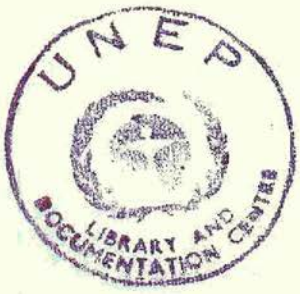
UNITED NATIONS ENVIRONMENT PROGRAMME

MICROBIAL DEGRADATION OF XENOBIOTICS

Practical Manual



Pushchino 1990

UNITED NATIONS ENVIRONMENT PROGRAMME

USSR COMMISSION FOR UNEP
Centre for International Projects GosKomPrirody

USSR ACADEMY OF SCIENCES
Centre for Biological Research

UNEP Training Course

"Microbial Technologies for Degradation of Persistent Pollutants"

MICROBIAL DEGRADATION OF XENOBIOTICS

Practical Manual

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Pushchino 1990

FOREWORD

The present manual is conceived as the training component of the project "Microbial Technologies for Degradation of Persistent Pollutants" in order to allow the firsthand acquaintance and acquisition of certain practical skills in modern microbial techniques aimed at abating environmental levels of persistent pollutants.

It embraces a follow-up set of laboratory exercises to a lecture course treating similar topics that focus on: methods of extraction of persistent pollutants from soil and their qualitative and quantitative analyses; establishment 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 of 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 utilized strains under selective and non-selective conditions.

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CONTENTS

STUDY OF PARTICULAR FEATURES OF THE PESTICIDE SORPTION IN SOIL

V.P. Sukhoparova and N.D. Ananyeva

The behaviour of xenobiotics in the environment is in many respects determined by processes of pesticide adsorption and desorption by various soils as well as by such phenomena as volatilization, photo- and thermal decomposition, migration and microbial conversion. Under natural conditions, it is hardly possible to single out the effect of a specific ecofactor. Therefore, while investigating detoxication of pollutants, researchers have to resort to ecotoxicological modelling, i.e., imitation of the effect of a specific ecofactor on pesticides present in soil or in other media. This laid the foundation for developing experimental ecotoxicological simulation models of pesticide behaviour in the environment. The description of individual components of a proposed model, i.e., that of imitated processes is given in papers of M.S. Sokolov (1, 2). Basic ecofactors and processes which control the behaviour of toxicants in environments fall into two classes of the model components. The first embraces evaporation and co-evaporation, insolation, hydrolysis, sorptional interactions with organomineral sorbents and migration. The second class of the model components includes effects of biological factors: uptake of pesticides by plants; conversion and degradation in water, soil and biota; effects on microorganisms, soil enzymes, and other processes.

Below we shall dwell in sufficient detail upon certain peculiarities of one of the model elements, namely the study of sorptional interaction of pesticides with soil.

While conducting this investigation we shall use the following definitions:

- sorption: a common term denoting adsorption and absorption processes;
- kinetics of sorption: time required for reaching an equilibrium in the sorbate-sorbent system;
- adsorption isotherm: graphic dependence of the quantity of the pesticide adsorbed versus its concentration;
- phase ratio: solid-liquid (S/L) ratio of the sorbent mass and the volume of sorbate solution.

I. Adsorption of Pesticides

1.1. Treatment of Soil Samples

The soil to be studied is dried to the air-dry state, then sieved to separate it from plant residues and solid rock fragments, and finally ground with a pestle and passed through a 1 mm mesh sieve.

1.2. Preparation of Pesticide Solutions

Solutions of pesticides are prepared using distilled water. Heating on a water bath may be necessary to increase the solubility of the compounds under study. The lower limit of a pesticide concentration is assumed depending on the sensitivity of an analytical technique, whereas the upper one is determined by the toxicant solubility. The initial pesticide concentration (C_0) is measured by analyzing an aliquot (small portion) of the solution.

1.3. Experimental Routine Manipulations

An air-dried soil sample (0.2-2.0 g) is introduced into a 100 ml flat-bottom flask together with a certain portion of pesticide solution and placed on a shaker for a preset time. The suspension obtained is centrifuged and an aliquot of the supernatant (precipitate-overlying liquid) is taken for analysis. The quantity of the toxicant absorbed by

the soil (x) is calculated from the equation:

$$x = \frac{V \cdot C_0}{g} (1 - \frac{C}{C_0})$$

where C_0 is the initial pesticide concentration, mM; C is the pesticide equilibrium concentration in supernatant, mM; g is the sample mass, g; v is the solution volume, ml.

Experimental results suggest that the optimum solid/liquid phase ratio (S/L) is 1:10 for the soils with low organic matter content, whereas it appears reasonable to assume the S/L ratio to be 1:100 for the high-humus soils. As a rule, variation of the starting concentration does not affect the time a system requires for attaining an equilibrium. The time required for centrifugation is 15-20 min at 4000 g for the low-organic matter soils and 25-30 min at 14000 g for the high-organic matter ones.

1.4. Problem of the Adsorption Kinetics Studies

Knowledge of the adsorption kinetics is indispensable for elucidating the mechanism of pesticide adsorption by soils. The forces of attraction that are inherent in solids may be of two types: physical and chemical. They predetermine the occurrence of either physical adsoption or chemosorption. Physical adsorption is known to allow for a system to attain equilibrium much faster compared to chemosorption. Therefore, studying peculiarities of pesticide adsorption by various soils calls for knowledge of the time required to establish equilibrium in the system sorbate-sorbent. For this, the amount of the adsorbed compound is determined after intervals of 0.25, 0.5, 1, 2, 4, 6, 8 ... hours following the onset of the phase interaction, i.e. that of soil and pesticide solution. Measurement of the kinetic process parameters is carried out with a constant S/L phase ratio. Here is one example.

1.5. Study of the Adsorption Kinetics of Arylamidic Pesticides: Linuron and Monolinuron

Constituents and parameters of the system:

- soils: soddy-podzolic and meadow-alluvial (humus content is 1.5% and

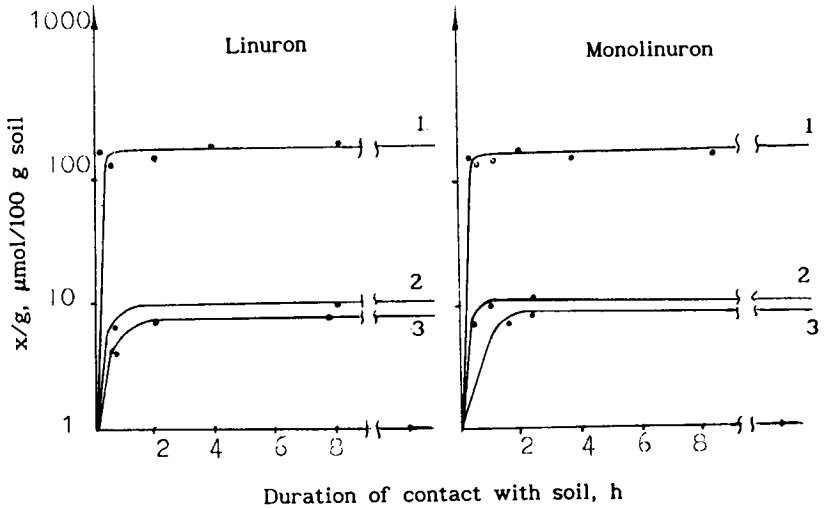


FIGURE 1. Time course of herbicide sorption by humic-peaty (1), soddy-podzolic (2) and meadow-alluvial (3) soils.

1.8%, respectively), and humic-peaty (organic matter content - 73.8%);

- phase ratio (S/L): 1:10 for soddy-podzolic and meadow-alluvial soils, and 1:100 for humus-peaty ones;
- sample mass: 2 g for soddy-podzolic and meadow-alluvial soils, and 0.2 g for humic-peaty ones;
- pesticide solution volume: 20 ml;
- starting pesticide concentration C_o: 0.05 mM;
- experiment temperature: 23°C.

Experiments showed that in the sorbate-sorbent system (humic-peaty soils) equilibrium is attained in 15-30 min after the onset of phase interaction, whereas during the sorbate uptake by the soddy-podzolic and the meadow-alluvial soils the equilibrium is established in 1.0-1.5 h after the phases were brought in contact (Fig. 1). The rapid onset of the sorptional equilibrium observed in this experiment may be explained by the prevalence of physical adsorption (Van der Vaalsian, dipole-dipole and dipole-ion interactions; hydrogen bonds). Formation of

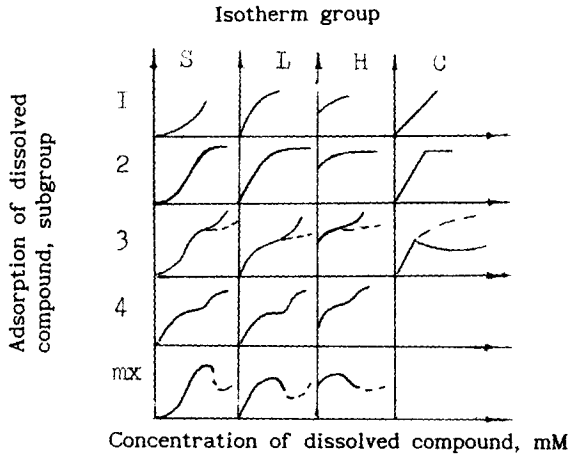
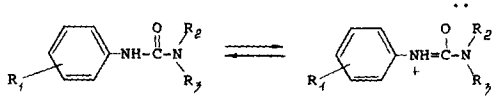


FIGURE 2. Classification of adsorption isotherms from solutions according to Jiles (3)

these bonds is often preceded by redistribution of the charge in the herbicide molecule as illustrated by the following scheme:



Moreover, values of the time required for the onset of sorptional equilibrium are indispensable for plotting pesticide adsorption isotherms.

1.6. Plotting of Pesticide Adsorption Isotherms

Quantification of the processes of pesticide adsorption by soil is usually made with the help of adsorption isotherms which show graphic dependence of the amount of a compound adsorbed by soil at its equilibrium concentration in solution. Interpretation of adsorption isotherms calls for the use of Jiles' classification referenced to in (3). A great diversity of experimental isotherms are divided into four groups, that in turn fall into subgroups, and they are examined on the basis of specific contributions to the entire adsorption process by

different interactions (dissolved substance-sorbent, dissolved substance-dissolved substance, dissolved substance-solvent and possible orientation of adsorbed molecules on the sorbent surface) (Fig. 2).

2. Pesticide Adsorption by the Soil Solid Phase

Under static conditions within the range of concentrations commonly found in soil solutions, the adsorption of pesticides by the solid phase of soil is described by the Freundlich's equation:

$$x/g = k\ c^{1/n}$$

where x is the quantity of sorbed pesticide, mM; g is the soil mass; C is the sorbate equilibrium concentration in solution, mM; k is the sorptional capacity constant; $1/n$ is the sorption rate constant.

It is more convenient to use Freundlich's equation in the logarithmic coordinates, where it is written as:

$$\lg\frac{x}{} = \frac{1}{}\lg C + \lg k$$

i.e. in the general form it represents an equation of regression:

$$y = Ax + B$$

Calculations making use of the regression equation and those of k and $1/n$ are performed with the least-squares technique. The correlation index (r) pointing to the measure of the adsorbed pesticide dependence upon its equilibrium concentration is obtained using the formula:

$$r = \frac{\sum(X - \bar{x})\cdot(Y - \bar{y})}{[\sum(X - \bar{x})^2\cdot(Y - \bar{y})^2]^{1/2}}$$

The error of the correlation and regression index evaluation is found from the following relations:

$$S_r = \left(\frac{1 - r^2}{n - 2}\right)^{1/2}; \qquad S_{reg} = S_r\left(\frac{\sum(Y - \bar{y})^2}{\sum(X - \bar{x})^2}\right)^{1/2}$$

The confidence intervals are

$$r \pm t_T\cdot S_r;\ 1/n \pm t_T\cdot S_{reg}.$$

The error of the departure from regression is

$$S_{yx} = S_r[\sum(Y - \bar{y})^2]^{1/2}$$

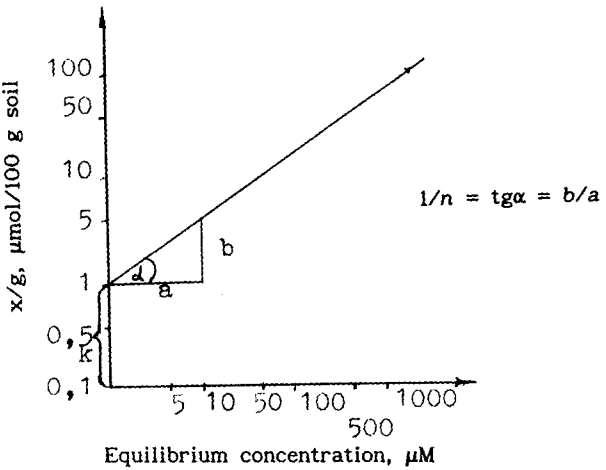


Figure 3. Graphical method for calculating the constants $1/n$ and k in Freundlich's adsorption equation.

In addition to calculating the "k" and "$1/n$", a graphic method referenced to in (5) is used. By plotting x/g values as the ordinate and those of equilibrium concentration as the abscissa a straight line is obtained whose slope corresponds to the $1/n$ value and the intersection with the ordinate - to the k value (Fig. 3).

3. Pesticide Uptake by Soils with High Adsorption Capacity

This process is well described by the linear isotherms which are governed by Henry's equation:

$$x/g = k \cdot C$$

where x is the sorbed pesticide quantity, mM; g is the soil mass, g; C is the equilibrium concentration of sorbate in solution, mM; k is the partition coefficient indicating relative distribution of sorbate between the solid and liquid phases of the system.

An example of plotting the isotherm of adsorption of linuron and monolinuron by the soddy-podzolic, meadow-alluvial and humic-peaty soils follows.

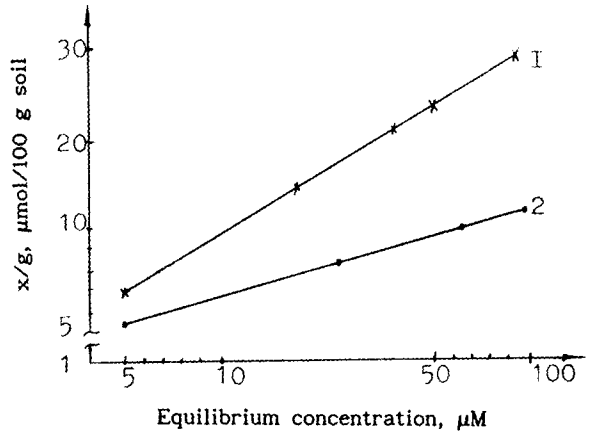


FIGURE 4. Adsorption isotherms for soddy-podzolic soil (according to Freundlich). 1, linuron; 2, monolinuron.

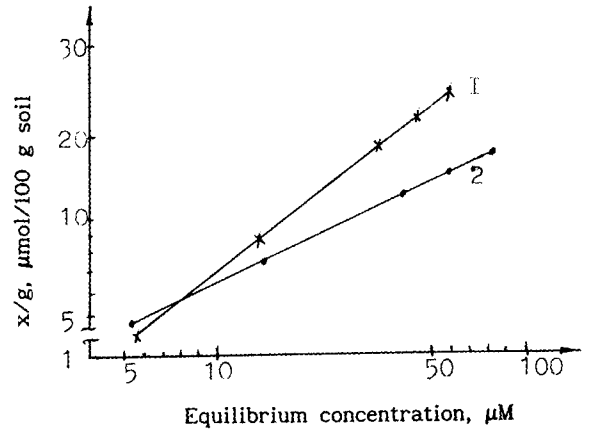


FIGURE 5. Adsorption isotherms for meadow-alluvial soil (according to Freundlich). 1, linuron; 2, monolinuron.

System parameters:

- C_0: 0.01, 0.025, 0.05, 0.075, 0.1 mM;
- duration of the phase contact: 2 h;
- S/L: 1:10 for meadow-alluvial and soddy-podzolic soils;
- S/L: 1:100 for humic-peaty soil;

- solution volume: 20 ml.

