolism of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) and its breakdown products. *Appl. Env. Microbiol.* 49:509-516.
Tsoy T.V., Kosheleva I.A., Zamarayev V.S., Trelina O.V., Selifonov S.A., Zakharova M.Yu., Starovoitov I.I., Boronin A.M. 1988. Cloning and expression of a gene of *Pseudomonas putida* contolling catechol 2,3-oxygenase activity in cells of *Escherichia coli. Genetika.* (in press) (in Russian).
Unger .P., Gunsalus I.C., Sligar S.G. 1986. Nucleotide sequence of the *Pseudomonas putida* cytochrome P-450cam gene and its expression in *Escherichia coli. J.Biol.Chem.*
Warcoin J. 1984. Production of catechol 2,3-oxygenase by means of yeasts, plasmid for the implementation thereof and application. PSTWO 84/04539. "Transgene".
Worsey M.J., Franklin F.C.H., Williams P.A. 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. *J. Bacteriol.* 134:757- 764.
Yamaguchi M., Fugisawa H. 1978. Characterization of NADH-cytochrome c reductase, a component of benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* 253:8848- 8853.
Yen K.-M., Gunsalus I.C. 1982. Plasmid gene organization: naphthalene/ salicylate oxidation. *Proc. Nat. Acad. Sci. USA* 79:874-878.
Yen K.-H., Gunsalus I.C. 1985. Regulation of naphthalene regulation genes of plasmid NAH7. *J. Bacteriol.* 162:1008-1013.
Zukowski M.M., Gaffney D.F.,Speck D., Kauffman M., Findelli A.,

Wisecup A., Lecock J.-P. 1983. Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. *Proc. Nat. Acad. Sci. USA* 80:1001-1105.
Zukowski M.M., Miller L. 1985. Hyperproduction of an intracellular heterologous protein in a SacUh mutant of *Bacillus subtilis*. *Gene* 46:247-258.

MICROBIAL TECHNOLOGIES FOR THE ENVIRONMENT PROTECTION AGAINST XENOBIOTICS

L.A. Golovleva

In recent decades, the environment has received a tremendous flux of synthetic compounds which are used by man as insecticides, dielectrics, retardants, detergents, etc. Originally, the use of these anthropogenic substances appeared beneficial and, owing to their stability, low inputs were required for producing fairly positive effects. Later, it has become apparent that the environmental toxicity and persistence of such compounds is a real hazard to both man and animals. This brought on the agenda the burning issue of the environment decontamination. The United States alone had to arrange for over 3,500 burial sites for metallic containers with residual chemicals. Leaks from the corroded containers are a continual source of pollution of the biosphere. The problem is much more dramatic in the regions where compounds of the type of 'orange mixture' had been sprayed for a long period over large areas for defoliation. The restoration of such areas contaminated with polychloroaromatic compounds is an extremely difficult task. These examples serve to explain the ever-growing interest in the screening of active microbial strains and the 'breeding' of novel microbial forms using genetic engineering techniques.

Another approach also appears very exciting to many biotechnologists, and that is the substitution of the xenobiotics, applied nowadays in fairly high concentrations, by antibiotics, mycoherbicides and bacterial toxins which are efficient in amount of a gram per acre. Let us consider in greater detail these two aspects of ecological biotechnology.

The discovery of biodegradation plasmids and the possibility of their transfer to various strains (Table 1) opened up new vistas for constructing novel efficient strains to degrade petroleum, DDT, polychlorobiphenyls, 2,4,5-T and many other products. The ability of plasmids to exchange genetic material and to interact resulted in the development of a new method of molecular selection termed the plasmid-assisted molecular breeding for the biodegradation of persistent toxicants.

Some examples presented below include the culture of *P. testosteroni* which carries a plasmid DNA of 67 kDa and is capable of degrading alkylbenzene sulphonate. The plasmid encodes the desulphonating enzymes and five of the enzymes of meta-cleavage pathway (Fig. 1).

TABLE 1. List of some biodegradation plasmids.

Plasmid	Degradation pathway	Origin	Size (MDa)	Reference
Cam	camphor	con.*	300	Chakrabarty, 1980
OCT	*n*-octane	non-con.	250	Chakrabarty, 1980
Sal	salicylate	con.	55, 48, 42	Chakrabarty, 1980
NAH	naphthalene	con.	46	Chakrabarty, 1980
XYL-K	xylene/toluene	con.	90	Chakrabarty, 1980
2HP	2-hydroxypyridine	?	63	Weinberger, 1979
TOL	xylene/toluene	con.	90	Chakrabarty, 1980
NIC	nicotine/nicotinate	con.	n.d.**	Chakrabarty, 1980
	6-aminocyclohexanoic acid			
pOAD2	dimer	non-con.	29	Negoro *et al.*, 1980
pJP1	2,4-D	con.	58	Chakrabarty, 1980
pAC8	xylene/toluene	con.	76	Chakrabarty, 1980
pAC21	p-chlorobiphenyl	con.	65	Chakrabarty, 1980
	3-chlorobenzoate	con.	68	Chatterjee *et al.*, 1981
pAC27	3-and 4-chloro-benzoate	con.	59	Chakrabarty, 1981

con.* - conjugated; n. d.** - not determined.

The plasmid called ASL was readily transferable to strains of other *Pseudomonas* species or to plasmid-free strains of *P. testosteroni*. The transfer was brought about under physiological conditions simulating those in activated sludge, soil, natural water bodies (Cain, 1981). The discovery of plasmids which specify oxidation of the long-chain hydrocarbons suggests that oxidation of the alkyl chain in alkylbenzene sulphonate may be plasmid-encoded and that successful developments in engineering microbial strains with enhanced ability to degrade persistent detergents is not a matter for the distant future.

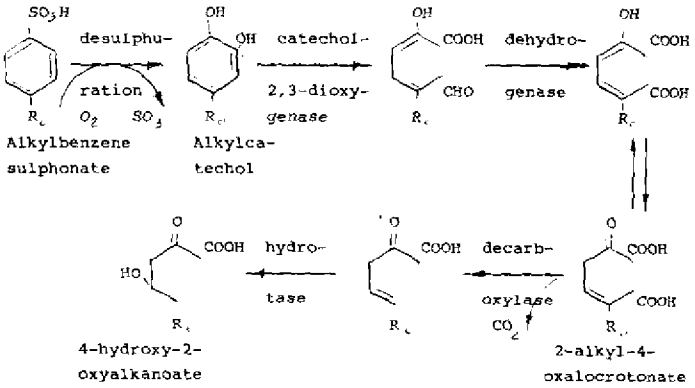


FIGURE 1. Degradation of alkylbenzene sulphonate by *P. testosteroni* PtSI (Cain, 1981)

Chloroaromatic acids can also be degraded by pure cultures, though these are rather rarely found. Their degradative characters may be enhanced with genetic means. Knackmuss and co-workers from the Göttingen University showed that introduction of the *P. putida* mt-2 cells carrying TOL-plasmid into a chemostat with *Pseudomonas sp.* B13 capable of growth on 3-chlorobenzoate (3-CB) led to the emergence of cells able to develop not only on 3-CB and 4-CBA but also on 3,5-dichlorobenzoic acid (Knackmuss *et al.*, 1981). Chlorocatechols, whose cleavage is always a problem in the oxidation of chloroaromatic acids proved to be degradable by isoenzymes already present in B13 cells.

Of interest is the progress on cloning of genes which encode the enzymes of carbofuran degradation by a culture of *Achromobacter* by the US Department of Agriculture in Beltsville (Kearney *et al.*, 1985). The authors performed cloning of the plasmid and chromosomal DNAs into the cosmid vector pCP13 and hope that the information derived from these experiments will enable a good insight into the way in which degradative functions emerge and are distributed among microbial populations in the soil.

An obvious example of the use of biodegradation plasmids to construct strains with enhanced ability to degrade xenobiotics is the work on designing strains capable of degrading DDT, kelthane and methoxychlor carried out at the Institute of Biochemistry and Physiology of Microorganisms (USSR) (Golovleva *et al.*, 1984).

TABLE 2. Degradation of DDT, methoxychlor and kelthane by *Pseudomonas aeruginosa* 640x, BS816 and BS827 (cultivation during 14 days).

Insecticide	Residue, % of Control		
	640x	BS816	BS827
DDT	32	11	40
Methoxychlor	82	35	32
Kelthane	68	13	9

TABLE 3. Dioxygenase activities in *P. aeruginosa* 640x and constructed strains BS816 and BS827

Source of carbon	Enzyme activity, mol/min·mg protein								
	metapyrocatechase			pyrocatechase			protocatechoate-3,4 oxygenase		
	640x	BS816	BS827	640x	BS816	BS827	640x	BS816	BS827
Naphthalene	-	44.0	286.4	-	7.2	0	0	0	0
Salicylate	-	52.2	155.4	-	5.0	0	0	0	0
Benzoate	0	16.9	10.3	13.2	117.6	89.8	24.0	0	0
p-Hydroxybenzoate	0	9.1	30.5	0	0	0	379.7	469.9	591.0
Homogentisate	0	62.9	5.6	0	1	0	0	0	0
Gentisate	0	5.4	9.5	4.0	1	0	0	0	0
Glycerol	0	3.3	28.3	0	0	0	0	0	0

The strain *P. aeruginosa* 640x was found to cometabolize DDT but the process was very slow. Thorough analysis revealed that the strain lacked the essential enzymes of aromatics metabolism (Table 1). Researchers from the Laboratory of microbial extrachromosomal heredity joined in the work, and new 640x-based strains were constructed which carried biodegradative plasmids specifying synthesis of enzymes absent from the parent culture. Two resultant strains *P. aeruginosa* BS816 and BS827 showed activities of both enzymes of interest - metapyrocatechase and salicylate hydroxylase (Table 2), the strain BS816 showed greater degradation of DDT (Table 3) and, what is of the utmost interest, the strains degraded kelthane - a DDT analogue (Table 2). In two weeks kelthane was completely degraded by the strain BS827, whereas the parent strain produced insignificant degradation of the insecticide.

Released in soil, the strain BS827 produced drastic activation of kelthane degradation. Experiments were carried out during 1985-1987 on test-grounds enclosed with frames in parallel with laboratory model experiments with the soil samples (Fig. 2).

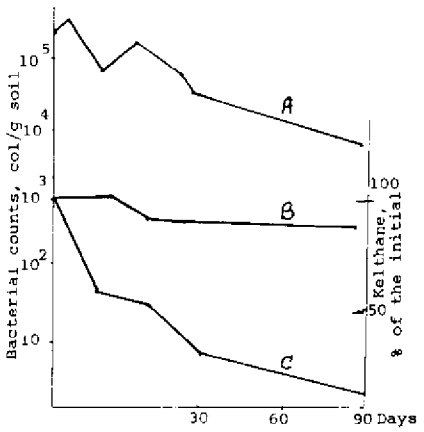


FIGURE 2. Bacterial counts for *P. aeruginosa* BS827 and dynamics of kelthane depletion in soil ecosystem.
A - kelthane-free soil; B - soil free of strain BS827; C - soil with strain BS827 and kelthane.

In 3 months the kelthane residue in soil accounted for 72%, whereas in experiments with BS827 the pesticide residue did not exceed 8% after the same period. Studies of the strain viability in natural ecosystems showed that it was dependent on the pesticide availability in soil (Table 4). In control experiments, the plasmid was deleted in 43% of the strain population in 13 days, while after 40 days the whole population lost the plasmid. In contrast, in experiments with kelthane from 72 to 100% of cells preserved this plasmid. A closer study of the strains isolated from the soil indicated that the plasmid was not always stable in them: in parallel with variants in which the plasmid was either totally preserved or deleted, there were cases with deletion of the Nah^+ or Sal^+ characters.

TABLE 4. Viability in soil of *P. aeruginosa* BS827 carrying plasmid pBS3

Experiment duration, days	Strain phenotype	Cell percentage in the population	
		with kelthane	without kelthane
1	Nah^+Sal^+	100.0	100.0
13	Nah^+Sal^+	83.0	39.0
	Nah^+Sal^-	13.0	19.2
	Nah^-Sal^+	4.0	1.8
	Nah^-Sal^-	0	43.0
17	Nah^+Sal^+	72.0	19.6
	Nah^+Sal^-	0	18.7
	Nah^-Sal^+	11.0	13.1
	Nah^-Sal^-	17.0	48.6
41	Nah^+Sal^+	100.0	0
	Nah^+Sal^-	0	0
	Nah^-Sal^+	0	8
	Nah^-Sal^-	0	92.0

Emergence of phenotype variants of the strain BS827 after its introduction in soil suggested mutations had occurred in the plasmid. This suggestion was supported by results of the plasmid DNA electrophoresis in the agar gel (Fig. 3).

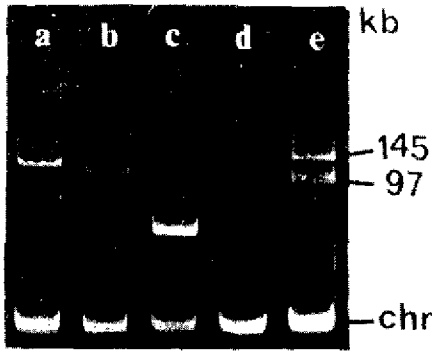


FIGURE 3. Agarose gel electrophoresis of plasmid DNAs.
Molecular masses of plasmid DNAs are indicated in kb.
Lane **a** – BS827 DNA; lane **b** – BS1000 DNA; lane **c** – BS1001 DNA;
lane **d** – BS1002 DNA; lane **e** – marker plasmid DNA.

The loss of the Nah or Sal characters is clearly seen to coincide in the electrophoregram with the decrease in the pBS3 mass. Phenotype variants of the strain BS827 were found to be less effective in kelthane degradation compared to the strain BS827 itself, the rate of kelthane degradation being particularly low with the deletion in the strain of the Nah character. Analysis of enzymic activities in the strain variants with deletion of some specific characters showed the following correlation: in variants with poor or zero kelthane degradation the metapyrocatechase and salicylate hydroxylase were virtually not available (Table 5). Consequently, despite the fact that the soil-released strain – degrader of a xenobiotic can survive and bring about degradation for a long period, some variants may in time emerge which would be either devoid of degradative properties or display much poorer degradation ability compared to the parent strain. The possibility of the emergence of variants with unpredictable characters is not ruled out. This possibility should be always kept in mind while deciding upon the advisability of the use of specific microbial species for abating levels of xenobiotics in the environment.

Thorough studies carried out in a number of laboratories with TOL, SAL, pAC25, pAC27, and pAC28 plasmids which encode resistance to antibiotics showed that in the course of selection they may undergo certain structural rearrangements leading to formation of novel

plasmids, the latter emerging by incorporation of genes from other plasmids. Thus, hybridization of ^{32}P-labelled DNA of pAC25 plasmid with EcoRI fragments of restriction of SAL, TOL and PAC30 plasmids clearly pointed to a certain homology of pAC25 with SAL, TOL and, to some extent, pAC30 plasmids. Plasmids of the pAC25 type may apparently emerge as a result of recombination of various fragments from plasmids of the SAL and TOL type which specify biodegradation of analogous nonchlorinated compounds, whereas a homology with the pAC30 plasmid may be in the region of replications, maintenance or transfer of plasmid genes.

TABLE 5. Activities of key enzymes of the oxidation of aromatic compounds in *P. aeruginosa* BS827 and its variants.

Phenotype	Enzyme activity, nmol/min·mg protein									
	Metapyrocatechase			Pyrocatechase			Salicylate hydroxylase			
	Substrate			Substrate			Substrate			
	*BA	PCA	Gl	BA	PCA	Gl	BA	PCA	Gl	SA
Nah^+Sal^+	8	72	141	500	0	0	12	0	4	70
Nah^+Sal^-	0	3	18	63	0	0	0	0	0	0
Nah^-Sal^+	0	0	0	290	0	87	0	0	6	18
Nah^-Sal^-	0	0	0	181	0	0	0	0	0	0

* BA - benzoic acid, PCA - protocatechoic acid, Gl - glycerol, SA - salicylic acid; concentration of substrates - 10 mM.

Our 5-month experiments in a soil with mixtures of *Pseudomonas* strains containing different biodegradation plasmids clearly indicated that at least two plasmids TOL and pBS271, which control degradation of toluylate and ε-caprolactam, respectively, could be transferred to other *Pseudomonas* strains free of plasmid DNA (Table 6).

Taking into account that under natural conditions new plasmids emerge by union of genes from other plasmids and that they interact thus broadening the set of xenobiotics sensitive to microbial attack, there appears a possibility to engineer novel strains in the laboratory under strictly selective conditions when the persistent xenobiotic is the sole source of carbon. The example is degradation of 2,4,5-T brought about by researchers from the Illinois University (Kellogg *et al.*, 1983). In the

beginning, laborious attempts to isolate a strain able to degrade 2,4,5-T were unsuccessful. The authors succeeded in isolating the culture *Pseudomonas cepacia* AC1100 which was capable of utilizing 2,4,5-T as the sole carbon source in concentration of 1.5-2.0 g/l. The culture has been isolated after prolonged cultivation in the chemostat of a mixture of *P. putida* strains carrying the plasmids CAM, TOL, SAL, pAC21, pAC25 and others, with microbial samples of soils from different polluted areas. A mixed culture isolated from the fermenter had further been maintained for a long period in flasks with increased concentrations of 2,4,5-T.

TABLE 6. Selection of transconjugants by D-plasmid markers amongst all strains released to soil.

Donor strain	Plasmid	Selective medium for selection of transconjugants	Titre of transconjugants in soil samples 2,45-T	2,4,5-TCP	Control
BS853	pBS256	3-CB, streptomycin	-	-	-
BS848	pBS271	Cap, rifampicin	-	-	2 10
		Cap	10	-	-
PpG9(TOL)	TOL	*m*-toluate+rifam-picin	2 10	10	10
BS817	pBS2	Sal+streptomycin	-	-	-
		Nah+streptomycin	-	-	-
BS856	pBS4	Sal+streptomycin	-	-	-
		Nah+streptomycin	-	-	-

The pure culture isolated by the authors proved to be capable of degrading 2,4,5-T in samples of different garden soils. Over 90% of the introduced toxicant was degraded in 4 days in the experiment, whereas only unessential removal of the pollutant was found in the control. Degradation of 2,4,5-T was accompanied by the release of chloride. Various soil samples showed similar results for both low (100 μg/g soil) and high (1070 μg/g soil) concentrations of the herbicide.

The results obtained enabled the authors to conclude that thanks to plasmid interactions it becomes possible to breed mixed cultures and to isolate later therefrom pure microbial cultures using the method of plasmid-assisted molecular breeding (PAMB). It is believed that such

strains may be efficient in local clean-up of areas highly polluted with toxicants of the type of 2,4,5-T. Moreover, the PAMB technique may enable selection of strains able to degrade chlorinated dioxins, while the release of these strains into polluted soil and water may be instrumental in tackling the problem of removal of these toxicants from the environment.

The second most important approach in ecological biotechnology which has been developed intensively in recent years is the replacement of persistent pesticides by biological means of plant protection, i. e. antibiotics, mycoherbicides, and bacterial toxins produced by microorganisms, as well as inoculations of bacterial biomass capable of effective protection of plants against various disease vectors. Inoculation of plant seeds and roots with such bacteria as *Bacillus sp.*, *Agrobacterium rediobacter* K-84, *Pseudomonas sp.*, *Chaetomium sp.* and others was shown to render the plants disease-resistant. The experiments were conducted under field conditions. Many *Pseudomonas* species were found to produce antibiotics and toxins that are also very effective as preparations for plant protection. These are heterocyclic nitrogenous compounds of the type of phenazines and pyrrolnitriles. Bacteria of the *Pseudomonas* genus can produce iron-binding compounds termed siderophores. These compounds were also shown to protect plants and contribute to their growth. A great number of fungi were investigated for their mycoherbicidal activity, and many of them have been found to be efficient in abating specific weeds that are highly resistant to conventional chemicals.

Thorough studies conducted by researchers from the Merck Sharp company resulted in the discovery of very interesting compounds - avermectins (Fig. 4). This group embraces eight closely related macrocyclic lactones isolated from a novel streptomycete - *Str. avermetilis*.

They exhibit potent activity against a number of important human, animal and plant parasites. 22,23-dihydroavermectin B_1 (ivermectin) is widely used as an endectocide in animals where it is highly effective against most pathogenic nematodes and arthropods. Ivermectin is also currently under investigation for treatment for human infections with

the filarial worm *Onchocerca volvulus*, the causative agent of human onchocerciasis which often leads to blindness. Avermectin B2 (abamectin) is under development as an agricultural pesticide and also it is toxic to all arthropods. At some level it is generally more toxic against acarine species and less potent against insects. Recent studies aimed at modification of avermectin B1 have resulted in the discovery that 4-epiamino-4-dioxyavermectin is approximately one hundred fold more active than the initial avermectin against the southern armyworm *Spodoptera cridania*.

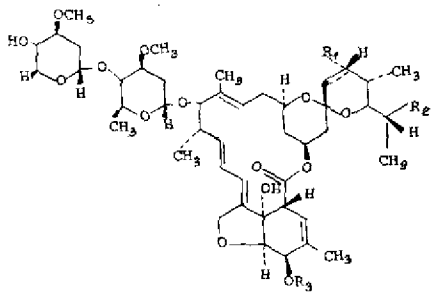
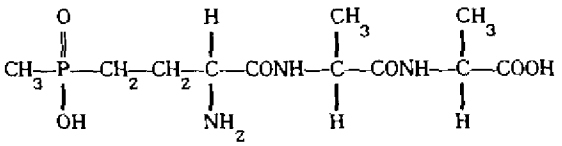


FIGURE 4. Structure of avermectins.

Avermectin	R1	R2	R3
A1a		C2H5	CH3
A1b		CH3	CH3
A2a	OH	C2H5	CH3
A2b	OH	CH3	CH3
B1a		C2H5	H
B1b		CH3	H
B2a	OH	C2H5	H
B2b	OH	CH3	H

Another interesting preparation is bialaphos which has been developed by the Japanese firm Meiji Seika. It is synthesized by *Streptomyces hygroscopicus*. This is the first herbicide ever produced by fermentation. Its structure is rather unusual and, in general, it is quite a unique tripeptide phosphinotriacylalanylalanine:



The fungus *Phytophthora palmivora* is being effectively utilized in the USA against weeds on citric plantations. The preparation is manufactured by Abbott Co. under the name of 'devine'. Another commercial preparation manufactured in the United States by Upjohn Co. on the basis of *Collototrichum gloesporoides* fungus is called 'collego' and widely used for abating wild herbs in rice and soya-bean fields. The devine is applied as a liquid suspension of chlamydospores in concentration of 6.7 10^4 spores/ml in the proportion of 2.5 l/ha. The collego is employed in the form of dry powder containing 15% sporous and 85% inert ingredients and sprayed from airplanes, or using specially equipped tractors, in quantities of 25–40 g/ha.

In Australia, the rust fungus *Puccinia chondrillina* proved to be efficient for combating weeds in wheat fields.

The fungus *Fusarium solani* isolated from infected seeds and germs of the Texas pumpkin showed good herbicidal activity. Detailed studies conducted in field and greenhouse conditions pointed to the fungus applicability for abating the Texas pumpkin - a vicious weed on soya-bean plantations. The fungal preparation is applied as a liquid suspension in greenhouse experiments or as a granulated material of sand and corn meal containing 5% of fungal mass. The fungus displayed herbicidal activity for 12 months, but after this period its biomass in soil was no longer sufficient for killing sprouts of Texas pumpkin.

Utilization of biological means for plant protection faced the biotechnologist with a number of problems related, in the first place, to the need for production of important quantities of biomass; secondly, to methods of release into soil of the microorganisms themselves and of the substances synthesized by them; thirdly, to problems of their safety to man and the environment. There are proposals for the use of sodium alginate gel for preparing tablets with fungal mycelia, or with conidia and fungal mycelia, etc. The future of this field of biotechnology appears bright but progress will depend on the joint effort of molecular biologists, plant physiologists, ecologists, microbiologists and biotechnologists. These and other aspects of the problem will be examined in greater detail in the lecture of Dr. D.N. Chermensky.

REFERENCES

Cain R.B. 1981. Microbial degradation of surfactants and 'builder' components. In: *Microbial Degradation of Xenobiotic and Recalcitrant Compounds* (Leisinger T., Cook A., Hütter R., Nüesch J., eds.) N.Y.-L.: Acad. Press P. 326.

Campbell W.C., Burg R.W., Fisher M.H., Gybos R.A. 1984. The discovery of ivermectin and other avermectins. In: *Pesticide Synthesis through Rational Approaches*. Ch.1, p. 5. Amer. Chem. Soc., N.Y.

Chakrabarty A.M. 1980. Plasmids and dissimilation of synthetic environmental pollutants. In: *Plasmids and Transposons: Environmental Effects and Maintenance Mechanisms* (Stattard C., Rozee K.R., eds.) N.Y.-L.: Acad. Press, p. 21.

Chakrabarty A.M., Friello D.A., Bopp L.H. 1978. Transformation of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. *Proc. Nat. Acad. Sci. USA* 75: 3109.

Chatterjee D.K., Chakrabarty A.M. 1981. Plasmids in the biodegradation of PCBs and chlorobenzoates. In: *Microbial Degradation of Xenobiotic and Recalcitrant Compounds* (Leisinger T., Cook A.M., Hütter R., Nüesch J., eds) N.Y.-L.: Acad. Press, p. 213.

Golovleva L.A., Pertsova R.N., Boronin A.M., Grishchenkov V.G., Baskunov B.P., Polyakova A.V. 1982. Degradation of polychloroaromatic insecticides by *Pseudomonas aeruginosa* carrying biodegradation plasmids. *Mikrobiologiya* 51:973 (in Russian).

Karns J.S., Duttagupta S., Chakrabarty A. 1983. Regulation of 2,4,5-trichlorophenoxyacetic acid and chlorophenol metabolism in *Pseudomonas cepacia* AC 1100. *Appl. Environ. Microbiol.* 46:1182.

Karns J.S., Duttagupta S., Chakrabarty A. 1983. Metabolism of halophenols by 2,4,5-trichlorophenoxyacetic acid-degrading *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 46:1176.

Kilbane J., Chatterjee D., Karns J., Kellogg S., Chakrabarty A. 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 44:72.

Knackmuss H. 1981. Degradation of halogenated and sulfonated hydrocarbons. In: *Microbial Degradation of Xenobiotic and Recalcitrant Compounds* (Leisinger T., Cook A., Hütter R., Nüesch J., eds.) N.Y.-L.: Acad. Press, p. 190.

Negoro S., Shinagawa H., Nakata A., Kinoshita S., Hatozaki T., Okada H. 1980. Plasmid control of 6-aminohexanoic acid dimer degradation enzymes of *Flavobacterium sp.* KJ72. *J. Bacteriol.* 143:238.

Walker L., Connick W. 1983. Sodium alginate for production and formulation of mycoherbicides. *Weed Sci.* 31:333.

Weinberger M., Kolenbrander P.E. 1979. Plasmid-determined 2-hydroxy-pyridine utilization by *Arthrobacter crystallopoides*. *Canad. J. Microbiol.* 25:329.

SORPTION AND PHASE DISTRIBUTION OF PESTICIDES IN SOIL

V.P. Sukhoparova and N.D. Ananyeva

The process of sorption-desorptional interaction of pesticides with the soil is for researchers a subject for continual studies. A large body of knowledge has been accumulated to date on problems of pesticide adsorption by the soil solid phase. Various aspects of the pesticide sorption-desorption have received a detailed coverage in numerous reviews and papers rich in substantiated experimental results. In this lecture we shall briefly examine the role played by the physico-chemical properties of pesticides and by the basic characters of the soil sorptional complex (such as the content of organic matter and clay minerals) in the process of pollutant interaction with soil. We shall also attempt to feature the actual state of another, no less important aspect that is inseparably linked with the sorption problems, namely the phase distribution and redistribution of pesticides in soil, and the methods that are likely to provide us with both qualitative and quantitative information on the forms of pesticide occurrence in soils.

The phenomenon of sorption-desorption interaction of pesticides with soil is an exceedingly complex one. It is affected by both the structure and properties of the sorbates, and the character of a soil, or rather the soil absorbing capacity. The sorptional capacity of soil is in a great measure determined by the nature of functional groups and

substituents, position of substituents with respect to functional groups, the character of molecular charges, and the polarity of molecules. Thus, studies of the arylamidic herbicides sorption revealed that their sorptional properties depend on the structure of the aromatic part of the molecule. The introduction of various substituents into phenyl radical causes a shift of the electron density, and, subject to this, the sorptional characters of toxicants are weakened or enhanced. Those of herbicides, for instance, are strengthened by the fixation of halides. Table 1 features a classification of pesticides based on the characters of pesticide linking in the course of adsorption.

TABLE 1. Classification of pesticides based on their behaviour during adsorption (1)

Group	Class	Name
Ionic: cationic	Dipyridyls	Diquat, paraquat
alkaline	Sym-triazines	Atrazine, propazine, simazine, prometryne.
	Sym-triazoles	Amitrole
Acidic:	Chlorophenoxy-acetic acid	2,4-D
	Benzoic acid	Dicarbam, amiben
	Picolinic acid	Cycloram
	Phenols	Dinoseb
Non-ionic:	Organohalogenic hydrocarbons	DDT, endrin, dieldrin, lindanee, heptachlor, toxaphene
	Phosphororganic compounds	Parathion, diazinon
	Phenylcarbamates	Propham, chlorpro-pham, carbaryl
	Phenylurea	Fenuron, monuron, linuron
	Anilide	Propachlor, propanil
	Phenylamide	Diphenamid
	Thiocarbamates	Eptam, CDEC, aldicarb
	Benzonitriles	Dichlobenil

The extent of adsorption is also affected by important characteristic of pesticides such as solubility: adsorptional retention of compounds weakens with growing solubility.

Thorough studies of the sorption of pesticides by soils enabled

researchers to develop methodological approaches to its prediction for natural conditions. Thus in 1969, Hance (2) proposed an equation for predicting the sorption of compounds capable of forming hydrogen bonds:

$$\lg k = 0.067(P - 45N) - 0.65$$

where k is the constant from Freundlich's equation; P is the parachore; N – number of substituents in a molecule which are capable of forming hydrogen bonds.

Comparison of the results obtained by the above method with those from experiments, however, gives a poor - or rather an unsatisfactory correlation.

In a recent study, an attempt was made to use the method of molecular connectivity aimed at dovetailing information on chemical structure of a pesticide molecule (molecular size, ramification of lateral chains, availability and saturation of heteroatoms) and experimental data (distribution index in the system octanol:water, adsorption coefficient, organic matter content). Yet, 46 pesticides referred to different classes exhibited poor correlation (3).

The above studies stress once again the complexity of pesticide sorption by the soil and the dependence of this process on numerous factors.

Thus it may be stated that sorption of pesticides by soil is in a certain measure controlled by their physico-chemical properties and by specific features of chemical structure. This process is also essentially affected by the properties of a soil and this will be the subject of the discussion that follows.

Sorption of pesticides by the soil is known to be regulated by its sorptional capacity which in turn depends upon the characters of constituent colloids: organic, mineral and organo-mineral ones.