The experimental results obtained in the studies with arylamide herbicide sorption show that the adsorption of monolinuron and linuron by the soddy-podzolic and meadow-alluvial soils are described by Freundlich's equation (Figures 4, 5) (6). In this case adsorption isotherms obtained for a concentration (C_0) range of 0.01-0.1 mM apparently fall under the L-class of the second subgroup according to the classification of Jiles. The classical interpretation of this group of isotherms is based on the assumption that the sorptional layer is monomolecular and that a plateau on the isotherm corresponds to filling the monolayer.

Table 1 gives an example of the statistical treatment of experimental results.

TABLE 1. Constants of the Freundlich adsorption equation (soddy-podzolic and meadow-alluvial soils).

Compound	1/n	k	r	S
		Soddy-podzolic soil		
Linuron	0.52 + 0.05	2.88	0.988 + 0.09	0.022
Monolinuron	0.39 + 0.05	2.45	0.982 + 0.01	0.020
		Meadow-alluvial soil		
Linuron	0.94 + 0.11	0.71	0.981 + 0.11	0.041
Monolinuron	0.48 + 0.17	1.66	0.917 + 0.24	0.050

Isotherms of arylamide herbicide adsorption by humic-peaty soils are linear in the concentration (C_o) range of 0.01-0.1 mM and obey Henry's equation (7, 8). They may be referred to as isotherms of C-class of the first subgroup. Here the sorptional capacity of the sorbent is unlimited and proportional to the sorbate concentration in solution (Fig. 6).

TABLE 2. Constants of Freundlich's desorption equation (humic-peaty soils)

Compound	1/n	k	r	S
Linuron	1.84 + 0.08	93.4	0.997 + 0.045	0.01
Monolinuron	0,42 + 0.02	8.9	0.997 + 0.045	0.46

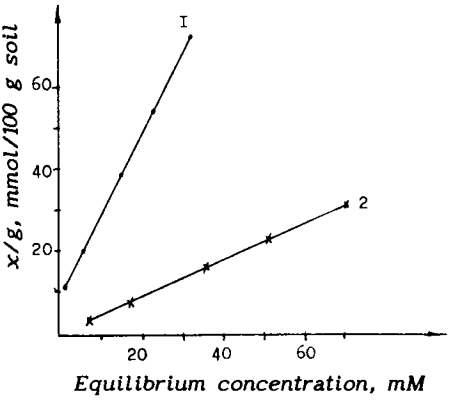


FIGURE 6. Adsorption isotherms for humic-peaty soils (according to Freundlich). 1, linuron; 2, monolinuron.

II. Pesticide Desorption from Soil

1. General Outline

Adsorption isotherms are quantitative (not qualitative) characteristics of the process of pesticide sorption. In order to investigate qualitative regularities of sorptional interaction (uptake forms), one should assess particular features of desorption of a compound from the sorbate-sorbent complex.

1.1. Sample Preparation

Investigation of the pesticide desorption under static conditions is preceded by saturation of the soil sample with a toxicant whose concentration is maintained at the same maximum level as it was during the isotherm measurements, and with the same phase ratio and the duration of S-L phase contact. A soil sample saturated with a pesticide is dried to the air-dry state.

1.2. Experimental Routine Manipulations

Pesticide-saturated soil samples (0.2-2.0 g) are added to 100 ml flask together with the appropriate volume of distilled water, a solvent or

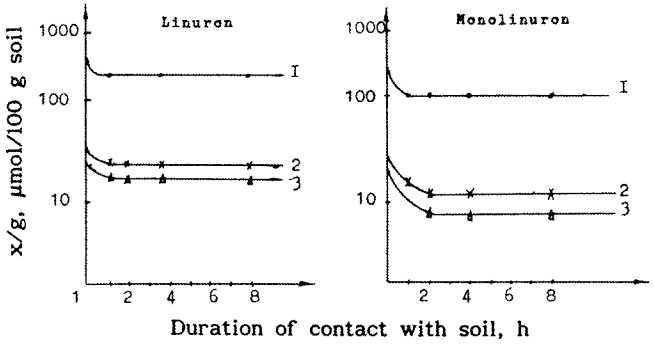


FIGURE 7. Time course of pesticide desorption in humic-peaty (1), soddy-podzolic (2) and meadow-alluvial (3) soils.

mixture of solvents, then placed on a shaker and agitated (desorption is taking place) for 0.25, 0.5, 1, 2, 4, 6, 8 .... hours, and finally centrifuged. Further, the sample is analyzed for pesticide residues remaining after desorption and desorption-resistant pesticide quantities are plotted on a graph versus time of phase contact.

An example follows with the description of pesticide desorption from the sorbate-sorbent complex.

Components and parameters of the system:

- pesticides: linuron and monolinuron;
- soils: meadow-alluvial, soddy-podzolic and humic-peaty;
- S/L: 1:10 for meadow-alluvial and soddy-podzolic soils;
- S/L: 1:100 for humic-peaty soils;
- volume of extracting agent: 20 ml.

Figure 7 shows the kinetics of desorption of linuron and monolinuron by water from soddy-podzolic, meadow-alluvial and humic-peaty soils. The desorption process is basically completed after the first two hours and the equilibrium established.

1.3. Study of the Forms of Pesticide Adsorption by Soil

The pesticide binding to soil may characterize to some degree the qualitative steps of the pesticide sorption by soils (Table 3). A native

soil treated with a pesticide contains a portion of the toxicant in soil solution; it is termed the non-sorbed or dissolved form. In addition, part of the pesticide is sorbed by soil reversibly (reversibly sorbed form). In this portion three other forms may be specified: poorly, moderately and firmly sorbed residues. They are identified by the appropriate selection of solvents differing by their physico-chemical properties. Numerous classes of pesticides form in soil the so-called 'bound residues' which can be extracted by a mixture of organic solvents as well as by rigourous treatments, e.g. acidic or alkaline hydrolysis. An example aimed at illustrating the above considerations follows.

1.4. Identification of the Forms of Linuron and Monolinuron Binding to Soils

Experiment setting:

- S/L: 1:10 for meadow-alluvial and soddy-podzolic soils;
- S/L: 1:100 for humic-peaty soil;
- volume of extracting agent: (water, acetone, or a mixture of solvents
- acetone:hexane:water, 5:5:3), 20 ml.

An estimation of the desorbing properties of the above solvents shows that water can extract 40 to 60% of the compounds. In some instances acetone gives an additional amount of reversibly sorbed compounds. The greatest number of compounds, including dissolved toxicants, are effectively extracted by mixtures of polar and non-polar solvents.

Thus the use of extracting solvents with differing extractive capacities is advisable for characterizing the forms of sorption.

Investigation of pesticide sorption by various soils often calls for experimenting with varying phase ratios, sorbent humidity, pH of suspension, and temperature aimed at elucidating their possible effects on the sorption of xenobiotics. For information on the practical recommendations on carrying out experiments of this type the reader is referred to Knyr's paper (5).

TABLE 3. Adsorption forms and bonding characters of soil sorbents with non-polar non-ionogenic pesticides and products of their transformation.

Form of compound in soil and extent of its adsorption	Method of extraction and/or identification of sorbed compound	Bonding character
1. Non-adsorbed (remains in solution)	Direct analysis of soil solution (supernatant, eluant) or of toxicant extract in a non-polar solvent	-
A. Physical and physico-chemical adsorption		
2. Reversibly sorbed:	2. Extraction:	Electrostatic, dipole-dipole and dipole-ionic interactions; formation of a complex with hydrophobic part of sorbent or by electric charge redistribution; hydrogen bonds
a) poorly sorbed	a) with water (at saturation temperature)	
b) moderately sorbed	b) with hot water or an organic polar solvent	
c) firmly sorbed	with a mixture of polar and non-polar solvents (including extraction after ultra-sonic treatment)	
B. Chemosorption		
3. Bound (immobilized):	3. Fission of chemical bonds:	
a) hydrolysable	a) hydrolysis upon heating	Formation of new chemical compounds (including organo-mineral ones) via covalent and coordination bonds (exchange of ligands)
b) non-hydrolysable	b) chemical decomposition of mineral soil skeleton and organo-mineral compounds (xenobiotic + clay) treated with HF	
	b') pyrolysis of bound labelled compounds and CO analysis	
	b'') analysis (after extraction, see 2.b above) of C-labelled sorbate in the complex sorbate-sorbent	

TABLE 4. Desorption of herbicides from soils by various extractants.

Compound	Extraction of reversibly sorbed compounds, %			Content of hydrolysable soil-bound compounds, %
	combination of solvents	acetone	water	
	Soddy-podzolic soil			
Linuron	84	57	46	16
Monolinuron	80	53	35	20
	Meadow-alluvial soil			
Linuron	100	65	60	0
Monolinuron	100	60	45	0
	Humic-peaty soil			
Linuron	100	26	42	0
Monolinuron	100	36	46	0

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ANALYSIS OF RESIDUAL PESTICIDES AND THEIR METABOLITES IN WATER AND SOIL

Z.I. Finkelshtein

Effective pest control in modern agriculture requires a very broad and ever-increasing spectrum of substances which belong to various classes of chemical compounds and are collectively called pesticides. Halogenated, and primarily chlorinated, organic compounds, organophosphorus substances, urea-based compounds, derivatives of carbaminic, thio- and dithiocarbaminic acids, symmetrical triazines and many others are widely used to this end. However, even within any of these groups the pesticides differ by their composition and structure, and, consequently, by their properties and mode of analysis. Therefore, the relevant literature carries descriptions of hundreds of techniques used for qualitative and quantitative analyses of pesticidal residues in soil.

The present work is aimed at a practical demonstration of the analysis of pesticidal residues exemplified by the fungicide named ridomil (active substance - metalaxyl). It belongs to the acylalanine group of pesticides which is presently under thorough investigation in the Laboratory of enzymatic degradation of organic compounds (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences). Some aspects of chromatographic methods are considered using

examples of the organochlorine herbicide methoxychlor (DDT analog) and the organophosphorus insecticide phosalone.

1. Sampling Procedure

The soil sample is uniformly spread over a sheet of paper. Small stones, pieces of wood and other alien objects are removed, and the sampling is performed by quartering: the sample is divided into four parts along two diagonal lines, two parts are removed, while the two remaining are mixed, and the operation is repeated. After this is completed, soil samples of 50-100 g are taken. They should be air-dry, i.e., have ~ 3% moisture. In case a damp soil is prepared for analysis, a small sample is also taken for measuring moisture content by drying to a constant weight at 40-60°C (air-dried state).

2. Extraction of Pesticides from Soil and Water

a) Ridomil extraction.

Air-dried soil (50-100 g) is placed in a stoppered 500 ml flask, moistened with an equal volume of water, and after 2-3 h, a 100-200 ml mixture of acetone:0.05N calcium chloride solution (1:1) is added. This mixture was selected as a result of assays on the extractivity of ridomil from soils with various organic solvent mixtures. Calcium chloride is added to the water to repress co-extractants which would otherwise interfere with further analysis. The flask is placed on a shaker for two hours, then the slurry is poured into centrifuge tubes made of glass or a polymeric material (resistant to solvents!). After centrifugation the liquid phase is poured into clean flasks, and the soil is transferred from the centrifuge tubes to another 500 ml flask, re-extracted with 100 ml of acetone mixed with 0.05 N $CaCl_2$ solution, shaken for one hour and centrifuged.

The combined extracts are transferred to a separating funnel, 100-200 ml of chloroform is added, and the ridomil re-extraction is performed. This operation is repeated twice. The chloroform extracts are brought together, dried by adding anhydrous sodium sulphate and concentrated by evaporating the solvent in a rotary vacuum evaporator at 35-38°C.

The ridomil is isolated from water or the culture liquid by extracting it thrice with chloroform.

b) Methoxychlor extraction

The procedure for isolating methoxychlor from soil is similar to that described above but is performed with a moist soil using a mixture hexane:acetone (1:1) as the toxicant extractant and followed by redistribution of methoxychlor in hexane. Hexane:acetone mixtures of varying ratios are often used to extract various organochlorine pesticides (OCP) from soil. The extraction of OCP with hexane:acetone mixtures frequently requires a specific clean-up of the extract depending on the type of soil. After concentrating the extract by evaporation, purification is carried out by shaking it with concentrated sulphuric acid until the extract is clarified.

c) Phosalone extraction

A soil sample (100 g) is added with 100 ml of water and 50 ml of chloroform and placed on a shaker for 3 hours. The suspension is passed through a folded paper filter or the soil is separated by centrifugation and washed twice with the water:chloroform mixture (10:2). The chloroform phase is separated from the filtrate and the water phase extracted twice with fresh portions of chloroform.

3. Determination of Pesticides by Thin-Layer Chromatography

Thin-layer chromatography (TLC) is the easiest of all chromatographic methods to perform, does not require any expensive and sophisticated equipment, and is well suited for determining residual quantities of pesticides. At present, many manufacturers produce ready-made chromatographic plates with a layer of various sorbents fixed on a metal foil, plastic or glass.

In the present study the Silufol UV 254 plates are used obtained from the Czechoslovakian firm "Kavalier". These plates have a layer of silica gel containing a luminophor fixed on to aluminium foil (size 15x15 cm); starch is used as a binder. The silufol is easy to cut to give plates of any size required.

The sample under investigation is applied on a chromatographic plate 1.5 cm up its bottom edge (the start line). In the qualitative analysis, samples are applied on a TLC plate with a common capillary prepared by elongating a thin-wall capillary tube in the flame of a glass blower's burner. Once used, such a capillary is discarded. In order to evaluate the component concentration in a sample, the spotting is performed with calibrated capillaries applying 1-20 μl from the thoroughly measured volume of the sample solution in a polar solvent. Sample application should produce starting spots of 2-4 mm in diameter. An exceedingly large starting zone will produce oversized spots on the chromatogram liable to diffusion and, consequently, overlapping with the neighbouring ones. The layer of sorbent should not be seriously damaged by contact with the capillary, although a slight disturbance does not affect the resolution. In the analyses of mixtures, the spots should be spaced 1-1.5 cm apart. Different quantities of standard compounds, necessary for the identification and quantitation of the toxicant under study, are applied to the same plate.

Development of plates is carried out in a chromatographic chamber into which the mobile phase (solvent system) is poured 10-20 min before the operation starts. The plate's edge with the sample spots above it should be submerged into the mobile phase to about 0.5 cm. After the solvent front has ascended 10-12 cm, the plate is removed from the chamber and placed into a fume hood for a few minutes to allow the solvent to evaporate. Then the plate is examined under UV light and sprayed with a chromogenic reagent. Then R_f (ratio of the distances travelled by a zone and the mobile phase from the origin)) of the studied compound is measured, which should be identical to that of the standard. Other absorbing spots, that may be indicative of the pesticide metabolites formed in soil, are also marked. Evaluation of the approximate quantity of the toxicant is made by comparison of the areas of spots from the solutions with those of standard solutions.

EXAMPLES

1. Evaluation of Residual Ridomil

Extracts from soil samples concentrated by evaporation of the solvent are dissolved in 0.4 ml of acetone, and a 10–12 ml aliquot of the solution is applied to a plate. Standard solutions of ridomil are also applied to the same plate in quantities of 1–10 to 20–50 μg. The plate is developed in the solvent systems hexane:acetone (5:2) or benzene:diethyl ether (1:1). After drying, the plate is examined under UV light for the presence of absorption zones with R_f equal to that of ridomil. By comparing the areas of such zones with the standard ones, one can make an approximate evaluation of the ridomil content in the sample.

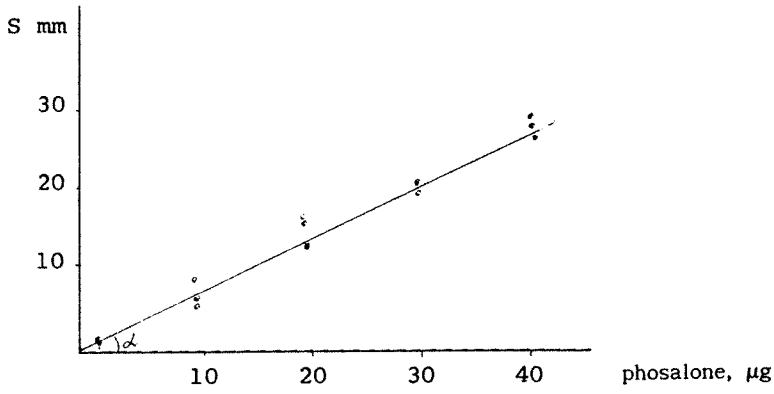
2. Evaluation of Residual Methoxychlor

The solvent systems hexane:acetone (6:1) and benzene:dioxane:acetic acid (45:5:1) are used for developing the methoxychlor spots. After examining the chromatogram in UV for methoxychlor and its metabolites, a specific and very sensitive to chloro-containing compounds $AgNO_3$-based reagent is used (0.1 g $AgNO_3$ dissolved in 1 ml of water and added with 10 ml of 2-phenoxyethanol, 190 ml acetone and one drop of 30% hydrogen peroxide solution). After thorough spraying with this reagent, the plate is placed against long-wave UV light for 10 min. The zones of chloro-containing compounds are revealed as grey spots against a lighter background. Quantitative and qualitative evaluation of methoxychlor in the sample is performed by comparing chromatographic mobility of the zones and by measuring areas of the coloured spots.

3. Evaluation of Residual Phosalone

Phosalone-containing samples and standard solutions are applied to a plate following the procedure described in Example 1. The plate is developed in the solvent systems carbon tetrachloride:ethanol (5:1) or benzene:acetone (5:1). The insecticide is revealed with $PdCl_2$ solution (0.5 g $PdCl_2$ in 50 ml of water added with 50 ml of acetone and two drops

of conc. HCl). Phosalone is observed as bright yellow zones against a grey background with R_f equal to 0.56 and 0.93 in the first and the second systems, respectively. Spot areas of the samples and those of standard solutions are measured and used for plotting a calibration graph as spot area versus phosalone concentration.



The phosalone concentration is calculated using the formula:

$$q = K \cdot \frac{SbV}{an \cdot 1000}$$

where q is the phosalone concentration in the sample, mg; S is the spot area, mm ; a is the volume of the aliquot applied to the plate, μl ; b is the total solution volume, ml; n is the soil sample weight taken for analysis, g; V is the total soil sample weight, g; $K = 1/tg\alpha$ (from the calibration graph).

4. Preparative Isolation of Pesticidal Metabolites

In addition to a residual pesticide, some of its metabolism products are often detected in the soil samples under study. They generally have R_f values different from that of the pesticide. When this is the case, the sample aliquot is applied to 1-2 plates, and standards of the pesticide metabolites are spotted nearby. The plates are chromatographed in the solvent systems of differing polarities. Comparison of their R_f values and methods of detection may provide preliminary information on the structure of metabolites in question. Some specific agents may prove to

be effective for detecting various functional groups, e.g. diazotized benzidine for phenolic groups, Fe (III) chloride/ferricyanide for amines etc.

Reliable identification of metabolites may be effected using physico-chemical methods such as chromato-mass- and mass-spectrometry, infra-red (IR), nuclear magnetic resonance (NMR), and proton magnetic resonance (PMR) spectroscopy. These methods are efficient for establishing the structure of a compound, but they require pure individual substances in quantities of 0.2-0.5 to a few milligrams. A preparative variation of TLC is well suited for the purpose. It is convenient to use high-performance precoated silica gel plates from Merck which have a thicker silica gel layer as compared to to Silufol plates, and they permit efficient separation and evaluation of greater sample quantities.

Silufol plate that will be used for quantitation purpose should be washed. To do this, it is placed in a chromatographic chamber filled with ethanol or the solvent system to be used. The solvent is allowed to ascend to the upper edge of the plate, before the plate is removed, and left for 2-3 hours for drying. Then the air-dried plate receives the whole of the sample applied as 'strips' across its breadth leaving just 1.5-2 cm margins. Standard solution or a mixture of standards are spotted on the margin at one edge of the plate. The plate is chromatographed. Then a narrow strip of the plate containing the standard compound spot and the edge-adjoining part of sample is cut off and treated with the detecting reagent. The location of the compound under study is marked with a pencil or a needle. The compound zone is then scraped off, or the plate is cut into small pieces, put into a flask and extracted with a polar solvent (ethanol, acetone etc.). The solvent is decanted after 30-60 min and the extraction repeated. The assembled eluates are filtered to remove the sorbent particles and is then concentrated. Then the minimum quantity of the concentrated solution is applied to a plate (with the standards) which is then chromatographed in a system of solvents with different polarity. If the isolated substance is a single compound, it is submitted for identification by physico-chemical methods. When some impurities are

present, chromatography is repeated in a system sensitive to their presence. In some cases such a purification should be repeated several times, but the sample must be dealt with carefully, since a portion of dissolved compounds - metabolites - may be lost in the course of elution. They are generally present in very low quantities, and therefore their subsequent identification may be rendered difficult.

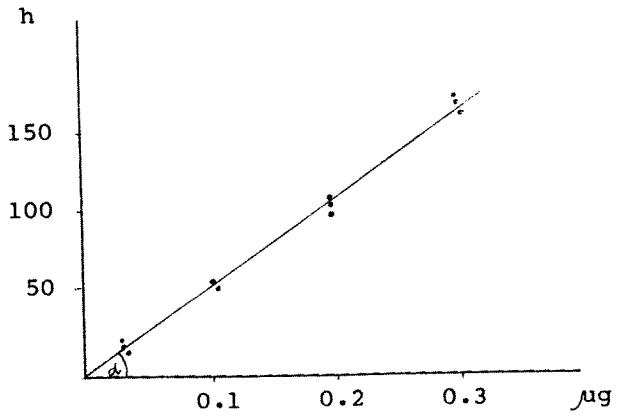
5. Determination of Residual Pesticides by Gas Chromatography

Gas chromatography (GC) is more sensitive, effective and precise compared to TLC and; therefore, is widely used for analyzing residual quantities of pesticides. But, unlike TLC, this method requires rather expensive equipment and necessitates a preliminary study of its principles (selection of sorbent, stationary phase, preparation and conditioning of columns etc.). A gas chromatographic installation consists of the following units: a cylinder containing the carrier gas is connected via pressure regulator with the gas pretreatment unit, in which the gas flow rate is measured, and with the input system. One end of the column, which is inside the column-oven, is connected to the gas input system (the injector), while the other - to the detector. The sample is injected with a microsyringe through a rubber seal in the injector port. The evaporated sample is transported by the carrier gas through the column. Sample components are selectively retained by the stationary phase, each being eluted after a specific retention time and recorded by the detector. Signals from the detector are amplified and recorded as peaks on the chromatogram.

a) Determination of residual ridomil by GC

Analysis is performed using the Pye Unicam GCD instrument with the flame-ionizing detector (FID). After conditioning the glass column (0.9 m long, 2 mm in inner diameter, packed with 1.5% OV-101 on Supelcoport 80-100 mesh), carrier gas is passed into the system at a flow rate of 50-60 ml/min. Then the chromatograph is switched on and the temperature set to 200°C for both the injector and detector, and 180°C for the column. Normal FID operation requires a hydrogen flow rate of 50-60 ml/min and an air supply. The total gas flow rate is 250-300 ml/min.

Then the detector is switched on and the chromatograph actuated; 1 ml sample, taken from the solution prepared as described in the TLC procedure, is introduced with a microsyringe. The detector responds to the appearance of the studied compound in carrier gas by recording a peak on the recorder chart. Identification of the peak is carried out by comparing the retention time of the sample to that of the standard. For ridomil, its magnitude is 2 min 50s. For quantitation of ridomil, a calibration graph should be plotted. Ridomil solutions of 0.05, 0.1, 0.25 and 0.5 μg/μl are prepared and introduced in the chromatograph. The height of the peaks or their areas ($S = h\mu_{0.5}$), where $\mu_{0.5}$ is the peak breadth multiplied by 0.5 height. Then the calibration graph is plotted as the peak height versus ridomil concentration.



The ridomil concentration is determined from the graph and its concentration in the sample is calculated from the following formula:

$$q = \frac{1}{\mathrm{tg}\alpha} \cdot \frac{hbv}{an \cdot 1000}$$

where h is the peak height; a is the injected volume, μl; b is the total solution volume, ml; n is the soil sample weight taken for analysis, g; v is the total weight of the soil sample, g; $\mathrm{tg}\alpha$ is the slope of the calibration curve.

The method allows determination of ridomil concentrations down to 10^{-8} g/l.

b) Determination of methoxychlor

The chloro-containing insecticide methoxychlor is determined chromatographically using the Pye Umicam 304 model with the electron-capture detector. The same column is used as in the ridomil determination (see 1).The column temperature is 200°C, those of detector and injector are 250 and 220°C, respectively. The carrier gas rate (high purity nitrogen) is 30 ml/min. Quantitative determination of methoxychlor is analogous to that of ridomil described above.

ISOLATION OF ACTIVE MICROBIAL STRAINS CAPABLE OF DEGRADING PERSISTENT POLLUTANTS

R.N. Pertsova

In recent times the environment has become the ultimate recipient of numerous toxic and persistent compounds and this flux is still increasing. More intensive degradation of xenobiotics has thus also become a necessity that brings to the forefront the task of searching for microbial strains capable of degrading recalcitrant foreign substances both in the laboratory and under natural conditions of

biocenoses.

The purpose of the present work is to make, using certain examples, a practical review of the basic methods employed for isolating microbial strains, which are active degraders of persistent pollutants.

This may be achieved by bringing about the following major steps: preparation of soil samples, isolation of active strains of soil microorganisms and assessment of their degradative ability.

In this study bromoxynil was selected as a model xenobiotic.

Bromoxynil or dibromocyanophenol is a foliar contact herbicide applied to rice and cereal fields for abating broad-leafed weeds. It is referred to the class of halogenated herbicides.

1. Sampling Technique

Soil samples are obtained by digging with a spade or a soil blade thoroughly cleaned and wiped with ethanol. Ten samples each of 0.5 kg are taken from the area of 100-200 m. The samples are thoroughly mixed in a sterile sack, then an average sample of about 1 kg is taken and placed into a sterile oil paper bag put into another bag of sacking. The latter is tied up firmly and in turn placed into a larger plastic bag with a label indicating location of the sampling site and details of the relief, vegetation pattern, agrotechnical background and soil type. The sample is stored in a refrigerating chamber. For training purposes, soil samples are taken from a test ground where the soil has been in a long-term contact with bromoxynil.

1.1. Sample Preparation for Analysis

A well mixed soil sample is spread over a dry glass surface wiped with a cloth wetted with alcohol and sterilized in the flame of a gas burner. The soil is again thoroughly mixed with a palette-knife and evenly distributed over the glass. Rootlets and other foreign objects are removed using tweezers. Small portions are taken from many different places on the soil surface to give a soil sample of 1 g upon weighing in a sterile porcelain dish previously tared.

2. Preparation of Nutrient Media

Nutrient media are needed for enrichment, isolation and subsequent preservation of microorganisms as well as for culturing microbial strains with the view of investigating their metabolic routes. The medium should include all components required by the cell for synthesis and energy purposes, i.e., sources of carbon and nitrogen, ash-contained elements, and microelements. As to the physical state, liquid and solid media are distinguished.

Liquid media are useful for elucidating physiological and biochemical peculiarities of microorganisms, biomass production, accumulation of metabolites etc.

Solid media are used for isolating pure cultures for diagnostic purposes, culture maintenance, bacterial counting and for a number of other purposes. To solidify, liquid media of specific composition are amended, prior to sterilization with 1.5% of agar.

The elemental requirements of various microbial groups are usually met with common sets of mineral salts.

In the present work two media are used: Eroshin's 8-E medium as the mineral one and a medium for culturing *Pseudomonas* strains; 50 mg of bromoxynil are added per 1 l of each medium.

Composition of Eroshin's 8-E mineral medium:

Mineral salt	Concentration, g/l
NaCl	0.5
$MgSO_4 \cdot 7H_2O$	0.8
KH_2PO_4	0.7
$(NH_4)_2HPO_4$	1.5

Phosphates are dissolved separately in 200 ml of distilled water and, upon cooling, the phosphate solution is mixed with the rest of the salts (800 ml).

Composition of *Pseudomonas* culture medium:

Mineral salt	Concentration, g/l
$(NH_4)_2SO_4$	4.0
KH_2PO_4	1.5
Na_2HPO_4	1.5
$MgSO_4 \cdot 7H_2O$	0.2
$FeSO_4 \cdot 7H_2O$	0.001
$CaCl_2 \cdot 2H_2O$	0.01
Yeast extract	0.003

The media thus prepared are poured out in portions of 100 ml into 750 ml flasks, plugged with cotton-gauze stoppers, wrapped with solid paper on which the composition and amount of medium are written, and sterilized for 30 min at 1 atm.

To prepare solid media, the melted agar-amended media, after sterilization, are supplemented with a bromoxynil solution to give a concentration of 50 mg per 1 litre of medium. The solution is prepared by mixing equimolar quantities of bromoxynil and KOH: 0.1 g of bromoxynil and 0.5 ml of 0.1M KOH are dissolved in 25 ml of water with heating and stirring, and diluted to 50 ml volume. Portions (~20 ml) of the melted agar medium containing bromoxynil are poured out into Petri dishes, and when the medium solidifies, the dishes are marked.

3. Isolation of Active Microbial Strains

3.1. Screening of Active Strains in Microbial Culture Collections

Following a review of the relevant literature, individual microbial species or genera are selected that are capable of specific conversions of xenobiotics. Strains of a particular species or genus are given preference compared to other cultures in microbial collections and are tested with regard to their ability to transform and utilize a toxicant as the sole source of carbon.

For the present work, such strains were selected from microbial cultures collected and maintained in the Laboratory of enzymatic degradation of organic compounds (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences).

Cultures of *Pseudomonas aeruginosa* 640x, *P. putida* 87, *P.aeruginosa*

BS827, *P. putida* 13xf, and *Alcaligenes faecalis* 192x are known to bring about dehalogenation of the aromatic ring in various xenobiotics. Such cultures may therefore be used for the purpose of screening. In preliminary assays these cultures are reinoculated from the slant agar layers, on which they are maintained on slants of beef-peptone agar (BPA), grown for 24 hours and tested as to their ability to utilize the xenobiotic under study as the sole carbon source on solid media (see Section 4.1) and on liquid media (Sections 4.2 and 4.3).

3.2. Isolation from Natural Sources

To isolate active microbial strains from natural sources, samples are taken from soil, water, sludge and sewage, preferably from those that have been in a prolonged contact with the xenobiotic in question or at least with one of its structural analogues. Inoculations from natural sources are made on the solid media containing the compound under study as the sole source of carbon.

In this work, samples to be tested are from the soil treated with bromoxynil (see Section 1). Soil samples undergo a special treatment for destroying soil aggregations and desorbing microbial cells from soil particles. From one of the two previously sterilized 250 ml flasks (one filled with 100 ml of tap water and the other kept empty) 0.4–0.8 ml of water are used to moisten 1 g of the soil in a porcelain dish to give a paste by triturating the soil with a finger covered in a rubber glove. The the paste-like soil is quantitatively transferred to an empty flask, using the whole volume of sterile water. The trituration and transfer of the soil are effected in the proximity of a gas burner flame. The soil suspension is agitated on a shaker for 5 min, left for 30 s to allow the gross particles to sediment, and immediately after it is used for subsequent dilutions, while remembering the previous 100-fold ($1:10^2$) dilution. For further dilutions, 9 ml portions of tap water are pipetted into dry test tubes, both tap water and utensils used have to have been sterilized. Then 1 ml of suspended matter under study is added into each test tube. After a thorough stirring, the 1000-fold ($1:10^3$) diluted suspension is ready. Inoculations are made from both dilutions. 0.05 ml of stirred suspension from the 100- or 1000-fold dilutions is added to

the agar surface in Petri dishes. The aliquot is spread with a palette over the medium surface. 4-6 parallel inoculations are carried out for each dilution.

Growth of microbial colonies is checked at 3, 5 and 7 days.

3.3. Enrichment Techniques

One of the distinctive features of microorganisms is their exceptionally high ability to adapt to changing environmental conditions. This character is put to good use for maintaining enriched cultures in microbial processes which have to occur under varying stresses of xenobiotics.