At present, the general consensus is that organic part of the soil is a major contributor to the process of pesticide adsorption. Pesticides are basically organic substances and their molecules display a high affinity to soil humus compared with the mineral portion of the soil. This is evidenced by numerous studies conducted with various classes of pesticides.

Sorptional binding of arylamidic herbicides (fenuron, monuron, arezine, metobromuron, diuron, linuron, chlorobromuron) correlated well with the content of organic matter in soil, whereas the presence of clay minerals had little effect on their adsorption (4). Adsorption of an organophosphate, azinphos-methyl by 19 soil samples (pH: 4.5–7.9; organic matter: 0.03–13.1%; clay: 8.7–62.0%) correlated with the content of organic matter, the clay fraction contributing only after removal of the soil organic matter (6). Investigation of the distribution of simazine over granulometric fractions of the soddy-podzolic soil revealed that after a 4-month interaction of the toxicant with the soil, 73 to 83% of the simazine had accumulated in the finest fraction (0.001 mm), which accounted for about 0.1% of the total soil mass (7).

The specific surface of soil particles was reported by many authors not to play a decisive role in simazine sorption. The distribution of the toxicant over the soil fractions correlated with that of humus. This afforded a conclusion on the prevalent role of humus in simazine sorption by podzolic soils.

Sorption of pesticides by the organic portion of soil depends not only on the content but also the qualitative composition of humus (8–10), and in peaty soils, on the measure of peat decay (11). Thus a study of arylamidic herbicide sorption by the meadow-alluvial, soddy-podzolic (humus content: 1.8 and 1.5%, respectively) and humic-peaty (organic matter content – 73.8%) soils showed that the amount adsorbed increased with the percent of organic matter. Yet, the bonds formed by arylamidic herbicides with the humic-peaty soil, which are rich in organic matter, exhibited rather low strength. In this case, the use of a mixture of solvents with the differing polarities for desorption purposes allowed nearly a quantitative extraction of herbicides from the 'sorbate/humic-peaty soil' complex (12). This observation suggests that the widely held opinion that 'the more organic matter in soil, the stronger sorptional retention of pesticides' (13–14) is not quite correct. Such a viewpoint appears to be valid only for comparison of soils apparented by their genesis and quality of organic matter. One should distinguish between the soils containing the organic substance commonly termed humus and those rich in crude organic matter

of which peaty soils are an example.

While studying the process of pesticide sorption by soil, one should bear in mind the significance of the soil mineral part. Thus, a study of propanil sorption by montmorillonite revealed that the herbicide is not only adsorbed at the surface but also finds its way into the interstitial space, i.e. is sorbed irreversibly (15). The uptake of paraquat by the clay minerals proved to be irreversible, and microorganisms did not degrade it (16). Isouron is better sorbed by soils rich in montmorillonite as compared to those high in kaolinite. A direct correlation was traced between the content of the argillaceous montmorillonitic fraction and the extent of toxicant adsorption (17). The sorptional capacity of the montmorillonitic group was found to be 10- 15 times that of kaolinite. Therefore, the idea that organic matter plays a dominant role in the sorption of xenobiotics by soils with high content of kaolinite compared to those rich in montmorillonite (18) does not appear groundless. The dominant factor in soil-pesticide interaction is attributed to the colloidal particles of the soil forming the organic and mineral fractions (19), which are called the 'colloidal complex' of the soil (1). With the above examples we attempted to show that in nearly all cases the authors apparently tried not only to investigate the peculiarities of pesticide sorption-desorption by various soils but also to elucidatie the role of major components of the soil solid phase in xenobiotic binding. Knowledge of the contribution of specific components of the soil absorbing capacity may become instrumental in unravelling the mechanism of pesticide residue binding with the soil. A methodic approach was developed to this end : it consisted in treating the soil with appropriate reagents, which would decompose or extract particular components of the soil absorbing capacity. After such a treatment the soil samples are utilized for investigating sorptional mechanisms and elucidating the role of specific components in pesticide binding. Thus, in studies of atrazine sorption, use was made of a native soil: its humus-free portion obtained by the treatment with hydrogen peroxide (according to Hedroitz), the mineral part free of sesquioxides (after a treatment with hydrogen peroxide and subsequent removal of R_2O_3 following Jackson's technique) and humic acids isolated from native

soils by extraction with 0.1 N sodium hydroxide solution followed by reprecipitation with sulphuric acid. Organic matter was shown to be the principal sorbent of atrazine in soddy -podzolic and chernozemic soils, while in the krasnozem the involvement of sesquioxides (whose content is high in krasnozem) was essential, since their removal resulted in a drastic decrease in the sorption extent (20).

Adsorption of atrazine by a sandy clay and a loamy soil was also noticeably inhibited by the removal of the soil organic matter followed by that of iron and aluminium oxides (21).

Thus preparation of soil sorbents of varying sorptional capacity by specific treatments of the soil makes it possible to demonstrate the quantitative role of individual components of the soil in the binding of xenobiotics.

In this context, a few questions arise: in what way can one identify the pesticide bound to organic components of the soil, what part of it is sorbed by mineral particles, and how to quantify its distribution in the soil? Under natural conditions, a pesticide is known to be distributed over the soil constitutive phases (let us call it the phase distribution) and becomes eventually bound to them. This fact predetermines its further behaviour in local environments: herbicidal effect, accessibility to microorganisms, persistence, degradation rate etc. The xenobiotic may be degraded or transformed by microorganisms provided it is uniformly distributed in the soil liquid phase and is not bound to its organo-mineral colloids. Pesticide residues sorbed by the soil solid phase were shown to remain practically unaffected by soil microorganisms (22). Plants also take up from the soil mainly the dissolved portion of herbicide, i.e. that contained in the soil liquid phase. The binding of pesticide residues lowers appreciably their accessibility to plants.

Numerous studies suggest that the rates of xenobiotic removal from soil (during vegetation period) vary depending on the residence time of the toxicants in soil. The rate of removal of HCCH which resided in soil for a long time is ten times less when compared to that of recently applied HCCH (23). The authors attribute this to an enhanced sorptional fixation and to changes in the character of its localization in the

soil. Another example shows that during the first several months after application of lindane, the highest rate of its removal from soil was observed. After three months, the soil contained only 10% of the applied quantity of lindane and this percentage did not drop further for quite a long period (23, 24). It may be suggested that there occurs a redistribution of xenobiotics amongst soil components (phase distribution), which is the cause of the different residence times of their residues.

Analysis of the relevant literature and experimental data obtained by the authors support the idea that xenobiotics are not, as a rule, totally degraded, and their residues, on the average, may persist for rather a long time in quantities of 10 to 20% of the applied amount. It is noteworthy that numerous data underline the differing characters of toxicant binding to the soil solid phase, thus implying various forms of pesticide linking with components of the soil sorptional complex. From now on we shall use the term 'form of pesticide' for designating specific types of pesticide binding to soil. But what will be the fate of pesticide residues and to what soil constituents are they strongly bound? What factors are responsible for high persistence of pesticides in the soil? In what manner does the redistribution of a pesticide occur? All these questions are intimately related to our understanding of the state of the pesticide in the soil and, first and foremost, to its phase distribution amongst the organic and mineral particles of the soil.

To date, two basic trends (or rather two lines of studies) have been observed in an endeavour to give substantiated answers to the above questions.

The first line of research is based on investigating the dynamics of pesticide removal from the soil during the vegetation period, estimating the rate constants criteria such as T_{50}, T_{95}, and T_{99}, T_{mpc} (*mpc* - max. permissible conc.) and revealing any correlation of the latter values and residue levels, with soil characters and various ecofactors. The investigation of the dynamics of pesticide disappearance, though an important and necessary condition in the studies of pesticide persistence, is unable to explain the origin and

identify residues bound by soil components. All such experiments, though labour- and time-consuming, are advisable and should be encouraged. The first step in the studies of particular features of pesticide degradation consists in determining the kinetic characteristics (rate constants and various T criteria). We believe, however, that the dynamic pattern should be supplemented with characterization of the toxicant phase distribution (also dynamic) in soil. Therefore the second trend, or the next step, is called for, and it should deal with studying the fate of xenobiotics in the soil. This concerns the identification of various forms of xenobiotic binding and establishing their phase distribution in soil. This line of research is intermingled with unravelling the problem of bound (non-extractable) pesticide residues, i.e. identification of the portion of residual pesticides that resist isolation by conventional extraction techniques. It should be pointed out that a uniform methodological approach to investigating pesticide forms in the soil has not yet been developed. Neither is there a general consensus in interpreting the data on phase distribution of xenobiotics in soil.

Our earlier studies (12) showed that the poorly, medium and strongly adsorbed portions of pesticides may be extracted from the soil with properly selected extractants differing by their physico-chemical characters, while the use of specific techniques for treatment of soil samples enabled the identification of the bound form of adsorbed compounds.

Desorption of arylurea-derived herbicides and chlorinated anilines from humic-peaty, soddy-podzolic and meadow-alluvial soils with various extractants under static conditions showed that water extracted from 18 to 60% of the poorly bound pesticides, acetone - 19 to 65% of the medium bound and a mixture of solvents with differing polarities from 50 to 100% of the strongly bound forms. The content of bound hydrolysable toxicants in these soils was 16 to 48%, depending on the type of soil and the character of compounds. Such classification of pesticidal forms made it possible to show varying strength of arylamidic herbicide residue binding with soil but did not provide sufficient data for quantitative assessment of the role of various components in pesticide

sorption, i.e. for elucidating the mechanism of binding. Moreover, the concept of 'bound residues' presupposes the necessity of identification of the soil sorptional complex components to which they are bound and which may be the cause of irreversible xenobiotic sorption.

A successful attempt to elucidate the role of some soil compounds in the binding of simazine may be examplified by the isolation of five forms under which it is present in the soil (25):

1. Free: is extracted from soils by displacement with saturated zinc chloride solution whose density is higher than that of simazine;
2. Reversibly sorbed: is isolated by treatment with 1M KOH solution;
3. Irreversibly sorbed: is extracted by the mixture of organic solvents - chloroform:methanol (1:1);
4. Simazine included into molecular composition of humic substances: is extracted from soils with 2% NH_4OH followed by gel chromatography of the complex;
5. Simazine as a constituent of plant residues: is extracted with the mixture chloroform:methanol (1:1) after isolation of plant biomass from soil and hydrolysis.

The above forms of simazine correlate with their residence times in the soil:

free - from 1 to 20 days;
reversibly sorbed - from 20 to 150 days;
irreversibly sorbed - from 15 to 700 days;
humus-bound simazine - depending on soil humus characters;
plant residue-bound simazine - from plant death to complete decay after burial in the soil.

In the method proposed by Yu.V. Kruglov (26), the free simazine is extracted by chloroform from soil suspensions prepared on the basis of chernozem, serozem, zheltozem, peat, and soddy-podzolic soils. Herbicide residues bound to humates are extracted with 0.5 M sodium hydroxide solution. Herbicide residues bound to mineral portion of the soil were evaluated by difference technique. The author classified the pesticidal forms as free, organic matter-bound and mineral residue-bound.

The above examples demonstrate that researchers make arbitrary classifications of the pesticide forms. To our mind, the scientific

effort aimed at tackling the problems relevant to sorption, binding, distribution and redistribution of xenobiotics must concentrate on developing uniform analytical techniques which would allow isolation of the soil solid components to which pesticide residues are bound. In addition, it is necessary to identify the free and weakly bound toxicant forms that are mainly contained in the liquid phase of the soil; precisely this portion of pesticide controls its uptake by plants, degradation rates, transformation, migration etc.

Numerous studies clearly indicate that the organic matter of soils plays a major part in xenobiotic binding. Hence, the use of such 'mild', for organic matter and pesticide residue bound to it, extractants as sodium pyrophosphate and sodium hydroxide deserve primary attention. Utilization of drastic treatments of soils leading to destruction of the organic compounds is hardly advisable for performing the phase analysis.

In addition to organic colloids the soil sorptional complex also includes a mineral portion composed of argillaceous minerals and amorphous colloids, mainly iron, manganese and aluminium hydroxides. Therefore, a need is felt for assessing the contribution of mineral part of the soil complex to the sorption of toxicants. For certain varieties of soils it may be decisive. It is noteworthy that a good number of reagents are applicable to the isolation of iron, manganese and aluminium hydroxides.

Utilization of conventional and new selective extractants for separating constituents of the soil sorptional complex permits isolation of the bound pesticide forms.

In this connection, we attempted to work out a method for the phase analysis of pesticides based on selective extraction of soil components together with pesticides bouynd to them. As a result, the following classification of the forms of pesticide distribution in the soil was proposed:

1. free (poorly bound);
2. exchangeably sorbed;
3. organic matter-bound form;
4. oxide- and hydroxide-bound form;
5. mineral residue-bound form (27);

Figure 1 schematizes the dynamics of the linuron forms' distribution in the soil.

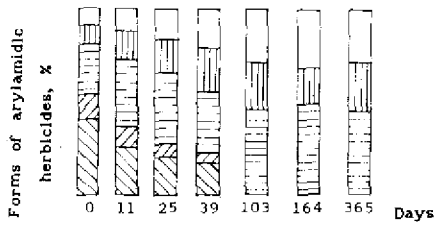


FIGURE 1. Dynamics of the distribution of arylamidic herbicides in a grey forest soil.

- free form;
- exchangeably sorbed form;
- form bound to organic matter;
- form bound to oxides and hydroxides;
- form bound to mineral residue.

It turned out that three months after the herbicide application, the free and exchangeably sorbed forms were no longer detectable. The portion of herbicide bound to organic matter, hydroxides and soil mineral residue increased during the vegetation period. After a three-month period, only firmly bound pesticide residues were extractable, and they resided in the course of one year.

Figure 2 features the depletion of the herbicide with the decreasing content of the free and exchangeably sorbed forms (total) and the disappearance of the portion bound to organic matter (total). The character of the two depletion curves (I and II) is identical, i. e. the observed depletion of herbicide content is determined by the decreasing quantities of its free and exchangeably sorbed forms. This process terminates rather rapidly, essentially at the end of 1.5-2.0 months after the application of the toxicant to soil. It should be stressed that the availability of free and exchangeably sorbed forms provides an explanation for the highest herbicidal effect and the possibility of the intensive degradation of the pesticide by microorganisms during the

first months following its application. After 1.5-2.0 months, virtually all the herbicide still residing in the soil is represented by the bound form, whose depletion is very slow.

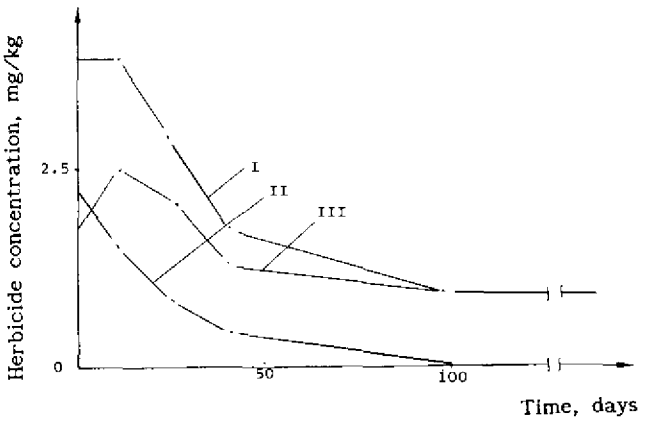


FIGURE 2. Dynamics of arylamidic herbicide concentrations in a grey forest soil.
I - total herbicide content;
II - content of free and exchangeably sorbed forms;
III - content of forms bound to organic matter, hydroxides, and mineral residue.

Thus, the isolation of pesticide forms and studies of their dynamics enables one to identify the character of their binding to soil sorbents and the cause of differing residence times of pesticides in the soil, and to predict the period of their intensive effect on biota. The latter statement appears extremely important from the economic viewpoint as it contributs to the efficient and safe application of chemicals for plant protection.

REFERENCES

1. Reutse K., Krystya S. 1986. *Abating Soil Pollution.* Agropromizdat, Moscow, p.156-176 (in Russian).
2. Hance R.J. 1969. An empirical relationship between chemical structure

and the sorption of some herbicides by soils. *J. Agr. Food Chem.* 17: 667-668.
3. Gerste L., Helling C. 1987. Evaluation of molecular connectivity as a predictive method for the adsorption of pesticides by soils. *J. Environ. Sci. and Health* 22:55-69.
4. Grover R. 1975. Adsorption and desorption of urea herbicides on soil. *Can. J. Soil Sci.*, 55:127-135.
5. Krogmann H., Maass V., Scharpenseel H.W. 1985. Radiometrische Untersuchungen zu Sorptionverhalten and Abbau von C-PCB sowie C-Picliram in verschiedenen Boden. *Z. Pflanzenernahr und Bodenk.* 148:248-259.
6. Sanchez C.M., Sanchez M.J. 1984. Adsorcion de azinfosmetil por suelos. *Agrochimica* 28:148-158.
7. Syunyaev Kh.Kh., Khegay T.A., Fokin A.D., Rachinsky V.V. 1984. Radioindication study of the simazine sorption by soils. *Izvestiya TSKhA* 2:69-74 (in Russian).
8. Hance R.J. 1969. The adsorption of linuron, atrazine and EPTC by model aliphatic adsorbents and soil organic preparations. *Weed Res.* 9:108.
9. Spiridonov Yu.Ya., Shestakov V.G., Matveyev Yu.M., Shabanov A.K. 1981. Sorption of herbicides by major components of the soil. *Agrokhimiya* 3:121-127 (in Russian).
10. Spiridonov Yu.Ya., Shestakov V.G., Matveyev Yu.M., Kozlova I.I., Umnov A.M. 1984. Sorption of herbicides by major components of the soil. Report 6. Sorption of Terbacillum by soils. *Agrokhimiya* 11:126-129 (in Russian).
11. Morita H. (1976) Linuron adsorption and the degree of decomposition of peats as measured by rubbed fibre content and pyrophosphate index. *Can. J. Soil Sci.* 56:105-109.
12. Sukhoparova V.P., Sokolov M.S., Vasilyeva G.K. 1984. Particular features of herbicidal arylureas and chlorinated anilines in the sorptional interaction with soil. *Agrokhimiya* 7:88-103 (in Russian).
13. Polubesova T.A. 1982. Certain regularities of the adsorption-desorptional interaction of pesticides with soil. *Agrokhimiya* 11:120-126 (in Russian).
14. Maier-Bode H., Hartel K. 1981. Linuron and monolinuron. *Res. Rev.* 77:365.
15. Trubin A.I., Sokolov M.S. 1975. On propanil sorption by montmorillinite. *Agrokhimiya* 3:128-132 (in Russian).
16. Burrs R.J., Audus I. 1970. *Weed Res.* 10:49.
17. Imose J. *et al.* 1982. *The Fifth Intern. Congr. Pest. Chem.* Abstracts. Kyoto, Japan.
18. Golovkin G.V., Volovnik L.L. 1976. Sorption of pesticides by soil components. *Khimiya v selskom khozyaystve* 9:48-57 (in Russian).
19. Sethi R.K., Chopra S.L. 1977. Adsorption and behaviour of pesticides in soils. *Pesticides*, 9:15-25.
20. Khlebnikova M.V., Konchits V.A. 1981. Atrazine adsorption by soil adsorbents. In: *Prediction of Pesticide Behaviour in the Environment.* Proceedings of the Soviet-American Symposium. Yerevan, Oct. 1981. Gidrometeoizdat, Leningrad, p.72 (in Russian).
21. Huang P.M., Grover R., McKercher R.B. 1984. Components and particle size fractions involved in atrazine adsorption by soils.

Soil Sci. 138:20-25.
22. Sokolov M.S., Galiulin R.V. 1982. Biodegradation factors of nonvolatile pesticides in soil. In: *Ecological Implications of the Use of Agrochemicals (Pesticides).* Pushchino, ONTI NCBI, p.130-137 (in Russian).
23. Kretova L.G., Fokin A.N., Rachinsky V.V., Borzilov V.A., Dragoluybova I.V. 1985. Effect of the hexachloran residence time in soil on linkage of its residue. In: *Proceedings of the Goskomgidromet Institute of Experimental Meteorology.* No.13/118, p.3-10 (in Russian).
24. Virchenko Ye.P., Bobovnikova Ts.I. 1985. The behaviour of HCCH isomers in soils. In: *Proceedings of the Goskomgidromet Institute of Experimental Meteorology.* No.13/118, p.18-23. (in Russian).
25. Syunyaev Kh.Kh. 1984. A radioindication study on the simazine migration in podzols and chernozems. *Doctorate thesis synopsis.* Timiryazev Agricultural Academy. Moscow, 15 p. (in Russian).
26. Kruglov Yu.V., Kuznetsova I.A., Gordienko S. 1983. Interaction of the herbicide simazine with humic substances. In: *Reports on Humus,* v.II. Prague, p. 481-484 (in Russian).
27. Sukhoparova V.P., Oreshkin V.N., Strekozov B.P., Perfilova N.V. 987. Evaluation of the total phase distribution of pesticides in soil and study of the dynamics of the occurrence of pesticidal forms. *VDNKh Prospectus.* ONTI NCBI, Pushchino, 4 p. (in Russian).

QUANTITATION OF PESTICIDE RESIDUES AND METABOLITES IN SOIL

Z.I. Finkelshtein

Studies of the fate of pesticides in natural environments involve certain specific features:

- low quantities of toxicants applied per unit soil area: this is in particular characteristic of pesticides of the third generation used at concentrations of a few grams per hectare;
- capacity of pesticides and their metabolites to sorption by the soil components: this creates problems for their extraction from the soil and subsequent quantitative analysis;
- a broad spectrum of chemical compounds used to produce pesticidal effects.

Therefore, reliable identification and quantitation of pesticides in soil call for selective, highly sensitive and universal analytical techniques.

High diversity of the structure of pesticides predetermines the use of various methods for their analysis that differ even within specific groups of toxicants. The most common pesticides are the chlorinated organics, organophosphorus compounds, as well as derivatives of symmetric triazines, phenoxyalkanoic acids, urea, carbamates, thio- and dithiocarbamates, pyrethroids etc.

Nonetheless, whatever the group of pesticides, the basic procedure of pesticide residue analysis in soil is common including the following steps.

1. Sample conditioning which embraces soil sampling technique, extraction of pesticides and their metabolites from soil followed by purification and concentration of extracts.
2. Identification and quantitation by chromatographic methods.

1. Preparation of Samples

1.1. Sampling Technique

Sampling is a crucial step in the determination of residual quantities of pesticides in specific environments. Negligent manipulations may be the cause of incorrect analytical results.

Sampling is performed on several different locations of the soil area under study. The quartation technique is used for taking the average sample of about 1 kg from which smaller samples are taken for laboratory investigation. It is advisable to use the minimum weight of soil to spare time during sample treatment and also to save reagents, but a sufficient amount is required to operate within adequate sensitivity limits of the analytical technique employed. Usually, soil samples of 10 to 100 g are sufficient.

The amount of a pesticide residue is commonly expressed in grams or milligrams per 1 kg of air-dry soil. In order to minimize losses of toxicants it is recommended to analyze most recent soil samples with natural moisture levels. Parallel sampling is performed for measuring the moisture content in the soil. The soil is also analyzed for determining pH, organic matter content as well as some other properties.

1.2. Extraction of Pesticides and Their Metabolites from Soil

Extraction of toxicants with an appropriate organic solvent or a mixture of solvents at room temperature is well suited for most analyses. Rarer cases call for distilling with water vapour or extraction in a Soxhlet apparatus.

In most cases pesticide residues are extracted from a moist soil. Extraction from the air-dry soil is practiced only for water-insoluble persistent toxicants. The ratio of soil mass to solvent volume is usually 1:(1–2.5). The extraction is carried out by intensive shaking of

a soil sample with a solvent for 30–90 min. Thereafter the soil is separated by centrifugation, another portion of the solvent is added and the operation is repeated. The two supernatants are combined. If needed, the third extraction may follow.

Depending on the soil type, isolation of pesticides shows specific features, as soils differ by their agrochemical properties and, consequently, display varying retention capacities for pesticides that in turn are characterized by specific chemical structures. The composition of clay minerals, humic substances, the texture of the inorganic part of the soil and other characters are known to essentially affect the extent of pesticide sorption by soils.

A large body of data can be found in literature on the binding to soil of 3-chloraniline, 4-chloraniline, dichloraniline, 2,4-dichlorophenol which are metabolites of the pesticides referred to the phenylureas, phenylcarbamates and other groups. These metabolites form covalent bonds with the soil humus. The size of the soil clay fraction influences sensibly the sorption of aldrin, malathion, and parathion.

Assays are usually performed aimed at checking whether the pesticide extraction is complete: a preset quantity of a pesticide is introduced into a soil. After thorough mixing, the sample is analyzed following the procedure described later in this work. A parallel analysis of the pesticide-free soil is also carried out. The yield (%) of the pesticide introduced is calculated by comparing the applied and found quantities. This factor is used to correct the analysis results. One should also bear in mind the fact that the aging of soil samples results in enhanced capacity to pesticide sorption. Therefore, in addition to fresh applications of pesticides, it is advisable to stage experiments for checking the extractability of toxicants under conditions of prolonged contact of pesticides with a sterilized soil.

The choice of an appropriate solvent for the pesticide extraction is a very important step. An ideal extractive should dissolve the toxicant under study well but must not extract foreign compounds (i.e. be selective), be easily accessible, sufficiently pure, and have a low boiling temperature.

The most frequent solvents for the pesticide extraction from soil are hexane, petroleum ether (light fraction), chloroform, ethyl acetate etc. or mixtures with alcohols (methanol, isopropanol) and ketones (acetone) in various proportions. The effect of pesticide extraction is usually better if it was preceded by moistening the soil with a ixture of water and acetone. The choice of a solvent is somewhat facilitated by keeping to the rule: that a polar solvent extracts polar compounds. While studying toxicants of low polarity, one should remember that metabolites are in general more polar than their parent compounds. It is thus advisable to give preference to mixtures of solvents having differing polarities.

1.3. Purification and Concentration of Extracts

While extracting pesticides from the soils rich in humus, the resultant extract usually contains many co-extractants that interfere with the analysis. Such extracts should be subjected to purification. One of the cleanup techniques most commonly employed is a redistribution of the extract components in an appropriate system of solvents. Good results are obtained for the extraction of some pesticides by amending the water-acetone extractive mixture with calcium chloride. Extracts of some persistent pesticides are purified by oxidation of accompanying compounds with concentrated inorganic acids.

The most popular technique is purification of the extract by column chromatography on silica gel, alumina, activated carbon, florisil. Herein, nonpolar compounds are eluted from a column packed with one of the above adsorbents by a nonpolar solvent, whereas the more polar ones - by a mixture of nonpolar and polar solvents. For the extracts that are only slightly coloured, the purification step may often be omitted.

Concentration of the extracts is carried out by evaporation using a rotor vacuum system with a water bath at 35-40°C. For removal of the more volatile solvents, preference is given to spontaneous evaporation at 20-30°C in a flow of air or nitrogen. Potential loss of the pesticide under study is minimized by concentrating the extracts down to 5-10 ml volume prior to their purification. Further concentration is carried out to obtain volumes of 0.2-0.5 ml. Total evaporation of the solvent should

be avoided, as it is fraught with a substantial loss of the toxicant under investigation.

2. Chromatographic Identification and Quantitation of Pesticides and Their Metabolites

A broad spectrum of physico-chemical techniques is in use for separating complex mixtures of pesticides, and subsequent qualitative and quantitative analysis of the isolated components. Of these, chromatographic methods are the most widespread. The emergence of numerous novel chromatographic techniques and the improvement of those in use have permitted to single out variants such as column, paper, thin-layer and gas chromatography. These chromatographic procedures allow effective isolation of individual components from complex pesticide mixtures. Chromatographic separation is a process in which the constituents of a mixture are repeatedly redistributed between two immiscible phases, one of which is the stationary phase while the other is the mobile one. Separation of compounds occurs either owing to their ability to bind to the sorbent surface (adsorptional chromatography) or due to their partition between the immobile (solid or liquid) and mobile (liquid or gaseous) phases (partitional chromatography) (Table 1).

2.1. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is the simplest and readily accessible method for qualitative and quantitative analysis of pesticides that can be easily performed in any laboratory. It is a variation of liquid chromatography brought about in a thin layer of a sorbent fixed on a solid support. Depending on the character of retention of the components under separation by the sorptional surface, TLC may be subdivided into adsorptional, partitional, ion-exchange, molecular sieve and affinity chromatography.

Without indulging into theoretical considerations relevant to TLC which are described in sufficient detail in numerous monographs, let us examine its practical applications for the analysis of pesticide residues.

TABLE 1. Variations of chromatographic methods

		Stationary phase	
		Solid substance (adsorption)	Liquid (partition)
Mobile phase	Gas	Gas adsorptional chromatography	Gas partitional chromatography
	Liquid	Liquid adsorptional chromatography	Liquid partitional chromatography

Separation of a mixture of compounds on a chromatographic plate (development of a chromatogram) may be performed in any glass vessel of suitable size fitted with a lid and called a chromatographic chamber. A system of solvents is in the bottom of the chamber. It is advisable to operate with chambers saturated with the vapours of the solvent system used. The establishment of equilibrium conditions is favoured by the use of chambers with tight lids or by fixing straps of filter paper to three vertical sides of the chamber. Saturation of the chamber with the solvent vapour enables one to avoid the 'edge effect' under which a compound exhibits lower chromatographic mobility in the middle of a chromatogram as compared to that near its edges (Fig. 1a).

The appropriate choice of a solvent system is critical in the separation of compounds by TLC. Various organic solvents display different abilities to elute specific compounds due to various types of interactions.

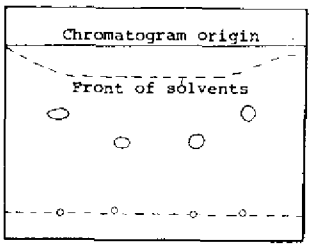


FIGURE 1a. Illustration for the 'edge effect' due to the development in a chamber unsaturated with vapours of solvents.

The simplest means of obtaining a system with the required elution capacity is to mix two solvents with the differing polarities. Determination of their proportion is carried out experimentally, since variation of the elution capacity of solvent mixtures is not linear. A solvent system even with close polarities does not necessarily provide an efficient resolution of a specific group of compounds. Tentative selection of an appropriate solvent system usually calls for the use of Stahl's method according to which a number of spots of the mixture of compounds under study are applied to a chromatographic plate. Thereafter a fairly small quantity of the solvent system is brought to the spot centres with a pipette. The miniature circular chromatograms obtained are helpful for deciding about the system that provides the optimum resolution.

The next step in the analysis of pesticides is the detection of individual compounds on a chromatogram. The coloured compounds do not require any special detection procedure but unfortunately this is not the case with most pesticides. In the first place, if the plate contains a luminophore, it can be examined under UV-light. The spots of organic compounds with cyclic structures or conjugated double bonds are revealed as absorption zones. Thereafter, the detection may be carried out by spraying chromatograms with a suitable reagent that may be of a general-purpose character or specific for certain elements of functional groups. Literature carries extensive lists of reagents that produce colour reactions when specific elements or functional groups are present.