The enrichment cultures are mixed microbial populations grown in liquid media where the xenobiotic to be studied is used as the sole source of carbon or when it is amended with an additional, better digestible substrate.

Such cultures are serviced with microbial sources by natural systems (soil, water, sludge etc.) that have hitherto been in contact with xenobiotics structurally similar to the one under study.

Sugars, organic acids, hydrocarbons, aromatic compounds and other substances may be used as readily metabolizable substrates.

Under these conditions, the need for maintaining enrichment cultures compels microorganisms to adapt to higher, as compared to the initial, concentrations of xenobiotics.

In the present work, to maintain enrichment cultures, flasks with 100 ml of liquid medium (see Section 2) are sterilized, soil samples are taken (Section 1) and prepared for analysis (Section 1.1). Then a set of marked flasks receive each 1 g of soil sample and the amount of bromoxynil to give a final concentration of 50 mg/l, while the other set of flasks are added with bromoxynil (50 g/l) and amended with additional substrates (g/l): ribose, 2; sodium benzoate, 2; and glycerin, 2.

Culturing is carried out at 29°C: on a shaker, under aerobic conditions without stirring and under limited aeration.

The enrichment cultures maintained on a shaker are reinoculated on a 10-day basis during the first month, then -monthly, whereas the cultures without stirring are inoculated every month from the beginning.

During reinoculations, 10% (volume) of the starting enrichment culture is taken and compensated for by the corresponding additions of fresh medium.

In the enrichment cultures the concentration of the toxicant is controlled as well as the isolation of pure cultures on the agar-amended medium with the xenobiotic as sole source of carbon.

Determinations of bromoxynil and its metabolites are carried out following their extraction. The biomass intended for analysis is separated from the liquid culture by centrifugation and destroyed in the MSE disintegrator for 3 min. Bromoxynil and the metabolites are extracted separately from the culture liquid and cell homogenate. After acidification to a pH of 2 with 0.1M HCl, the extraction is made with an equal volume of chloroform, the chloroformic layer is separated by centrifuging for 20 min at 10°C and 5,000 *g*.

The solvent is evaporated in a rotor evaporator at 25°C. The residue obtained is subjected to either quantitative analysis by thin-layer chromatography (TLC) for the presence of bromoxynil and its metabolites or determined quantitatively by gas chromatography (GC). In the former case, Silufol UV-254 plates are used. Identification of the compounds in question is effected by comparison of the compound mobility with those of the standards: bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and its derivatives, (3,5-dibromo-4-hydroxybenzoamide and 3,5-dibromo-4-hydroxybenzoic acid). Comparison is made using 3 systems of solvents:

1. benzene:dioxane:acetic acid (90:15:4);
2. hexane:ethylacetate:acetic acid (90:10:0.1);
3. benzene:dioxane:acetic acid (90:10:2).

The chromatographic mobility of bromoxynil and its metabolites in the solvent systems used in this work are the following:

Compound	Rf values in solvent systems used		
	1	2	3
3,5-dibromo-4-hydroxybenzonitrile	0.8	0.15-0.2	0.65-0.7
3,5-dibromo-4-hydroxybenzamide	0.2	0	0.25-0.3
3,5-dibromo-4-hydroxybenzoic acid	0.45	0	0.5-0.55

Brominated compounds are detected with the following reagent:

silver nitrate	-	0.1 g
water	-	1.0 ml
acetone	-	190.0 ml
2-phenoxyethanol	-	10.0 ml
hydrogen peroxide	-	1 drop

After wetting with the reagent, the chromatographic plate was exposed for 15 min to the long-wave UV-rays. Bromine-containing compounds appear as grey spots against colourless background. The sensitivity of the reaction for bromoxynil and its derivatives is 0.5 μg per spot.

Phenolic hydroxyls are detected by spraying the plate with a solution of diazotized benzidine.

Identification of bromoxynil and its metabolites is achieved by spectrophotometry and chromato-mass-spectrometry which are discussed.

The quantitative determination of bromoxynil and its metabolites is carried out in the form of methyl esters by gas chromatography. Methylation is performed in methanol with ethyl ester solution of diazomethane. Analysis is conducted by a chromatograph with an electron capture detector in a glass 180x0.2 cm chromatographic column filled with Chlorosorb W/HP (OV-phase - 1.5%); carrier gas rate - 30 ml/min, column temperature - 200°C, those of injector and detector, 200°C and 220°C, respectively.

4. Assessment of the Degradation Capability of Microorganisms

While assessing the degradative abilities of microbes, one should bear in mind that xenobiotics may be transformed by microorganisms through different routes. These may be:

1) utilization of the toxicant as the sole carbon source, as, for example, is the case with 3-chlorobenzoic acid utilized by the strains *Pseudomonas* sp. B13 or *P. putida* 87;
2) use of a fragment of the molecule, i.e. bioconversion, observed during degradation of 2,4-dichlorophenoxyacetic acid, where microorganisms develop at the expense of the dicarbonic fragment of the molecule;
3) utilization of the xenobiotic molecule by microorganisms in the

presence of other, readily metabolizable, substrates (cometabolism).

4.1. Auxanography

The simplest and most rapid method for evaluating the degradative capabilities of microorganisms is the assessment of their ability to grow utilizing a xenobiotic on solid agar media. The method has been given the name of auxanography. To evaluate, using this approach, the microbial capacity for growth, a microbial culture is grown on a slant BPA or any other metabolizable substrate for 24 hours, one culture loop is thoroughly suspended in 1 ml of sterile tap water. Then the whole loop of the suspension is taken and streaked on a solid medium with the xenobiotic as the sole carbon source. In parallel, an inoculation is made on the control carbon source-free agar medium. The growth is controlled in 1, 3, 5 and 7 days. Special attention should be paid to the grade of reactants' purity, agar in particular: they must be organic matter-free, even in trace quantity.

The present work offers an assessment of the growth of cultures isolated from natural sources (Sec. 3.2) as well as those of enrichment cultures (Sec. 3.3) and microbial collection strains (Sec. 3.1).

4.2. Bioconversion

In the course of bioconversion, no radical changes occur in the xenobiotic molecule as the result of utilization by microorganisms of various aliphatic substituents in the aromatic ring or heterocycles. The product of bioconversion - a metabolite - may, like the parent molecule, also prove to be recalcitrant to a total degradation, as the microbial attack-resistant part of the molecule remains intact.

The present work exemplifies the above statement with the octyl ester of bromoxynil - bromoxynil-octanoate. Microbial cultures utilizing the C fragment of the bromoxynil molecule do not affect the aromatic ring of the compound and convert the octyl bromoxynil ester to bromoxynil.

An active microbial culture is inoculated into sterilized flasks with 100 ml of liquid mineral medium for culturing pseudomonads, grown

for 48 hours in aerobic conditions, extracted as described in Section 3.3 for enrichment cultures, and analyzed with TLC by comparing the extract with the standards: bromoxynil and bromoxynil octanoate.

4.3. Cometabolism

Under the term of cometabolism are included the processes based on a coupling of the metabolic routes of two substrates, the metabolism of one either stimulating or predestining that of the other. The process of cometabolism involves thus two substrates: the term of 'substrate' or 'target substrate' is attributed to the compound whose conversion is either the purpose or the problem whereas the compound, that provides for or stimulates the conversion, is assumed as a 'co-substrate'. Relations between the metabolic pathways of substrates, specifically xenobiotics, and those of co-substrates may differ essentially.

Under conditions of cometabolism, transformations are brought about by introducing two compounds: the target substrate - a xenobiotic, and the co-substrate - readily metabolizable compounds. Numerous substances belonging to different classes of chemical compounds (hydrocarbons, aromatic and aliphatic acids, alcohols, sugars etc.) are capable of serving the purpose of co-substrates.

The present work proposes a practical demonstration of the metabolic changes in bromoxynil effected by *P. putida* 13xf in the presence of ribose.

Flasks with 100 ml of liquid medium for culturing pseudomonads are sterilized, the culture *P.putida* 13 xf is grown in matrices with 2.0 g of benzoate for 24 hours, and a sterile 10% ribose solution is prepared as well as that of bromoxynil.

The above culture is washed off the matrix with sterile medium into a sterile flask. Under sterile conditions, the flask intended for culturing is filled with bromoxynil solution to have its concentration of 50 g per 1 litre of medium, ribose solution to give 2 g of ribose per 1 litre of medium, and the culture suspension. Cultivation is carried out on a shaker, in the dark, at 29°C. After 7 days, the biomass is centrifuged, cells are disintegrated for 3 min, residual bromoxynil and its metabolites are extracted from the cell homogenate and culture

liquid after acidification (pH 2) with 0.1M HCl, and qualitative and quantitative analyses are performed as described for enrichment cultures in Section 3.3.

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GENETICS OF BIODEGRADATION

T.V. Tsoy, V.V. Kochetkov, and R.N. Pertsova

I. Methods of D-plasmid Detection

In order to provide sufficient evidence in support of a plasmid character of genetic biodegradation systems in bacteria it is necessary to use the whole complex of biochemical, molecular-biological and genetic methods. Some of these are described below for the purpose of practical demonstration.

1. Method of Visualization of Plasmid DNAs

It is advisable to start a study of microbial strains capable of degrading xenobiotics with assays aimed at revealing the availability of plasmids. The procedure used in the present work is a modification of the Eckhardt's method (Eckhardt, 1978), used for screening bacteria of the *Pseudomonas* genus, that implies the following manipulations:

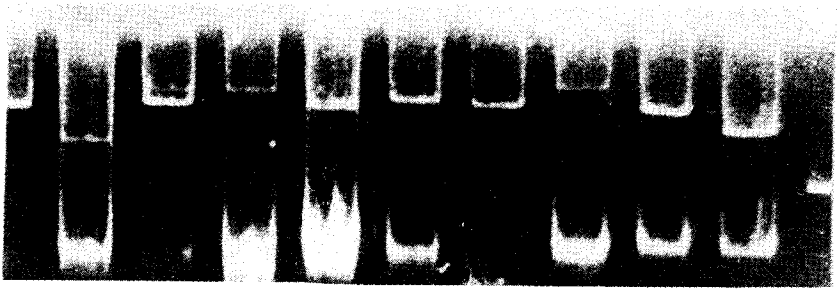
1. Material from a single bacterial colony is added to 5-10 ml of LB-medium and incubated overnight on a shaker at 30°C.
2. 1% agarose is prepared using the following procedure: 1 g of agarose Bio-Rad, 10 ml of 10 x TAE, dilute with distilled water to 100 ml.
3. Agarose is melted on a boiling water bath, the vertical electrophoresis unit is prepared for operation. Spacers and combs 3 mm. thick

are used. The number of wells is determined by the number of samples. All the volumes indicated below are calculated for the 10-well, 3mm thick comb. When a 20-well comb is used all the volumes are reduced by half.

4. Fill the electrophoresis unit with melted agarose cooled to 60-65°C, install the comb. In 2-3 min the upper chamber of the unit is filled with 1 x TAE (this will facilitate pulling the comb out later on) and the gel is allowed to polymerize for 45-60 min.
5. Transfer 100-400 µl of the 'overnight' culture into an Eppendorf tube and centrifuge for 1 min in the Eppendorf centrifuge.
6. Decant the supernatant, leaving the bacterial cell precipitate in the tube. Bacterial cells are resuspended in 400 µl of 20% ficoll prepared on 1 x TAE. The cells are thoroughly stirred in a mixer.
7. Prepare a lysis mixture. For this mix 150 ml of the base of the lysis mixture with 50 µl of lysozyme (1 µg/ml) and 5 µl of RNA-ase (1 mg/ml).
8. Pull the comb carefully out of the gel. Cautiously dry the wells using a Pasteur pipette or a peristaltic pump.
9. Add into the well 10 µl of lysis mixture prepared as indicated in 7.
10. Bring in 10 µl of cell suspension in 20% ficoll (see 6). Mix the cells carefully with the lysis mixture directly in the well.
11. Add 30 µl of SDS-mixture.
12. Spread carefully 100 µl of the covering mixture to form a covering layer.
13. The well is carefully closed with 1% agarose (50°C), 1xTAE is poured into the upper and lower chambers of the unit.
14. Perform electrophoresis for 30 min at a voltage of 20 V, followed by 2-2.5 hour electrophoresis at 100-120 V. Upon termination of electrophoresis the bromophenol blue should be practically withdrawn from the gel.
15. Colour the gel in a solution of ethidium bromide (0.5-1.0 µg/ml) for 10-15 min, thereafter wash the gel off with distilled water.
16. Photograph the gel in reflected or transmitted UV-light of 240-260 nm wave-length using the red-orange filter.

Example. Below is reproduced an electrophoregram of the naphthalene degradative plasmids from the strains of *P. putida* and *P. fluorescens*

obtained using the above procedure.



Note. a) The above conditions do not always provide for a good lysis of bacterial cells. If this happens, select lysis conditions by increasing concentrations of SDS and lysozyme. Various modifications of the alkaline lysis may also be used (Maniatis *et al.*, 1982).

b) Composition of LB medium and the procedure for its preparation as well as for TAE buffer, components of lysis mixture, SDS mixture for the covering mixture are described in the Appendix.

2. Elimination of D-plasmids

Like the method of conjugational transfer, this technique enables establishment of a plasmid character of genetic biodegradative systems in bacteria. The present work makes use of a modification of the Rheinwald method (Rheinwald *et al.*, 1973).

1. The culture under study is grown in the LB medium to obtain a density of 10^8 cells/ml.
2. A set of test tubes are filled with the LB medium, each subsequent tube containing double the amount of mitomycin C compared to the previous one (1, 2, 4, 8, 16 μg/l etc).
3. The studied culture is introduced into the above test tubes to reach a density of 10^4 cl/ml. The same cell density is created in the control, mitomycin C-free test tube.
4. The culture is grown on a shaker for 36-48 hours in the dark, as mitomycin C is light-sensitive.
5. Choose the test tube with a subinhibition concentration of

mitomycin C. If the culture proves to be resistant to all mitomycin concentrations selected for the experiment, repeat everything from the beginning with increased mitomycin C concentrations.

6. From the test tube with subinhibiting concentration of mitomycin C, perform 4 standard 10-fold dilutions with 0.85 % NaCl solution. Inoculate 0.1 ml from each (0,1,2,3,4) dilution on Petri dishes with LB agar. Dilutions are stored at 4°C.
7. After incubation select for subsequent analysis the dishes with sufficient number of well isolated bacterial colonies. If necessary, inoculate additional dishes from suitable dilutions stored at 4°C.
8. Using sterile needles or tooth-picks print several hundreds of colonies on dishes with synthetic medium B + corresponding carbon source and on dishes with medium B + glucose.
9. After incubation select the imprints that do not grow on the first medium but develop on medium B + glucose, clean them to obtain individual colonies and check them once more on the above media.
10. The eliminates thus prepared are checked for the presence of plasmid DNA in order to eliminate the possibility of selection of plasmid mutants.

Note. a) For low frequency of elimination which requires the check-up of a large number of colonies, it is advisable to use the replica method (Clowes and Hayes, 1968).

b) Composition of the medium and procedure for its preparation is described in the Appendix.

3. Conjugational Transfer of D-plasmids

Most D-plasmids known to date are of conjugative origin. This fact allows the use of the conjugation method for the detection of such plasmids. For this purpose various modifications of the Dann and Gunsalus method are usually employed (Dann and Gunsalus, 1973).

1. Mix the donor and recipient cultures in the volume ratio 1:10 (or 1:1) and incubate the conjugational mixture in standard conditions for 1-2 h at 30°C. Use the donor and recipient cultures with a density of 10^8 cells/ml.
2. Inoculate 0.1 ml of the conjugation mixture (or its corresponding

dilutions) on the elective agarized medium B with the corresponding carbon source. The donor and the recipient are also inoculated on the same medium for control.

3. Incubate dishes at 30°C for 2 to 5 days depending on the rate of transconjugants' growth.
4. The transconjugants obtained are cleaned in the same elective medium and checked on the recipient markers for the presence of plasmid DNA. The frequency of conjugational transfer is calculated as the ratio of the number of transconjugants and that of donor cells in 1 ml of conjugational mixture.

Note. In some cases the conjugational transfer is carried out on the LB agar by mixing in Petri dishes the donor and the recipient, 0.1 ml of each. After incubation for 2-4 (sometimes 6-8) h, the cells are washed off with 0.85% NaCl solution, and inoculated on the corresponding elective media. When wild-type strains are used as donors, the recipients may be plasmid-free, preferably the auxotrophic strains *P. putida*, *P. fluorescens* and *P. aeruginosa* strains resistant to rifampicin and streptomycin.

Appendix

1. Medium LB

For 1 litre:	- tryptone	10 g
	- yeast extract	5 g
	- sodium chloride	10 g

Sterilization by autoclaving.

2. Tris-acetate buffer (TAE)

Concentrated 10xTAE

For 1 litre:	- tris	48.4 g
	- acetic acid	11.4 ml
	- 0.5M EDTA, pH 8.0	20.0 ml

Working solution 1 x TAE

0.04M tris-acetate
0.002M EDTA

Concentrated 10xTAE is sterilized by autoclaving and stored at 4°C.

3. Base of the lysis mixture

For 10 ml:	-bromophenol blue	5 mg
	- ficoll	2.5 g
	- 10 x TAE	1 ml

Dilute with distilled water to volume of 10 ml and store at room temperature.

4. Lysozyme

Aqueous solution of lysozyme (concentration 1 mg/ml) is stored as aliquots at -20°C. To be used directly for preparing the lysis mixture.

5. RNA-ase

It is prepared on 0.4M acetate buffer (pH 4.0) in concentration of 1 mg/ml. To be heated for 2 min at 98°C, and stored at -20°C.

6. SDS-mixture

For 10 ml:	- SDS	20 mg
	- ficoll	1 g
	- 10 x TAE	1 ml

Dilute with distilled water to 10 ml. and store at room temperature.

7. Covering mixture

For 10 ml.	- SDS	20 mg
	- ficoll	500 mg
	- 10xTAE	1 ml

Dilute with distilled water to 10 ml. and store at room temperature.

8. Synthetic medium B

For 1 litre:	- KH_2PO_4	1 g
	- K_2HPO_4	10 g
	- $NaNO_3$	3 g
	- $FeSO_4 \cdot 7H_2O$	0.05 g
	- $MgSO_4 \cdot 7H_2O$	0.05 g
	- $CaCl_2 \cdot 6H_2O$	0.05 g
	- $CuSO_4 \cdot 5H_2O$	0.008 g
	- H_3BO_3	0.004 g
	- $ZnSO_4 \cdot H_2O$	0.02 g
	- $MnSO_4 \cdot H_2O$	0.03 g
	- $Na_2MoO_4 \cdot 2H_2O$	0.003 g
	- $NiCl_2 \cdot 6H_2O$	0.0005 g
	- agar	15 g

The following solutions must be prepared separately and sterilized by autoclaving:

1) phosphates and sodium nitrate;
2) ferrous sulphate;
3) all other microelements.

All the components are to be mixed immediately before pouring out the medium and the carbon sources are to be added to the medium in concentration 1-2 g/l.

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II. Methods of Plasmid DNA Analysis

Modern analysis of the genetic systems of biodegradation is hardly feasible without using some techniques of molecular biology and genetic engineering. Of these, the basic methods are the isolation of DNAs, restriction analysis and DNA mapping, hybridization of nucleic acids on solid supports, molecular cloning of DNA.

While investigating the structural and functional organization of the biodegradation plasmids, it is important to combine in vitro analysis with in vivo functional data. Thus, the D-plasmid genetic mapping is not confined to the physical DNA mapping; it relies essentially on the results of restriction and mutation analyses and may make use of a comparative analysis with functionally apparented plasmids that have already been characterized using, for instance, DNA-DNA hybridization. A more detailed study of the structure and functions of plasmid DNAs calls for methods of transposon mutagenesis, gene cloning and analysis of their organization and expression, complementary analysis of the functions of plasmids and their various mutants as well as that of cloned genes.

For the first-hand view of the methodology of plasmid DNA analysis, three practical exercises including the most frequent techniques are proposed below.

1. Isolation of Plasmid DNA

A large number of different methods are in current use for isolation and purification of plasmid DNAs depending on the particular research tasks. Preferences given to specific techniques depend on such factors as the size of the plasmid, its replication capacity etc. Methods widely used for the fast isolation of plasmid DNAs are suitable for restriction mapping, subcloning of the DNA fragments in plasmid vectors, hybridization experiments, determination of nucleotide sequences etc. Of these, the most universal is the method of alkaline lysis of small volumes of bacterial cultures (Birnboim and Doly, 1979; Maniatis *et al.*, 1982). An account is given below for isolating plasmid DNAs from small volumes of bacterial cultures by the alkaline lysis. This procedure may

be efficiently employed for isolating plasmids of different sizes (up to 150 Kb) from many Gram-negative bacteria.

1. Introduce one bacterial colony in 5 ml of a medium containing, if required, an antibiotic. Incubate under intensive shaking overnight at the appropriate temperature (30, 37, 42°C etc.).
2. Precipitate cells by centrifugation in glass or plastic 10-ml tubes at 4,000 *g* at 0 to 5°C).
3. Remove the supernatant very carefully using a pipette.
4. Resuspend the centrifuged cells by shaking in 300 µl of the ice-cooled solution containing 50 mM of glucose, 10 mM EDTA Na, 25 mM Tris HCl, pH 8.0, and 2-4 mg/ml of lysozyme, the latter to be added immediately before use.
5. Incubate for 5 min at room temperature.
6. Add 600 µl of the fresh, ice-cooled NaOH 0.2M solution containing 1% SDS (sodium dodecylsulphate). The tube is stoppered with parafilm and its contents are well mixed by turning the tube upside down 2 to 5 times (without shaking!). Place the test tube in ice for 5 min.
7. Add 450 µl of ice-cooled solution of potassium acetate (pH 4.8) prepared in the following way: 60 ml of potassium acetate 5M solution are added with 11.5 ml of glacial acetic acid and 28.5 ml of water thus making the solution 3M in potassium and 5 M in acetate. The tube is stoppered with parafilm, carefully turned upside down several times during 10 s and left to cool in ice for 5 min.
8. Centrifuge for 20 min at 0°C (5,000 *g*).
9. Transfer the supernatant into a clean glass or plastic test tube.
10. Add 1.35 ml of phenol saturated with 0.1M Tris HCl, pH 8.0. The tube is stoppered with parafilm and shaken. After 5 min centrifugation (0°C, 4,000 *g*), the upper phase is transferred with a pipette to another test tube.
11. Add 2.7 ml of ethanol at room temperature.
12. Centrifuge for 15 min (0°C; 5,000 *g*).
13. Remove the supernatant with a pipette. Leave the overturned tube for some time on a paper towel to let the remaining liquid trickle down.
14. Add 1 ml of 70% ethanol, shake the test tube and transfer its content with a pipette into an Eppendorf tube for subsequent

centrifuging in the Eppendorf centrifuge for 5 min.

15. Remove the supernatant and repeat washing with 70% ethanol.
16. Remove again the supernatant, add 1 ml of 96% ethanol and let the tube stand for 20–30 min at room temperature. Repeat centrifuging.
17. Remove the supernatant. Dry the precipitate for 5–10 min in a vacuum concentrator (can be replaced by air-drying for 30–40 min).
18. Dissolve the precipitate in 150–200 μl of TE buffer (10 mM Tris HCl, pH 8.0, 0.5 mM EDTA Na) containing the pancreatic RNA-ase (50 μg/ml) free of DNA-ase admixtures and shake it fast.
19. An aliquot of 5 μl of DNA is mixed with 1 μl of electrophoresis dying agent composed of 60% glycerin, 0.01% bromophenol blue and 0.1% xylene cyanol.
20. Introduce the samples into the horizontal electrophoresis gel (0.8% agarose in the electrophoresis buffer containing 90 mM of Tris base, 90 mM of boric acid, 2 ml of EDTA, pH 8.3).
21. Electrophoresis is conducted at the electric field voltage of 80–100 V. Upon electrophoresis termination, dye the gel in the 0.5 μg/ml ethidium bromide water solution for 10–15 min. Wash the gel in water and visualize it in UV-light. The DNA concentration may be determined by comparing the control DNA samples of known concentrations measured spectrophotometrically.

2.Restriction Analysis of Plasmid DNA

Restriction endonucleases (restrictases) are the enzymes that recognize specific sequences (restriction sites) in two-stranded DNA and cleave the DNA molecule on such sites. Modern methods of molecular gene cloning have been developed as a result of the discovery of these enzymes (and extrachromosomal elements of heredity - plasmids and phages). Since in every specific DNA molecule each restrictase can recognize only certain sites (Maniatis *et al.*, 1982), in the simplest cases it is possible to draft the physical map (i.e., to establish the reciprocal location of the recognition sites for various restrictases and spacings between them) using individual and common treatments of plasmid DNA with various restrictases followed by electrophoretic separation of the DNA fragments produced in agarose or polyacrylic gel.

In more complex situations, when one has to deal with large plasmids, as in the case of biodegradation plasmids, that have a large number of recognition sites for the most commonly used restrictases, additional mapping techniques are called for. This may be a comparative restriction analysis of deletion variants of a plasmid that have lost part of the DNA sequence, restriction analysis of an individual plasmid DNA fragments isolated from the gel or cloned on a cloning vector with the known restriction map, etc. Thus, for example, the physical map was established for the nah region of the naphthalene biodegradation plasmid NPL-1 containing genes that encode the naphthalene degradation enzymes (Tsoy *et al.*, 1986; Boronin, 1987).

With the purpose of first-hand acquaintance with the principles of the restriction analysis, the following practical exercise is proposed.

1. An Eppendorf test tube is filled 15 ml of sterile and deionized water, 2 μl of 10-fold restriction buffer in order to have the final volume of restriction mixture composed of 50 mM of Tris HCl, pH 7.5, 100 mM of NaCl, 10 mM of $MgCl_2$, 1 mM of dithiothreitol, 100 μg/ml of bovine serum albumin. Add 0.5 μg of DNA of the known plasmid pBR322 in volume of 2 μl in TE buffer (see Section 1). Add 1 ml (containing no less than 3 activity units) of restrictase EcoRI.
2. In a second test tube the same components are introduced except for EcoRI being replaced by the restrictase SalI.
3. In a third test tube the same components are introduced but using the restrictase PstI.
4. The fourth test tube is with the same components but using jointly two restrictases EcoRI and SalI.
5. The fifth test tube contains the same components but using jointly EcoRI and PstI.
6. The sixth test tube is with the same components but using SalI and PstI.
7. All test tubes are incubated for one hour at 37°C.
   The reaction is arrested by heating the tubes for 10 min at 65°C.
8. Add to each test tube 4 μl of electrophoresis dye (see Section 1, step 19) and introduce the samples into the wells of electrophoresis gel (see Section 1, step 20).

9. Electrophoresis is conducted in a 60-100 V regime until the bromophenol blue dye migrates to the 4/5 of the gel length.
10. The gel is dyed and visualized in UV-light as described in Section 1, step 21).
11. As molecular size markers, use the standard mixture of HindIII DNA fragments of the phage brought onto the gel in parallel with the restriction samples. Determine, based on the standard fragment sizes, the size of DNA fragments produced as the result of the pBR322 DNA treatment with the restrictases EcoRI, SalI, PstI, EcoRI+SalI, EcoRI+PstI, PstI+SalI by comparing values of the run length for the fragments produced with those for the standard HindIII - phage fragments. The size of the fragments is calculated based on the fact that molecules of the linear two-stranded DNA move in the gel with the rate inversely proportional to the decimal logarithm of their molecular mass
(Maniatis *et al.*, 1982).
12. Based on the standard fragment sizes (λ-HindIII) plot the standard curve on the semilogarithmic scale. Use the curve for calculating the fragment sizes formed based on their run length in each sample. Using the size and the number of fragments produced as the result of single and double hydrolyses brought about by restrictases make a circular map for pBR322 and compare it with that described by Maniatis *et al.* (1982). To establish the map, use the trial and error method as well as logical reasoning (Maniatis *et al.*, 1982). If the comparison of the drafted map with that known for pBR322 reveals some discrepancy search for an error source (incomplete hydrolysis by one of the enzymes may be the most probable case) and repeat the experiment.

3. Hybridization Analysis of Plasmid DNAs

Various modes of DNA-DNA hybridization are an efficient tool for localizing specific sequences in the DNA molecule. Of these, the most widely used is the method of blotting-hybridization of restriction DNA fragments transferred on the nitrocellulose filter or other solid supports with the subsequent incubation in the hybridization medium containing the radioactive DNA-probe. Initially the method was developed

by Southern (1975), other variations followed soon afterwards (Rigby *et al.*,1977; Meinkoth and Wahl, 1984).

The blotting-hybridization technique is widely used in the genetic studies of biodegradation, specifically for elucidating evolutionary links among various D-plasmids as well as for localizing biodegradation genes and other functions using as radioactive probes the DNA fragments known to contain the required genes. This may be exemplified by the analysis of the structural and functional organization of the plasmid pBS286 when the hybridization of P-DNA probes of the NAH7 plasmid restriction fragments containing various nah-genes was used for making the genetic map of the pBS286 nah-region (Tsoy *et al.*, 1986).

For the first-hand view of the possibilities offered by the blotting-hybridization technique, the following practical exercise is proposed.

The bidirectional DNA transfer from the agarose gel on nitrocellulose filters is carried out based on the procedure described by Meinkoth and Wahl (1984).

1. Portions of 3 μg of the p286 plasmid DNA are treated with the restrictases EcoRI, BamHI, XhoI and subjected to electrophoresis as described in Section 2.
2. After electrophoresis and the DNA dying with ethidium bromide the gel is photographed. For an easier analysis of the results, a ruler is put alongside the gel before photographing. For further manipulations, rubber gloves should be used.
3. All needless parts of the gel should be cut off with a scalpel and removed.
4. The gel is placed in 0.25M HCl for 10-15 min (all treatment steps are performed at room temperature).
5. Carefully decant the acid and cover the gel with the denaturation solution of 0.5M NaOH and 1.5M NaCl. Keep the gel in the solution for some time with a slight shaking.
6. Remove the denaturating solution, rinse the gel with distilled water and neutralize it by adding 0.5M Tris-HCl (pH 7.0) and 1.5M NaCl. Neutralization is carried out twice for 15 min each.
7. Cut out sheets of filter paper the size of 0.5 cm larger than that

of gel. Prepare 2 piles of the filter paper sheets 2-3 cm high.

8. Cut out 4 sheets of Whatman 3 MM paper of the size 0.5 cm larger than that of gel and 2 sheets of of nitrocellulose paper (BA 85, 0.45 μm, "Schleicher & Schuell") precisely the size of the gel. Put them into the 20 x SSC solution (1 SSC contains 0.15M NaCl and 0.03 M sodium citrate).
9. Put one pile of filter paper on a glass surface. Top it with one sheet of Whatman 3MM soaked with the 20 x SSC. Cover it with a nitrocellulose filter also wetted in the 20 x SSC solution. Carefully top the filter with the gel and cover it with the second sheet of nitrocellulose filter. Pay attention there are no air bubbles between the sheets of gel and nitrocellulose. Put on the top 3 Whatman sheets and a pile of filter paper. Cover the whole pile with a piece of polyethylene topped with a glass and a 0.5 kg weight.
10. The transfer is brought about for 1-2 h at room temperature.

After the DNA transfer, the filters are washed for 10-15 min in the 2 x SSC solution, air-dried first, then dried in a vacuum oven for 1-3 h at 80°C. Thereafter the filters are ready for further use.

Nick-translation of DNA is carried following the procedure described by Rigby *et al.* (1977) in compliance with the recommendations formulated by Meinkoth and Wahl (1984).