One of the basic characteristics of the TLC is the Rf value which represents the ratio of the distance between the spot centre and the origin (a) to that between the solvent front and the origin (b) (Fig. 1b).

Rf values vary within the range of 0 to 1. The Rf is not a physical constant and is dependent on a number of factors:

- quantity of the sample applied;
- distance between the origin and the bottom edge of the plate;
- depth of immersion into the solvent system;
- extent of the chamber saturation with vapour of solvents;

- purity grade of solvents;
- thickness of the sorbent layer;
- activity of the sorbent layer;
- temperature and humidity of ambient air.

All these should be accounted for while using the tabulated data.

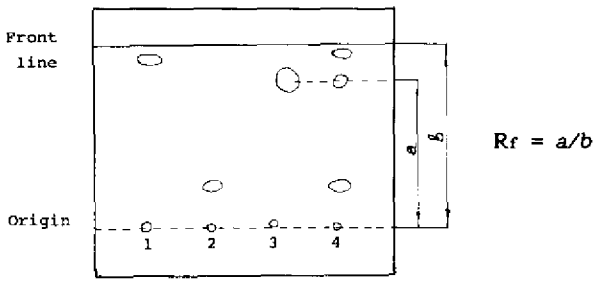


FIGURE 1B. Resolution pattern of a mixture of compounds in an adsorbent thin layer.
1, 2, 3 - individual compounds; 4 - a mixture of compounds.

To identify specific components of the mixture under analysis, some standards of compounds, that may presumably be present in the mixture, are also applied to the chromatographic plate. Compounds may be considered identical if their chromatographic mobilities (Rf values) coincide in 2 or 3 systems of different polarity and when they show the same colour character upon treatment with specific reagents.

To obtain quantitative data on the content of a pesticide in a sample, a number of spots are made on the plate with a gauged capillary by applying aliquots of pesticide extracts and another set of spots are made on the same plate by aliquots of pesticide solutions of known concentration.

A semi-quantitative evaluation of the pesticide content in the sample is made by visual comparison of the toxicant and standard with regard to their spot areas or colour intensity. The spot areas can be measured and used for determination of the toxicant concentration from a calibration graph. The error of such an evaluation amounts to 10-20%. The accuracy of such determination may be increased, but in this case the procedure is complicated by the use of various physico-chemical

detection techniques (densitometry, spectrophotometry, fluorometry etc.).

TLC is well suited for the analysis of pesticides labelled with radioactive markers (radioautography). This facilitates both the identification of the pesticides and their metabolites, and quantitation of pesticide residues.

2.2. Gas–Liquid Chromatography

The method of gas–liquid chromatography (GLC) is particularly suitable for the analysis of residual quantities of pesticides. Its basic principles are as follows. The prepared sample is introduced with a microsyringe through a rubber seal into the injector unit of a chromatograph where it is evaporated and transported by a flow of a carrier gas along the column packed with a support – a solid inert particulate material whose granules of duly selected size are coated with a thin film of a liquid phase. The column is placed in an oven at a preset temperature or the temperature varies between certain limits (programmed temperature).

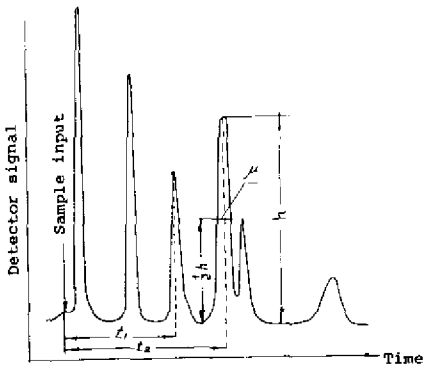


FIGURE 2. A differential chromatogram.
t_1, t_2 are the retention times for compounds 1 and 2; h is the peak height; $\mu_{0.5}$ is the peak width at 1/2 height; $S = h \cdot \mu_{0.5}$.

The liquid phase selectively retains individual components of a mixture of studied compounds transported along the column in the flow of

inert carrier gas. The sample components resolved in the column are eluted after different retention times and recorded on a potentiometric recorder in the form of peaks of differential chromatogram, the abscissa indicating the retention time and the ordinate - the detector signals (Fig. 2).

The GLC technique is highly sensitive enabling determination of compounds present in quantities of 10^{-10} to 10^{-13} g due to detectors that are selective with regard to organic compounds containing halogens, phosphorus, sulphur, nitrogen – all being common functional constituents of pesticide molecules.

This type includes the electron capture detector (ECD), thermo-ion detector (TID) and flame-photometric detector (FPD).

Another unit of GLC chromatograph crucial for the successful resolution of components is the chromatographic column. Routine analyses of pesticides make use of glass columns (0.3–2.0 m x 2 mm), packed with an inert solid support coated with a thin layer of immobile liquid phase. Those in most frequent use for the analysis of pesticides are methylsilicone liquid phases (OV-1, OV-101, SP-2100, SE-30, C-200), which are particularly well suited for analyzing nonpolar pesticides. At the same time, their use for investigating compounds with polar groups is not ruled out.

Polar compounds are fairly well chromatographed on the polyester phases PEGA and DEGS. Correct determination of the quantity of liquid phase to be applied to a solid support is as important as the choice of the liquid phase itself. For modern detectors it is recommended to utilize columns with no more than 2 to 5% of the liquid phase, since the efficiency of such columns has been shown to increase with the diminishing amount of liquid phase. Packed columns are readily obtainable from manufacturers but liquid phase loading and column packing present no problem using vacuum and vibration. The column should be conditioned to remove the phase of volatile compounds by passing a flow of the carrier gas through the column and maintaining the latter at a conditioning temperature ($t_{cond.}$) which is stated in the relevant catalogues, being usually somewhat higher than the maximum working temperature of the phase. For methylsilicone phases it is about 260°C

and does not exceed 230°C for DEGS. The duration of conditioning of the column is not less than 16 hours. During conditioning the èxit end of the column should be disconnected from the detector.

For the routine analyses of residual quantities of pesticides, capillary chromatographic columns (5-100 m x 0.2-0.3 mm) are not recommended, but they are given preference for the separation of complex pesticide mixtures, since they permit more effective resolution and rapid analysis.

As a rule, most pesticides are sufficiently volatile for direct injection from soil extracts into the chromatograph but those having carboxyl groups (e.g. phenoxyalkanoic acids) have to be converted in more volatile derivatives prior to their injection. Various methylation and silylation reactions serve this purpose.

For determination of residual pesticides, an extract aliquot of 0.5-1.0 μl is injected. Introduction of exceedingly high volumes or concentrated samples may result in the overloading of the column and detector. The input temperature is usually some 10-20°C higher than that of the column temperature.

A peak of the toxicant under study is identified using values of the retention time ($t_{ret.}$), i.e. the period of time between the moment of sample injection and the appearance of the peak maximum on a chromatogram. It should agree with the $t_{ret.}$ of the pesticide standard (Fig. 2).

Quantitation of pesticides using GLC is based on the proportional dependence of the peak height (or its area) and the toxicant concentration in the sample. The height of the peak (h) is the easiest parameter to measure. The area of the peak is calculated by multiplying the peak height by the peak width ($\mu_{0.5}$) measured at its half-height (Fig. 2). Compared to its height, the area of the peak is less sensible to variation of chromatographic parameters thus making this mode of calculation more universal. Several methods are currently used to derive quantitative data: absolute calibration technique, method of internal standard and comparison technique. Absolute calibration is based on determining the relationship between the peak height (or its area) and the pesticide content in the sample. A calibration graph is plotted

based on the injection of equal volumes of preset concentrations of the pesticide under study. For determining pesticide concentration in a soil sample, aliquots of its extracts are injected into the chromatograph, and the peak height (area) is measured; the concentrations of the pesticide are determined from the calibration graph. This method suffers the necessity of daily verification of the calibration. Many other techniques, having their own advantages and drawbacks, have been reported in literature.

During the past decades, the Laboratory of enzymatic degradation of organic compounds (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences) has gained substantial experience in the application of chromatographic techniques to studies of the pathways and conditions of microbial degradation of various pesticides. In this context, the dynamics of degradation of the herbicide molinate (a thiocarbamate) has been under a thorough investigation. Quantitation of the toxicant was performed using GLC. Fig. 3 shows the time course of the molinate depletion.

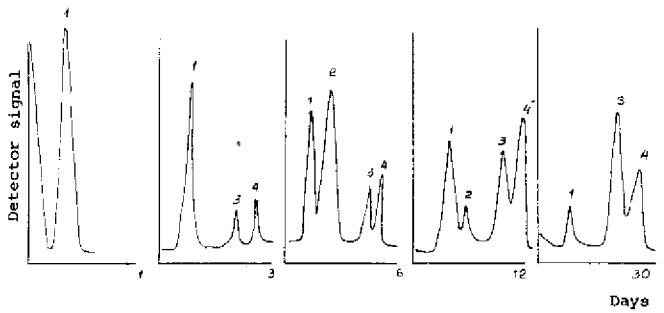


FIGURE 3. Dynamics of molinate bioconversion. 1 – molinate; 2 – molinate epoxide; 3 – keto-derivative of molinate; 4 – molinate hydroxy-derivative.

This work led to the isolation of products of its bioconversion by TLC techniques, and their identification was carried out by methods of chromato-mass-spectrometry and IR-spectroscopy.

Further investigation of molinate metabolites revealed that its keto-derivative proved to be more toxic than the parent herbicide.

Degradation of kelthane (DDT analogue) resulted in simpler metabolites compared to the parent compound, metabolites (Fig. 4) that could apparently be involved in the major pathway of microbial metabolism.

Transformation of the organophosphorus insecticide, phosalone gives 2-amino-5-chlorophenol, that condenses to form more complex and persistent compounds which are a potential hazard to the environment.

In conclusion it should be noted that techniques designed to quantitate the analysis of pesticides are continually being improved. Recent literature describes novel techniques in high performance thin layer chromatography (HPTLC) characterized by the use of precoated plates with a layer of a sorbent composed of a uniform fraction of very small particles. A variation of HPTLC has become competitive with GLC and HPLC in relation to the efficiency and sensitivity.

Use of high performance liquid chromatography (HPLC) has also gained importance in the analysis of residual pesticides. The most conventional method of liquid column chromatography has been 'rediscovered' owing to development of highly effective columns and improvement of detection techniques. This method allows separations and analyses of nonvolatile compounds without derivatization, thermally unstable substances as well as compounds of high molecular weight. Since HPLC is not a destructive technique, it is well suited for semi-quantitative and quantitative analysis as well as for quantitation of trace amounts of pesticides.

Recommended Literature

1. *Analytical Methods for Pesticides and Plant Growth Regulators. Modern Analytical Techniques.* 1986. V. XIV (G. Zweig, J.Sherma, eds.) Academic Press.
2. McNair H.M., Bonelli E.J. 1967. *Basic Gas Chromatography.* Consolidated Printers, Oakland, California.
3. Shellard E.J. 1984. *Quantitative Paper and Thin-Layer Chromatography.* Academic Press.
4. Kirchner J.G. 1978. *Thin-Layer Chromatography.* V.1, 2. J.Wiley & Sons.
5. Klisenko M.A., ed. 1983. *Determination of Microquantities of Pesticides in the Environment, Feed and Food Stuffs.* Moscow, Kolos, (in Russian).
6. Klisenko M.A., ed. 1984. *Quantitation of Microamounts of Pesticides.* Moscow, Meditsina (in Russian).

7. Sharshunova M., Shwartz V., Mihalec Ch. 1980. Thin-Layer Chromatography in Pharmacy and Clinical Biochemistry. V. 1, 2. Moscow, Mir (in Russian).
8. Batora V., Vitorovic S., Thier H.P., Klisenko M.A. 1981. Development and evaluation of simplified approaches to residue analysis. In: *Pure and Applied Chemistry*. 53:1051-1080.
9. Hemingway R.J., Aharanson N., Greve P.A., Roberts T.P., Thier H.P. Improved cost-effective approaches to pesticide analysis. 1984. In: *Pure and Applied Chemistry*. 56:1131-1152.
10. Roberts T.R., Klein W., Still G.C., Kearney P.C. *et al.* 1984. Nonextractible pesticide residues in soils and plants. In: *Pure and Applied Chemistry* 56:945-956.
11. Ambrus A., Thier H.P. 1986. Application of multiresidue procedures in pesticides residues analysis. In: *Pure and Applied Chemistry* 56: 1035-1062.

MICROBIAL DEGRADATION OF CHLORINATED AROMATIC COMPOUNDS

R.N. Pertsova

Organochlorine pesticides are among the most efficient and widely spread classes of chemicals used for plant protection. These include herbicides - chlorinated derivatives of phenoxyacetic acid (2,4,5 -T; 2,4-D, MCPA); insecticides - DDT and its analogues (kelthane, methoxychlor, methiochlor), chlorinated derivatives of cyclohexane (lindane, heptachlor) etc. Utilization of these pesticides, however, creates potential risks for the biosphere. This is due to high recalcitrance of chlorinated compounds: DDT, for example, persists in the environment for up to 20 years, while aldrin - for up to 12 years.

Many chlorinated organic compounds undergo only slight changes in the environment, and their metabolites migrate in the biosphere together with the parent pesticide.

It is also noteworthy that environmental transformation of chlorinated pesticides may result in formation of metabolites which are no less toxic and at times prove to be even more hazardous.

One such example is the herbicide lindane (γ-hexachlorocyclohexane) capable of producing its α-isomer which displays carcinogenic characters.

Chlorinated pesticides have been found in soil, water, plants, animals,and even the human body, despite the fact that the recent years

have seen substantial reduction in the scope of application of these pesticides, the drop in concentrations in local environments is insignificant. Therefore, the need for abating their levels in the environment is nowadays an important problem.

The present lecture outlines one of the perspective approaches to solving the problem of the environment clean-up from chlorinated pesticides based on their microbial degradation. A review is also presented of recent progress in screening and selecting microbial strains capable of degrading chlorinated pesticides, investigating routes and mechanisms of pollutants bioconversion by active cultures, as well as novel microbial technologies for cleaning the environment of persistent toxicants.

Resistance of chlorinated organics to microbial attack is related to three basic causes: (*i*) absence in most soil-based microorganisms of enzymes capable of biotransforming such compounds, (*ii*) no transport into the cell and (*iii*) formation of metabolites toxic to microorganisms. For example, *Pseudomonas putida* mt-2 grown on 3-chlorobenzoate produced halogenated catechol. The 3-chlorocatechol produced could not be metabolized by the culture, as it inactivated the enzyme required for further metabolism of the 3-chlorocatechol. Other metabolites of chloroorganic compounds are also known to exhibit biocidal properties. Yet, soil microorganisms exist which are able to degrade chlorinated pesticides. These are, as a rule, mixed cultures of 2-4 strains, and in rarer cases - pure cultures.

Later, there will be more discussion of active strains, but for the moment, let us consider in greater detail the possible metabolic routes for chlorinated organic compounds.

Dehalogenation is the most important initial step of the degradation of chlorinated organic compounds. Enzymic mechanisms operating during dehalogenation have not yet been adequately studied. Nonetheless, the information available on the subject allows one to single out a few reaction types: (1) reductive dechlorination, (2) dehydrochlorination, (3) hydrolytic dechlorination.

1. Reductive Dechlorination

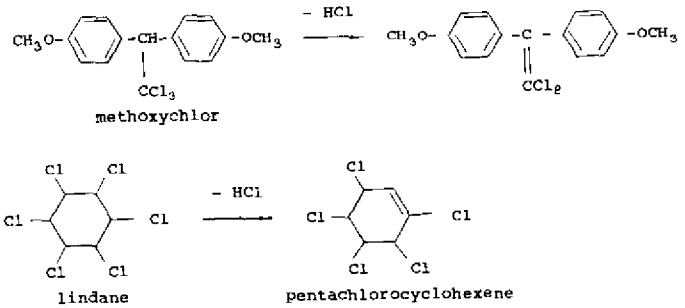
This reaction involves the substitution of a halogen atom by hydrogen. A classical example of reductive dechlorination is microbial transformation of DDT:

Reductive dechlorination has also been reported to occur in chlorophenols, chlorobenzoates, and chlorophenoxyacetic acids.

2. Dehydrochlorination

This reaction is accompanied by simultaneous removal of a halogen atom and a hydrogen from the neighbouring carbon atom.

Such a mechanism was proposed as the initial step in the degradation of DDT-analogues and lindane:



3. Hydrolytic Dechlorination

This is a nucleophilic substitution of a halogen by a hydroxy group. Like other dechlorination reactions, this route of enzymic dehalogenation offers removal of a halogen without affecting the

aromatic ring. Hydrolytic dehalogenation is known to occur in 3- and 4-chlorobenzoates.

Dehalogenation is followed by another no less important step in the degradation of polychlorinated organic compounds, i.e. the cleavage of aromatic rings. Fission of bicyclic compounds such as DDT and its analogues starts, as a rule, with the cleavage of one benzene ring to produce aromatic acids, mainly benzoic acid. Such a route has been described for DDT metabolites - *bis*(4-chlorophenyl)methane and chloro-diphenylmethane.

Under aerobic conditions, oxidation of benzoic acid may be induced by the benzoate-oxidase system leading to catechol or hydroxylation may be brought about by hydroxylases to form protocatechuic acid.

During aerobic decomposition of catechol and protocatechuic acid, the following types of reactions are possible:

- ortho-cleavage (intradiolic) between two hydroxylated carbons;
- meta-cleavage (extradiolic) between one hydroxylated carbon and the neighbouring hydroxyl-free carbon.

The products of benzene ring fission may be utilized by the cell as growth substrates. Such an enzymic combination is very unusual for microorganisms and is rather rarely found under natural conditions. Therefore, the search for microorganisms capable of degrading chlorinated organics is a laborious task. Isolation of such active strains is effected from natural sources that have been in a long-term contact with the chlorinated compound under study or with one of its structural analogues. It is assumed that in places of contact of the natural microflora with the pollutant, active microbial forms capable of assimilating the xenobiotic emerge owing to its rapid adaptation. The microflora is sampled from such polluted sources and inoculated on solid media having the toxicant as the sole source of carbon and energy. Such selection provides strains capable of utilizing mono- and dichloroaromatics. To date, no strain has been found directly in

polluted environments that could directly utilize three or more chlorinated aromatics.

In addition to the active microbial strains found in natural sources, an effort is made to isolate highly degradative strains from enrichment cultures. To obtain an enrichment culture, a liquid mineral medium containing the toxicant under investigation is inoculated with a sample of known mass or volume taken from a polluted natural source. The pollutant may be present in the medium either as a sole source of carbon and energy or in combination with additional substrate.

The most frequent situations are when the chloroaromatic compounds cannot be utilized by the microflora as the sole source of carbon, but nonetheless they undergo biodegradation in the presence of additional substrate, i.e. in conditions of cometabolism. Cometabolism is based on provision of basic processes responsible for bioconversion of the target substrate (a chloroaromatic) with energy, cofactors, metabolites on different levels - transport of substrates, biosynthesis of enzymes, their functioning at the expense of incorporation an additional organic compound. Cometabolism is related to particular features of the regulation of peripheral metabolism based on metabolic links between bioconversion of one substrate (xenobiotic) and utilization of another (cosubstrate). As a cosubstrate, representatives of numerous chemical groups may be used: sugars, alcohols, hydrocarbons, aromatic and aliphatic acids etc.

The compound studied is initially introduced in the concentration which produce no toxic effect on representatives of microbial species known from the literature to be the most frequent agents of its biotransformation. In time, the initial concentration is gradually increased. When the process commences in conditions of cometabolism, the concentration of the cosubstrate is decreased proportionally, while increasing the concentration of the chloroaromatic. The overwhelming majority of active strains capable of degrading various chloroaromatic pesticides have been isolated using the enrichment culture technique; a few examples are given below.

Active strains of *Corynebacterium sp., Flavobacterium peregrinum, Pseudomonas sp., Achromobacter sp.* have been isolated by the method of

enrichment culture from soil, water, sludge, and sewage (Table 1).

TABLE 1. Active strains isolated by enrichment culture technique.

Substrate under degradation	Strain	Source
2,4-D	*Corynebacterium sp., Flavobacterium peregrinum, Pseudomonas sp., Achromobacter sp.*	Liquid sewage, soil, activated sludge
MPCA	*Alcaligenes paradoxus*	Soil
2,4,5-T	*Brevibacterium sp.*	Soil
DDT, methoxychlor, kelthane	*Pseudomonas aeruginosa, Actinobacter calcoaceticus, Alcaligenes sp.*	Soil

These cultures are able to degrade 2,4-D. The same technique was employed for isolating a strain of *Brevibacterium sp.* capable of co-oxidizing 2,4,5-T, and the strain *Pseudomonas aeruginosa* 640x co-metabolizing DDT. The described method of enrichment culture enables isolation of active microbial strains from the environment.

Recently, a novel technique for the genetic design of microbial strains with enhanced ability to utilize chloroaromatic compounds has been developed based on progress in genetic engineering. The method is based on the possibility of assembling in one strain the degradative genes from several strains which are responsible for specific steps of metabolic pathways on different levels of the xenobiotic degradation.

Such a way of engineering novel microbial strains is feasible due to the fact that the enzymes involved in the metabolism of chlorinated organics are encoded by plasmids with a few exceptions. At present, some 10 plasmids are known which are responsible for the degradation of chloroaromatic compounds (Table 2).

Employment of appropriate genetic techniques permits the transfer of plasmids, and consequently parts of metabolic pathways, from one strain to another. This approach develops from the research carried out in recent years by Knackmuss (3), Chakrabarty (8) and in the Laboratory of enzymatic degradation of organic compounds (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino).

TABLE 2. Plasmids controlling biodegradation of chloroaromatic compounds

Plasmid	Substrate under degradation	Plasmid size (MDa)	Reference
pJP1	2,4-D	51	Fisher *et al.*, 1978
pJP , pJP and pJP	2,4-D, 3CB, MCPA	37, 51, 83	Don a. Pemberton, 1981
pAC21	*p*-chlorobiphenyl	65	Kamp a. Chakrabarty,1979
pAC25, pAC27, pAC28,pAC29	3CB, 4CB, 3,5DCB	68, 59, 50, 50	Chatterjee *et al.*, 1981

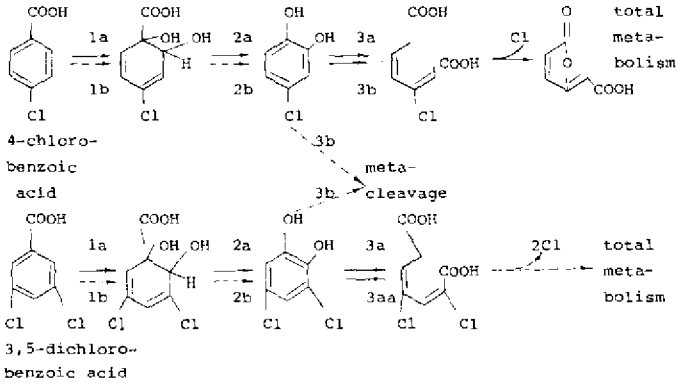


FIGURE 1. Metabolism of 4-chlorobenzoic acid and 3,5-dichlorobenzoic acid by transconjugants of *Pseudomonas sp.* B13. (a) - enzymes of *Pseudomonas sp.* B13; (b) - enzymes of *P. putida* mt-2; (1a) - benzoate 1,2-dioxygenase; (1b) - toluate 1,2-dioxygenase; (2a,2b) - dihydroxybenzoate dehydrogenase; (3a) - pyrocatechase I; (3aa) - pyrocatechase II; (3b) - metapyrocatechase.

Knackmuss *et al.* engineered a hybrid strain that united the ortho-pathway of cleavage of the aromatic ring by *Pseudomonas sp.* B13 with a relatively unspecific toluate dioxygenase of *P. putida* mt-2. The strain was constructed on the basis of *Pseudomonas sp.* B13 utilizing 3-chlorobenzoate to which the TOL plasmid of *P. putida* mt-2 was transferred. It is interesting to note that reposing cells of the new strain metabolized 4-chloro- and 3,5-dichlorobenzoate in addition to

utilization 3-chlorobenzoate (Fig. 1).

The same strain, *Pseudomonas sp.* B13, and the strain *Alcaligenes sp.* A7, which degrades phenol via the meta-pathway and is inactive towards chlorophenols were used for obtaining transconjugant strains. A novel strain utilized phenol via the ortho-pathway, metabolized 2, 3, 4-chlorophenols and 3-chlorobenzoic acid.

The use of genetic engineering techniques enabled Chakrabarty *et al.* to obtain the culture *Ps. cepacia* AC 1100 capable of utilizing 2,4,5-T as the sole source of carbon. To obtain the culture, various bacterial strains were used, isolated from sewage treatment works, and *Pseudomonas spp.* carrying biodegradation plasmids - TOL, SAL and pAC25. The cultures were maintained in a chemostat and supplied with 2,4,5-T as the sole carbon source. After prolonged cultivation, a mixed culture was isolated which assimilated 2,4,5-T, while inoculation of the mixed culture on solid agarized media gave a pure culture utilizing 2,4,5-T.

Researchers from the Laboratory of enzymatic degradation of organic compounds (Pushchino) were successful in obtaining the hybrid strain *P. aeruginosa* BS827 by transferring the plasmid of biodegradation of naphthalene and salicylate pBS3 in the strain *P. aeruginosa* 640x capable of co-metabolizing DDT. The new strain was more effective in degrading DDT compared to the wild one and, moreover, was able to degrade kelthane - a DDT analogue.

As mentioned above, a very important aspect in the degradation of chloroaromatic compounds is the study of metabolic pathways. Degradation of certain xenobiotics is known to produce metabolites that are more toxic, mutagenic and teratogenic than the parent compound. Hence, the necessity for a thorough investigation of possible metabolic routes in degradation of xenobiotics by specific active strains. One of the most studied chloroaromatic herbicides is 2,4-D. A whole range of cultures representing the genera *Pseudomonas, Alcaligenes, Arthrobacter, Achromobacter* were isolated and found to convert or utilize this compound, whose metabolism appears to be adequately studied (Fig. 2)

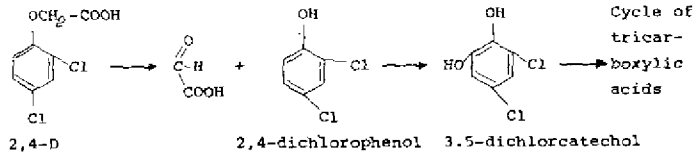


FIGURE 2. Bacterial metabolism of 2,4-D.

Much effort has been devoted to research into the microbial degradation of DDT. To date, no strain has been isolated capable of utilizing DDT as the sole carbon source. Total degradation of DDT was successful only in conditions of cometabolism. Cometabolic degradation of DDT has been studied in sufficient detail using the culture of *P. aeruginosa* 640x.

The initial steps of the microbial degradation of DDT were found to require anaerobic conditions, whereas terminating reactions starting with hydroxylation and cleavage of the aromatic rings proceed better under enhanced aeration. Specific cosubstrates are needed for different steps of dechlorination. For example, dehalogenation of the aliphatic fragment of the DDT molecule stands in need of an additional substrate – hexadecane, while that of aromatic fragments proceeds better with glycerol as cosubstrate. The metabolic routes were established for microbial degradation of DDT by *P. aeruginosa* 640x (Fig. 3).

Studies of the microbial degradation mechanisms for persistent xenobiotics are conducted, as a rule, in laboratory conditions on liquid media with pure cultures. In real conditions pollutants are found everywhere: in soil, water, air, sludge etc. Their properties may change somewhat in time: they are growing more persistent. The cause of the persistence of chloroaromatic compounds in soil may be the lack of conditions that favour the microbial degradation. Of these, the most essential are: accessibility of pollutants to microbial enzymes, which is determined by the extent of their sorption by soil particles and colloids, availability of organic compounds acceptable as energy substrates for the microflora, moisture content, aeration conditions and some other factors.

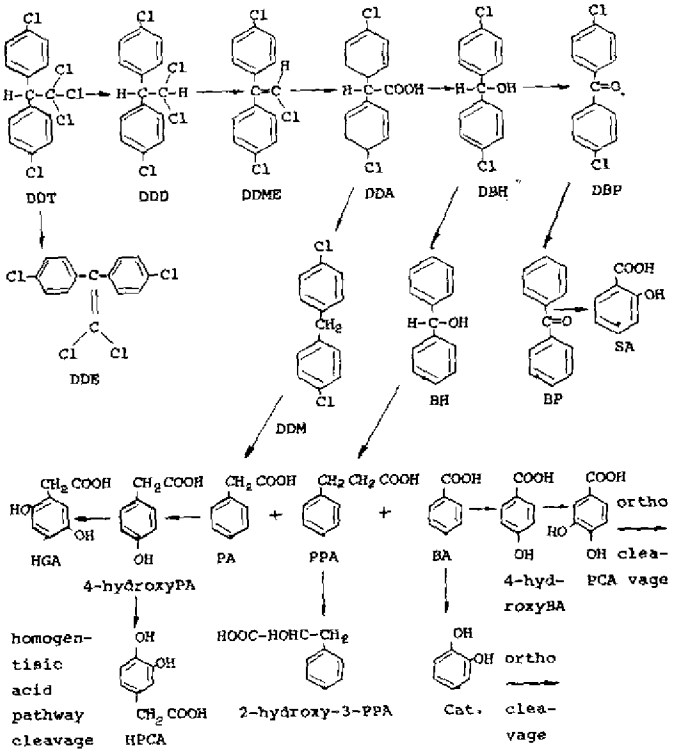
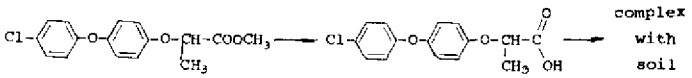


FIGURE 3. Pathways of DDT degradation by *P. aeruginosa* 640x.

The sorption of pesticides by soil constituents is a very complex process which is the sum total of the effects of many other processes. The mechanisms of the sorption of chloroaromatic compounds by soils, including the effects of clay fractions and soil organic matter have received sufficient coverage in numerous papers and reviews. Therefore, in the present study, the accent will be placed on assessing the effect of the chlorinated aromatics sorption by soil, on the measure of success of the microbial attack on these pollutants, since more often than not, the adsorption of chloroaromatics renders them highly insensitive to, or totally independent of, microbial activities. In a homogeneous nutrient medium, propanil is readily utilized by microorganisms, whereas in

conditions of microbial attack on the bentonite-herbicide complex, only the portion of the propanil desorbed into solution was degraded, while some 20-30% remained inaccessible to microbial cells. The above inference is in full measure valid for metabolites of chlorinated aromatics. Thus, the degradation of the herbicide dichlofop-methyl was studied in three soils: loam, sandy loam and silty soil. In all three cases, the herbicide was transformed into an acidic derivative and adsorbed by soil particles. In the first two soils the derivative was further metabolized, whereas in the silty soil the acidic metabolite formed a soil-bound complex and could be extracted only through heating with an alkaline solution:



The problem of the effect of organic matter on microbial degradation of chloroaromatic compounds has two aspects: on the one hand, the organic matter may prove to be a sorbent for pesticides, and on the other to serve as a source of carbon and energy providing for increased microbial populations and/or a source of factors required for bringing about primary steps of the biodegradation of xenobiotics. Usually, soil contains very low concentrations of chloroaromatic compounds and they cannot provide for a sufficient development of microbial populations even when utilized as the sole source of carbon and energy by some microbial groups. Therefore, the concentration in the medium of low-molecular-weight organic compounds readily accessible to microorganisms may become the limiting factor of the degradation. An additional supply of organic matter often enhances the degradation of chloroaromatic compounds. Literature describes the stimulating effect of the additions of green mass of alfalfa or peas on the degradation of lindane and methoxychlor, or of cattle manure, rice hulls and straw on the degradation of DDT and heptachlor. It should be noted, however, that cases were reported in which such readily metabolizable substrates as glucose had a low efficiency and sometimes even an inhibiting effect on the degradation of some pesticides.