1. 10-20 μCu α-^{34}P-deoxycytidine triphosphate (NPO "Izotop", USSR) are introduced into an Eppendorf test tube and dried in a vacuum concentrator.
2. Add into the test tube with the dried radiomarker, 13 μl of sterile and deionized water, 4 μl of buffer composed of 50 mM of Tris-HCl (pH 7.5), 6 mM of $MgCl_2$, 10 μg/ml of gelatin, 20 μM of desoxythymidine phosphate. Introduce 1 μl of DNA of the recombinant plasmid carrying the cloned gene nahC from the plasmid pBS286 (Tsoy *et al.*, 1988).
3. Add 0.05-0.1 units of DNA-ase I *E. coli* activity in 1 μl, let the tube stand for 2 min at room temperature and introduce 0.5-1 unit of DNA polymerase I *E.coli* activity in the 1 μl volume. Incubate the reaction mixture for 1-3 h at 12-14°C.
4. Stop the reaction by adding 2 μl of 1M NaOH and 2 μl of 2% SDS, 250 mM of EDTA.

5. Prepare a column packed with Sephadex G50. For this, a sterile Pasteur pipette with an elongated tip packed with glass wool is packed with Sephadex previously soaked in the elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM of EDTA Na , 150 mM NaCl, 0.05% SDS). Wash the column in 3-10 ml of elution buffer.
6. Introduce a sample and carry out the elution with the above buffer. Collect the 250 µl fractions in previously prepared Eppendorf test tubes (the first fraction remains in the column volume, 1 ml). Collect 12-15 fractions.
7. After calculating the incorporated radioactivity with Cherenkov's method (using the MarkII counter with the open canal), the radioactive fractions are assembled.

DNA-DNA hybridization

1. A dried nitrocellulose filter with immobilized DNA is placed in the prehybridization medium composed of 5 x SSC 2 x Denhardt solution (6 x SSC, 0.2% ficoll, 0.02% polyvinylpyrrolidol, 0.02% bovine serum albumin). Incubate for 0.5-1.0 h at 65°C in a glass crystallization dish.
2. Measure the volume of P-DNA fraction and denaturate DNA by adding 1M NaOH to obtain a final concentration of 0.1 M. Now the radiomarker is ready for further use.
3. Prepare the hybridization medium. Introduce into a glass or polypropylene test tube 600 µl of 20 x SSC, 60 µl of 100 x Denhardt, 100 µl of the denaturated calf thymus DNA (6 g/ml), 3-5 10 cpm of P-DNA, dilute with water to 3 ml.
4. Take the filter out of the prehybridization medium blot it with a paper towel, insert into a polyethylene bag, pour some hybridization medium into the bag and seal it. Incubate for 12-14 h at 65°C.
5. Open the bag, pull the filter carefully out and make three one-hour washings with 2 x SSC and 0.1% SDS.
6. Wash the filter 3 times for 15 min with 2 x SSC.
7. Air-dry the filter and expose it on the PM-1 or PT-1 film at -70°C.
8. Analyze the autograph by comparing, in matching scales, its spots to the restrictional fragments on the gel photograph after electrophoresis. Determine the restriction pBS286 fragments which hybridize with

the recombinant pBS955 plasmid and, based on the map of the pBS286 nah-region, localize the cloned nahC gene in the pBS955.

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III. Survival, Growth and Stability of Plasmid-Carrying Strains under Natural Conditions

The cleanup of the environment from persistent pesticides has become an urgent problem. One of the perspective directions in modern ecological biotechnology is the genetic engineering of strains endowed with enhanced capabilities of degrading persistent pollutants. In a number of laboratories strains have already been constructed that are capable of degrading chemicals previously considered as resistant to microbial attack. These strains harbour, as a rule, plasmids that are involved in the biodegradation.

After successful laboratory assays active microbial strains may be tested for their ability to degrade pollutants under environmental conditions.

The purpose of the present work is to provide the first-hand acquaintance with the techniques used to study survival and resistance of genetically constructed strains, and the stability of plasmids under natural conditions.

Basic steps of this work comprise:

- preparation of a soil ecosystem;
- preparation of the genetically constructed strain *Pseudomonas aeruginosa* BS827 to be used as inoculation material;
- release of *P. aeruginosa* BS827 in the soil ecosystem;
- introduction of kelthane in the soil;
- sampling technique;
- assessment of the capacity of *P. aeruginosa* BS827 to survive in the soil;
- assessment of the plasmid pBS3 stability in the strain *P. aeruginosa* BS827.
- analysis of the kelthane residue in the soil.

Preparation of a Soil Ecosystem

Degradation studies are conducted in natural soil ecosystems which are the 1.5 x 1.5 m soil plots bordered with metallic or plastic sheets

driven into the soil 50 cm deep. The grass cover of the soil is removed, the soil is tilled 10 cm deep. Three soil lots or 'lysimeters' are prepared: one for experimentation and two for control.

Preparation of the Inoculum

The strain *P. aeruginosa* BS827 used as inoculation material was constructed on the basis of the plasmid-free strain *P. aeruginosa* 640x into which the plasmid of naphthalene and salicylate degradation pBS3 had been transferred as endowing the strain with the ability to grow on naphthalene (Nah^+ character) and salicylate (Sal^+ character). For the purpose of genetic labelling, a rifampicin-resistant (rif^r) variant of this strain had been obtained with a mutation in the chromosomal gene (Golovleva *et al.*, 1982).

The strain intended for inoculation is grown in the beef-peptone broth (BPB) with rifampicin at final concentration of 100 g/l. The stock solution of rifampicin is prepared by dissolving 100 mg of rifampicin in 10 ml of 0.1M KOH. To obtain the above rifampicin concentration, 99 ml of BPB are amended with 1 ml of the stock rifampicin solution. For inoculating, the strain is washed out from a BPA slant with 3-5 ml of BPB, the cell suspension is transferred into a flask, cultured for 18 h, centrifuged for 15 min at 5,000 g, and washed with 0.03 M phosphate buffer (pH 7.5). The washed cells are suspended in water to give the optical density of 0.17 corresponding to 1 10 cells per one ml.

Release of *P. aeruginosa* BS827 in a Soil Ecosystem

The saprophytic soil microflora are counted prior to the release of the strain, then the constructed microorganism is inoculated in the quantity equal to the microbial numbers of the soil microflora, i.e. some $1 \cdot 10^6$ to $1 \cdot 10^7$ cells per 1 g of soil.

The strain is inoculated into the surface soil layer of 0-3 cm. The uniformity of microbial distribution is reached by thorough mixing of the soil.

The total amount of cells to be released depends on the surface area of the ecosystem and the abundance of microbial flora.

Here is an example for calculating the total numbers of the

released microorganism: the total volume of soil, into which the strain is inoculated, makes up 150 cm x 150 cm x 3 cm = $67.5 \cdot 10^3$ cm^3; its specific density is 1.5 g/cm^3. Hence the total soil mass is 101,250 g. The abundance of the soil microflora is $5 \cdot 10^{11}$ cells/g soil, 101,250 g of soil thus contain $5 \cdot 10^{11}$ cells. Consequently, $5 \cdot 10^{11}$ cells of the strain are required for inoculation.

The cell suspension contains after dilution $1 \cdot 10^9$ cells/ml. To have the total microbial population of $5 \cdot 10^{11}$ cells/ml, one should take 500 ml of such suspension. The experimental lysimeter is divided into 4 equal rectangular parts, and 500 ml of suspension diluted with 1.5 l of water are brought evenly into the soil using a watering pot. The soil is mixed with a glass spatula to a depth of 3 cm.

Introduction of Kelthane in the Soil

Kelthane is introduced in the quantity of 50 doses usually supplied in agricultural practice, i.e. 50 mg of active substance per 1 kg of soil (concentration is calculated for the active compound in the kelthane formulation). It is introduced into soil as emulsion diluted with 2 l of water and uniformly sprayed on the soil surface. The soil is thoroughly mixed to the depth of 3 cm.

Two other lysimeters are used for control: one for observing the strain in the kelthane-free soil and the other for control of residual kelthane in the absence of the strain. The first one received the same amount of microbial suspension while the other only the emulsion of the pesticide. Tap water is added in both controls instead of microbial suspension and xenobiotic emulsion.

Sampling Technique

Soil samples are taken immediately after the release of microbial strain in the soil and in 1, 3, 7, 14 etc. days since the inoculation day. Samples of 100 g intended for microbiological analysis are taken observing sterile conditions from 10 different points of the soil surface horizon to the 5 cm depth. The samples are mixed, an average sample is taken, prepared for analysis, diluted 10-fold 6 times in succession (1:10) and inoculated on the appropriate media.

Assessment of the Capacity of *P. aeruginosa* BS827 to Survive in Soil

Microbial counting for the strain *P. aeruginosa* BS827 is carried out by inoculating it on BPA with rifampicin (100 μg/ml) which is prepared by amending 500 ml of melted BPA with 5 ml of stock rifampicin solution of 10 mg/ml. The melted medium is poured in sterile Petri dishes which are kept on a horizontal surface till the agar sets. The dishes with solid BPA medium with rifampicin are inoculated with 0.05 ml of the thoroughly mixed suspension (10^{-4}– 10^{-5} dilution, 5 parallel). The suspension is spread over the medium surface with a sterile spatula. The dishes are kept at 29°C. The cell growth is controlled in 1-3 days. After cultivation, the developed microbial colonies are counted and the most probable number of microorganisms is calculated for 1 g of soil at $P_{0.95}$ (confidence interval). All results of microbial counting are tabulated as shown below. Confidence intervals obtained by recalculating data on different dilutions are compared for drawing a conclusion on the reliability of results obtained.

Table form: Number of the colony forming units (CFU) on BPA with rifampicin and calculation of the most probable quantity of microorganisms per 1 g of soil.

Dilution	Number of experimental runs	Number of CFU on each dish	$\sum x$	$\bar{x}$	$\sigma\bar{x}$	Most probable number of cells per 1 g dry soil at $P_{0.95}$

It should be kept in mind that the accuracy of the method depends on the number of counted colonies and not on the experiment repetitions. The best dilution is that where the inoculation on a solid nutrient medium provided for a growth of 50 to 100 CFU.

The most probable number of microorganisms contained in 1 g of dry soil at the reliability level of 95% ($P_{0.95}$) is calculated using the formula:

$$N = (\bar{X} \pm 2\sigma x)K\frac{1}{V}$$

where $\bar{X} = \Sigma x/n$ is the average number of CFU after inoculation from individual dilutions; $\sigma x = \pm(\Sigma x)^{1/2}/n$ is the mean squared deviation; K

is the dilution used for inoculation, ml; V is the volume of suspension taken for inoculation, ml; Σx is the total number of counted CFU for specific dilution; n is the number of parallel experimental runs;

Assessment of the Plasmid pBS3 Stability in *P. aeruginosa* BS827

The number of colonies grown on BPA with rifampicin is used the for determination of bacterial numbers for the strain BS827 in soil. The growth on the medium with rifampicin does not reveal the availability of the plasmid in the studied strain. Detection of the plasmid pBS3 or its deletion variants calls for additional inoculations of the grown colonies on selective media. For subsequent inoculation, the dishes with developed colonies are thoroughly examined. The most appropriate dilution is determined and no less than 100 colonies resulting from this dilution are checked for their ability to grow on naphthalene and salicylate.

The medium with salicylate is prepared by treating 500 ml of melted mineral agarized medium with 5 ml of 10% sodium salicylate solution.

For microbial growth on naphthalene, Petri dishes are filled with a mineral agarized medium free of the carbon source. Naphthalene is applied in the form of crystals to the inner face of the Petri dish lid.

For inoculation, each of the 100 selected colonies is suspended in sterile 0.5M phosphate buffer (pH 7.0) and transferred, using a replicator, onto three dishes with: naphthalene, salicylate and BPA. During inoculation every dish is labelled on the outer bottom face. The inoculated dishes are cultivated for 24-48 h.

After 48 h the dishes are examined. The availability of the intact plasmid or its deletion variants are determined in the following manner: if the clone shows simultaneous growth on both naphthalene and salicylate, this means that it harbours the intact plasmid pBS3. When it grows only either on salicylate or naphthalene this means that it contains only the Sal^+ or the Nah^+ fragments of the plasmid.

Analysis of Kelthane Residue in the Soil

The kelthane concentration is determined in the experimental and control lysimeters. An average soil sample is taken similarly to that for

microbiological analysis, but here the observance of sterile conditions is not rigorously required. A 10 g sample from the average sample is placed in a flask, mixed with 20 ml of water and let stand for 24 h. The soil suspension is acidified to pH 2.0 with 0.1M HCl and extracted three times with equal volumes of diethyl ether. The ether extracts are assembled, washed with the equal volume of distilled water to remove water-soluble compounds and dried with anhydrous sodium sulphate, the solvent being eventually removed into a rotary evaporator. The residue is dissolved in heptane, and the kelthane content in the sample is determined by gas chromatography. The analysis is performed using the a 304 Pye Unicam with the electron capture detector and a glass column (120 x 0.2 cm); the stationary phase OV-101 - 1.5% on 'Supelcoport W/HP', 80-100 mesh; carrier gas - nitrogen at a flow rate of 40 ml/min; temperature of the injector and column - 210°C, that of the detector - 220°C.

The qualitative analysis follows the quantitative one and makes use of thin-layer chromatography on Silufol UV-254 plates. Heptane is totally removed in a rotor evaporator, the residue is dissolved into 400 µl of acetone, and 10 µl of extracts from samples taken from the experimental and control lysimeters are spotted together with 10 µl of the stock kelthane solution, on the chromatographic plates using calibrated capillaries. The plates are developed in a mixture of solvents: *n*-heptane:ethylacetate:acetic acid (30:10:0.1 by volume) and the chlorinated and phenolic compounds are detected with appropriate solutions. The chlorinated compounds are determined with the reagent composed of 0.1 g of silver nitrate, 1 ml of water, 190 ml of acetone and 10 ml of 2-phenoxyethanol. After spraying with the reagent, the plate is exposed to the long-wave UV for 10-15 min. The chlorine-containing compounds are revealed as grey spots against white background (Szokolay and Madaric, 1969).

The phenol-containing compounds are detected with diazotized benzidine (Kirchner, 1981). The reagent is prepared immediately before spraying the chromatograms by mixing equal volumes of solution A:(5 g of benzidine in 14 ml of conc. HCl diluted with H_2O to 1 l). and B: (10 g of $NaNO_3$ in 100 ml H_2O). The phenolic compounds show up as yellow

brown spots.

Identification of the detected compounds is carried out by chromato-mass-spectrometry. Separation conditions: glass column (180 x 0.2 cm); solid support - Chromosorb W/HP, 100-120 mesh with 3% of the OV-101 phase. Temperature of the injector - 250°C, that of the column - 90-280°C, heating gradient - 10°C/min. The carrier gas flow rate - 20 ml/min. Mass-spectra are recorded under an ionization potential of 70 eV. Compounds are identified by comparing the mass-spectra recorded with those of the standard compounds.

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EVALUATION OF THE PESTICIDE TOXIC EFFECT ON SOIL MICROFLORA

Yu.V. Kruglov and L.N. Paromenskaya

One of the most important factors of intensive agriculture is chemical protection of crops which is effected by utilization of chemicals known as pesticides. Their application is increasing and has become quite massive to date. The annual world output of pesticides has reached some 3 mln tons and hundreds of new toxicants are being synthesized and proposed for future uses.

Pesticides are physiologically active compounds. Therefore their massive application has already resulted in some undesirable consequences. These in turn bring to the forefront an acute problem of the ecotoxicological evaluation of pesticides and control of their fate in the environment.

As pesticides are chiefly used in farming practices, the soil ultimately proves to be their recipient irrespective of the manner of application. Once in the soil, pesticides are retained there for a certain time and, among other things, interact with the soil microflora.

The role of microorganisms in maintaining soil fertility and its self-purification of xenobiotics is well known. Therefore, there is an urgent need for information on the significance of soil microbial communities and an assessment of the possible consequences of pesticide uses. The use of a uniform approach to such an evaluation also appears

to be extremely important.

Pesticides are known to affect soil microorganisms both directly and indirectly.

Direct effects are related to pesticide toxicity and, as a rule, have a selective character. Indirect effects are due to changing technologies of mechanical soil tilling, variation of the functional status of plants and redistribution of plant residues over the soil profile. In this context, we believe that a soil-microbial assessment of possible consequences of pesticide applications should include two basic steps.

The first step can be carried out in laboratory conditions. Its major task is to elucidate toxic stresses caused by pesticides, to detect the most sensitive indicators to act as guidelines.