Aeration is also one of the essential factors for the degradation

of chloroaromatic pollutants. Various pollutants are attacked by microorganisms only under specific conditions of oxygen supply. In certain natural soil environments, aerobic, anaerobic and microaerophilic conditions may exist. Deficient oxygen status or even anaerobic conditions may emerge inside individual soil clots, as the microorganisms residing on their surface absorb oxygen but do not allow it to diffuse inside the soil clots. Moistening of soil also contributes to the removal of some air and inhibition of aerobic microbial processes. In flooded soils, e.g. in rice fields, only the uppermost soil layer enjoys a satisfactory supply of oxygen. Going deeper in soil, the Eh values drop to 100-110 mV, thus creating conditions for reduction processes.

For certain chloroaromatic pollutants, aerobic conditions are indispensable for bringing about microbial degradation, e.g. the rate of removal of trichlorobenzenes from soil is essentially enhanced under aerobic conditions. On the other hand, anaerobic conditions are preferred for degradation of polychloroaromatic compounds. Heptachlor, endrin, aldrin and lindane are better degraded in anaerobic microzones. However, total degradation of highly persistent pollutants of complex structure, such as DDT and methoxychlor requires alternation of aerobic and anaerobic conditions. In the course of DDT degradation by *P. aeruginosa* 640x, the degradative steps which follow its bioconversion to benzophenone and benzohydrol require conditions of intensified aeration.

The rate of methoxychlor degradation increases 70-fold under alternating conditions of aeration from the anaerobic to aerobic.

Hence, modern means of increasing microbial degradation of chloroaromatic pollutants in soil are the introduction of additional sources of organic matter for stimulating the development of active microflora and flooding or tilling of the soil for changing the soil aeration regime. All these efforts are aimed at inducing the development of the autochthonous soil microflora.

Recent years have witnessed the development of totally new approaches, e.g. the use of genetically engineered bacterial strains to degrade chloroaromatic pollutants in natural environments. Both successes and failures have been reported in relation to their use for

the cleanup of the biosphere. Thus the strain *P. aeruginosa* BS827 carrying the plasmid pBS3 for the soil ecosystem purification of kelthane (DDT analogue) has been constructed in the Laboratory of enzymatic degradation of organic compounds (Pushchino) and tested in natural soil conditions. The strain was found to be viable in soil and capable of degrading kelthane in concentrations 1 to 100 μg per 1 kg of soil (Fig. 4). It is of course premature to speak of large-scale applications of such strains. Before the final conclusion is made, a thorough investigation should be carried out with regard to their behaviour in various ecological niches, possibility of transfer of their characters to representatives of autochthonous soil microflora, stability of the character responsible for degradation of the xenobiotic and many other aspects. This approach opens up broad promising vistas.

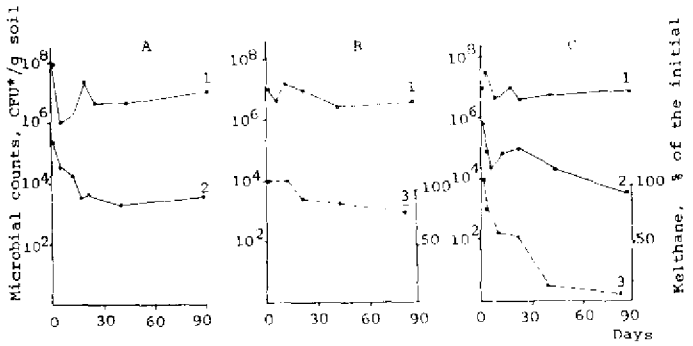


FIGURE 4. Numbers of *P. aeruginosa* BS827 (2) and dynamics of kelthane depletion (3) in a natural soil ecosystem.
1 - total population of microorganisms; A - without kelthane; B - without strain BS827, C - strain BS827 with kelthane.

* CFU - colony-forming unit.

RECOMMENDED LITERATURE

1. *Pesticide Microbiology.* 1978. Hill I.R. and S.Y.L. Wright, eds. Academic Press, New York.
2. *Microbial Degradation of Xenobiotics and Recalcitrant Compounds.* 1981. Leisinger T., R. Hütter, A.M. Cook and J. Nüecsh, eds. Academic Press, New York.
3. *Biodegradation and Detoxification of Environmental Pollutants.* 1982.

Chakrabarty A.M., ed. CRC Press, Inc., Boca Raton, Florida,
4. *Microbial Degradation of Organic Compounds*. 1984. Gibson D.T., ed. Marcel Dekker, Inc., New York.
5. *Progress in Pesticide Biochemistry and Toxicology*. V .4. 1985. Hutson D.H., T.R.Roberts, eds. John Wiley & Sons.
6. *Microbial Decomposition of Chlorinated Aromatic Compounds*. 1986. EPA /600/2-86/090. US Environmental Protection Agency, Office of esearch and Development, Hazardous Waste Engineering Research Lab., Cincinnati, Ohio.
7. *Pesticide Science and Biotechnology*. 1987. R. Greenhalgh and T.R. Roberts, eds. Blackwell Scientific Publications.
8. *Plasmids in Bacteria*. 1985. D.A. Helsinki, S.N. Cohen, D.B. Clewell, D.A. Jackson, A.Hollaender, eds. Plenum Press.
9. *Biotechnology. V. 8. Microbial Degradation*. 1986. W. Schonbarn, ed. VCH.

CHROMATOGRAPHIC-MASS-SPECTROMETRIC IDENTIFICATION OF XENOBIOTIC BIODEGRADATION PRODUCTS

V.M. Adanin

1. Introduction

The identification of pesticide degradation products derived from chemical, photochemical and metabolic processes is an essential component in defining a compound's toxicology and environmental behaviour. In general, minute amounts of sample are sufficient for analysis by mass-spectrometric methods (M), and the information obtained includes molecular weights as well as structural details.

Some of the most important spectrometric improvements in recent years are sophisticated inlet systems employing capillary gas chromatography and high-pressure liquid chromatography on-line to mass spectrometer, various methods of ionization, fast scanning of full spectra, heightened sensitivity, and computers specially designed for handling MS data.

In this lecture we do not propose to deal in detail with the various instrumental designs of mass spectrometers, or with the interpretation of mass spectra of different types of molecules. Such information is available in textbooks.

We hope that this introductory "paper" on MS will give the

reader some insight into the potentialities of MS in microbiological research.

2. Methods of Ionization

2.1. Electron Impact

The basic principle of MS is the separation and registration of atomic masses. The ionization procedure takes place in the ion source of the instrument and can be accomplished in several ways.

In an electron impact (EI) ion source held at high vacuum ($\sim 10^{-7}$ torr) the vaporized sample is bombarded with a beam of electrons, the energy of which can be varied from about 10 to 100 eV. The spectrometers are usually operated at 70 eV, a value which yields spectra of good reproducibility and of high diagnostic value. Most of the ions formed will be singly positively charged:

$$M + e \longrightarrow [M]^{+\cdot} + 2e$$

If sufficiently excited, the M ions decompose to form a variety of charged and neutral species.

Reactions involving only a simple cleavage of the molecular ion radical ($M^{+\cdot}$), or a successive series of such simple cleavages are often accompanied by reactions that involve cleavage of two bonds followed by rearrangements. The overall behavioural result of electron impact on the molecules in the ion source is conveniently illustrated by a graph (mass spectrum) in which the abscissa indicates the ratio of mass to charge (m/z) and the ordinate indicates the relative abundance of the ions produced (Fig. 1).

Although electron ionization (EI) is widely used in the characterization of pesticides, it has serious limitations in the analysis of their degradation products. Such compounds are usually more polar, have lower vapour pressure, and are often thermally unstable, and thus difficult to volatilize, or are structurally unsuited to the production of abundant molecular ions. These problems can be circumvented to some extent by derivatization or by use of lower ionization energies, although with lowered sensitivity.

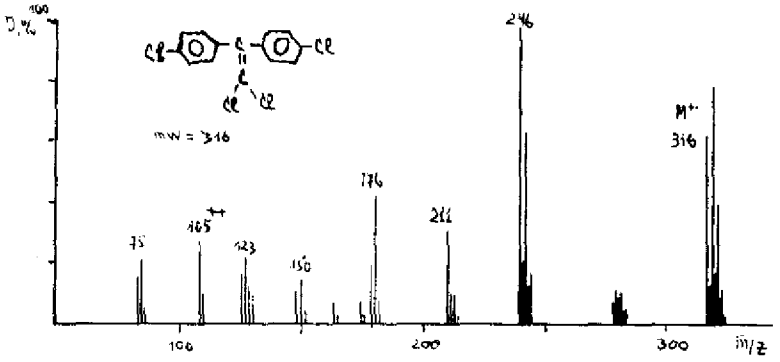


FIGURE 1. Electron ionization mass spectrum of DDE.

2.2. Chemical Ionization

An alternative ionization technique, chemical ionization (CI), provides mass-spectral information that often complements that from electron ionization. Chemical ionization is of the so-called soft ionization techniques: i.e. it achieves ionization of the analyte without transferring excessive energy to the nascent ions. The chemical ionization method relies on ion-molecule reactions that yield new ionized species. In the case of EI-ionized methane, the collision of cations with neutral molecules yields the formal CH_5^+ ion Fig. 2).

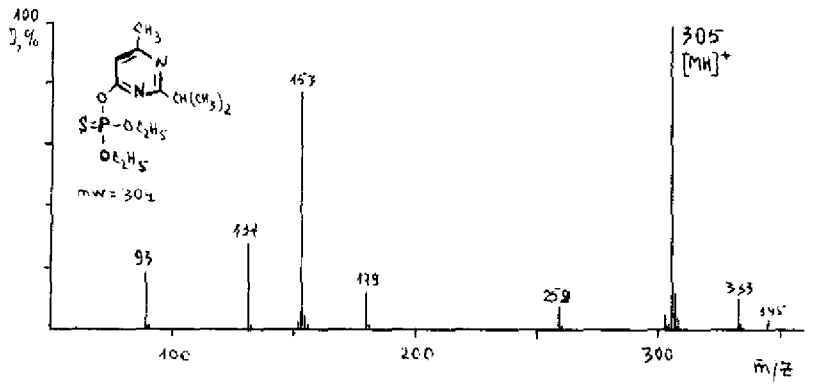


FIGURE 2.. Methane chemical ionization mass spectrum of diazinon.

This reactant ion in turn ionizes the analyte, usually by proton transfer, to give the "quasimolecular ion", MH^+. Various reactant gases have been used, including methane, isobutane, hydrogen and ammonia. These reagent gases sometimes are used as carriers in gas chromatography–mass spectrometry (GC–MS). The quasimolecular ion MH^+ is less likely to fragment, since it is lower in energy than M^+. Thus CI is useful for assigning molecular mass in cases where the analyte exerts at least a low vapour pressure in the source and is not thermally labile. In single-ion monitoring (SIM) applications the MH is frequently chosen for quantitation due to its intensity. Thanks to advances in design and vacuum technology there exist several commercial instruments that can perform EI, CI, and negative-ion chemical ionization (NCI) with only simple adjustments or almost simultaneously. These and other methods described below are most useful when used in combination.

Disadvantages of CI include highly temperature-dependent spectra, which may result in poor reproducibility. Further, the relatively high pressure in the ion source used in CI means that contamination of the ion source tends to be more pronounced than in EI. The high purity of reactant gases is an important prerequisite for reducing the risk of contamination and minimizing the background spectrum.

2.3. Field Ionization

Field ionization (FI) of a molecule occurs when the molecule interacts with a strong electric field (10^7 –10^8 V/cm). Sufficiently high fields can be produced in sources where sharp blades, wires, or metal points form the anodes. Removal of an electron from a molecule in an electric field is accompanied by a much smaller transfer of internal energy to the molecular ion than in the case of EI. Because of the high relative abundance of the molecular ion in FI, mass spectra obtained by EI and FI are complementary. In field ionization the gaseous sample is passed near the electrical field, while field desorption (FD) utilizes a sample coated on the emitter which is usually a wire coated with microneedles consisting of organic polymers and graphitized regions. In FD only $M^{+\cdot}$ ions are formed, and consequently this technique is of particular value for determinations of molecular weight (Fig. 3).

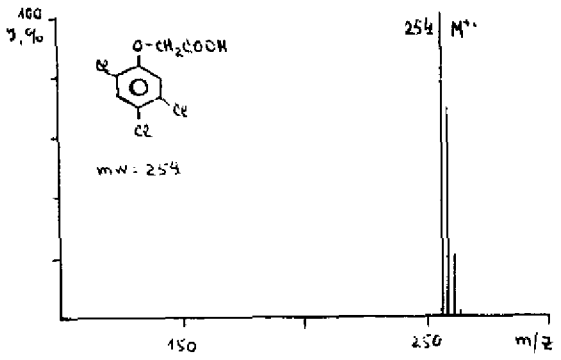


FIGURE 3. Field desorption mass spectrum of underivatized 2,4,5-trichlorophenylacetic acid (2,4,5-T).

FD is an extremely sensitive method and is of great advantage for trace analysis. Minimum amounts detected are in the range of 10^{-11} g. FD is particularly useful for organic molecules of low volatility or thermal instability and shows great promise for the analysis of polar pesticide metabolites.

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2.4. Fast Atom Bombardment (FAB) Ionization

In secondary ion mass spectrometry (SIMS), the sample to be analyzed is placed on a metal surface and bombarded with primary ions of high kinetic energy (usually Ar^+). At the metal surface, ion-molecule and charge exchange reactions produce secondary ions, which may be of several types including M^+ , MH^+ , $[M-H]^-$, $[M+metal]^+$ and fragment ions.

A related but simpler technique (FAB) uses fast neutral atoms for bombardment with the sample loaded in an involatile liquid or on a solid matrix. The sputtering of surface molecules from a liquid sample on impact within a high-energy atom beam produces positive, negative, and neutral species. The process of ion ejection is not yet well understood.

A great variety of matrices and bombardment gases have been examined. Thioglycol, triethanolamine, and many other compounds are

effective alternative matrices to glycerol. Molecular ion intensity is also affected by the reactant gas, decreasing in the order Xe ⇒ Ar ⇒ Ne.

FAB has been compared with FD in both positive- and negative- ion modes and has been found to provide a greater abundance of fragment ions, which may aid structure elucidation.

FAB has also been used successfully in combination with mass spectrometry-mass spectrometry (MS-MS). The latter removes the glycerol "background" and can serve as a separation stage for each component of a mixture.

SIMS and especially FAB are being increasingly recognized as useful methods for the ionization of thermally unstable, involatile, and multifunctional molecules. Simplified instrument designs for FAB, including the use of quadrupoles and their increased availability will undoubtedly enhance the importance of this technique for the analysis of pesticides and their transformation products.

3. Separation of Ions

3.1. Magnetic-Sector Analyzers

On acceleration of the ion beam out of the ion source, it is focused by a series of lenses or slits and passed through an electric potential which further focuses the beam according to its energy (electric sector). The beam is then passed through a magnetic field for mass separation. This combination constitutes a double-focusing instrument suitable for high-resolution mass spectrometry (HR-MS). HR-MS is particularly useful for determination of elemental composition by exact mass measurements with an accuracy of a few parts per million.

Other analyzer arrangements include single-focusing designs in which the electric sector is omitted (low resolution), and reverse geometry in which a magnet precedes the electric sector. An advantage of the latter is achieved when selected masses are focused into the field-free region (and the electric sector) by manipulation of the magnetic field. Some of the selected ions decompose, giving metastables with different kinetic energies, they can be separated by the electric sector. This technique, termed MIKES (mass-analyzed ion energy

spectroscopy) is effective in the examination of mixtures, since ions characteristic of a given component can be transmitted. The metastable species characterize the substance without interference.

In a modification of the MIKES technique, the mass-selected ion is collisionally excited by passage through a collision cell containing an inert gas at high pressure ($\sim 10^{-5}$ torr). This modification, known as collision-induced dissociation (CID-MIKES), aids in differentiating structural isomers.

3.2. Quadrupole Analyzers

Another type of analyzer is based on the use of quadrupoles for mass separation. Quadrupoles are based on a completely different principle than that of magnetic sector instruments. Four parallel rods are arranged in pairs so that between each pair of opposite and electronically connected rods a direct-current (dc) voltage is applied with a superimposed radiofrequency (rf) field. Under the influence of these fields, ions acquire complex trajectories, and under constant rf/dc changes in the voltage, ions are separated according to mass. The quadrupole filter allows only one mass to pass through the system at any point in the scan. Its capabilities are similar to those of a single-focusing magnetic sector instrument (i.e. maximum resolution 2000, high mass 1200 amu) but it does not provide data on metastables. Quadrupoles are particularly useful for GC-MS since the full mass range can be scanned in milliseconds. They are also amenable to computerization for precise control of the rod voltages.

Quadrupoles have proven very useful in the development of tandem mass spectrometry or MS-MS, a technique in which separation of components in mixtures is carried out by ionization and several stages of mass separation rather than by chromatography. Although not yet utilized widely MS-MS should prove to be more selective, more sensitive, and faster than techniques with prior gas chromatographic (GC) or liquid chromatographic (LC) separation. Initially ions are separated, producing a primary mass spectrum from which a single ion is selected to obtain a secondary or daughter ion spectrum.

Any of the present range of ionization techniques can be used to

ionize the sample prior to the first stage of mass analysis.

The tandem mass spectrometry was recently used in the analysis of mycotoxins in biological samples at the sub-parts per million level.

4. Introduction of Sample

4.1. Direct Inlet Probe

Modern mass spectrometers usually provide several means of introducing the sample into the ion source. The simplest is the direct inlet probe technique. It utilizes a small glass or gold crucible attached to a metal rod. The rod may be advanced through an airtight seal connected to a vacuum lock so that the opening of the crucible closely approaches the bombarding electron beam. The inlet probe is designed for accurate, rapid heating, by which a small quantity (μg or less) of sample can be vaporized and introduced into the beam of electrons. Precisely constructed direct inlet systems permit controlled, fractionated distillation in the source and thus consecutive analysis of components in a mixture.

4.2. Gas Chromatograph

The most important operation for the pesticide analyst is the introduction of sample provided as a chromatography eluent from GC and more recently from HPLC. When packed GC columns are utilized, the high gas flows required necessitate the use of molecular jet separators at the interface. These differentiate between sample and carrier so that enrichment of the analyte relative to the carrier gas takes place.

The use of capillary columns (0.25–0.3 mm) and now wide-bore capillaries (0.5 mm) enhances the usefulness of GC-MS, allowing for direct introduction of the sample into the ion source in addition to increased chromatographic resolution.

In CI-MS the ionization gas can be used as a carrier or introduced directly to the source as a make-up gas. GC-MS is of particular importance in the regulatory analysis of pesticides and has been applied to a variety of pesticide degradation products.

4.3. Liquid Chromatograph

The high-performance liquid chromatography (HPLC) has advantages over GC in that compounds are not exposed to excessive heat, less sample cleanup is required, and derivatization is usually not necessary. The first successful commercially available LC-MS interface was the transport or moving-belt system. The operating principle of the moving-belt can be separated into three distinct steps: deposition of the eluent, removal of the solvent in vacuum and volatilization of the sample into the ion source. The other popular mode of on-line LC-MS has been called direct liquid introduction (DLI). DLI LC-MS often uses a split such that only 1 -5% of the total effluent is introduced into the mass spectrometer. Only CI mass spectra are available from DLI LC-MS experiments, which often provide desired molecular weight information.

Recently developed thermospray LC-MS interface promises to be useful addition to the range of MS methods applicable to analysis of xenobiotics. In most instances the base peak in the thermospray mass spectra corresponded to the protonated molecular ion species $[M+H]^+$ (Fig. 4).

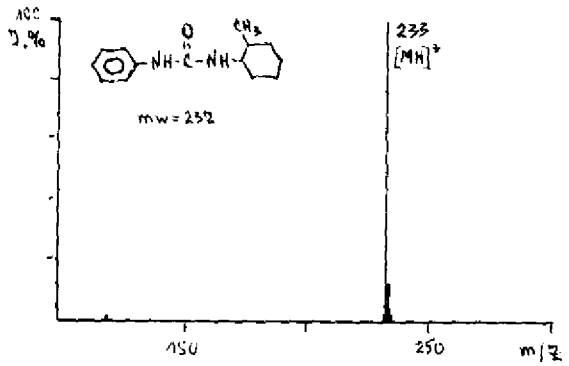


FIGURE 4. Thermospray of mass spectrum of siduron.

5. Use of Computers

The coupling of mass-spectrometry to computerized systems considerably enhances their utility and ease of operation. The computer can

coordinate scanning control, data acquisition, and data processing. During the 1970s, computers were used to cope with the great volume of data produced during analysis by GC–MS.

The process of converting information acquired by the computer (digitized scan/time/intensity data) into the mass spectrum requires mass calibration using a reference compound. These substances vary, depending on ionization mode, mass range, and inlet. Perfluorokerosene (PFK) is normally used in magnetic sector instruments (m/z 40–800) and heptafluorotributylamine in quadrupoles (m/z 40–600) for EI-MS and CI-MS. Calibration at higher masses utilizes substituted triazines.

In addition to obtaining low-resolution spectra, the data system can be used to determine accurate masses for establishing elemental composition. With the mass spectrometer operating at high resolution (>8000), the sample is admitted and ionized simultaneously with the reference material. The data system locates the reference signals and recalibrates, taking any variations into account. The accurate masses of abundant ions are computed by interpolation between adjacent reference ions, which are then subtracted, leaving the accurate mass spectrum of the sample. This technique is suitable for GC–MS given peaks of sufficient width.

Repetitive or multiscan data processing is typical for the analysis of chromatographic eluents (GC–MS and LC–MS) but can also be applied to thermal separation of substances on heating the direct introduction probe. The trace obtained is the total ion current (TIC) chromatogram, equivalent to the chromatogram obtained with a conventional nonspecific detector as far as peak retention times but not areas. The computer sums the ion abundances and normalizes and plots them against time or scan number to produce the TIC. Quantification of chromatographic peaks can be carried out with certain limitations. The TIC can be relieved of background signals by subtraction of the appropriate spectra, usually those adjacent to the peak of interest. Mass chromatograms obtained by plotting specific masses against time can be used to check the homogeneity (purity) of chromatographic peaks. Single-scan data can be compared with a library of reference spectra in a computer library search. Most commercial data systems have libraries stored on magnetic

discs or tape containing several thousand reference EI spectra – e.g., the National Bureau of Standards compilation (USA) contains 38,785 complete spectra. Libraries based on other ionization methods are not generally available.

In the usual mass scanning mode, also termed full scan or total mass scanning, a mass range (e.g. 50-450 amu) is scanned repetitively. Alternatively, the mass spectrometer can be pre-programmed to detect only certain masses in a technique known as single-ion monitoring (SIM-MS) or multiple-ion detection (MID-MS). Quadrupole instruments are particularly suited to this application, since they are not subject to hysteresis problems as magnetic instruments. In fact, quadrupoles operate at maximum sensitivity in this mode, since the detector acquires data only at masses of interest. Accordingly, Mirex and its degradation products, for example, are analyzed with a detection limit of 500 fg by SIM, whereas a detection limit in the low picogram range is achievable by MID.

Mass spectrometry is widely employed as confirmatory technique in pesticide residue analysis. A full spectrum of the suspected residue provides the greatest amount of structural information and specificity; however, a limited-mass scan including diagnostic ions or MID of three or more ions may suffice.

6. Quantitation Procedure

Mass spectrometry methods can be used to obtain quantitative information in addition to their powerful contribution toward structure elucidation. Quantitation by MS is carried out by using calibration graphs of the ion abundance of internal or external standards, much as peak areas are used in GC.

The method of standard additions has been used in quantitative mass spectrometry, and absolute calibration by external standards may often give high precision and accuracy. Best results, however, are obtained with internal standards, where calculations can be based on ratios rather than absolute measurements. The substances which are used as internal standards may be homologs (with similar substituents) or

isotopically labeled materials (^{13}C, ^{2}H, ^{18}O). Homologs require previous chromatographic separation (GC, LC), and those yieding common fragment ions are preferred for ion-monitoring techniques. A substantial mass difference (3 amu) between the isotopically labeled analyte and actual analyte is necessary, particularly for substances with broad isotope clusters (e.g., polychlorinated molecules). Such standards may also function as carriers that decrease the loss of ultratrace quantities on chromatographic adsorbents and elsewhere.

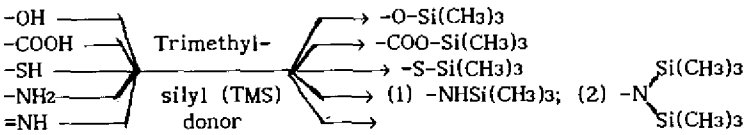
Recently, atrazine, lindane, pentachlorophenol, and diazinon have been determined in water using standards containing stable isotopes.

The highest sensitivity for quantitation is achieved with single- or multiple-ion detection. The abundance of one or more selected abundant ions is acquired in real time as components emerge from the chromatographic system.

7. Chemical Derivatization

Modification of chemical structure by introduction of substituents in the reactive groups of a molecule has the same goals in MS as in gas chromatography applications. Improvement of volatility or thermal stability of the sample can be accomplished, and enhanced chromatographic resolution can be obtained by a variety of reactions.

Silylation is probably the most commonly used procedure, applicable to acids, phenols, alcohols, and amines (Scheme 1).



SCHEME 1. Preparation of silylated derivatives.

Advantages of such type of derivatization include improved volatility, easy removal of the protective group, and mass spectra that often exhibit abundant molecular ions or intense characteristic fragments (e.g., $M-CH_3$). Other commonly used derivatizations involve

alkylation, acylation, and isotopic labeling.

In addition to improving the efficiency of sample introduction into the ion source, new substituents may increase the abundance of M^+ with groups of low ionization potential, or direct fragmentation toward more intensive ions, suitable for SIM, or to fragments that provide structural information.

8. Application Examples

The use of mass spectrometry to identify and quantitate organic pesticides is continually expanding, and the rate of appearance of new reports increases each year.

In spite of its limitations, electron-impact MS (EI-MS) continues to be the principal method for identification of degradation products, mainly due to the availability of instrumentation and ease of operation.

Numerous studies have used EI-MS combined with GC introduction for analysis of the chlorinated insecticides, probably because of their thermal stability. Mercapturic acid derivatives of chlorophenyl metabolites of lindane have been characterized by GC-EI-MS after derivatization to the trifluoroacetylated butyl esters. SIM was successfully utilized to determine hexachlorocyclohexane isomers in aquatic environments. Thus, parts per trillion levels have been established using deuterated internal standards.

When SIM was used for the analysis of the chlorinated pollutants, the molecular-ion cluster areas are a fairly constant fraction of the total ion current, essentially regardless of the chlorine number, thus simplifying the determination of relative concentrations.

Many studies have analyzed the chlorophenoxy herbicides and their degradation products. A direct interface EI-GC-MS has been used for 2,4-D analysis in air samples to obtain maximum sensitivity and reproducibility. The base peak used for SIM allows for sensitivities below 1 pg.

The presence of a strong molecular ion, characteristic in chemical ionization MS (CI-MS), is an important requirement for structure elucidation.

Aldicarb, butocarboxime, and their metabolic sulphoxides and sulphones have been analyzed by GC-CI-MS, although aldicarb and its metabolites are subject to decomposition in the column. Fragmentation patterns are useful for differentiating aldicarb and butocarboxime.

Carbaryl is difficult to analyze by GC in environmental samples, but GC-CI-MS (ammonia) has been successful at sub-parts per million levels with quantitative results in close agreement with those obtained by HPLC.

Various mass spectrometry techniques were compared in the analysis of di-(2-ethyl hexyl)phthalate metabolites. EI was nonspecific, with each metabolite revealing the familiar m/z 149, 163, and 181 ions, methane CI provided molecular weight data (i.e., MH^+ , $[M+29]^+$, etc.), in the oxygen NCI mode data complementary to methane CI was obtained; and CID-MIKES was specific enough to differentiate isomers.

The involatile herbicide hexazione has been examined by FAB- MS. Recently a procedure has been reported that combines TLC and FAB-MS. Direct analysis of chromatographic zones should prove useful for a variety of polar and thermally labile materials that cannot be separated by GC.

Recommended Literature

Analytical Methods for Pesticides and Plant Growth Regulators. Vol. XIV. Modern Analytical Techniques. G. Zweig, J. Sherma, eds. New York, Academic Press. 260 p.

Applications of New Mass Spectrometry Techniques in Pesticide Chemistry. 1987. J.D. Rosen, ed. N-Y. John Wiley & Sons, Inc.

Biochemical Applications of Mass Spectrometry. 1980. First Suppl. Volume. G.R. Waller, O.C. Dermer, eds. New York. Wiley Interscience. 1279 p.

Biochemical Applications of Mass Spectrometry. 1972. G.R. Waller, ed. New York. Wiley Interscience. 872 p.

Gas Chromatography - Mass Spectrometry. Applications in Microbiology. 1984. G. Odham, L. Larsson, P.A. Mardh, eds. N. Y. Plenum Press. 444 p.

Hamming M.C., N.G. Foster. 1972. *Interpretation of Mass Spectra of Organic Compounds.* New York. Academic Press. 694 p.

Sherma J. 1987. Pesticides (Review). *Analytical Chemistry* 59:18R-31R.

Watson J.T. 1985. *Introduction to Mass Spectrometry.* New York. Raven Press. 351 p.

MICROBIOLOGICAL ASPECTS OF THE PURIFICATION OF INDUSTRIAL WASTE WATERS

R.M. Alieva

Uncontrolled disposal of toxic synthetic substances aggravates the potential hazards of man's impact on the environment. Anthropogenic stresses on ecological systems are caused, in the first place, by channelling to the environment synthetic organic compounds and heavy metals. Such substances display, as a rule, rather high toxicity to, and persistence in, local environments.

The self-purification capacity of the biota is heavily affected by the ever-increasing influx of deleterious industrial wastes.

Biochemical treatment of waste waters using activated sludge as the major technogenic agent of the effluents' clean-up has gained ground in disposal of the agricultural and communal sewage, which is characterized by a relatively stable composition. The industrial waste waters, however, carry high concentrations of oxidation-resistant compounds, and this makes conventional sewage treatment technologies somewhat obsolete.

Microbial technologies for the purification of waste waters, when extrapolated from agricultural and communal sewage to industrial wastes, which contain relatively high quantities of toxic organic compounds and heavy metal ions, prove to be low-efficient. They call for development of novel, research-based methods, since the oxidative potential of the

microbial community in activated sludge is no longer sufficient.