The second step embraces field experiments aimed at providing factual material for the soil-microbiological evaluation of proposed agrotechnical measures among which pesticides are no more than an agronomical, and often not major, component of the system that determines the character and intensity of microbial processes occurring in the soil.

Elucidation of the original causes of changes imposed on the soil microflora is of an essential significance to substantiate a practical set of agrotechnical measures. For example, the cause of a negative effect of some 'minimum' pesticidal treatments on the microflora of loamy soils under conditions of sufficient moistening does not reside in toxic properties of the herbicide 2,4-D, but rather in the variation of the soil air-water regime. Consequently, a negative evaluation should be attributed not to the herbicide but to the wrong mechanical treatment of these soils.

1. Evaluation of the Pesticide Toxicity for Soil Microorganisms

The principle of thresholds is assumed as a basis for ecotoxicological evaluation of pesticides. It implies that the extent of the effect of chemicals on the organism depends on their concentration in the medium, doses applied and time of contact. For every toxicant there exists a low limit (threshold) of concentration below which its toxic effect is no

longer detectable.

The primary effect of pesticides on the individual organism is determined by their chemical composition and focuses on a vitally important physiological factor that is commonly called the 'target'. Thus, the basic 'target' for phenylurea derivatives is the photosynthetic mechanisms of plants, whereas acetylcholine esterase is the target for organophosphorus insecticides.

The concepts formulated by hygiene experts and toxicologists for assessing toxic effects produced by chemical and physical agents are in general also valid for the ecosystem. The latter in its entirety, is characterized by a specific structural organization (biocenosis), a flow of energy and a turnover of substances. The soil microflora is part of the terrestrial ecosystem with all the attributes inherent in it. For a primary evaluation, it is therefore important to determine those loads or concentrations of pesticides which cause substantial changes in basic parameters of the ecosystem. This predetermines the choice of indices (or indicators) for the soil-microbial evaluation of pesticide toxicity. Table 1 gives an idea of the set of indicators that can be used for this purpose.

Concentrations of toxicants at which the numbers of microorganisms are reduced or the rates of biochemical processes inhibited by 50% are assumed as criteria for evaluating the toxic effect of pesticides on the soil microflora (RC_{50} and IC_{50}, respectively).

To calculate RC_{50} and IC_{50} values laboratory experiments are staged using pesticide concentrations which increase in a geometrical progression. The experimental procedure is described below. Values of RC_{50} and IC_{50} are determined graphically or by calculation.

In the first case, based on the results of microbial and biochemical soil analysis, a graph is plotted using pesticide concentrations as abscissa and as ordinate the number of microorganisms or rates of corresponding biochemical processes expressed as percentage of those in control experiments (pesticide -free experiment version). The graph is further used for determining values of RC and IC (Fig.1).

In the second case, to calculate RC_{50} (IC_{50}), use is made of the the Berens equation (Lakin, 1966):

$$IC_{50} = A + \frac{(50 - a)d}{b - a}$$

where IC_{50} is the pesticide concentration (in soil) reducing the microbial numbers or inhibiting biochemical process by 50% ;

A is the pesticide concentration reducing microbial numbers or inhibiting a process by a% ;

B is the pesticide concentration reducing microbial numbers or inhibiting a process by b% ;

a is the inhibition below 50% (at concentration A);

b is the inhibition above 50% (at concentration B);

d is the difference of two concentrations producing a% and b% inhibitions.

For example: A = 25 mg/kg; a - 30% ; b - 60% ; d - 50 mg/kg

$$IC_{50} = 25 + \frac{(50 - 30) \cdot 50}{60 - 30} = 58.3 \text{ mg/kg}$$

Maximum concentrations of pesticides are preset as determined by production needs and sanitary-hygienic regulations. Evaluation of 'harmfulness' is therefore made by comparison with PC values and may be expressed as:

$$K_s = \frac{IC_{50} (\text{or } RC_{50})}{PC}$$

This ratio may be conditionally regarded as the index of reliability or safety (K_s).

Results of analyses are registered in the toxicological chart in the form of calculated values of IC_{50} and K_s. This is exemplified below with the chart of atrazine ecotoxicological evaluation in soddy-podzolic soil (Table 1).

Characterization of IC_{50} and Ks values for each of the indicators makes it possible to single out the most sensitive ones and thus determine 'ecological targets'.

The 'deleteriousness' of the pesticide for a 'target' is evaluated using the following gradation:

Ks	Inhibiting effect
below 1	Powerful inhibitor (sterilizer)
1 to 10	Moderate inhibitor
10 to 100	Poor inhibitor
over 100	Practically nontoxic

TABLE 1. Chart of soil-microbial evaluation of chemical compounds

Pesticide: atrazine; hydrophilic powder; atrazine content, 50%.
Active substance: 2-chloro-4-ethylamino-6-isopropylamino sym-triazine.
Soil: soddy-podzolic, light-loamy, humus content - 2.8%; pH, 6.2.

Ecological index	Indicative microorganisms and processes	IC50	Ks
Energy flux	Soil respiration (CO_2 emission)	600	100
Nitrogen cycle	Nitrification	600	100
	Nitrogen fixation (heterotrophic)	600	100
	Nitrogen fixation (phototrophic)	5	1
Phosphorus cycle	Mineralization of organic phosphate	-	-
Carbon cycle	Emission of CO_2	600	100
	Decomposition of cellulose	600	100
Biocenosis	Saprophytic bacteria (BPA)	600	100
	Saprophytic actinomycetes (SAA)	600	100
	Saprophytic fungi(Chapek's medium)	600	100
	Microscopic algae	1	1
	Microfauna	-	-
	Symbiotic systems:		
	a) leguminous-rhizobial symbiosis		
	- nodule bacteria	600	100
	- leguminous plants (lupine, soy-beans, peas, clover, haricot)	5	1
	- nodule formation	5	1
	- nitrogen fixation (symbiotic)	5	1
	b) mycorhiza	-	-
	c) lichens	-	-

Conclusions:

1. Algicide (powerful inhibitor of algae), represses nitrogen fixation by blue-green algae.

2. Represses nodule formation on roots of leguminous plants.
3. Reduces symbiotic nitrogen fixation to levels inhibiting growth of leguminous plants.

Recommendations:

1. It is not advisable to utilize atrazine for leguminous crops or for their forerunners.
2. It is not recommended for use in agroecosystems where it is necessary to maintain high production of microscopic algae that fix molecular nitrogen.
3. It may be utilized for abating algal growth in industrial water reservoirs.

The chart also carries conclusions that indicate the main 'targets' and the category of their safety against the pesticide toxic effect.

The laboratory ecotoxicological evaluation is a preliminary one and is far from pretending to be field-valid. Nonetheless, it allows one to have an idea regarding the pesticidal effects on soil microorganisms by singling out more vulnerable components of an ecosystem. The chart may also be used for screening of toxic compounds thus facilitating the subsequent choice of basic indices for field experiments. The latter step may also help one to avoid spending too much time and even money when assessing possible consequences of pesticide application in farming practices.

1.1. Sampling Technique

For laboratory experiments the soil is sampled from an arable field 0-20 cm deep. The moist soil is air-dried, passed through a 3-mm (size of pores) sieve and stored in Kraft-sacks or boxes.

Before the soil sample is used in the experiment, its moisture content and water capacity are measured. A weighed sample of soil is spread on a glass or plastic dish to form a thin uniform layer and wetted with water to get 60% of the total moisture capacity. Following the adopted version, the pesticides under study are introduced into the sample in the form of aqueous solutions, emulsions or fine suspensions,

if the moistening step is included as described above. Granulated preparations are evenly dispersed over the surface of moistened soil. Then the soil is thoroughly mixed and placed into 0.5–1.0 l flasks, stoppered with pieces of polyethylene film, weighed and incubated at 26–27°C. The experiment is repeated 2–3 times. Pesticide concentrations are calculated on the soil weight basis. For evaluation of most toxicants with production-required doses not exceeding 10 kg/ha the following geometrical sequence of concentrations is recommended: 0 (control) – 1–5–25–125–625 mg/kg. In general, quantities of the pesticide released to the soil should remain within the range of 1–100 times its concentration required for crop cultivation.

The soils used in the experiment should also be characterized as regards their type, humus content and pH.

1.2. Assessment of the Pesticide Effect on Soil Microflora

Microbiological analysis of the soil is carried out after 5 and 30 days of incubation. Average samples of 10 g from each flask are transferred to flasks filled with 90 ml of sterile water. After 5-min agitation, the soil suspensions are allowed to sediment for 15–20 sec., then they are subjected to successive 10-fold dilutions. The number of dilutions depends on the type of soil studied and generally does not exceed 7. Individual sterile pipettes are used for every of the successive dilutions. For microbial counts, the soil suspensions are inoculated on agarized media following the immersion technique. For this purpose, 1 ml of soil suspension from a particular dilution step is transferred onto a Petri dish, covered with an agarized medium (about 15 ml), cooled to 40°C and thoroughly agitated by circular movements. When the agar has set, the Petri dishes are inverted and incubated at 27°C. Counting of the saprophytic bacteria is achieved using soil or beef-peptone agar (BPA), fungi are enumerated on the Chapek medium amended with streptomycin, and actinomycetes are counted on the starch-ammoniac agar (SAA).

The optimum conditions for microbial counts on agarized media are 20–100 colonies per Petri dish.

1.3. Counts of Microscopic Algae

Evaluation of the algicidal effect of pesticides is brought about using the microalgae-overgrown glass plates (Hollerbach and Stina, 1969; Stina, 1972). 50 g of the soil treated with pesticides at concentrations, specified by the experiment selected, is placed on a Petri dish and carefully moistened to preserve a rough surface of the soil sample. Overmoistening and surface smoothing are undesirable. Then the soil surface is covered with glass plates sterilized in a gas burner flame while pressing on them slightly with fingers. The Petri dishes are then incubated under permanent illumination with fluorescent lamps ($2\text{-}3 \cdot 10^3$ lx).

One week after treatment of the soil sample with herbicides, the plates are analyzed, and the analysis is repeated 4-6 times at weekly intervals. Microbial populations are counted on 3 surfaces (one glass plate from each Petri dish). The covering glass plates are carefully (to avoid damaging microbial overgrowth) taken off the soil surface. Big clots of soil are removed by carefully tapping the plate, or with a needle or tweezers. The glass plates are superposed on microscopic slides with a drop of tap water in-between them and systematically examined under a microscope. No less than 100 vision fields should be examined. Counting is performed with differentiation of the morphologically individualized species. Quantitative evaluation is based on the frequency of occurrence of particular species on the microbe-overgrown plates and expressed as percentage of the total number of fields of vision studied or in the form of the following scale:

5 - Massive accumulations of algae are ubiquitous in more than 75 cases.
4 - Individual cells and groups of algae with rare massive accumulations are typical for 50 to 74 observations.
3 - Individual cells and groups in 25 to 49 cases.
2 - Individual cells and rare groups in 5 to 24 cases.
1 - Individual cells in less than 5 cases.
0 - Not found.

While analyzing the species composition of the algal community, a description should be provided of the algal forms most sensitive to pesticidal effects and the dominant ones.

The identification of the algae present, including species and

genus, should be performed using standard determination references for fresh-water algae. Certain indices widely used in ecology (Oudum, 1975) are recommended as integral indicators characterizing the species composition of algocenosis.

The index of species diversity is calculated from the ratio **(S - 1)/logN**, where S is the number of species found in soil (on algae-overgrown glass plates); N is the algal counts in soil; logN is the natural logarithm.

The coefficient of similarity of two versions under comparison (control - pesticides) is **2c/(A + B)**, where c is the number of species common for both experiment versions; A is the number of species in the control; B is the number of species in the experiment pesticide-treated soil.

1.4. Nitrogen Mineralization in Soil

The mineralization process is derived from the nitrification capacity of a soil. To measure the rate of nitrate accumulation, 50 g soil samples containing different concentrations of pesticides are moistened to the 60% level, amended with 300 mg of lupine flour, thoroughly mixed and transferred to Petri dishes, 250 ml Erlenmeyer flasks or wide-neck bottles. The soil is incubated for 14-15 days in a thermostat at 27°C. The nitrate concentrations are determined prior to and after incubation by any of the methods recommended in literature. The nitrification capacity is calculated from the difference between concentrations found and expressed as in mg of nitrate nitrogen per 100 g of soil during 14 days.

1.5. Soil Respiration

The intensity of soil respiration is determined by the absorption of oxygen and emission of carbon dioxide. Several methods are in current use for determination of carbon dioxide. In this work the following technique is recommended. Soil samples of 25 g (totally dry weight basis) are placed in nylon gauze bags. The bags are loaded on a metallic netting in a special flask used for studying seed respiration. The bottom of the flask is covered with 20 ml of 0.1N NaOH. The flasks are

stoppered to be air-tight and incubated for 3-5 days at 27°C. When the experiment is over, NaOH solution is poured out into another flask. The respiration flasks are washed 2-3 times with small portions of distilled water that are added to the alkaline solution. Then the NaOH solution is treated with 2 ml of 50% $BaCl_2$, 2-3 drops of 0.1% alcohol solution of phenolphthalein and titrated with 0.1N HCl.

The quantity of carbon dioxide produced per unit time in the course of soil respiration is calculated from:

$$CO_2 \text{ (in mg/g·24h)} = \frac{(V_1 - V_2)\cdot 2.2}{mt}$$

where V_1 is the volume of 0.1N HCl used for titrating initial 0.1N NaOH, ml; V_2 is the volume of 0.1N HCl for titrating 0.1N NaOH after incubation, ml; m is the soil mass, g; t is the incubation time, days.

1.6. Degradation of Cellulose

The bottoms of sterile Petri dishes are covered with sterile discs of filter paper (8 cm diameter, weighed). The paper filters are covered with nylon or glass wool on which 25 g of the 60%-moistened soil is spread. The dishes are placed into a humid chamber and incubated at 27°C. The experiment should be 5-fold reproducible. Development of cellulose-degrading bacteria on the filter paper at the flask bottom is observed after 10, 20 and 30 days of incubation. Percentage of cellulose decomposed is calculated after 30 days. The soil is poured out of the dish, the fabric is carefully taken off, the remnants of the paper filter are scraped off the bottom, air-dried and weighed. The degree of cellulose degradation is evaluated from the difference of starting and resulting weights of paper and expressed in mg per dish or in percentage of the starting weight. Should there be a need be for more detailed studies, the community of microorganisms that develop on the paper filter is analyzed. To this end, the character of cellulose degradation and pigmentation is described, and the dynamics of the development of bacteria, fungi and actinomycetes are characterized. Samples are taken for microscopic analysis, and microbiological analysis is done by inoculating paper samples with the appropriate nutrient media.

1.7. Evaluation of the Soil Nitrogen Fixation Capacity

To assess the activity of nitrogenases, the 60%-moist soil samples of 5-20 g are poured into 20-50 ml flasks. The flasks are plugged with rubber stoppers and screwed with plastic lids with a small perforation. Acetylene, in an amount of up to 10% of the flask volume (partial pressure of 0.1 atm.), is injected with a syringe into the flasks that are further incubated at 27°C. One day after, 2 ml volume of Nessler reagent is injected to arrest the reaction. The ethylene produced is quantitated by gas chromatography using gas samples of 0.5-1.0 ml. Measurements are performed using the flame-ionizing detector and a stainless steel column, 4 mm in inner diameter, 1 m long, and packed with chromaton amended with 10% α-oxydipropionitrile. Carrier gas - nitrogen with a flow rate of 50 ml/min; air flow rate - 400 ml/min; hydrogen flow rate - 40 ml/min; the column is thermostatted at room temperature (22°C); the ethylene peak is eluted after 12th s Acetylene reduction is calculated using the following equation:

$$C_2H_4 \text{(in nM/100g soil·24h)} = \frac{H_s C_{st} V \cdot 100}{H_{st} V_s}$$

where, H_s is the ethylene peak height for the flask sample, mm; H_{st} is the ethylene peak height for the standard, mm; C_{st} is the ethylene quantity in the standard, nM; m is the soil mass, g; V is the flask volume, ml; V_s is the sample volume for chromatography, ml.

The nitrogenase activity is expressed in nanomoles (nM) of ethylene per 100 g of soil per 24 h.