An urgent need is thus felt for elaborating ecologically sound strategies of the use of microorganisms for neutralizing noxious substances prior to their release to the environment. Effort aimed at tackling the problem should, first and foremost, focus on screening active microbial strains, which display high degradative ability, their comprehensive investigation and the development of a novel system of their utilization. Such an endeavour, if successful, will provide protection of the activated sludge microflora from highly toxic components of the sewage.

Specific chemical industries, for example those producing synthetic rubber and a number of other organic compounds based on divinyl and α-methylstyrene, discharge highly toxic effluents. These waste waters have a complex chemical composition, α-methylstyrene and crotonic aldehyde being amongst the most noxious because of their toxic characters and present at high concentrations due to imperfection of certain technological steps. Their concentrations in waste treatment plants are many times higher than tolerated doses, and the activated sludge in the aeration tanks is obviously unable to provide for effective utilization of these toxicants.

The above problem has been for a few years one of the basic lines of applied research at the Institute of Microbiology and Virology of the Kazakh SSR Academy of Sciences (Alma-Ata).

In this context, the present lecture presents the fundamental background, experimental material, and the experience gained as a result of the effort to develop more efficient microbial techniques to be used in waste treatment plants for abating drastically levels of the above pollutants.

I. Inhibitory Effect of the Sewage Toxic Components on Microbiocenosis in the Activated Sludge

The formation of microbial populations in waste treatment plants occurs under the influence of various physico-chemical factors (aeration conditions, temperature, pH etc.) and the chemical composition of

organic and mineral substances under treatment. With this in mind, we undertook analysis of the waste treatment plant, which suggested division of the whole system into 4 specific ecological zones. Physiological studies of the basic microbial groups revealed that these zones are in the main populated by aerobic heterotrophic microorganisms (bacteria, fungi, yeast and actinomycetes). Major attention was paid, however, to characterization of the aeration tanks that are the site of basic degradation processes.

The activated sludge is a cenosis composed of bacteria, fungi, yeast and protozoa with the heterotrophic bacteria present in large quantities. Among these, the predominant are Gram-negative, nonsporous members of the genera *Pseudomonas, Alcaligenes, Xanthomonas, Zoogloea, Proteus, Escherichia, Sphaerotilus* and *Azotobacter.* Gram-positive bacteria are represented by the genera *Bacillus, Micrococcus, Staphylococcus, Lactobacillus, Listeria, Corynebacterium.* In addition, two fungal strains of *Geotrichum sp.* and yeast *Rhodotorula and Cryptococcus* were isolated. Among bacteria, the most dominant are the genera *Pseudomonas, Alcaligenes, Xanthomonas, Zoogloea* with frequent occurrence of the genera *Bacillus, Corynebacterium, Micrococcus.*

The microflora of the activated sludge is composed of different groups of protozoa which are sensitive biological indicators and respond to changing environmental conditions. In the waste treatment plant, the activated sludge is populated by the following groups of protozoa: *Aspidiscka, Arcella, Monostyla, Vorticella, Opercularia, Sphaerophria.*

A distinctive feature of the industrial sewage is that it is the recipient of substantial quantities of toxic technogenic compounds that create stresses on the activated sludge microflora.

Pulse discharges of α-methylstyrene, crotonic aldehyde and mercury ions to the activated sludge prove to be fatal for specific microbial groups, and these most vulnerable 'ecological targets' gradually disappear (Table 1).

Studies of the effect of the above toxicants at concentrations exceeding the tolerated levels showed that 6–12 hours after contact with the pollutants, the occurrence for many microorganisms falls. The species composition of the sludge microflora is reduced, *Pseudomonas and*

Bacillus sp. are becoming dominant in the sludge microbiocenosis, while the microfauna is totally destroyed.

TABLE 1. Effect of toxic compounds on the qualitative composition of microflora in activated sludge

Control	α-Methylstyrene	Mercury ions	Crotonic aldehyde
Pseudomonas	*Pseudomonas*	*Pseudomonas*	*Pseudomonas*
Alcaligenes	*Alcaligenes*	*Alcaligenes*	
Xanthomonas			
Zoogloea	*Zoogloea*		
Escherichia			
Proteus			
Asotobacter			
Spaerotilus			
Bacillus	*Bacillus*	*Bacillus*	*Bacillus*
Staphylococcus			
Listeria			
Lactobacillus			
Corynebacterium	*Corynebacterium*	*Corynebacterium*	
Micrococcus		*Micrococcus*	
Cryptococcus	*Cryptococcus*	*Cryptococcus*	

Hence, the major consequence of the interaction of activated sludge with industrial effluents is the reduction of the microbial community, qualitative changes effected in the sludge, and inhibition of biochemical activity. These point to the fact that the system is overloaded with toxic pollutants and is no longer able to process the discharged compounds.

Following the above statement, the strategy aimed at averting grave perturbations in the activated sludge biocenosis should focus on improving the existent technologies of sewage clean-up and preventing accidental or uncontrolled influx of toxic compounds in sewage treatment plants.

2. Microbial Degradation of Toxic Organic Compounds

Some 500 microbial strains belonging to various taxonomic groups have been assayed for their ability to utilize xenobiotics as the sole carbon source in the attempt to find microorganisms that could grow at the

expense of α-methylstyrene and crotonic aldehyde.

Screening in microbial culture collections failed to provide any positive results thus giving an impetus to a search for active strains in natural biocenoses. At present, the search for such microbes may acquire a purposeful character provided it makes use of a microflora adapted to specific substrates. Conditions for such an adaptation may arise in the activated sludge and industrial waste waters as the result of long-term adaptation by the microbial population to heavy concentrations of these compounds, in other terms, by their microevolution.

The use of conditions which developed in sewage from the mentioned industries permitted the isolation of more than 50 microbial strains capable of degrading α-methylstyrene and crotonic aldehyde in the activated sludge and waste waters.

The degradation of crotonic aldehyde was studied using the strain *Bac. pumilus* 153 which exhibited the highest oxidative characters. Intensive growth of this culture was accompanied by proportional degradation of crotonic aldehyde.

The first 3 days showed degradation of some 630 mg/l or 78% of the total crotonic aldehyde. Further, a decline in bacterial growth, and consequently the aldehyde degradation rate, was observed, the pollutant being completely oxidized after 5-6 days. The use of a mixed culture understandably curtailed the period of crotonic aldehyde degradation.

Growth of these cultures on industrial effluents containing ethanol, butanol and ethyl acetate showed that the bacteria adapted to such conditions were capable of oxidizing the crotonic aldehyde in concentrations up to 1.0 g/l within 2 days.

Studies of the metabolic routes in the crotonic aldehyde oxidative degradation by the cultures *Bac. pumilus* 153 and *Bac. megaterium* 148 pointed to hydration of its double bond to form two molecules of acetaldehyde followed by production of acetic acid, which was utilized by the microorganisms in constructive metabolism.

Oxidation of crotonic aldehyde may be represented as:

$$CH_3\text{-}CH{=}CHO + 2HOH \longrightarrow 2CH_3\text{-}CHO \longrightarrow 2CH_3\text{-}COOH$$

Investigation of the dynamics of α-methylstyrene (AMS) bacterial oxidation by the strains *P. aeruginosa* 8 and 13 showed active growth of the cultures on this substrate. Its initial concentration of 2 g/l was completely oxidized within 24 h, while 4 g/l enhanced bacterial growth, and in 2 days the toxicant was no longer detectable in the medium (Fig. 1).

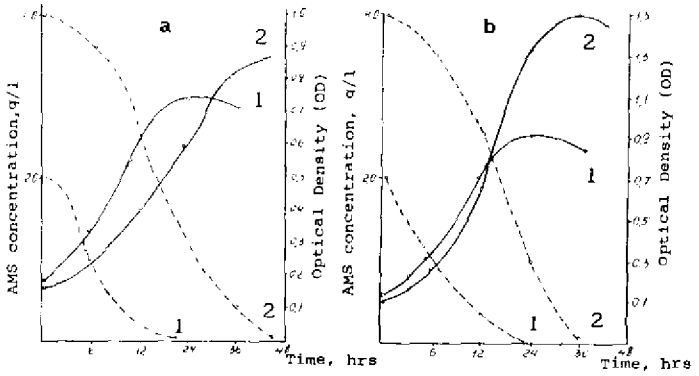


FIGURE 1. Dynamics of AMS oxidation and growth of *P. aeruginosa* 6(a) and *P. aeruginosa* DS 13(b) in the liquid medium 8-E.
—— biomass production on 2 g/l (1) and 4 g/l (2) of AMS;
--- AMS depletion from 2 g/l (1) and 4 g/l (2).

The oxidation was shown to proceed best at 28°C, pH 6.5-8.5, under conditions of aeration and the toxicant concentration of 2-4 g/l.

The most distinctive feature of the microorganisms isolated directly from the sludge or waste treatment plant is their high oxidative activity during degradation of α-methylstyrene and crotonic aldehyde. Of these, over 30 cultures were found to utilize 2 to 10 g of α-methylstyrene and toluene, and about 20 strains capable of oxidizing 0.8 to 1 g of crotonic aldehyde per 1 litre of medium. A microbial collection was established based on the genera *Pseudomonas* and *Bacillus*, which included strains able to utilize the most toxic waste products from acetaldehyde and synthetic divinyl rubber production: α-methylstyrene, crotonic aldehyde and toluene.

In addition to high oxidative potential in relation to α-methylstyrene and crotonic aldehyde, the isolated cultures also

developed, in the course of adaptation, other unique distinctive characters compared to their microbial analogues isolated from natural environments. Thus, nearly all sludge-isolated strains of the *Pseudomonas* genus were remarkable for their enhanced ability to grow on aromatic hydrocarbons (toluene, biphenyl, naphthalene) and to display poor growth on oxidized aromatic compounds. They proved to be tolerant to high concentrations of mercury.

3. Investigation of the Pathways of α-Methylstyrene Peripheral Metabolism by Plasmid Carrying *Pseudomonas* Strains

Many xenobiotics are transformed by microorganisms into metabolites that may prove to be more toxic and recalcitrant compared to the parent substrate.

Peripheral metabolism of α-methylstyrene (AMS) was best studied for the strains *P. aeruginosa* 8 and 13, and *P. acidovorans* 9.

Physico-chemical analysis gave evidence for 8 compounds as metabolites of AMS degradation by *P. aeruginosa* 8 and 13 which utilized AMS as the sole source of carbon and energy at concentrations of 2 .to 10 g/l.

Study of the culture growth dynamics revealed that the basic process of AMS bioconversion occurs in the exponential phase of 6-12 hour duration (Fig. 1).

Thorough examination of the data obtained suggested two different routes be operative in AMS catabolism. The principal oxidative route starts with hydroxylation of the aromatic ring to form (cis)-2,3-dihydroxy-1-isopropenyl-6-cyclohexene and 3-iso-propylcatechol followed by the ring cleavage via the meta-route with the metapyrocatechase of broad substrate specificity to form 3 aliphatic ketoacids differing by their carbon chain length (C_7-C_9) (Fig. 2).

Elucidation of the structure of the above acids was complicated by their involvement into subsequent metabolism.

The minor route of AMS degradation is related to the accumulation of α-phenylacrylic acid and α,α-phenylmethylglycol which are the products of the isopropenylic fragment oxidation. At the same time,

acetophenone was produced via α-methylbenzylic alcohol but these metabolites did not accumulate in the stationary phase.

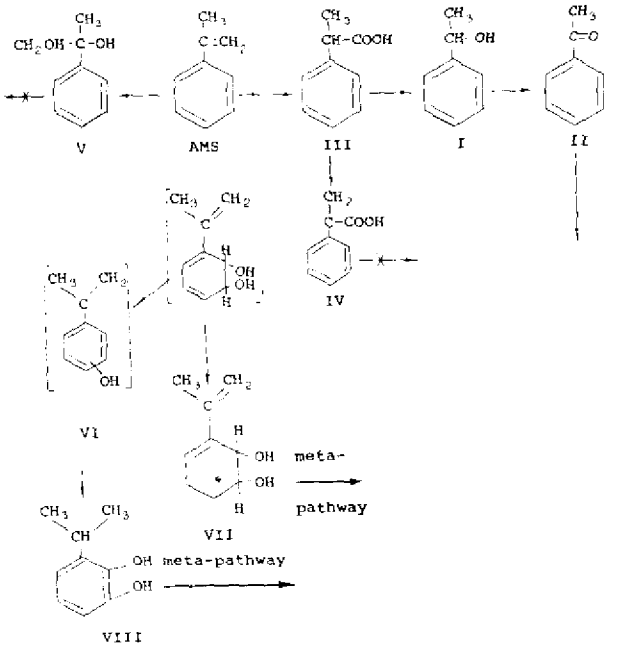


FIGURE 2. *Primary steps of AMS degradation by P. aeruginosa 8 and 13. I, AMS; II, acetophenone; III, α-phenylpropionic acid; IV, α-phenylacrylic acid; V, α,α-phenylmethylglycol; VI, (oxy)-isopropenylbenzene; VII, cis-2,3-dihydroxy-1-isopropenyl-6-cyclohexene.*

Under conditions of the bioconversion of washed cells, acetophenone disappeared rapidly from the medium giving no metabolites. The key enzyme of the aromatic ring cleavage - metapyrocatechase was induced by acetophenone. Acetophenone is apparently further catabolized by the culture via the ring meta-cleavage.

Metabolism of AMS by *P. aeruginosa* 8 and 13 is characterized by a diversity of routes for the primary enzymic attack on the molecule. This pattern is indicative of the oxidation of xenobiotics and was outlined in earlier works devoted to oxidative degradation of xylenes, pseudocumene, ordram, and DDT (Skryabin and Golovleva, 1976).

The dynamics of AMS oxidation by *P. aeruginosa* 9 was characterized by poor microbial growth, though the substrate disappeared totally after 2 days. Analysis of the culture liquid pointed to the availability of 2 products: α-methylbenzylic alcohol and acetophenone thus confirming that AMS degradation by *P. acidovorans* 9 is a typical microbial bioconversion. Analysis of the key enzymes which bring about oxidation of the aromatic ring showed the availability of nearly all enzymes involved in the ring cleavage.

Summing up the above example, it may be inferred that the appropriate choice of microbial cultures adapted to highly toxic conditions permits total oxidation of the toxicant, as is the case with AMS, whereas establishment of metabolites and degradation routes may be used as the guide-lines in staging similar studies (Fig. 2).

4. Investigation of the Plasmids Responsible for the Biodegradation of α-Methylstyrene and Toluene by *Pseudomonas* Strains

Enzymic systems responsible for the oxidation of alkylbenzenes are known to be controlled by plasmid genes (Boronin and Skryabin, 1985). Investigation of 11 strains able to degrade AMS showed the availability of a plasmid DNA with molecular weight of the order of 130 MDa. Cultures of these strains on beef-peptone agar and nutrient broth did not lead to the emergence of plasmid-free versions in microbial population. Neither strain treatment with subinhibiting concentrations of mitomycin *C* (20 μg/ml) provided plasmid-free variants. However, *P. aeruginosa* DC-13 showed a drop in the molecular weight of plasmid DNA. Herewith, variants with deletions of the plasmid were losing their ability to catabolize AMS and toluene as well as their resistance to mercury ions. The frequency of occurrence of such variants made up 20%, on the average. The absence of reversions to the initial phenotype speaks in favour of the deletion loss of genetic determinants.

5. Biotechnology of Local Purification of Waste Waters

Thorough study of microbial cultures able to degrade AMS and toluene

suggested the use of the most active strains for the clean-up of industrial sewage. However, the microbial technology intended for purification of local waste waters to be efficient, requires high concentrations of degradative microorganisms. It was therefore necessary to develop techniques of immobilization of active strains in order to establish a stabilized technological system. In this context, two directions were given first priority: aggregation of microbial cultures into flocculi (bulking of activated sludge) on divinyl-styrene latex and cell immobilization on solid supports.

The production conditions limit the choice of materials used for fixing microbial cells. Preference is given to solid materials such as glass fibres, porolon (porous polyvinyl chloride) etc. As supports for immobilizing microorganisms, porolon, glass cloth and bead- or 'hedge-hog'-shaped glass fibre were assayed. The best results were obtained using porolon and glass cloth. Model experiments were carried out in a biochemical sewage treatment pilot plant under conditions of continuous culture using active degradative strains immobilized on glass cloth. Results of these experiments illustrate the feasibility of waste water microbial purification of crotonic aldehyde and AMS in concentrations 1 to 2 g/l.

5.1. Local Industrial Sewage Purification of Crotonic Aldehyde and AMS

Under industrial conditions, microorganisms capable of degrading crotonic aldehyde and AMS were immobilized on inert supports and used in an installation for local purification of industrial effluents (Alieva *et al.*, 1985). The installation contains strips of porolon or glass cloth, their dimensions being predetermined by those of the waste water discharge canals starting in the production shop.

The effluents discharged from the acetylene hydration unit are rich in crotonic aldehyde and acetylene (flow rate - 10 m^3/h, at 30°C), whereas the of acetaldehyde rectification unit discharged the waste water at 40–50°C with the flow rate of 150 m^3/h.

5.2. Clean-up of the Effluents from the Acetylene Hydration Unit.

The installation includes a microbial purification unit composed of

tail-strips of porolon on which active degradative bacterial cultures of the genus *Pseudomonas* were earlier immobilized. The unit of microbial purification thus prepared is installed in the shop discharge canal. Use of this unit permitted the total removal of the aldehyde from the waste water discharged (Table 2).

TABLE 2. Local waste water purification from aldehydes in the acetylene hydration production unit.

Purification step	Assay No.	Conc-n of components, mg/l						Purification effect, %	
		prior to purification			after purification			from COD***	from initial conc-n
		CA*	AA**	C_2H_2	CA	AA	C_2H_2	CA	
	1	25.6	159.4	132.8	0	0	15.9	88.7	100
First	2	48.3	192.5	166.1	0	0	20.7	91.3	100
unit	3	74.8	173.3	148.6	9.1	1.3	27.8	86.7	87.8
	4	103.7	128.7	153.3	23.2	6.2	11.9	74.8	77.6
Second	5	112.7	119.3	180.2	0	0	316.5	94.7	100
unit	6	173.4	168.7	136.8	0	0	22.4	90.8	100
	7	218.2	120.9	157.6	0.7	8.3	29.5	87.6	92.7
	8	252.8	138.8	98.7	28.1	13.6	11.4	81.3	88.9
Third	9	226.3	187.2	76.7	0	0	12.4	92.4	100
unit	10	276.4	167.5	123.8	0	0	18.9	89.8	100
	11	341.5	148.7	174.3	9.3	19.7	23.5	94.6	97.3
	12	360.8	170.6	86.7	21.6	68.5	17.3	91.9	94.0

* - crotonic aldehyde; ** - acetaldehyde;
*** - chemical oxygen demand.

Chemical production control gave some 88.7–91.3% for the effect of the clean-up. Modelling of the bulk discharges of crotonic aldehyde in concentrations up to 103 mg/l (4-fold increase compared to the tolerated dose) did not affect the purification efficiency. In the purified effluents, the aldehyde concentration did not exceed the maximum tolerated dose of 24 mg/l thus giving an aldehyde removal efficiency of 77.6% and 74.8% in terms of the chemical oxygen demand (COD). Further increase of the crotonic aldehyde concentration was impossible because of the risk of exceeding the maximum permissible concentration of the

pollutant in the final waste water discharge.

To enhance the purification effect, a second, and later a third, microbial purification units were installed in the waste water discharge canals. These of additional units raised the efficiency of purification to 92.7% thus making possible neutralization of bulk aldehyde discharges up to 340 mg/l while maintaining the purification effect at no less than 90% level, in a number of instances, the aldehyde was completely removed from the discharge.

Similar results were obtained for the effluents from the acetaldehyde rectification unit where purification was carried out at different temperatures. The data obtained showed that the purified discharge contained the aldehyde within tolerated limits even when the bulk discharge gave the 10-fold quantity. A 10°C temperature increase did not practically affect the bacterial activity level, whereas at 50°C the purification efficiency dropped only by 1-4%.

5.3. Waste Water Purification of AMS in the Synthetic Rubber Production

Similar installations were used for purifying effluents from the synthetic rubber production unit. The waste water was basically poluted with AMS, synthetic fatty acids (SFA) and latex. AMS concentrations in the effluent did not exceed 100 mg/l. However, during pulse AMS discharges, its concentration increased 6-fold. Oxidation of AMS was carried out by the mixed culture of *B. cereus* 3 and *P. aeruginosa* 6 and 8. Testing of the installation under industrial conditions showed a high purification effect in relation to AMS and other organic compounds (Table 3).

In emergency situations (300-600 mg AMS per litre), the toxicant concentration was rapidly reduced to a tolerance level with the purification efficiency of 99.6%. During a month-long test of the installation under industrial conditions, it received some 21,600 m^3 of waste water with AMS concentrations reaching at times 600 mg/l. Under such conditions the installation displayed reliable performance providing a 99.5% purification efficiency, i.e. practically total microbial oxidation of AMS.

TABLE 3. Efficiency of the installation for the local waste water purification from AMS.

AMS concentration, mg/l		Purification efficiency, %	
prior to purification	after purification	of AMS	according to COD
44.4	0.2	99.9	92.5
89.0	0.4	99.9	91.0
155.6	1.1	99.9	89.9
298.8	2.3	99.8	85.4
646.2	2.8	99.6	80.2

In conditions of increased industrial output additional pollutants, mainly highly toxic organic compounds and heavy metal ions, are incorporated into industrial sewage which, when discharged uncontrollably to the environment, perturbs the natural biocenoses. Increased public concern in relation to environmental protection requires principally new, research-based microbial technologies for the degradation of xenobiotics, the more so since the conventional techniques relying on the oxidative characters of the activated sludge microflora are no longer efficient because of repression of the enzymic systems and destruction of microbial populations by the increased contamination potential of the sewage.

Recent research shows that the highly toxic components of industrial sewage influence certain microorganisms; some hitherto active organic matter oxidizers are removed from microbiocenosis. This, in turn, leads to a decreased biochemical activity of the activated sludge and to an overloading of the system with pollutants and its inability to process the discharged toxicants.

On the other hand, selective conditions occurring in the activated sludge and sewage force the remaining microflora to form populations having the capability to degrade toxicants present in sewage.

The results from the sewage treatment plant operation in Alma-Ata are promising. Over 50 active microbial strains isolated from the industrial effluents not only displayed high degradative characters towards AMS, toluene and crotonic aldehyde but they also acquired some

unique properties not found in analogous strains of the *Pseudomonas* and *Bacillus* genera isolated from natural environments. The unique characters of such microbial strains are highly promising for developing novel microbial technologies with efficient degradation (1-10 g/l) of the toxicants discharged in industrial sewage. Immobilized on inert supports, these microorganisms bring about preliminary transformations of organic pollutants producing simpler forms that are further involved by a spontaneous microflora into metabolic processes which occur in the activated sludge.

REFERENCES

Alieva R.M., A.N. Ilyaletdinov. 1986. Realization of ecolgical principles in the microbial cleanup of industrial effluents. *Izv. AN SSSR, seriya biol.* 1:517-527 (in Russian).

Boronin A.M., G.K. Skryabin. 1985. Genetic aspects of the bacterial degradation of xenobiotics. *Uspekhi Mikrobiologii* 20:40-50 (in Russian).

Skryabin G.K., L.A. Golovleva. 1976. *Use of Microorganisms in Organic Syntheses.* Moscow, Nauka (in Russian).

RECOMMENDED LITERATURE

Advances in Biotehnological Processes. 1985. V. 5. A. Mirrahi, A. L. van Wezel, eds. Alan R. Liss, Inc., New York.

Biotechnology. V. 8. Microbial Degradation of Organic Compounds. 1986. W. Schonbarn, ed. VCH, D6940, FRG

Microbial Degradation of Organic Compounds. 1984. Gibson D.T., ed. Marcel Dekker, Inc. New York.

Plasmids in Bacteria. 1985. D.A. Helsincki, S.N. Cohen, D.B. Clewell, D.A. Jackson, A. Hollaender, eds. Plenum Press.

ECOLOGICAL ASPECTS OF THE MICROORGANISM-PESTICIDE INTERACTION

Yu.V. Kruglov

In conditions of intensive farming, pesticides prove to be the most efficient tool for plant protection from various pests and weeds. Owing to their application the efficiency of crop cultivation has increased tremendously, but at the same time problems related to the environment protection have also become more acute.

At the present time, annual world production of pesticides is about 3 mln tons of which nearly 50% are herbicides applied to combat wild vegetation.

Experts prognosticate that the level of world production of pesticides will be 8-10 mln tons by the year 2000. The extent of pesticide stress on the soil varies from country to country within the range of 300 g to 30 kg per hectare depending on the soil-climatic conditions, crop varieties and the level of agricultural production.

Pesticides are synthetic, i.e. man-made substances that display (with rare exceptions) very high physiological activity and are utilized in quantities of 0.5 to 10 kg/ha, often applied repeatedly to the same fields. Subsequent or simultaneous application of fungicides, herbicides, and insecto-acaricides results in superimposition of one type of pesticide on another.

The relatively high persistence of pesticides and their metabolites

allows for their environmental cycling from several days to years, thus creating an unprecedented potential of anthropogenic effect on naturally occurring processes.

Irrespective of the procedure, 60 to 100% of applied pesticides find ultimately their way to soil. Therefore, the soil is a sort of a depot, the basic venue of microbe-pesticide interaction and a continual source of pollution for other ecosystems, feed and food products. At the same time, of all the natural systems, the soil is the only one for which the permissible level of herbicides is not legally regulated in most countries.

A large body of literature has accumulated on the effects of pesticides on the soil microflora and biochemical processes, though most of the reports are of a descriptive nature. Interpretations of soil-microbial analyses are often conflicting and hardly useable for informative comparison.

Two monographs published in recent years on this subject (Pesticide Microbiology, 1978; Insecticide Microbiology, 1984) are not free of the above-mentioned shortcomings. Over the last decade, a number of reviews have appeared that present a fairly good factual and logical analysis of the experimental material accumulated to date. Therefore the purpose of the present lecture is to focus on methodological principles of the soil-microbial assessment of pesticide toxicity and analysis of the basic regularities underlying formation and activities of microorganisms upon toxicants' introduction in the soil. An evaluation of the subsequent effects of pesticide application on soil fertility, prognostication, monitoring and development of efficient and safe methods for chemical plant protection in agriculture will be hardly feasible without revealing such regularities and adequate understanding of the cause-and-effect relation in the behaviour of soil microorganisms in response to pesticide stress. Unfortunately, current practices of plant protection as well as environmental programmes take little into account of the results of microbial soil studies. Thus, methodology of the soil-microbial assessment of pesticides should be regarded as the key problem in this field.

The core of this problem consists in the fact that in soil we do

not deal with only one species, and even not with mixed cultures, of microorganisms but with their associations that function and evolve in conformity with the ecological statute.

In ecology, the basic unit is an ecological system which is characterized by a flux of energy, turnover of substances and specific structural organization. The soil microflora is part of the terrestrial ecosystem in which the role of specific microorganisms is determined by their place in the flux of energy and the cycles of elements.

Microorganisms occupy various trophic levels, though the principal flow of energy passes through saprophytes. The basic sphere of their activity is mineralization and humification of the products of exoosmos and organic matter of dead animals and plants entering the soil. These are closely related to processes of soil formation and plant nutrition.

While considering the soil microflora as a structural unit of the land ecosystem, we may also attempt to formulate the principal task of the soil-microbial assessment of pesticide toxicity and that of exogenic substances in general. It consists in determining the upper limits e.g. stress, concentrations, under which the energy flow is affected, the cycling of substances is perturbed, and the structural organization of microbiocenosis is upset. It also predetermines the choice of indicators (Table 1). These embrace basic parameters of the ecosystem including the soil capacity to a self-cleanup from pathogenic microbial forms and toxic chemicals.

The first three parameters are of certain interest from the ecological and agronomical viewpoints, while the next two (4, 5) are important from the sanitary and hygienic ones. With these indicators in mind, one can have a clear idea about the changes that take place in the soil biota under the influence of pesticides. This naturally implies the availability of quantitative criteria which are indispensable for the objective assessment of such changes.

Toxicological evaluation of chemicals is known to be based on the principle of thresholds which states that 'beyond a certain range of concentrations the chemical ceases to be poisonous and will no longer affect the organism in the manner hitherto inherent in it'.

TABLE 1. Soil-microbial evaluation of pesticide toxicity

Ecological and sanitary-hygienic indicators	Basic indicator micro-organisms and processes	Additional indicators
Energy flux	Soil respiration (CO_2 emission)	
Cycles of major elements	Nitrogen: nitrification, N_2-fixation	Ammonification, urease, protease
	Phosphorus: mineralization of organophosphates	Phosphatase
	Carbon: CO_2 emission, degradation of cellulose	Invertase, amylase
Biocenosis composition	Saprophitic bacteria, actinomycetes, fungi, microscopic algae, microfauna	Species diversity, dominant species, indicator species
	Symbiotic systems: - bacteriotrophic plants, - mycotrophic plants, - lichens	
Sanitary conditions	Pathogenic forms of micro-organisms	Antibiotic potential
Soil capacity for selfpurification	Persistence of pesticides, kinetics of detoxification	Bioconversion of pesticides, metabolites

Methodologically, the threshold character of an action may be revealed by analysis of the experimental curve 'effect on microflora versus pesticide dose' presented in its generalized form in Figure 1.

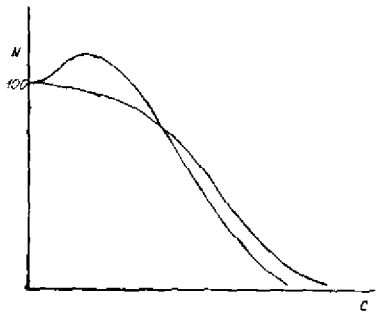


FIGURE 1. Effect of pesticides on the microflora and biochemical processes in soil (dose/effect curve).
N, microbial counts or rates of biochemical processes;
C, pesticide concentration.

It is seen that upon reaching certain concentrations, herbicides overcome the physiological resistance of microorganisms. The number and rates of biochemical processes in the soil drop drastically. It should be noted that the toxic effect of pesticides on the microflora disappears long before their concentrations in soil reach zero values. In a number of instances low concentrations produce a stimulating effect indicative of physiologically active compounds.

Quantitative assessment of the toxic effect reposes on the use of differing levels of the inhibiting action.

Taking into account the rather low accuracy of the soil and microbial analytical techniques and their lability with time and space, we assume for the criteria of toxicity such concentrations of herbicides (RC_{50}) at which they repress half the numbers of microorganisms or the rates of biochemical processes occurring in the soil.

As the maximum tolerated concentrations of pesticides in soil are preset as dictated by production needs, and sanitary and hygienic regulations, it appears advisable to express their 'harmfulness' with regard to individual soil-microbial parameters by comparison with the production-required concentration (PC). We define the RC_{50}/PC ratio as the reliability or safety index (K_s). By its meaning, it is similar to the index of the range of therapeutic effect used in pharmacology for evaluating medicinal properties of substances, and it is supposed to indicate how many times the inhibiting concentration of the herbicide exceeds the production-needed one.

As in the case of medicinal substances, the application of pesticides is a necessary measure caused by man's striving to create conditions favouring crop cultivation and thus obtain maximum yields of agricultural produce per area unit. Therefore, the application of pesticides in agriculture, as well as the use of curative substances in medicine, is fraught with certain risks. Regarding themicroflora as one of the critical factors of soil fertility, such hazards diminish with the increasing safety index (K_s).

For practical risk evaluation the following 'safety scale' is used:

Ks	Inhibitory Effect
below 1	powerful inhibitor (sterilizer)
1 to 10	moderate inhibitor
10 to 100	poor inhibitor
above 100	practically nontoxic

The use of this scale to assess the toxicity of herbicides provided results that are presented in Fig. 2. The effect of herbicides, referred to various classes of chemical substances, on the biota appears to be of a selective nature which makes it possible to single out the most toxic compounds and the most vulnerable components of the land ecosystem.

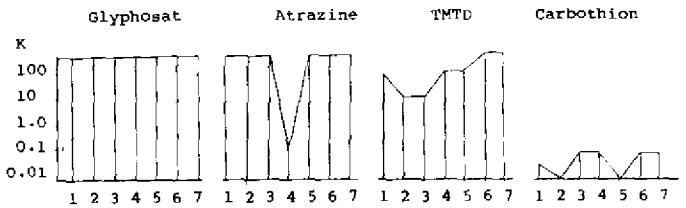


FIGURE 2. Diagrams of the soil-microbial evaluation of pesticides. 1, bacteria; 2, fungi; 3, actinomycetes; 4, microscopic algae; 5, nitrification; 6, degradation of cellulose; 7, soil respiration; Ks, safety coefficient.

Specific microbial groups and processes for which the safety index (Ks) is below 1, may be regarded as a sort of 'targets'.

Variation of other soil-microbial parameters will naturally depend on how closely they are linked to such targets. Thus atrazine, prometryne, monuron and other herbicidal inhibitors selectively repress the growth of microscopic algae. It seems that in the agroecosystems, where higher plants are the basic producers, the above herbicides will not substantially affect the production processes responsible for renewal and accumulation of organic matter in the soil. On the other

hand, if they find their way to primitive soils in which algae and lichens are the basic producers, they may lead to catastrophic consequences.

Inhibition of the nitrification processes by carbothion and ragor results in the accumulation of ammonium nitrogen and compensation for the loss of nitrogen fertilizers due to denitrification and leaching of nitrate under the conditions of intensive cultivation. Repression of nitrogen fixation by TMTD and carbothion blocks the sole natural source of replenishment of the soil nitrogen pool.

Yet the negative result *per se* does not provide sufficient reason for refusing to use chemicals to protect plants. A comprehensive and thorough analysis of experimental material is in order before the final conclusion can be drawn.

Long-term observations showed that the deleterious effect of pesticides on target microorganisms is reversible. The dynamics of the toxic effect may be characterized with the U-shaped curve (Fig. 3). After a time, depending on the pesticide type, the microbial numbers and intensity of biochemical processes in soil are restored. The amplitude of variation and the restoration rate depend on the toxicity and persistence of the pesticide, as well as the adaptation capacity of the microbial population. For powerful biocides, such as carbothion for example, the negative effect is more lasting compared to their residence time in the soil.

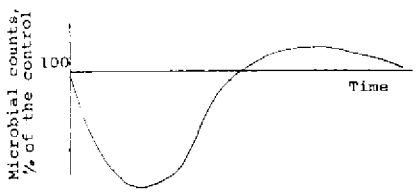


FIGURE 3. Dynamics of the pesticide toxic effect on soil microorganisms.

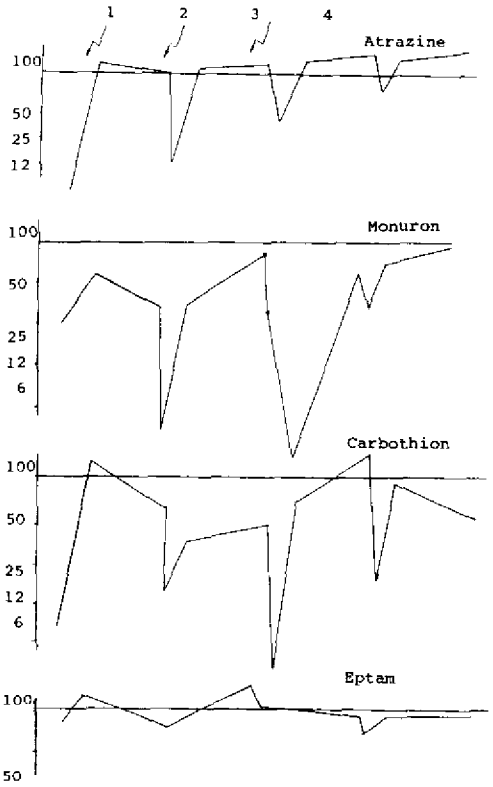


FIGURE 4. Dynamics of numbers of algal population in soil under repeated seasonal pesticide treatments.
I, 2, 3, 4 - years of treatments with herbicides.

After systematic application over several years, the inhibiting pesticidal effect is shown with a pulsating curve (Fig. 4). Here, the amplitude of the fluctuations in the numbers of microorganisms decreases after recurrent treatment of soil with pesticides. Hence, the negative effect of pesticides on the system indicators is reduced with repeated applications to soil. There occurs an adaptation of the microorganisms whose nature and causes will be dealt with later in this lecture.

In the case of highly recalcitrant pesticides (monuron) and their strong toxic effect on target microorganisms a different pattern is observed (Fig. 4). Complete restoration of the numbers of microorganisms is not attained by the end of vegetation period. The level of biological activity in soil is decreased accordingly. Therefore, the systematic

application of persistent pesticides is one of the most serious dangers to the soil biota and, consequently, requires a thorough ecotoxicological evaluation.

The reversible (or temporary) character of the pesticidal toxic effect on the indicator group of target microorganisms is due to a number of causes. First, the pesticide concentration in the medium does not remain constant: it is reduced as a result of the pesticide dispersion over the soil profile, its subsequent adsorption and degradation under the effect of physico-chemical and biological factors. Second, there occurs an adaptation of microorganisms to the herbicide in question. From the ecological viewpoint, the term adaptation should be understood here not only as the genetic or physiological adaptability of an individual microorganism or a species, but rather as specific changes in the population of microbial indicators and microbial community on the whole.

For the sake of illustration, Fig. 5 presents the results of a study of the species composition in populations of cyanobacteria and microscopic algae in a soil treated with herbicides. The frequency of occurrence is seen to diminish for many species one week after herbicide application, while part of them disappears totally from the algal cenosis. A predominant position is occupied by 1-2 species which are essential for the productivity of algal flora. Restoration of the species composition occurs slowly and is dependent on the toxicant persistence.

Such a situation is stabilized by repeated long-term applications of herbicides. The most vulnerable microbial species are merely 'thrown out' from the microbiocenosis as the result of regular herbicide applications. This leads to formation of a qualitatively new community whose distinctive features are a decreased level of species diversity, high index of predominance, absence of cyanobacteria responsible for molecular nitrogen fixation. (Fig. 6).

The major consequence of regular long-term applications of herbicides is a restructuring of the microbial community and, first and foremost, changes in the species composition of the microbial indicator group.

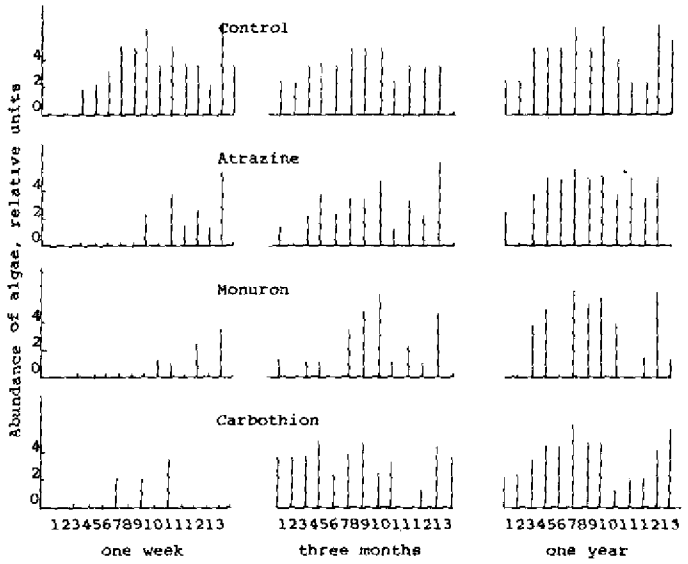


FIGURE 5. Effect of herbicides on the species composition of algae in soil.
1 - *Amorphonostoc punktiphorme*, 2 - *Anabaena sp.*, 3 - *Cylindrospherum sp.*, 4 - *Phormidium automnale*, 5 - *Ph. curtum*, 6 - *Ph. foveolarum*, 7 - *Chlamidomonas goeogama*, 8 - *Chllorococcum infusionum*, 9 - *Hormigium flacidum*, 10 - *Chloridella negecta*, 11 - *Hantzschia amphioxys*, 12 - *Navicua atoms*, 13 - *Nitschia palea*.

Stabilized variations in the species composition of the indicative group are the first warning symptoms pointing to an overloading of the soil with biocides. The index of species diversity proves to be very helpful for characterization of such an overdose.

The third important factor responsible for high stability of the soil as a biological system is its heterogeneity and multiphase composition, and hydrophobic properties of most herbicides.

Figure 6 features a scheme which characterizes the low-granular structure of a soil and the distribution pattern of microorganisms over the soil aggregations. Under conditions of deficient or 'normal' soil moistening, microorganisms concentrate mainly on the surface of soil

particles, microcolonies and films, and are incorporated into the organo-mineral gel where they are immobilized. Only a small microbial portion is distributed in the soil solution. Naturally, microorganisms display different attitudes towards physiologically active substances, as examplified by antibiotics and other natural compounds. Judging from the data of soil-microbial analysis, the total microbial stability increases, as a rule, ten to hundred-fold compared to that observed for pure cultures on synthetic nutrition media.

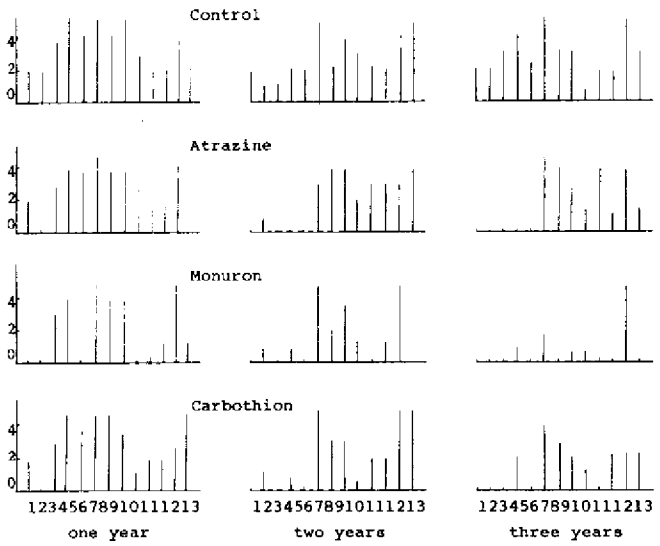


FIGURE 6. Effect of a long-term application of herbicides on the soil algoflora. 1-13 - the same species composition as in Fig. 5.

On the other hand, upon entering the soil, pesticides are adsorbed by the surface of soil particles and interact with the soil organic matter by forming with the latter sufficiently strong bonds. This also affects essentially the pattern of their effect on microorganisms. Our understanding of the character and extent of this effect are today far from satisfactory. An interesting phenomenon of pesticide-microorganism

interaction was observed in the course of direct microscopic investigation of soils. The pesticides which are poorly soluble in water exhibit a discontinuous distribution pattern observed as particles or films. Their diffusion to the surrounding solution becomes the centre of microflora formation.

Physico-chemical characters and toxicity of pesticides determine their specific influences on the microflora. In this case, at least two types of microbial formations can be singled out associated with the particles. The first type is characteristic of compounds which display a broad-range microbicidal effect (Fig. 7). Here, the development of microorganisms begins some distance away from the particles thus resulting in the formation of a sterile zone around these particles, the inhibiting effect being gradually decreased and ultimately transformed into a stimulating one on leaving the particles. The latter situation is characteristic of physiologically active compounds. Consequently, under heterogenic soil conditions the herbicidal effect on the same microbial community may be both positive and negative at the same time, such a situation leading to a spatial rearrangement of the microorganisms.

The second type is indicative of substances with a restricted selective effect. In this case the sterile zone does not form. Some time after the herbicide enters the soil, the surface of particles and surrounding space become the site of bacterial growth and agglomeration to form colonies similarly to the pattern observed for the growth of water-insoluble polymers (cellulose, chitin, synthetic rubber etc.). In such a case a bacterial monoculture usually forms and colonizes the surface herbicide. Accumulation of bacterial mass on the herbicide surface is very slow and varies for different disconnected particles. Long-term observations show that even after half a year, practically sterile crystals were found in soil among particles of atrazine overgrown with dense bacterial layers. An 'overgrowth' of herbicides with the mycelium forms of microorganisms has never been observed.

Actinomycetes and fungi 'avoid' contact with herbicides and display negative chemotaxis towards these compounds. The ability of mycelial microorganisms to actively escape the zone saturated with herbicides under heterogenic soil conditions underlies high resistance of the

microflora to agricultural chemical toxicants and in a certain measure explains low efficiency of chemicals in the protection of plants against the soil-based phytopathogens. It becomes clear from this, that the effect of the poorly water-soluble herbicides on the soil biota is restricted to a relatively small diffusion zone and implies the availability of a comparatively large volume of soil free of herbicides, or where they are present in low concentrations.

This idea may be illustrated with the following calculations. Under a pesticide dose of 1 kg/ha and a particulate mass of 10 μg, only one particle is available in one square cm of soil. Taking into account that the herbicide is distributed mainly over the soil layer 10 cm deep, only one particle will be allotted for 1 cm, i.e. the distance between individual soil particles is thousands of times that of their physical size.

The above relation is of crucial significance for understanding the high biological buffering capacity of soil with regard to chemical toxicants used in agriculture. These data also point to the need for revising or at least correcting the current concepts on the pathways of microbial degradation of herbicides in the soil based exclusively on the occurrence of such processes in aqueous solutions.

This is not exactly the case in reality. The example of the atrazine behaviour in soil indicates unequivocally that the surface of the pesticide particles becomes the site of formation of a specific microflora which displays an active development leading to the emergence of groups, colonies and films. Atrazine particles are covered with multicellular bacterial layers, reduced in size, lose their shape and finally disappear, a bacterial mass being left as its trace. In other terms, the degradation of pesticides is taking place.

The above observations evidence that the pesticides which resist as a solid phase and display hydrophobic properties are subject to microbial attack and degrade in the 'overgrowth' manner.

While investigating possible degradation routes for these compounds, one should also consider the studies of biological lesions. In the first place, the exoenzymes that catalyze reactions of oxidation and hydrolysis are the most important of all the biochemical mechanisms.

Low solubility and specific composition of such pesticides renders them incapable of inducing the enzyme synthesis. Their degradation appears to involve enzymes with a broad substrate specificity which in turn requires additional inducers and energy sources, i.e. the process can occur only when a growth substrate is available in the medium.

In the second place, microorganisms are capable of producing reactive products such as organic and mineral acids, sulphur oxides, hydrogen peroxide etc. Under their impact, hydrolysis, oxidation and destruction of the toxicants occur.

Thirdly, one should keep in mind that in the course of their activity, microorganisms can produce various organic solvents (methanol, ethanol, butanol, acetone etc.) which will under local effects, transfer pesticides into solution thus contributing to their redistribution and degradation in the soil.

Hence, microorganisms can induce partial transformation or solubilization of water-insoluble pesticides, the former occurring indirectly, via intermediation of the reactive products of microbial activities without direct involvement of specific enzymes.

This makes it possible to explain the involvement of microorganisms in the pesticide degradation, though they have no enzymes capable of attacking the toxicants. This mechanism differs from both catabolic and cometabolic routes and should occupy an adequate position in the assessment of the role of microorganisms in the pesticide degradation under natural conditions.

In terrestrial ecosystems, the soil microflora is intimately related to other components of biocenosis and, in the first place, to plants that constitute the principal group of producers. It is precisely plants that are the basic nutrition energy sources for saprophytic microorganisms in the metabiotic cycle of the total turnover of substances.

Replacement of agrotechnical methods for combatting weeds by the chemical ones results in essential changes in the character of their growth and residue distribution over the soil profile.

Under agronomical techniques, weeds are buried in the soil and become a continual nutrition and energy source for the soil

microorganisms. Analysis of the experimental material and the information published in literature on the subject shows that under the average field contamination with weeds of 0.1 to 0.5 kg/m^2, some 1 to 5 tons of fresh vegetation is buried into the soil per hectare. This provides for a fairly high level of life activities of heterotrophic microflora, when the total mass of weeds is 10 to 100 times less under chemical protection of plants. Subject to this, the organic matter pool of the soil deteriorates leading in turn to reduced rates of microbiological processes.

This mechanism remains sometimes unnoticed by researchers in the studies of herbicide application to soils for crop protection. But its effect is clearly felt in the course of repeated herbicide treatments for protecting perennial plants.

Long-term soil-microbial studies carried out by the author on plantations of tangerine-trees in the Abkhazian SSR (5 years), on spruce forest-plantations in the Leningrad region (8-9 years) and in fields of a medicinal herb (*Diascorea caucasian*) in conditions of Moscow region (3-4 years), provided basically similar results. The total repression of weeds with herbicides leads, in the long run, to a reduction of major parameters of the soil biological activity. The microflora acquires specific features indicative of the soils that have been black-fallowed for long periods. The total biogenic capacity of the soil is deteriorated, the relative numbers of sporous bacteria and fungi are reduced, while the population of humate-degrading bacteria and nocardia increases. Along with this, there occurs a decrease in the humus content and the ammonium and nitrate forms of nitrogen are accumulated.

In the herbicidal version of the experiment, losses of humus were due to a shift of the processes of decomposition-synthesis of humic compounds to the decomposition side because of a drastic decrease of the quantity of plant residues supplied to the soil by weeds. Such humus losses were also due to a functional rearrangement of carbon nutrition for the microbial community evidenced by increased numbers of humate-degrading bacteria and nocardia.

Table 2 illustrates the results of an experiment in a tangerine plantation under subtropical conditions, where a formulation of

herbicides including atrazine, monuron and region was applied for a period of 5 years.

The data obtained suggest that systematic herbicide applications for perennial plant protection ultimately affect the soil fertility. In this case, the use of organic fertilizers acceptable for a specific crop (manure, compost, siderates, intermediate crops etc.) as well as rational combination of agronomical and chemical methods for combatting wild herbs is crucial for the soil protection and maintenance of its fertility.

TABLE 2. Effect of a 5-year seasonal application of a set of herbicides on the microflora and enzyme activity in soils of tangerine groves.

Soil, assay variants	Horizon, cm	Bacterial counts, 1000/g soil: Saprophytes Total	Saprophytes Spores	Nocardia	nitrifying	denitrifying	ACD *	Enzymes: catalase, **	urease, ***	Humus %	% of control
Podzolic:											
Control	0-15	1050	159	7.0	10.0	250	90	7.0	170	4.03	
Herbicide	-	1470	117	32.1	5.0	60	1	2.1	67	3.53	88
Control	15-30	1780	311	2.0	8.0	110	10	4.1	126	3.26	
Herbicide	-	530	53	41.2	1.5	6	1	2.0	-	3.00	91
Alluvial:											
Control	0-15	1000	170	-	20.0	25	10	2.2	475	4.36	
Herbicide	-	530	67	-	1.0	6	1	1.9	120	3.57	82
Control	15-30	3660	530	-	4.0	250	10	2.4	232	2.50	
Herbicide	-	920	130	-	1.0	6	1	2.2	16	2.22	89

* - ACD - aerobic cellulose-degrading bacteria;
** - catalase activity expressed in O_2 mg/g;
*** - urease activity expressed in μg/g.

Thus, analysis of the above results suggests that herbicides affect microorganisms both directly and indirectly.

The direct effect is due to herbicide toxicity, it bears a selective character and is restricted to individual components of microbiocenosis regarded as 'targets' in the association of soil microorganisms. The nature and extent of this effect on the soil biota depends, in the main, on the place and role of the 'target' in the

cycling of substances.

The indirect effect is explained by variation of conditions of nutrition and energy supply for the microorganisms as the result of a reduced flow of plant residues to the soil. In this situation, complete reduction of biological activity and functional restructuring of carbon nutrition of the soil microflora can occur. This, in the final analysis, may lead to a more intensive loss of humus and deterioration of the soil fertility as a whole.

From the above considerations it follows that prognostication of the possible consequences of herbicide application should include the following steps: specification of the 'target', ecological analysis of its role in the ecosystem, and examining the ecotope status as a result of changes in the technology of crop cultivation.

The high sensitivity of microbial indicators provides a real background for discussing the problem of soil-microbial monitoring of biospheric pollution. In this connection two approaches stand out. The first one is concerned with the character and extent of soil pollution with herbicides, which may be clarified by analysis of the microbial indicator group structure using the index of species diversity as the integral indicator.

Disappearance of certain species from the biocenosis and a decreasing index of the species diversity may be regarded as a signal of soil pollution with exogenic substances, which allows one to undertake appropriate steps in order to identify and eliminate the source of pollution.

This method has been mostly developed for inhibiting photosynthesis and may in the first place be used in the biosphere reserves.

The second approach is based on the utilization of highly sensitive test-cultures of microorganisms for experimental determination of herbicides in the soil.

In our studies we isolated and selected a number of microbial strains, and developed a few modifications of the algological method for determining herbicidal derivatives of the sym-triazines and phenylurea directly in aqueous solutions and soils by circumventing the laborious and expensive process of toxicant extraction with organic solvents.

Figure 7 presents nomograms which reflect the relation between the toxic effect of herbicides and their concentrations in the soddy-podzolic soil. The curves permit quantitation of these substances in the soil. A set of experiments showed that the sensitivity and accuracy of determinations of the herbicides which inhibited photosynthesis using the algological method were equal to those in other biological methods in which higher plants are used as test cultures, but the former method is 5-10-fold faster.

The basic value of the method is in the fact that it allows rapid evaluation of the phytotoxicity of the herbicide and its metabolites even in combinations with various natural compounds that accumulate in the soil, thus making possible amendments to the technology of their application.

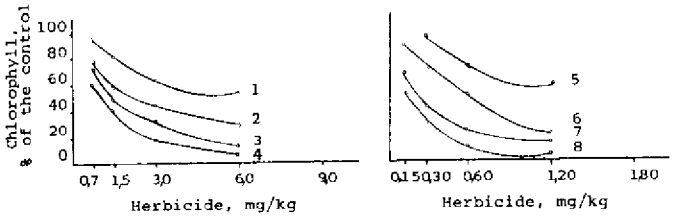


FIGURE 7. Effect of herbicides on the growth and chlorophyll production in *Ch. vulgaris* (method of 'paper discs'). 1-linuron, 2-arezine, 3-diuron, 4-monouron, 5-chlorazine, 6-simazine, 7-atrazine, 8-prometryne.

Figure 8 shows results of the experiment in which the herbicide diuron was determined by a chemical method, while its phytotoxicity was evaluated using the algological method. The chemical analysis suggests that plant residues enhance the diuron decomposition. However, the phytotoxicity was increasing during the 4-month observation. The explanation may be found in the synergetic or total effect of diuron, plant residues and products of their degradation.

Similarly, the algological method allows determination of the herbicidal metabolite toxicity. Specifically, degradation of chlorazine to trietazine and simazine was accompanied by the gradual increase in

phytotoxicity from 4 to 25 times. Transformation of phenylcycloureas to the corresponding derivatives of phenyl-methylureas produced an increase of several orders.

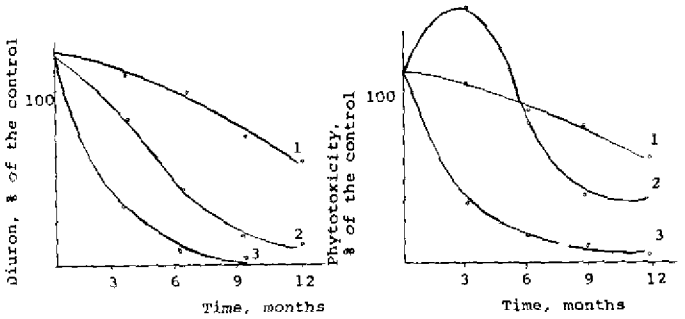


FIGURE 8. Dynamics of diuron degradation and phytotoxicity of soil. 1 - soil, 2 - soil + 2% lupine green mass, 3 - soil + 2% manure.

Thus, the use of indicative microorganisms in monitoring the biosphere provides better ecological information compared to the used chemical analyses. Therefore, utilization of biological methods should be regarded as one of the most important steps in the ecotoxicological soil monitoring, microbiological methods, in particular, playing the key role.

Analysis of the herbicidal effect on symbiotic systems calls for special attention. In this context, the leguminous-rhizobial symbiosis is of the utmost significance from both fundamental and practical viewpoints.

Physiologically active by their nature, herbicides can affect both partners in this symbiosis. Accordingly, their effect on the character of symbiotic relations will be displayed differently. This also implies different approaches to the development of technologies for their rational and efficient application.

Table 3 carries experimental results pertinent to herbicidal toxicity of 55 strains of nodule bacteria. The herbicidal effect is

dependent on the chemical composition of the toxicant and on the type of bacterial strain, such a variability being fairly high for herbicides of the atratone type.

TABLE 3. Sensitivity of nodule bacteria to various herbicides.

Herbicide	Diameter of sterile zones, mm	Number of strains, %			
		0	1-5	6-10	over 10
Simazine		100	0	0	0
Atrazine		96.4	3.6	0	0
Propazine		89.3	10.7	0	0
Prometryne		63.6	30.9	5.5	0
Chlorazine		56.3	37.5	6.2	0
Trietazine		63.6	3.6	16.4	16.4
Atraton		9.1	36.4	9.1	45.4
Eptam		100	0	0	0
Tordon		97.2	0	0	2.8
Diuron		90.0	9.7	0	0
Monuron		93.5	3.2	3.1	0
Methurin		69.7	21.2	9.1	0
TMTD		0	20.6	48.3	31.0
Carbothion		0	0	0	100.0

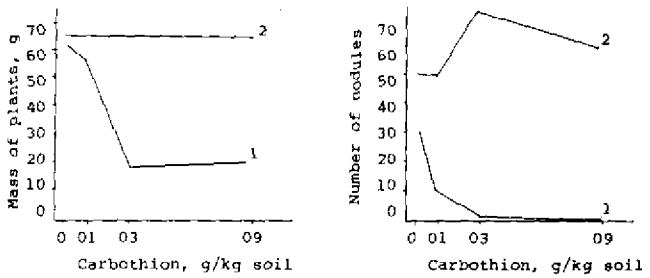


FIGURE 9. Effect of the soil treatment with carbothion on the efficiency of leguminous-rhizobial symbiosis.
1 - spontaneous infection of lupine with nodule bacteria;
2 - inoculation of lupine seeds with a preparation of nodule bacteria. Lupine seeds were sown 8 weeks after the soil treatment with carbothion.

Carbothion exhibits a very strong herbicidal character. Its

application to soil in concentrations 2-3 times below the PC (production-required concentration) level is lethal for all nodular bacteria. As the result, nodules do not form on the leguminous plant roots and the nitrogen fixation is thus blocked. For their nutrition, leguminous plants are forced to extract mineral nitrogen from the soil. If the soil is nitrogen-deficient, the crop productivity drops (Fig. 9).

The residence time of carbothion in soil is 4-6 weeks. Therefore, inoculation of leguminous seeds with nodule bacteria, 4-8 weeks after the herbicide was applied, produces a total restoration of symbiosis. In contrast, atrazine is not toxic for nodular bacteria. For example, for *Rhizobium* its half-lethal dose is 100 times the production concentration (PC). Released into soil, atrazine penetrates into the roots and translocates in the acropetal direction, concentrates in the foliage and starts inhibiting photosynthesis. Essentially, this comprises its herbicial effect. The consequence is that foliage as well as roots of the plant are depleted in monosaccharides, the amount and mass of nodules diminish, the molecular nitrogen fixation no longer occurs, and the plant productivity drops respectively. At high concentrations of atrazine, inoculation of plants with nodule bacteria produces a negative effect. This means that the nodular bacteria turn to a parasitic way of life (Fig. 10).

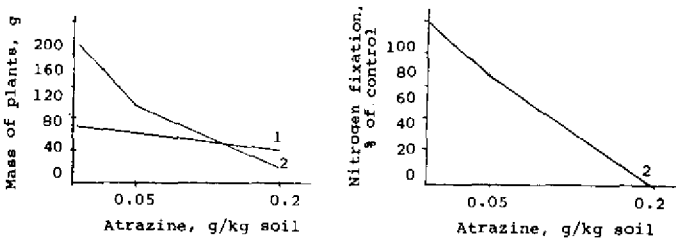


FIGURE 10. Effect of atrazine on the efficiency of leguminous-rhizobial symbiosis.
1 - lupine drown on sterilized sand: no nodule bacteria;
2 - lupine seeds inoculated with a preparation of nodule bacteria.

Herbicidal derivatives of phenoxyalkyl carboxylic acids are used for vegetating plants when the infection period ends and an active

growth of the meristem and the formation of bacteroidal tissues of nodules occur.

Experiments with 2,4-dichlorophenoxyacetic acid (2,4-D) with a radiomarker in the aromatic ring showed that herbicides of this group, once in the plant and propagating in the basipetal direction, penetrate inside tubercles and concentrate in the bacteroidal tissue (Fig. 11).

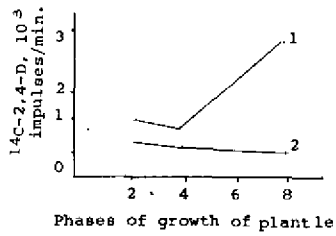


FIGURE 11. Distribution of C-2,4-D in roots of green pea plants. 1 - nodules; 2 - roots.

Capable of the auxin-type effect, the phenoxyalkanes tremendously affect the formation of nodules. Under the effect of herbicides, the growth cells of the bacteroidal zone are fragmented followed by their vacuolization, the cell wall is destroyed, and the bacteroidal tissue thus disintegrates. As a result, the leglobin content decreases, the activities of the succinate dehydrogenase and nitrogenase are reduced, and the extent of nitrogen fixation declines (Table 4).

Comparison of the results of anatomo-morphological and biochemical studies suggests that one of the basic mechanisms of the negative effect of phenoxyalkyl carboxylic acid derivatives on the scope of nitrogen fixation is the disintegration of the bacteroidal tissue of nodules.

On the other hand, experiments with unaffected nodules separated from the plants showed that the activity of nitrogenases is sharply reduced during incubation of nodules in solutions of 2M-4X and 2M-4XM. This suggests that phenoxyalkanes finding their way into nodules directly affect the physiological and biochemical apparatus of the symbiotic nitrogen fixation. Derivatives of phenoxyacetic acid and their phenolic metabolites are known to be disconnectors of the oxidative phosphorylation.

TABLE 4. Effect of herbicides 2M-4C and 2M-4CM on the functional state of nodules.

Assay variants	Mass of plants, % of the control	Nodules succinate (*OD units)	Nodules legoglobin (OD units)	N_2-fixation mg	N_2-fixation % of the control
Control	100	0.08	0.15	42	100
2M-4C 1-2 leaves	78	0.06	0.10	39	93
7-8 leaves	67	0	0.09	28	66
2-4CM 1-2 leaves	97	0.12	0.16	51	121
7-8 leaves	63	0	0.05	18	43

Based on the above statement, one may suggest that while concentrating in the bacteroidal tissue, phenoxyalkanes arrest ATP regeneration thus repressing the activity of nitrogenases which require energy of macroergotic bonds.

In the case of phenoxyalkanes, two complementary mechanisms may be considered in order to interpret their negative effect on the leguminous-rhizobial symbiosis. One of them is due to an abnormal growth of the meristem leading to formation of a malignant bacteroidal tissue, while the other is responsible for the disconnection of oxidative phosphorylation resulting in the inhibition of nitrogenase activity.

The effect of herbicides on symbiotic systems is of a diverse nature, the mechanism of this effect depending on the chemical composition of the toxicant.

In the extreme case, the symbiosis is disconnected, nitrogen fixation is arrested, and conditions emerge for the nodular bacteria to turn to parasitism, the fact enhancing the negative pesticidal effect on plant productivity.

In the ecological respect, this deprives the leguminous plants of their major advantage compared to other plants and undermines their competitivity for nutrition sources.

On the other hand, the use of herbicides (which apparently needs a more thorough study) for leguminous crops represses or annihilates the positive effect of the seed inoculation with preparations of nodule bacteria.

It follows that misinterpretation of the symbiotic character of leguminous plant nutrition may result in errors both from the ecological and economic viewpoints. Hence, all the herbicides proposed for uses with leguminous plants are to be tested with due regard to their potential effect on the leguminous-rhizobial symbiosis.

In conclusion, it should be noted from the above discussion that once pesticides are released into the soil, they become a continual man-made ecological factor that must be taken into account. Systematic applications of pesticides result in substantial changes in the soil microorganisms.

Such changes are due to both direct effects of pesticides on the microorganisms and specific variations of other components of the land ecosystem.

Some regularities in the behaviour of microorganisms discussed in the present lecture allow microbial uses for monitoring and prognostication on the possible consequences of the utilization of chemicals for plant protection as well as a safe application of pesticides in agriculture.

Recommended Literature

Insecticide Microbiology. 1984. Lal R.,ed.Springer, Berlin, 268p.
Pesticide Microbiology. 1978. Hill J.R., Wright S.J. L., eds. London: Academic Press. 844 p.
Domsch K.H. *et al.* 1983. An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. *Residue Rev's* 86:65-100.
Anderson J. 1973. A system for evaluating effects of pesticides on soil microbial activities. *Bull. Ecol. Res. Comm.*, Stockholm, 17:470-478.
Greeves M. 1978. Problems and progress in the evaluation of herbicide safety to the soil microflora. In: *Weed Res. Organization Seventh Report.* 1976-1977, Oxford, p. 95-106.
Simon-Silvestre G., Fournier J.C. 1979. Effects of pesticides on the soil microflora. *Advances in Agronomy* 31:7-92.
Berestetsky O.A., Voznyakovskaya Yu.M., Dorosinsky L.M., Kruglov Yu.V. *et al.* 1984. *Biological Basis of Soil Fertility.* Moscow, Kolos, 287 p. (in Russian).
Kruglov Yu.V., Paromenskaya L.N. 1986. Herbicides and leguminous-rhizobial symbiosis. *Izv. AN SSSR, ser.biol.* 2:238-249 (in Russian).

BIOLOGICAL ALTERNATIVES TO CHEMICAL PESTICIDES

D.N. Chermensky

I. Introduction

About one third of the potential crop harvest is lost annually as the result of adverse activities of pathogenic fungi, viruses, insects and weeds. Each year over 3 million tons of pesticides with the estimated cost of some $ 15 billion are utilized worldwide for the control of pest in agriculture. Increased quantities of chemicals applied to soils on a regular basis are nowadays the cause of mounting public concern. An extensive literature has accumulated reporting the negative effects of intensive use of chemicals in farm practices: accumulation of persistent residues in soil, perturbations in biocenoses, production of mutagenic and carcinogenic intermediates, unpredictable effects on non-target objects, emergence of resistant forms, etc.

In this context, the recent years witness a heightened expert attention being paid to the search for biological methods of plant protection. In contrast to chemicals, the biological means of plant and animal protection are more effective: they require essentially lower concentrations of pest toxicants and their tests are cheaper.

At present, agricultural chemists show interest in microbial metabolites. Picloram was synthesized some 30 years after the discovery

of fusaric acid, a natural phytotoxin, both compounds are derivatives of pyridine carboxylic acid. Yet the herbicide kayametone (methoxyphen) was synthesized after a pseudomonad metabolite had been shown to be lethal for plants at a concentration of 5 kg/ha. Hoechst received a patent for the Basta's herbicide glufosinate immediately after the discovery of phytotoxic properties of bialaphos, a metabolite of *Streptomyces* species.

Thus Foster's appeal "Never underestimate the power of microbe!" is becoming ever more popular. Some 15,000 tons of microbial metabolites are used annually in farming practices in Japan alone. In many instances, the application of biopesticides does not involve the necessity of the isolation of pure products – instead, live microbial cultures or dried powders are in common use. The above advantages enabled biopreparations to rapidly gain popularity within rather a short time. In a number of countries, some chemicals have already been totally discarded and replaced with bioformulations for the pest control in greenhouses.

According to recent predictions made in the USA, by 1990 the biological means of plant and animal protection will account for 50%, whereas at the present time their share in pest control does not exceed some 5–7% of the market.

II. Basic Biopreparations and Microbial Cultures Used for Plant Protection

Some biological means of plant protection have been known since early times. As early as 900s A.D., ants were used in China for protecting citrus plants. In Italy, Bassi used in 1834 bacterial preparations for protecting the silkworm from a disease caused by the fungus *Beauveria bassiana*. In 1872, the Russian microbiologist I. Mechnikov proposed the fungus *Metarhizium anisopliae* for insecticidal uses, and later his disciple I. Krasilshchik produced by culturing some 54 kg of the fungus spores during the warm summer season of 1884. In Florida, strains of *Aschersonia* were first successfully used for combatting the whitefly,

destructive in citrus groves. The above examples have become history. Turning to the present, one has to state that the world register comprises today no more than 34 marketed biopesticides of fungal, bacterial and viral origin (Table 1).

2. Bioinsecticides

2.1. Bacterial Insecticides

Of all microbial pesticides, the formulations based on *Bacillus thuringiensis* (Bt) account for about 90% of the biotoxicants manufactured in Europe. According to various estimates the annual production of Bt amounts to 7,000–10,000 tons. The actual level of Bt output appears to meet current demand but higher Bt supply will be in order if it finds uses for forest protection.

TABLE 1. Biological means of plant protection produced on industrial scale.

Microbial producers	Target	Trade name	Country
Bacteria:			
Bacillus popilliae	Japan beetle	Doome, Milky	USA
B. entimorbus		Spore	
B. sphaericus	Mosquito	Field trials	--"--
B. thuringiensis			
serotype 1	Flies	Muscabac	USA and
--"-- 3a, 3b (HD-1)	Caterpillars	Dipel, Biobit Bactospeine, Thuricide	European
--"-- 3a, 3b (NRD12)	--"--	Javelin	countries
--"-- 7	Wax moth	Certan	--"--
--"-- 14	Mosquito	Bactimos, Skeetal Teknar, Vectobac	--"--
Agrobacterium radiobacter	Crowngall	Galltrol	Australia
Bacillus subtilis	Seedling root disease	Quantum 4000	--"--
Streptomyces sp.	Fungal root disease	Mycostop	Finland
Fungi:			
Beauveria bassiana	Colorado potato beetle	Boverin	USSR
Erynia neoaphidis	Aphids	Field trials	
Hirsutella thompsoni	Citrus rust mite	Mycar	USA

Metarhizium anisopliae	Spittle bug	Metaquino	Brazil
Nomurea rileyi	Caterpillars	Field trials	
Verticillium lecanii	Aphids	Vertalec	Great
--"--	Whitefly	Mycotal	Britain
Trichoderma spp.	Plum silver leaf	Binab	Great Britain
Colletotrichum gloeosporioides	Northern joint vetch	Collego	USA
Phytophthora palminovora	Milk weed vine	Devine	USA
Protozoa:			
Nosema locustae	Grasshoppers	Noloc	USA
Nematodes:			
Steinernema feltiae	Termites	Spear	USA
Virus:			
Cydia (GV)	Codling moth	Decyde	USA
Heliothis (NPV)	Cotton caterpillars	Elcar	USA
Lymantria (NPV)	Gypsy moth	Gypcheck	USA
		Virin-GC	USSR
Mamestra (NPV)	Caterpillars	Field trials	
Neodiprion (NPV)	Pine sawfly	Virox	USA
--"--		Virin-Diprion	USSR
--"--		NPV	Finland
Spodoptera (NPV)	Caterpillars	Field trials	
Orgyia (NPV)	Douglas fir tussok moth	TM-biocontrol-1	USA
Dendrolimus	Pine beauty moth	CPV	Japan

Experimental samples of Bt-entomocides appeared as early as in the 1930s and were registered as marketed biopesticides in 1960 but they have been actively used only since early 1970s after the isolation of strains free of β-exotoxin. The Bt-insecticides are still some 20- 25% more expensive compared to chemical analogues, but their broad target effect and ecological safety single out the Bt preparations from other bacterial and fungal insecticides. For instance, "dipel" may be used for protecting over 200 crops from 50 insect species. Bacterial insecticides are remarkable for a number of reasons: their production may be carried out using conventional equipment for microbial cultivation in liquid culture, their spores are sufficiently stable to be separated and stored, and remain suitable for practical uses in the form of water suspensions. Though their efficiency is somewhat affected by certain negative factors: contamination with alien microflora, phagolysis,

development of resistance by certain insect species.

These days, much attention is being paid to studying Bt genetics. Successful attempt has been reported in cloning the gene encoding the synthesis of endotoxins in *Pseudomonas fluorescens* - a common natural soil culture. The transformed strain was also found to be incapable of producing recombinants with the genera *Bacillus, Erwinia,* and *Agrobacterium,* as the structural gene of endotoxin is localized on a chromosome. Monsanto projected to start production of the constructed strain in 1988-1990 but apparently such projects are too much optimistic, since the new culture showed poor viability under natural conditions.

At present, the US Environment Protection Agency (EPA) recommends the use of Bt-treatments on fields sown with maize, cotton, tobacco, vegetables, fruit and citrus groves, forests, plantations of sugar cane, alfalfa, as well as soya beans and grain (during storage).

Another interesting group of compounds embraces the piericidins. Piericidin is an insecticide synthesized by the actinomycetes - *Streptomyces mobaraensis* and *Str. pactum.* The metabolite resembles structurally ubiquinone (coenzyme Q) involved in the electron transport through the respiratory chain at its length between NADH-dehydrogenase and cytochrome *b.* The piericidin blocks the metabolic chain of reactions, thus being lethal for pests. Experiments with the larvae of domestic fly and gypsy moth showed a 100% death issue level for metabolite concentrations of 4 to 5 μg/larva. Piericidins were also active against aphids and mites, and repressed the activities of pathogenic fungi (*Trichophyton spp., Criptococcus neoformans, Aspergillus fumigatus, Candida albicans*) at concentrations of 2-20 μg/ml, whereas phytopathogens proved to be more resistant (up to 200 μg/ml).

The insecticide physostigmin is over 120 years old. This alkaloid was isolated for the first time in 1864 from seeds of *Physostigma venenosum,* later - from French beans, more recently - from the liquid culture of *Str. pseudogriseolus subsp. iriomotensis* and, finally in 1986 - from an unidentified actinomycete *Streptomyces* AH-4. The alkaloid of both plant and microbial origins killed the silkworm at a concentration

of 30-50 μg/g diet.

Polyenic macrolid antibiotics are well known to form links with styrenes of the membrane and thereby injure eukariotic cells affecting the permeability or resulting in fragmentation of the membrane surface. At concentrations of 1 mg/ml metabolites from *Str. grisuns* Tu599 and *Str. hygroscopicus* MA5285 were lethal to insects of the orders *Lepidoptera, Coleoptera, Heteroptera, Diptera.*

The macrolid antibiotic tetranactin was isolated in 1971 from *Str. aureus* S-3466 and by 1974 it was registered as a commercial bioinsecticide used in Japan to combat the red citrus mite destructive in citrus groves and tea plantations (Table 2). The insecticide displays a very low toxicity towards warm-blood animals and is practically harmless to plants. The tetranactin is capable of producing entomocidal effect at high humidity, as it displays characters of an ionophore and promotes the leakage of potassium ions from the insect biomembrane.

TABLE 2. Microbial products used for agriculture in Japan.

Name of metabolite	Year of registration	Target
Antifungal:		
blasticidin S	1961	rice blast
kasugamycin	1965	rice blast
polyoxins	1967	rice sheath blight, fungal diseases of fruit trees a. vegetables
validamycins	1972	rice sheath blight
mildiomycins	1982	powdery mildew
Antibacterial:		
streptomycin	1957	bacterial diseases of fruit trees and vegetables
oxytetracyclin	1957	--"--
novobiocin	1974	bacterial canker of tomato
Insecticidal:		
tetranactin	1974	mites of fruit and tea trees
Herbicidal:		
bialaphos	1984	a wide range of weeds
Growth regulators:		
gibberellins	1958	seedless grapes
Coccicidal:		
salinomycin	1978	coccidiosis of poultry

The first communication appeared in 1979 about yet another new insecticide with potentially high activity. A strain of *Str. avermitilis* produced no more than 9 μg of active compound per 1 ml of culture medium. Appropriate choice of a medium and selection of active strains after UF-irradiation of the parent strain permitted the yield increase up to 0.5 mg/ml medium.

Avermectins are a group of macrocyclic lactones. Eight individual compounds have been isolated which contain an aglycone and two carbohydrate residues of α-L-oleandrose. The aglyconic part of the molecule proved to be analogous to milbemycin isolated in 1974 from *Str. hygroscopicus subsp. aureolacrimosus* and to display insecticide and acaricide characters.

The highest activity against insects was shown by avermectin B_1 (or abamectin) which in concentrations of 0.02–0.24 μg/ml was lethal for various mite species, its half-life time in soil is 2, 5–3 days with the optimum dose range of 0.05–0.5 kg/ha. Abamectin is also an active toxicant towards other insect parasites: Colorado potato beetle, pea aphid, cabbage looper, corn earworm, armyworm. It was successfully tested against the spider mite – a parasite living on cucumbers.

Chemical modification of abamectin resulted in 22,23–dihydro–avermectin B_1 (ivermectin) which is widely used as an endectocide in animals where it is highly effective against most pathogenic nematodes and arthropods. Ivermectin is also currently under investigation for treatment for human infections with the filarial worm *Onchocerca volvulus*, the causative agent of human onchocerciasis which often leads to blindness.

Avermectin preparations are produced commercially since 1984 for animal treatment at recommended levels of 0.2–0.3 mg per 1 kg animal weight. If we assume the effect of earlier applied thiobenzene for activity unit, then on the average, avermectin is 10 and ivermectin 1000 times more active compared to thiobenzene.

At present, the relevant literature carries over 100 patents and publications devoted to chemical variants of avermectins, i.e. the second generation of avermectins is being put to use with still higher activities and a wider spectrum of targets. The effect of avermectins

consists in blocking the transfer of signals to motility neurons, i.e. in arresting impulses directed to insect muscles, whereas their preparations exhibit no fungicidal or bactericidal characters.

At the recent IUPAC Congress on chemistry of pesticides, an international prize was presented to R.A. Dybass in recognition of the effort of the Merck Sharp company research team on avermectins.

2.2. Fungal Insecticides

Most scientists agree that there exist over 400 species (some insist on 500) of entomopathogenic fungi capable of affecting the insect pests. At the present time, however, no more than 12 species are in practical usage of which only 6 have been registered as commercial bioinsecticides (Table 3).

Practically all pathogens are referred to the class of *Deuteromycetes* embracing about 250 fungal species. Two species alone, *Beauveria bassiana* and *Metarhizium anisopliae*, have about 700 hosts among insects of the orders *Lepidoptera, Coleoptera, Orthoptera, Hemiptera, Diptera*. By their activity fungal preparations are comparable with best chemical pesticides. At present, in addition to the targets indicated in Tables 1 and 3, *B. bassiana* and *M. anisopliae* are under study as insecticides against sugar cane pests, mosquito, termite, and grass hopper.

The fungal preparation known under the name of "Micar" is effective against six species of spider mites destructive of citrus trees and coco palms. However, in first large-scale applications, Micar did not live up to expectations, and even the advisability for its uses was brought into question. The failure might have been due to the unusually hot season or an unfortunate combination with an incompatible chemical fungicide.

A British preparation based on the fungi *V. lecanii* or *Cephalosporium lecanii* is permitted for uses against aphids destructive of the chrysanthema and vegetables grown in hothouses and conservatories. Successful genetic studies promise a higher entomocidal effect of this preparation. Researchers predict wide uses for the bioinsecticide in Europe and USA.

TABLE 3.. Fungal insecticides of small scale production.

Producer culture	Target	Production mode	Country
Aschersonia spp.	whitefly scale	submerged fermentation	USSR, Hooland Great Britain
B. bassiana	corn borer	semisolid/diphase fermentation	USA, People's Rep. of China
Conidiobolus obscurus	aphids	submerged fermentation	France, USA
Culicinomyces clavosporus	mosquito	submerged fermentation	Australia
Entomophthora grylii	grass hopper	submerged fermentation	USA
Erynia neoaphidis	aphids	in vivo/submerged fermentation	Great Britain
Lagenidium giganteum	mosquito	submerged fermentation	USA
Metarh. anisopliae	spittle bug	semisolid ferm-n	Brazil
--"--	mosquito	--"--	USA
--"--	field cricket	--"--	Australia
--"--	rhinoceros beetle	--"--	South Oceania
Nomurea rileyi	caterpillars	--"--	USA
Verticillium lecanii	aphids	submerged fermentation	Great Britain
--"--	whitefly	--"--	--"--
--"--	trips	--"--	--"--
Zoophthora radicans	spruce budworm	--"--	USA

Another fungus intended for the aphid control has been tested in West Europe in fields of winter wheat. The fungus *Erynia neaphidis* was causative of a heavy insect epizooty and proved to be more effective compared to *Entomophthora spp.* and apparently will soon be marketed.

Entomophthora fungi are the most promising fungal entomocides, as they are capable of maintaining their activity in different climatic zones. Their wide application in agriculture is impeded by production problems. The most active strains are obtainable exclusively by in vivo cultivation. These fungi may be readily cultured on common media, but in this case they display very low pathogenic characters.

The majority of entomopathogenic fungi used commercially (Table 3) are grown in liquid media during 48-96 hours, except for the strain *Nomurea rileyi* which requires for cultivation 160 or even 500 hours.

This mode of culturing does not always provide expected results. The blastospores or conidia produced are generally badly affected during separation and drying procedures and often prove to be unable to infect the target insect. *Lagenidium giganteum* makes the presence of certain styrenes, extractable from soya and hemp, a condition for producing active zoospores.

The method of solid phase fermentation (SPF) allows production of active and viable fungal preparations using inexpensive media: grain of cereals, waste products from food and feed processing. A moist sterile substrate is loaded on trays or in plastic bags, glass containers etc. followed by its inoculation with a specific fungal culture. The fungus shows active growth and in 5–15 days produces 10^6–10^{10} conidia spores per 1 g of substrate.

Major candidates for SPF are *N. rileyi, H. thompsonii, M. anisopliae, V. lecanii.* The SPF techniques also enable the use of light which stimulates higher yields, production of melanin (UV-protectors) and renders spores more viable.

It should be recognized, however, that technological standards of the promising SPF method still remain at laboratory level, while need is felt for wider uses of biopesticides.

The entomopathogenic fungi affect insects through either the gastric tract or lesions and the cuticula. The latter way is most frequent mode of penetration by pathogenic fungi. The entomocides synthesize a set of extracellular enzymes degradative of components of the cuticular layer: lipids, proteins, chitin. *B. bassiana* and *M. anisopliae* also secrete proteases toxic to insects.

Massive death of insects infected with *Conidobolus obscurus* is related to possible synthesis of toxins. In fact, certain entomopathogens synthesize low molecular weight toxins, e.g. bovericin, a cyclodepsipeptide. However, all attempts to isolate toxins from infected insects have so far been unsuccessful, except for one case, when a quarter of the lethal dose of destruxins was extracted from the silkworm infected with *M. anisopliae*. Destruxins are peptide metabolites synthesized by the fungi *M. anisopliae, Oospora destructer, Aspergillus ochraceus* and regarded as potential insecticides. The quantity of

destruxins synthesized by fungi was shown to correlate with the in vivo virulence of pathogens in relation to mosquito larvae.

3. Bioherbicides

3.1. Bioherbicides of Bacterial Origin

The list of herbicides of bacterial origin is fairly short and only one such preparation has virtually been commercialized. Two metabolites, pyoluteorin and its nitro derivative, were isolated from *Pseudomonas aeruginosa* grown on paraffins. Both compounds were found to display herbicidal and fungicidal effect, being lethal to plants at a concentration of 5 kg/ha. In 1972, two metabolites were isolated from an unidentified actinomycete and found to repress germination of shoots and root growth in rice, millet, alfalfa, tomato, swede. One of the two, anisomycin, inhibited the development of roots and seedlings in concentrations of 12 μg/ml and 50 μg/ml, respectively. The structures of metabolites from pseudomonads and actinomycetes proved to be very similar, and this fact gave an impetus to the search for a technologically economic synthesis of anisomycin and pyoluteorin analogues.

The screening of 347 microbial cultures with potential herbicidal effect conducted by Putnam and coworkers (University of Michigan) showed that 10-12% of the studied cultures repressed the growth of test plants (cress, cucumber, millet). Practically all "negative" isolates usually contained inhibitors of total metabolism analogous to cycloheximide. Only one strain - *Str. hygroscopicus* synthesized two active components with herbicidal properties. Field trials revealed that a dose of 2 kg/ha was sufficient for eliminating nearly all weeds. It should be noted that the above studies showed that 90% of all isolates from *Str. hygroscopicus* exhibited phytotoxic effect, in particular in relation to dicotyledons.

The best known herbicide of bacterial origin is bialaphos manufactured on industrial scale. It was isolated from *Str. hygroscopicus* and *Str. viridochromogenes* and intended for usage as a bactericide and fungicide (*Botrytis cinerea*), but in 1984 it was

marketed as a herbicide. The sodium salt of bialaphos is known under the commercial name of SF 1293 obtainable from Meijy Seika. Bialaphos is used as unselective herbicide against numerous broad-leafed weeds in concentrations of 0.05–1 g/ha. The preparation showed low phytotoxic effect on vegetables, fruit trees, and mulberry. It is readily degraded by the soil microflora showing the half-life time of 2–3 days. Combination of bialaphos with urea or ammonium sulphate enhances its activity. The herbicide represses activities of glutamine and glutamate synthases, i.e. the system of nitrogen fixation in plants. Symptoms of the disease are yellow leaves and defoliation.

Another metabolite containing the phosphinothricin group was isolated in 1984 from the recently found microorganism *Kitasatosporia phosalacinea*. Like bialaphos, the new toxicant phosalicine (L-phosphinothricyl-L-alanyl-L-arginine) is a herbicide with broad target effect against mono- and dicotyledons.

A dipeptide tabtoxin readily penetrating into plant cells was extracted from *Pseudomonas tabaci* which affects tobacco leaves. Under the action of peptidase it is easily transformed to tabtoxin – β-lactam which is a strict inhibitor of glutamate synthase – one of the key enzymes of the photosynthetic cycle of plants.

3.2. Mycoherbicides

The fungi affecting plants found broad usage in modern agriculture. Successful experiments on application of bioherbicides were carried out in Australia in 1979. The effort of microbiologists was focused on abating the skeleton weed abounding in wheat fields. Researchers have tested several strains of rust fungi and finally found a highly virulent Italian strain *Puccinia chondrillina*. In 1975, the economic effect of the strain application was about $ 18 mln.

Another fungus, *Fusarium lateritium*, proved to be effective in combatting two weed species in soya and maize plantations – the sida spinosa and the velvet leaf. Trials were conducted in hothouses. Best results were obtained with the fungal inoculum concentrations of $7.5 \cdot 10^5$ and $1.5 \cdot 10^6$ macroconidia/ml applied when the weeds developed seven

authentic leaves. The fungus *Alternaria cassiae* was equally effective against the sicklepod - a weed abundant in soya and cotton plantations and 26 other crops. The fungal preparation was inoculated twice a season (at concentrations of 9.4 and 4.7 kg/ha) and 90% of the weed was abated in 14 days.

The interest in mycoherbicides is growing with each coming year. In recent years, progress has been achieved in abating over 20 weed species. However, only two mycoherbicides have been marketed: Collego and Devine. It took 13 years the former to gain recognition, whereas the latter has been a success after 9 years.

Collego is widely used for combatting leguminous weeds (e.g. northern joint vetch or curly indigo) in rice and soya-bean fields. The formulation contains spores of *Colletotrichum gloeosporioides* (15%) suspended in an inert ingredient. The herbicide is applied once a season (94 l/ha, 2 10 spores/ml), the herbicidal effect being observed in 4-5 weeks. The mycoherbicide Devine (*Pytophthora palminovora*) is used in citrus orchards in the form of a suspension. The citrus trees are also treated once a year. The herbicide is not selective and is better avoided with melons, cucumbers, carrots, begonia, box-trees, oak-trees and palms.

4. Fungicides

4.1. Fungicides of Bacterial Origin

As early as 1950s, it was observed that treatment of seeds prior to their sowing with the culture *Bac. subtilis* produced a 40 %- increase of the yield of oats crop. Such experiments were carried out with different crops in various regions of Australia. Although there were rare negative reports pointing to affected plant foliage and decreased crop yields, it did not prevent the bacterial preparation from being marketed in 1984 under the name of Quantum 4000 intended for protecting the seed from diseases caused by the fungi *Rhizoctonia solani, Pythium spp., Fusarium spp.*

At the present time, various rhizospheric bacteria are widely used as herbicides. For example, *Agrobacterium radiobacter* synthesizes

nucleoside antibiotic agrocin 84 for protecting roots and seeds of plants from the crown gall. Field tests were successful in Australia but large-scale trials in peach groves in Greece were a failure, as the soil was infected with abundant population of the pathogen *A. tumefaciens*. The bacteria *P. putida* and *P. fluorescens* synthesize the ion-transport agents of the siderophore thereby protecting potato, beetroot, black radish from potential pathogens and contributing to higher crop yields.

The culture *Str. hygroscopicus* inoculated to soil two days before the sowing protected the green pea from infection with the fungus *Rhizoctonia solani*, because the actinomycete synthesized an antibiotic repressing the pathogenic fungus growth.

In Finland, the antifungal agent mycostop synthesized by *Streptomyces sp.* has totally supplanted chemical fungicides in greenhouses. The principal target of mycostop are fungi of the *Rhizoctonia* genus causative of rotting and diseases of roots. The active substance of the formulation is preserved in soil for nearly two years being protective for wheat, cauliflower, carnation, cucumber and tomato against mycopathogens.

Blasticidin S and kasugamycin are also effective biofungicides synthesized by streptomycetes and widely used in farming practices. Both are inhibitors of the protein synthesis, but in contrast to blasticidin S, kasugamycin has low toxicity. Blasticidin S is used in a concentration of 1–3 g/ha. Both fungicides are effective against pseudomonads, the fungi *Alternaria spp., Sclerotinia mali* and others.

Polyoxins that are inhibitors of chitin synthase are commonly used as fungicides for combating fungi (*Rhizoctonia solani, Pellicularia filamentosa*) causative of rice diseases. They are secreted by streptomycetes *Str. piomogenes and Str. cacaoi var. asoensis*. Polyoxin B is the most effective of the group members and is used against the fungus *Alternaria* affecting apple and pear trees.

Validomycins are aminoglucoside compounds synthesized by *Str. hygroscopicus var. limoneous* and widely applied in Japan (9,000 tons annually) to combat *Rhizoctonia solani*. These fungicidal preparations inhibit the biosynthesis of mioinositol required for the synthesis of obligatory glicolipids.

In 1982, another fungicide - mildiomycin was marketed as a basic means against the mildew disease.

Pimaricin is a polyenic macrolid effective against rotting of onion bulbs caused by the fungus *Fusarium oxysporum*. This fungicide represses the biosynthesis of ergostyrene in the fungal membranes.

4.2. Mycofungicides

Fifty species of pathogenic fungi are known to affect over 200 crops in the USA alone, and, as the consequence, farmers lose annually a $ 4 billion-worth agricultural produce. Treatments with chemical fungicides are rarely successful, as only a very small portion of the applied preparations reach the horizon of plant rhizosphere.

Nowadays, plants may be effectively protected by the use of fungi-antogonists. To this end, fungi of the genera *Penicillium, Trichoderma, Chaetomium and Gliocladium* are widely used. *Trichoderma spp.* applied in the form of a thick suspension or diatomaceous earth wetted with the fungal inoculum (140 kg/ha) protect sugar beet, haricot and green pea against infection with the fungi *Rhizoctonia solani, Sclerotinia, Pithium ultimum.*

A preparation of the fungus *T. harzianum* with the titre of 10 spores/g proved to be highly efficient against the fungi causative of the peach canker, grey mold of grapes and Dutch elm disease. Some of these fungal species maintain their activity during two years. Introduction of the fungal strains resistant to pesticides, e.g. *Trichoderma* T-1-R9 resistant to benomil, decreases the disease incidence by 20-60%.

5. Antibacterial Preparations

Numerous attempts were made to use antibiotics for protecting plants from pathogenic bacteria but unfortunately their bactericidal effect was found to be much lower compared to chemical means of plant protection. Plants are most frequently affected with the bacteria *Xanthomonas cryzae, X. citri, Ps. solanaceum.* An active search for efficient bactericides resulted in the discovery of cellocidin (activamycin),

ascamycin, xantostatin, nojirimycin, all being synthesized by streptomycetes.

III. Conclusion: the State of the Art and Perspectives

All biopesticides used presently for plant protection fall in two principal groups: (*i*) authentic biopreparations - viable forms of fungi, bacteria, viruses and (*ii*) microbial metabolites - toxins, antibiotics, growth regulators. In general, microbial means of plant protection are still difficult to access and more expensive compared to chemical analogues. But, compared to chemicals, biopreparations present many advantages, whereas their specificity permits a target approach, i.e. to direct selectively their action only on pathogenic species while avoiding ecological disbalance and undesired effects on animals, crops, useful entomofauna and, virtually, man as the ultimate consumer of agricultural produce. On the other hand, the release to soil of various microorganisms, that may affect not only pests but also other microorganisms populating agrobiocenoses, is the matter of concern to many biologists. This relates specifically to fungal preparations. Some of them are known as allergens (e.g. *B. bassiana*) others - to be capable of producing toxins, still others - to find new hosts. The latter statement may be exemplified by the use of the mycoherbicide based on *Cercospora rodmanii*. The fungus was intended against the water hyacinth, but in 1985 the State of North Carolina incurred the expense of $ 18 mln in struggling against this fungus that also affected maize, soya beans, coffee, sugar beet.

All this impels one to approach "live" preparations with sufficient precaution. Against this background, an increasing interest is taken in microbial metabolites, whereas the scope of publications devoted to the problem makes one think of the incoming "era of agricultural antibiotics", though certain microbial metabolites are not at all remarkable for their antibiotic characters.

In fact, metabolites synthesized by soil microorganisms are fairly safe and readily degradable by similar soil-based microbial populations.

Strategy of the screening of potent producers among microbial

cultures appears to be of special importance. During the epoch of medical antibiotics the selection of highly productive strains was principally based on their activity towards Gram-positive and Gram-negative bacteria. And who will say nowadays how many microbial producers endowed with remarkable antihelminthic, herbicidal, acaricidal or other effects passed unnoticed or were injustly wasted?

In this context, Luis Pasteur's maxim seems to be well in place: "Be very careful if you are looking for something. You may find it".

At present, the screening among microbial cultures, at least as it is done by Omura's team, reposes on 15 different tests, and such an approach provides a prompt effect.

Correct evaluation of basic agricultural pests is also a matter of high priority. In South East Asia countries, for example, the piriculariosis of rice is the principal target for assays of new fungicides. It has been calculated that effective measures taken for combating only four pests of crops will save some $ 286 mln by the year 2000.

According to predictions of some French experts for the year 2000, European countries will produce 100,000 tons of biopesticides which in turn will require for their formulation some 350,000 tons of glucose and starch as well as 1 mln tons of soya-beans. In all probability, just like now, the Bt-preparations will account for the highest proportion of all types of biopesticides. In the first place, the Bt-preparations were standardized as early as 1966 and 1971 and their activity is currently expressed in international units: 16,000 int. units/mg, recommended doses - 0.5-1.0 kg/ha. The fungal preparations have not yet been provided with adequate standards. In the second place, advanced technologies for cultivation of *B. thuringiensis* enable growth of bacteria in 30-150 m fermenters during 24 hours, the process being much more rapid and ecomonic compared to production of fungal preparations. At the market price level of 1975, the cost 1 kg of bacterial preparation was about $ 11.3 slightly exceeding the price of synthetic pesticides.

Thirdly, recent developments in biotechnology, and in particular in genetic engineering, provide sufficient grounds for hope that, compared

to fungal toxicants, bacterial insecticides will in time become more selective, effective and resistant to a wider range of weather and climatic conditions.

Recommended Literature

Klausner A. 1984. *Microbial Insect Control Biotechnology*. V.2, No.15, p.409-419.

Lynch J.M., M.H.Ebben. 1986. The use of microorganisms to control plant disease. *J. Appl. Bacter. Symp.* Supplement. p.1155-1265.

Misato T. 1984. Recent Development of Microbial Origin Pesticides. British Crop Protection Conference. Pests and Diseases. V. 3, 9B-1, p.929-936.

Omura S. 1986. Phylosophy of New Drug Discovery. *Microbial Review* 50: 259-279.

Stockdale H. 1985. *Microbial Insecticide Comprehensive Biotechnology*. M. Mao-Young, ed. V.3, p.949-964.

THEORY AND PRACTICE OF MICROBIAL WATER PURIFICATION

P.I. Gvozdyak

The basic function of microorganisms in nature is known to consist in mineralization of organic matter of plant, animal and microbial origin. In addition, they are also endowed with yet another remarkable and vitally essential character: to develop fairly rapidly capabilities to transform and ultimately destroy synthetic or, in other terms, anthropogenic compounds. This property of microbes is assumed as a basis of all microbiological methods developed for the cleanup of most polluted and noxious effluents that cannot be purified otherwise, i.e. using conventional chemical and physico-chemical techniques. The only means for their detoxification has been until very recently the thermal treatment ("burning"). For this, 1 m^3 of liquid sewage requires some 250-300 m^3 of methane and, consequently, a thermal sewage treatment plant "burning" no more than 100 m^3 of effluents a day consumes annually about 10 m^3 of natural gas.

Although effluent waters are very often highly polluted with natural toxicants (sewage from live-stock farms and meat processing plants, not to mention others), let us examine the microbial purification of effluents containing synthetic compounds. In the first place, synthetic substances are the products of man's economic activity, earlier never found in natural environments, and it will be very

interesting to see in what way microorganisms succeed in utilizing them as sources of carbon and energy. In the second place, such synthetic chemicals known under the impressive name of xenobiotics, borrowed from the Greek and meaning "alien to life", are in fact highly hazardous to the environment, as most of them exhibit mutagenic and teratogenic characters. In this light, the adequate cleanup of sewage is of an obvious and special significance. Thirdly, many xenobiotics prove to be recalcitrant and persist in the biosphere for years. Therefore, the development of microbial methods for the cleanupof industrial effluents from such toxicants will contribute to abating levels of environmental pollution and averting an uncontrolled flux of anthropogenic chemicals to the biosphere.

On the other hand, a topical problem in microbial purification of industrial effluents is the removal of microorganisms prior to the waste water discharge. Difficulties related to the withdrawal of microorganisms from large volumes of water are one of the major obstacles hampering broad application of microbial techniques for the cleanup of liquid sewage which otherwise are undoubtedly more advanced and efficient compared to the activated sludge process.

In this context, consideration of microorganisms capable of effective degradation of xenobiotics appears to be in order. Until very recently, many synthetic compounds were believed to be exceptionally resistant to microbial attack. By reviewing the relevant world literature, M. Alexander, an eminent American microbiologist, published the list of "nonbiodegradable" compounds (Alexander, 1973). The current literature as well as reference books on water purification problems abound in zero BOD (biological oxygen demand) values as characteristic of numerous synthetic chemicals. This implies that waste waters containing such compounds are unsuitable for biological purification.

Recent developments in microbiology suggest, however, that in principle all compounds, irrespectively of their natural or anthropogenic origin, are, in the final analysis, biodegradable. Such an appraisal of a degradative potential of microbial cultures is gratifying, but how to spot among the great diversity of microbial species the rare strains capable of effective degradation of specific

xenobiotics? Unfortunately, the empirical search for highly degradative strains has been and still is largely guided by intuition - a poor substitute for a solid scientific base. Neither researchers nor practicians dispose of an adequate body of factual material that would enable them the appropriate selection of highly degradative microorganisms with specific taxonomic status based on the composition and structure of compounds to be degraded. Therefore, individual researchers in their quest of microbial degraders have to rely on personal experience, general considerations and in rare cases on the existent scientific approaches.

In applied microbiology, the appropriate selection of a microorganism, studies of its properties and knowledge of physiology are highly conducive to its successful performance. The remaining task is to use or design a fermenter with suitable working parameters and to ensure steady-state conditions of cultivation. In modern microbial technologies of waste water purification the availability of an excellent degradative microorganism for a most vicious pollutant hardly provides even a 25% guarantee of success. Regrettably, the richest experience of industrial microbiology cannot be totally extended on microbial purification of waste water. The reason is not a tremendous volume of effluents to be treated hardly comparable with those of nutrient media in any large scale microbial process; this is neither due to problems of maintaining sterility as it may seem at the first approach (in fact, effluents from chemical industries, for example, are mostly sterile, the more so that there are too few volunteers for populating waste waters). The basic features that distinguish microbial purification of effluents from processes of industrial microbiology are a highly heterogenic composition of the liquid sewage and a very low, by industrial microbiology standards, concentrations of potential nutrients. Low content of organic matter in waste water is of course far from sufficient for providing for high rates of microbial growth, and this in turn implies a relatively low efficiency of sewage treatment plants: it is common knowledge that dilution rates should be equal or less than those of microbial growth - otherwise the microbial culture will be finally washed out from the bioreactor (fermenter). On the other hand, a

long-term stay of waste water in the installation would require bioreactors of unthinkable dimensions which is certainly not advisable.

The situation is also complicated by the fact that industrial effluents may contain any substances used in sewage treatment plants as well as intermediate and final products of their interactions. The "bunch" of water pollutants from only one factory (not to mention complex production units) may be composed of dozens or even hundreds of compounds in most various combinations and at times drastically differing concentrations (Yakovlev *et al.*, 1985). Consequently, any waste water treatment plant should maintain on a regular basis degradative cultures for the whole set of pollutants. Combining in one microorganism capabilities for degradation of numerous compounds found in industrial effluents is hardly possible theoretically and entirely unrealistic practically. In the bioreactor a community of microbial cultures should be maintained capable of degrading different chemical compounds, some microorganisms receiving each during certain time "its own" pollutant as a nutrient source, whereas others have to starve.

Finally, microbial aspects of biotechnology and waste water treatment technologies essentially differ by their objectives. Industrial microbiology is basically aimed at biosynthesis or bioconversion of organic compounds for yielding target products such as alcohols, acids, steroids, antibiotics or producing biomass, whereas the task of waste water treatment is to bring about total mineralization of pollutants achieving this in the best issue without accumulation of biomass. An urgent need is felt for a non-waste technological system for purification of effluents.

But how to achieve this ?

Let us turn to Nature. Roughly speaking, the biosphere as a whole is a closed non-waste cycle, by technological standards. However, every individual organism is releasing specific waste products contaminating with such excreta its own environment and thereby favouring the development of other organisms. As the result, there occurs a gradual change of organisms known as their succession. A good example of such succession is the transformation of a lake into a marsh and further into shrubs and forest. This is a temporary succession. Nature is in no

hurry. It has, contrary to humans, plenty of time. In our striving to step up this process we replace the succession in time by succession in space. To achieve it, we should provide for a consecutive change of organisms in waste water treatment plants in a manner that excretions and nutrient remnants from one organism are a diet for the second organism and what is left by the second is utilized by the third etc. in succession.

We assume the space succession of degradative microorganisms to be one of the basic (if not the principal) problems in microbiology of water purification.

The spatial succession is the lever which will enable us to overhaul the entire system of biological purification of waste water (from industrial to municipal effluents) and to set it on a solid footing.

The idea of space succession of degradative microorganisms is suggestive of a number of important practical consequences (Gvozdyak *et al.*, 1985).

1. The system of microbial purification of effluents must be a concurrent flow excluding any recycling of biomass.
2. In conditions of a tremendous difference between the rates of dilution by waste water and those of microbial growth the concurrent flow regime may be observed only when degradative microorganisms are immobilized in the bioreactor on water-insoluble solid supports.
3. As high as possible concentrations of degradative microorganisms should be distributed over the entire volume of the bioreactor.
4. Deep purification of waste water from organic pollutants may be achieved by the use of oligotrophic bacteria at the last steps of microbial process, as it is precisely oligotrophs that are well adapted to subsist on trace concentrations of organic compounds.
5. In order to pave the way for a total and reliable water purification, the spatiale succession of degradative microorganisms must gradually lead to a chain of aquatic organisms in which protozoa would feed on excessive bacteria; arthropods, filtrators and tech-fish would in turn feed on protozoa so that finally fish would turn out to be the only "pollutant" of water.

Naturally, after microbial destruction of organic pollutants microbial cells may be removed from the purified water by various methods. All current techniques fall into two groups: (*i*) those employing reagents and (*ii*) reagent-free.

The reagent methods include the following steps: coagulation and flocculation, flotation and foam fractionation, and liquid-liquid extraction.

The reagent-free methods comprise: gravitational precipitation, filtration, electrophoresis and electroretention.

A common disadvantage of reactant-employing techniques is the necessity to use specific materials and compounds. Such reactants must be nontoxic, not change the pH value of the medium, not affect the chemical composition of cells, be used in low concentrations, be highly active and inexpensive, be well suited for separation and recycling. Despite the above restrictions certain reagent techniques found rather important practical uses.

Methods of water purification based on coagulation and flocculation are employed on a larger scale. Herein the electric charges of cells are neutralized resulting in their agglomeration and sedimentation. A variation of coagulation technique is electrocoagulation – a very interesting and promising means of water purification using hydroxides of aluminium, iron and other metals produced by electrochemical dissolution of metal anodes.

Adsorption of microorganisms on solid surfaces has been a subject for studies since the close of the last century. In the Soviet Union, Zvyagintsev attempted a review and interpretation of numerous relevant data and succeeded in establishing the basic regularities of this process. Adsorption was found to be dependent on properties of microorganisms and adsorbents, composition and physico-chemical characters of the medium and conditions providing for a contact between cells and specific solid surfaces. Perspectives of the use of immobilized microorganisms for purification of industrial effluents make the studies on adsorption (adhesion) of microorganisms on solid supports very attractive and topical.

Foam fractionation and flotation techniques, electroflotation

included, are based on capabilities of gas bubbles (air, hydrogen, oxygen) to bring microorganisms to the surface of a liquid in the presence of surfactants. The resultant foam is separated and broken to provide a concentrated suspension of microorganisms and purified water. The flotation is intended for eliminating from liquids a coarse particulate matter as well as microorganisms present in the effluents.

Liquid-liquid extraction techniques repose on intensive treatment of water suspensions with immiscible organic solvents such as benzine, diethylketone, *n*-propanol, butanol etc. which results in adhesion of cells to the organic phase and their concentration on the water-organic solvent interface. The three phases thus formed are separated, and water and cells are liberated from organic solvent. Albertson (Sweden) proposed to use the two-phase systems based on water solutions of such polymers as polyethylene glycol, dextran and their derivatives for separating bacteria, viruses, cell fragments, membranes, nuclei, proteins, nucleic acids and other particles of biological origin. The author recommends this method for industrial isolation of viruses and enzymes, but it is hardly advisable for the purification of waste water.

The reagent-free techniques appear more attractive, as they do not involve the introduction of additional compounds and materials.

The gravitational separation is a method of long standing and still widely used nowadays. However, low sedimentation rates of bacterial cells in the gravitational field and a relatively high energy consumption restrict the scale of its application for removing microorganisms from the purified effluents.

The filtration techniques for separation of microorganisms are based on: (*i*) mechanical sieve-type retention of cells when the diameter of filter pores is less than the size of bacteria, membrane filters, filtration plates and (*ii*) electrostatic (adsorptional) retention when the filter pores are greater than the size of bacteria but the cells do not pass through the filter, since the positively charged filter walls of porcelain, paper, asbestos etc. attract and retain the negatively charged microbial cells.

In 1972, we observed for the first time the behaviour of a microbial suspension during its passage through a granular packing

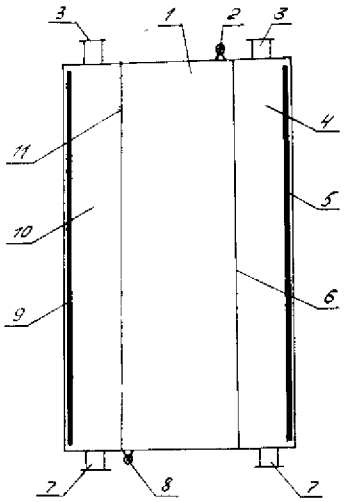


FIGURE 1. Installation for separation of microorganisms from the liquid phase by electroretention.
1 - middle chamber; 2, 8 - microbial suspension inlet and outlet; 3, 7 - water inlet and outlet; 4, 10 - electrode chambers; 5, 9 - electrodes; 6, 11 - membranes.

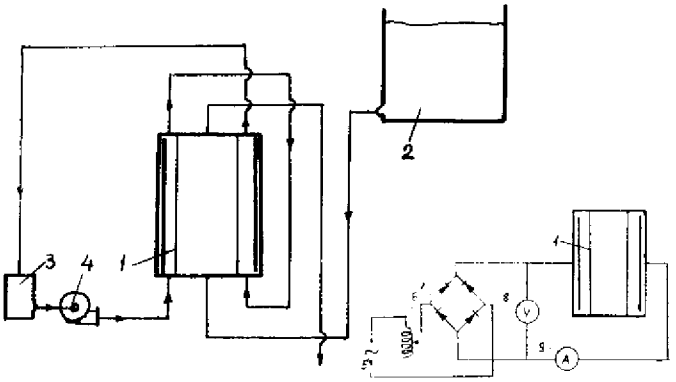


FIGURE 2. Hydraulic and electric schemes for the electroretention of microorganisms.
1 - three-chamber apparatus for electroretention; 2 - tank with a suspension of microorganisms; 3 - water bottle; 4 - pump; 5 - power supply; 6 - autotransformer; 7- rectifier; 8 - voltmeter; 9 - ampermeter.

placed into an electric field perpendicular to the liquid flow. Numerous experiments resulted in the discovery of a new phenomenon which we called "electoretention" and defined as "retention of particles within a range of dimensions (including certain real solutions of compounds) by polarized materials" (Rotmistrov *et al.*, 1978). Outwardly, the essence of this phenomenon consists of a layer of granular or porous material, that presents no obstacles for filtration of small size particles, but when placed in an electric field, it is transformed into a highly efficient filter retaining various colloidal particles and many substances forming real solutions.

The installation used for the studies of microbial retention is shown in Fig. 1.

The modes of power and liquid supply are fairly simple (Fig. 2). The apparatus for water purification is made of an insulating material (acrylic plastic, rubber, ebonite, teflon, etc.) and composed of three membrane-separated chambers: two electrode chambers and the middle working chamber. The working chamber is packed with granular, fibrous or porous dielectrics: ion-exchange resins, quartz sand, fragmented baked clay, cellulose or synthetic fibers, wool, silk, basalt or glass wool, polyurethane, various cotton tissues, etc. and the water containing microbial cells (up to 10^9 colonies per 1 ml) is passed through the packing. As the test-objects, microorganisms from different taxonomic groups were used characterized by a variety of forms and sizes, live and dead, Gram-positive and Gram-negative, forming capsules, sporous and nonsporous, flagellant and immobile (Table 1).

If a thick suspension of microorganisms is passed through a fresh packing composed, for example, of ion exchange resins, then a portion of the cells will be retained by the packing, as solid surfaces are in general capable of adsorbing microbial cells.

However, the adsorptional capacity of even best adsorbents is not high, whereas a rather thin layer (a few cm-thick) of granular or fibrous packing presents no obstacle for infiltration of tiny microbial particles. Therefore, in a few minutes the concentrations of microbial cells at the inlet and outlet of the chamber will become equal. But if an electric field is applied to the packing, it will be able to retain

incomparably larger quantities of microorganisms, many orders of magnitude higher than its starting adsorptional capacity. Microbial cells are released by switching off the electric field, and they are readily eluted as a viscous suspension in which the cell concentration may be 10 to 100-fold higher compared to the starting suspension. Repeated induction of the electric field results in the immediate retention of cells and their separation from the liquid (Fig. 3).

TABLE 1. Characterization of microbial cultures used in experiments on separation of microorganisms from liquids.

Microbial culture	Size, μm		Motility	Coloration, acc. to Gram
	Cells	Spores		
Aspergillus niger	10-300	2.5-4	-	+
Penicillium purpurogenum	3.5-200	2.5-4.5	-	+
Candida albicans	9.0-10	0	-	+
Candida tropicalis	6.0-7.0	0	-	+
Saccharomyces cerevisiae	5.0-10	0	-	+
Rhodotorula utilis	3.0-7.5	0	-	+
Bacillus cereus	1.3-4.0	0.9-1.3	+	+
Bacillus polymyxa	1.3-4.5	1.7-2.6	+	+
Bacillus mesentericus	0.6-6.0	0.5-0.9	+	+
Bacillus subtilis	0.6-4.0	0.6-0.9	+	+
Escherichia coli	0.5-1.3	0	+	+
Proteus vulgaris	0.5-2.0	0	+	-
Pseudomonas aeruginosa	0.5-1.2	0	+	-
Serratia marcescens	0.5-0.8	0	+	-
Staphylococcus aureus	0.5-0.8	0	-	+
Phage E. coli	0.12-0.2	0	x	x
Phage Ps. phaseolicola	0.07-0.15	0	x	x
Chlorella vulgaris	4.2-10	0	x	x
Anacytis nidulans	0.8-5.5	0	x	x

Thus, the packing may be used repeatedly for a long time without special regeneration.

The process of electroretention of microorganisms is dependent on the electric field strength (Table 2).

Increasing the filtration rates of microbial suspension leads to a poorer retention of cells. The extent of retention of microorganisms by polarized materials is inversely proportional to increasing salt

concentrations in purified water and somewhat drops with higher concentrations of microorganisms in a suspension. The material of the packing has also an essential effect on the retention process. In this respect, wool, for example, shows better characters than cotton and is much better, compared to glass cotton, whereas quartz sand has a lower retention capacity compared to silica gel or ion exchange resins.

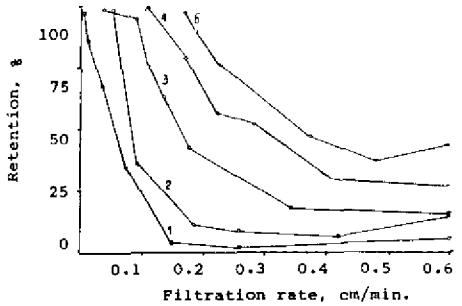


FIGURE 3. Effect of the electric field strength on the flow rate and retention of 24 h-culture of *P. aeruginosa*.
Packing: KY-2 + AB-17 (cationate and anionite resins) Membranes: ionites $[C]_{in.} = 1.2\ 10^9$ col./ml Electric field strength: 1, 2, 3, 4, 5, 15, 25, 37.5, 50 V/cm.

TABLE 2. Effect of the electric field strength on the retention of microorganisms (filtration rate, 0.2 cm/min).

Microbial culture	Cell size, μm	Retention of microorganisms, % under different field strength					
		5	10	20	30	50	(V/cm)
Saccharomyces cerevisiae (1 day)	4.0–12.0	4	11.3	32	50	66	
S. serevisiae (killed by autoclaving)	4.0–12.0	2.8	4.6	17.5	32.5	56.1	
Bacillus mesentericus (19 days, spores)	0.5–1.1	1.5	5.5	19.2	46	83	
Pseudomonas aeruginosa (1 day)	0.5–0.6	1.7	7	23	49	85	
Serratia marcescens (3 days)	0.5–0.6	2.3	3.9	13	20	45.2	

To obtain a sterile apyrogenic water used for preparing injection solutions, a filtration installation was devised with a 3.5 cm thick filtering layer of cotton gause (Fig. 4).

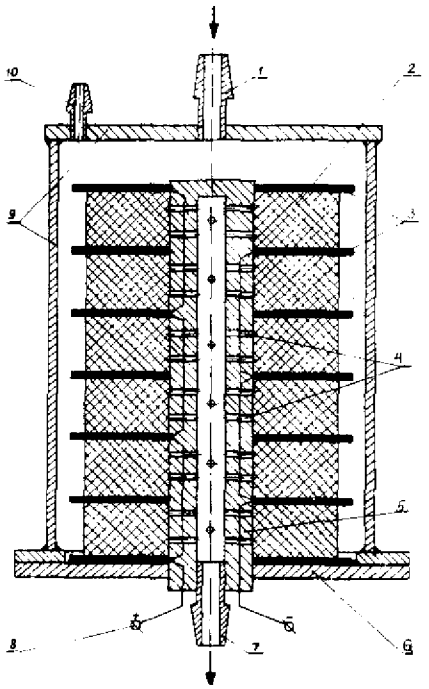


FIGURE 4. Sterilization filter.
1 - water input; 2 - cotton gause layer; 3 - electrodes; 4 - orifices for water infiltration; 5 - perforated pipe; 6 - filter bottom; 7 - sterile water outlet; 8 - terminals for power supply; 9 - filter lid; 10 - gas outlet.

But how does the retention of microbial cells by polarized materials occur? What are the basic driving forces of this phenomenon?

A special closed chamber with a monolayer of ball-shape silica gel 0.5 mm grains was devised for direct observation of the behaviour of microbial cells during suspension filtration in a heterogeneous electric field perpendicular to suspension flow (Fig. 5)

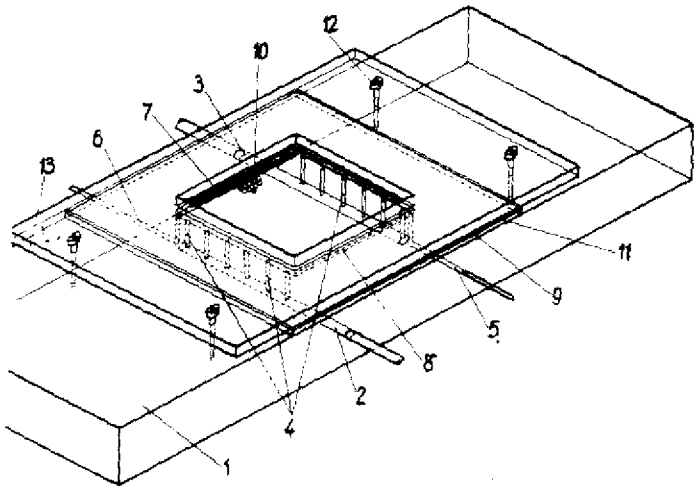


FIGURE 5. Chamber for studying the behaviour of microbial cells in the electric field.
1 - polyacrylate plate; 2,3 - canals for suspension; 4 - orifices; 5,6 - canals for electrodes; 7,8 - electrodes; 9 - rubber frame; 10 - silica gel balls; 11 - covering glass; 12 - stud bolts; 13 - polyacrylate frame.

During the suspension flow across the chamber without application of the electric field, only rare cells are adsorbed by the silica gel surface, while the bulk of microorganisms is entrained with the water flow. Application of the electric field sets the microbial cells in a motion different from the direction of water flow. Under a electric field (up to about 5 V/cm) such movements are not intensive and somewhat directed towards the anode. The cells are attracted to the silica gel surface with the cell accumulation around the points of contact of grains. Numerous small chains of microbial cells are formed and attached to the cathode-directed side of silica grains. Such chains contain 3-7 cells in the case of *Saccharomyces cerevisiae* and over 20 for *Bacillus subtilis*. In the inter-grain spaces hardly distinguishable circular movements of cells occur which become more visible with increasing strength of the electric field. In larger spaces formed by 4-5 balls of silica gel, 2-3 centres may be observed with microbial cells rotating

around them.

If a microorganism rotates clockwise around one centre, then the neighbouring one will induce the counter-clockwise movements. It is interesting that the arrest or inversion of the suspension flow does not affect the direction of microbial rotation, whereas the cells start to revolve in the counter direction the moment the polarity of the terminal is changed. Further increase of the electric field strength (up to 70-200 V/cm) compels the microbial cells to concentrate basically at spots of the grain contact, whereas their circular movement is repressed, and the inter-grain spaces become the sites of an intensive cell movement towards the anode.

After the electric current is switched off, the rotating cells as well as the bulk of cells accumulated on the silica gel surface are entrained by the water flow, andthe cell agglomerations promptly disintegrate.

Electrophoresis and electrostatic interaction are apparently the major cause of adhesion of microorganisms to the surface of packing material and formation of chain aggregations, whereas dielectrophoresis is contributable to the concentration of microbial cells in grain contact areas. Rotation of microbial cells in the inter-grain space appears to be caused by electrohydrodynamic fluxes the origin of which is still rather unclear. Removal of the electric field results in disappearance of all induced forces, and the cells are rapidly eluted from the chamber by a continuous water flow.

Retention of microorganisms is no more than a particular case of the discovered phenomenon. Recent experiments point to the possibility of similar separation from water (and other liquids) of colloids of clay minerals and other highly dispersed particles as well as many other soluble substances - proteins, nucleic acids, polysaccharides, humic substances and even organic compounds of a relatively low molecular weight, specifically organic dyes.

In this context, it appears fairly realistic and advisable to use the phenomenon of electroretention for a number of purposes, namely:
- separation of microorganisms,
- concentration of microbes and viruses,

- sterilization of liquids,
- production of apyrogenic water,
- separation of particles of biological origin,
- immobilization of enzymes and realization of enzymatic reactions,
- purification of liquids from various colloidal particles.

Removal of microorganisms is one of the prospective ways in solving the problem of the water cleanup from suspended organic matter which may be further utilized in industry and agriculture.

The use of the trophic chain of hydrobionts will enable a complete elimination of the suspended organic matter and thus obtain a water of full biological value and contribute to settling the problem of excessive biomass produced in the conventional activated sludge process.

Microbial purification of water is entering a stage of radical changes which undoubtedly will be of a direct relevance to both industrial and municipal sewage.

The above statement may be exemplified by the research carried out by Ukrainian scientists (Institute of Colloidal and Water Chemistry of the Ukrainian Academy of Sciences, Kiev) who developed effective microbial technologies for the cleanup of industrial effluents which are being successfully used nationwide in a number of chemical industries. The effluents in question are heterogenic as regards present pollutants: aliphatic and aromatic amines, anionic and nonionogenic surfactants, nitro products, methanol, ethylene glycols, penterythrite, etc. The common problem is that such waste waters cannot, for the moment, be purified by conventional techniques. The only way out is to "burn" them or pump into deep wells for burial. Recent industrial assays showed that aerobic and anaerobic microorganisms immobilized on solid supports are capable of degrading the above pollutants and thus detoxify industrial effluents. As a follow-up, installations for microbial removal of hexamethylene diamine from the so-called "dead water" resulting from the production of the chemical fibre Anid (nylon-66) was brought into operation. High concentrations of nonionogenic surfactants (up to 10 g/l) are removed by microbial purification of waste water from organic synthesis plants. Microbial technologies are also used for the cleanup of effluents from production of polyisocyanates, for the removal of

nitrocompounds, methanol, ethylene glycols and other toxicants. The installations used are of a simple design, relatively small size, ecologically safe and energy saving.

Thus, fundamental research on microbial purification of water lays foundation for broad practical utilization of highly efficient and ecologically sound methods for the cleanup of sewage.

REFERENCES

Alexander M. 1973. Nonbiodegradable and other recalcitrant molecules. *Biotechn. & Bioengin.* 15:611-647.

Gvozdyak P.I., G.N. Dmitrenko, N.I. Kulikov. 1985. Purification of industrial effluents by immobilized bacteria. *Khimiya i tekhnologiya vody* 7:64-68 (in Russian).

Rotmistrov M.N. P.I. Gvozdyak, S.S. Stavskaya. 1978. *Microbial Purification of Water.* Kiev, Naukova Dumka. 286 p. (in Russian).

Yakovlev S.V., Ya.A. Karelin, Yu.M. Laskov, Yu.V.Voronov. 1985. *Purification of Industrial Effluents.* Moscow, Stroyizdat. 336 p. (in Russian).

RECOMMENDED LITERATURE

Rehm, H.J., G. Reed. 1981. *Biotechnology. Vol.1. Microbial Fundamentals.* Weinheim. "Verlag Chemie". 520 p.

Rehm H.J., G. Reed, eds. 1986. *Biotechnology. Vol.8. Microbial Degradation* (W. Schonborn, vol. ed.). Weinheim, Verlagsgesellschaft VCH, 743 p.

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