Because of low nitrogen fixation rates in soil, it is recommended to treat the soil with glucose (as energy source, added in a quantity of 0.1% of the total soil mass) when evaluating pesticidal toxic effects.

No less than 8 parallel experimental runs should be performed to provide for a sufficient reproducibility of results.

2. Soil-Microbial Evaluation of Pesticidal Toxicity under Field Conditions

Under field conditions, analysis of the effect of pesticides on the soil microflora displays certain specific features. These may be due to the

fact that their application often entails substantial changes in the technology of cultivation as well as in the dynamics of recent organic matter and its distribution over the soil profile. All this, naturally, affects the air-water regime of the soil and the energy supply to the soil-based microbial processes. Therefore in reality, it is under field conditions that the soil-microbial evaluation of a pesticide is brought about considering the sum total of agrotechnical steps among which herbicides (pesticides) often appear far from being major technology affecting the soil microflora and its functional status. Under laboratory conditions, if a pesticide is found to produce no deleterious effects on basic biological factors, it means that it is totally rehabilitated as a non-toxicant towards soil microorganisms. If this is the case, another cause, that underlies variation of microbial soil processes, should be identified.

2.1. Technique of Soil Sampling for Analysis and Microbial Counts

In microbial and agrochemical studies, an average sample is derived from 5-10 and sometimes more individual samples.

Under a uniform treatment of a total field or plot with pesticides, the soil is sampled from the tilled horizon with a spade, soil auger or blade. Sterilizing may be omitted if the sampling tool was thoroughly washed shortly before use. Digging into the soil to be sampled several times proves to be sufficient in most cases. Depending on the task, the bulk of the sample is used (profile-wise) or it is divided into a number of 5-10 cm layers that are loaded and stored in polyethylenic or oil-paper bags. Representative soil samples are thoroughly mixed, and an average 200-300 g sample is taken and added to a broad-neck flask or a plastic bag.

After application of the pesticides, e.g., over rows of plants, under tree canopies, into ditches etc., samples are taken from these specific areas.

It is advisable to conduct microbiological analysis the same day the sampling is done. Since this is not always feasible, storage of samples in a refrigerator at +2 to +5°C should not exceed two days, all the samples being analyzed within one working day. Hence, a study

programme should be planned accordingly.

In the laboratory, the soil sample is thoroughly mixed, and the moisture content measured and used for microbial and biochemical analyses.

For the microbiological analysis, sample weights are taken corresponding to 10 g of absolutely dry soil with the view of simplifying subsequent calculations. Soil samples after weighing are poured into 250 ml flasks or bottles containing 90 ml of sterile tap water, and the first dilution is carried out. This is followed by subsequent 10-fold dilutions (up to 7 times), then soil suspensions are inoculatd on the appropriate media in the manner described above (see Section 1.2).

Microbial counts are performed on the following growth media:

1. beef-peptone agar - for sporous and non-sporous saprophytic bacteria;
2. starch-ammoniacal agar - actinomycetes and bacteria utilizing forms of inorganic nitrogen;
3. agarized Chapek medium with streptomycin - fungi.

Analysis of the soil microflora and microbial counts should be performed at least 3 times during the vegetation period. The first analysis is recommended 3-5 days after treatment, further - over the crop growth phases or 30-40 and 90-100 days after pesticide application.

2.2. Nitrification Capacity of Soil

Soil weights are taken from the average sample stored for the microbiological analysis (see above). Soil weights of 50 g are spread over Petri dishes, moistened to 60% and thermostatted. Incubation lasts 14 days at 27°C. Nitrates are determined prior to and after soil incubation. The rate of nitrate production is calculated as described in 1.4.

2.3. Nitrogen Fixation Capacity of Soil

Weights of soil from the averaged samples are placed into 50 ml screw-cap flasks and moistened to 60%. The flasks are rubber-stoppered, and caps with a small perforation at the centre are screwed on. Acetylene in amount of 5 ml is injected into the flasks with a syringe.

After 24-hour incubation at 27°C, 2 ml of Nessler reagent are added to stop the reaction. A 0.5-1.0 ml gas sample from flasks is chromatographed to determine the ethylene concentration as described above (Section 1.7).

2.4. Soil Respiration

The intensity of soil respiration under field conditions is determined from the emission of carbon dioxide using the method developed by Shtatnov. Porcelain evaporation bowls (10 cm in diameter) with 20 ml of 0.1N NaOH are placed on the surface of studied soil and covered with a glass hood (30 cm high, 30 cm in diameter). The hood is driven 5-7 cm deep into the soil. Hoods are also placed on a flat glass surface over control bowls and sealed with plasticine. After 4-12 h, the hood is taken off. An alkaline solution is poured in a flask together with the bowl rinsing water. 2-3 drops of phenol-phthalein are added and the solution is titrated with 0.1N HCl to decoloration. The rate of carbon dioxide emission is calculated using the following equation:

$$CO_2 = \frac{(V_1 - V_2)}{st} \quad (\text{in } mg/m^2 \cdot h)$$

where V_1 is the volume of 0.1N HCl used for titration of 0.1N NaOH from the control bowl, ml; V_2 is the volume of 0.1N HCl used for titration of NaOH solution from the experiment bowl, ml; s is the bottom area of the hood, m ; t is the incubation time, h.

2.5. Cellulose Degradation

The rate of cellulose degradation in soil is determined by the decomposition of a linen or a cotton tissue. The tissue is well washed prior to experiment. The starch test is carried out with the Lugol reagent. If starch is found, the tissue is boiled in slightly acidified water. The pretreated fabric is spread over glass plates (10x25 or 10x50 cm.; plates of other sizes may also be used), their position is fixed by wrapping the plates with nylon or glass wool. Instead of glass plates the plastic ones may also be used with the fabric fixed to one of their sides.

A spade is driven into the studied soil, and a vertical section obtained is smoothed with a soil blade. The plate is pressed to the soil section by its fabric-attached side and buried by a careful soil packing. In 1, 2 or 3 months the glass plates are taken out of the soil, cleaned of the clotted soil, and the protective netting removed. The character of the fabric disintegration over the profile is described and, if required, the remnants are photographed and weighed.

Should there be a need for characterizing the cellulose degradation rate over each of the soil horizons, the tissue is cut into corresponding pieces which are then weighed. The control tissue is cut out in the same manner. Results are compared, and the cellulose degradation rate is expressed as percentage of the starting tissue weight. At least 5 parallel experimental runs should be carried out to provide for the adequate reproducibility.

3. Preparation of Nutrient Media for Microbial Counts in Soil

3.1. Beef-peptone agar (for saprophytic bacteria)

A 500 g portion of fresh minced meat (free of bones, tendons and fat pieces) is covered with tepid water and maintained at 50°C for one hour. The extract thus obtained is drained through a multilayer piece of gauze, boiled for 30 min and filtered. The extract is treated with 5 g of NaCl and 10 g of peptone. The hot mixture is passed through a double paper filter, the filtrate volume is measured. Some water is added to restore the starting volume. The extract amended with 20 g of agar is heated on a water bath. Upon agar melting, the extract is poured out into flasks of required countenance and sterilized by autoclaving for one hour at 1 atm. Beef-peptone media may also be prepared from beef broth tablets produced by food industries. They are dissolved in hot water (200-300 ml) amended with 12 g of peptone and cooled in a refrigerator. The upper greasy layer is removed and the extract is passed through a folding paper filter. Tap water is added to give a total volume of 1.2 l. The pH is set at 6.8-7.0 followed by amending with agar (2% by volume) and sterilizing.

3.2. Starch-ammoniac agar (for actinomycetes and bacteria utilizing inorganic nitrogen)

$(NH_4)_2SO_4$	- 2 g
K_2HPO_4	- 1 g
$MgSO_4$	- 1 g
NaCl	- 1 g
$CaCO_3$	- 3 g
Soluble starch	- 10 g
Agar	- 10-20 g
Distilled water	- 1 l

Starch is thoroughly mixed with cold water and added to the medium.

3.3. Chapek medium (for fungi)

$NaNO_3$	- 3 g
KH_2PO_4	- 1 g
$MgSO_4$	- 0.5 g
KCl	- 0.5 g
$FeSO_4$	- 0.01 g
Sucrose	- 20 g
Agar	- 20 g
Tap water	- 1 g

Acidity of the medium is set with lactic or citric acid at pH 4.0 followed by 20 min sterilization at 0.5 atm.

For suppressing growth of bacteria the medium is amended (after sterilization) with streptomycin in concentration 20-30 g/l.

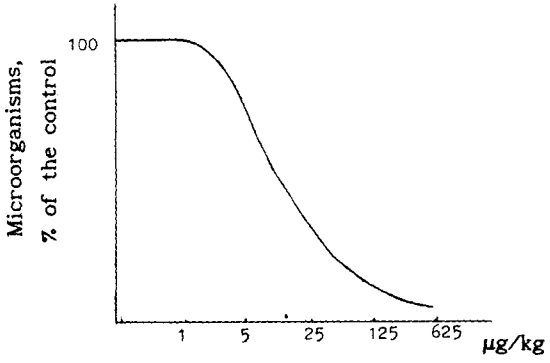


FIGURE 1. Graphical method for determining IC_{50} of pesticides for specific microbial indicators in soil.

ANALYSIS OF THE PRODUCTS OF MICROBIAL DEGRADATION OF XENOBIOTICS BY CHROMATO-MASS-SPECTROMETRY

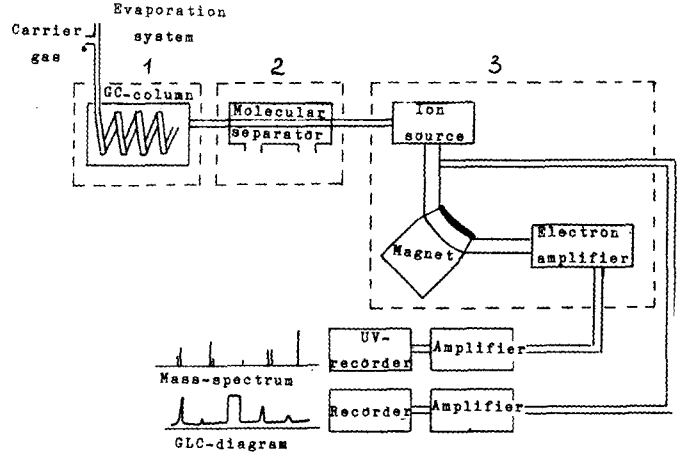
B.P. Baskunov

Chromato-mass-spectrometry has been successfully used, for several decades now, as a means of determining the composition of complex mixtures, establishment of the empiric formulae, and the functional and isotopic composition of organic compounds. In general, it allows researchers to obtain quantified data on specific functional groups, number and character of heteroatoms and cycles in the molecule.

The system of 'chromato-mass-spectrometer' embraces three basic units: gas chromatograph, separator and mass-spectrometer.

A sample is introduced into the uppermost part of the chromatographic column (injector port). The sample is evaporated there, mixed with the carrier gas (helium) and carried into the chromatographic column. The column is a spiral glass tubing filled with a solid support covered with a liquid (stationary phase) having a high boiling temperature.

Chromato-mass-spectrometry makes generally use of solid supports of the type of Chromosorb, high-performance Supelcoport AW-DMCS, W-HP (previously washed with an acid and treated with dimethyldichlorosilane) that are well suited for tackling various analytical problems. More rigid requirements should be met in selecting a stationary phase.



Analysis must be conducted in such a manner as to reduce to a minimum the stationary phase vapour pressure at the operation temperature of the column (column background). This requirement is met by nonpolar phases with a high upper limit of the performance temperatures: OV-101, OV-17, SE-30. Percentage of the stationary phase on the solid support does not usually exceed 5%. This provides for a high efficiency of the column, resolution rates, agglomeration resistance of the support, very low background level, which are of crucial importance for programming the column temperature.

Individual components of a sample under study are dissolved in the liquid stationary phase. An equilibrium is set between the gas and liquid phases. The rate of progression of a specific compound along the column depends on the character of interaction between the compound and the liquid stationary phase. Hence, differing interactions of specific components allow their resolution.

Before introducing the gas mixture to the mass-spectrometer, the amount of carrier gas must be reduced, because the mass-spectrometer operates at very low pressures (10 torr). The mixture of carrier gas and gaseous sample is fed to the two-step molecular separator for removal of the maximum quantity of the former. Because of different retention

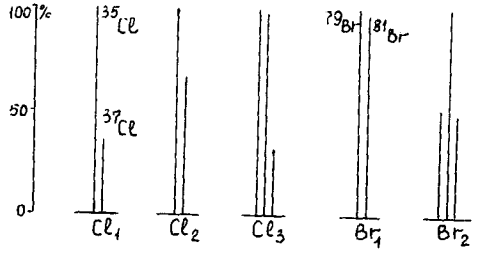
times, the components leave the column after specific time intervals and thus pass in strict succession through the separator to the mass-spectrometer.

Upon entering the ion source, the molecules of the sample components are bombarded with electrons: individual molecules lose an electron, and the emerging cations will thus have an excessive energy that in turn will force the molecular ion to break into a number of other ions having much lower and differing masses. Due to their individual masses, these ions may be sorted out when accelerated in an electric field and deflected in the magnetic one.

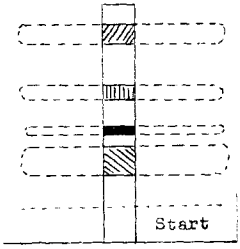
A mass-spectrum graph is thus obtained showing the distribution of ions as function of their masses. Corresponding signals are sent by the detector and recorded by the UV-recorder (mirror-galvanometer oscillograph).

A mass-spectrum is usually represented in the normalized form, i.e. as percentage of the maximum peak. Mass-spectra often show broadened peaks of low intensity which correspond to 'metastable ions' which are widely used for establishing fragmentation pathways. The magnitude of a metastable peak (m) is related to the value of the parent (a) and daughter (b) ions in a rather simple manner: $m = b / a$.

Many chemical elements are characterized by specific ratios of their natural isotopes. In this respect, Cl, Br and S are remarkable for having the most abundant isotopes that differ by two mass units. Owing to this, Cl and Br are readily distinguished in chlorinated and brominated organic compounds using their mass-spectra. Analysis of isotopic peaks in the field of molecular ion permits determination of the number of Cl and Br atoms in a molecule, as may be seen from the following example:



The instrument makes feasible the mass-spectrometric analysis of pure substances that are not available by gas chromatograph. A system such as a direct sample input is well suited for analyzing thermally unstable compounds. In some instances the system of direct input is used for rapid control of various extracts: for example, extracts of the spots obtained in thin-layer chromatography on Silufol plates. Following the spot resolution, the plates were placed into an aeration hood for removal of solvents. The corresponding zones were outlined using the ultrachemiscope (UV long-wave range). If the compounds under study showed no fluorescence, part of the plate was being sprayed with the appropriate developing reagents to obtain a stable coloured spot. The undeveloped zones which presumably contained interesting products were scraped off the plate and eluted. Elution may be carried out using a flask topped with a glass filter No. 3, 4 (10 mm in diameter) or a burette (50-70 x 5 mm) packed with glass wool as a filtering agent. The eluate is concentrated by evaporation under vacuum and analyzed by direct input or, if needed, via the gas chromatograph.



As stated above, gas chromatography (GC) is a method intended for analysis of volatile compounds based on the two-phase distribution of compounds under study. Nonetheless, the applicability of this technique may be expanded to include substances, which cannot be subject to direct analysis, by transforming them into volatile derivatives. For a satisfactory gas chromatographic analysis, such derivatives must be easily obtainable by fairly rapid reactions with quantitative yields and readily separable from the reaction products.

Aliphatic and aromatic carboxylic acids display a relatively low volatility and their direct analysis by gas chromatography is quite problematic. Esterification allows a fairly good quantitation of these

acids fostered by their better resolution.

Methyl esters of carboxylic acids are best suited to meet the above requirements: their synthesis poses no problem and provides high yields.

Esterification may be brought about in several ways, e.g.:

- by diazomethane method;
- under the effect of methanol and HCl;
- by specific marketed reagents that may be available from: Pierce, Cat No.49370 BF - Methanol 14% 100 ml, Cat No.49350 Methyl-8 25 ml in Pyridine.

Each of the above methods has its own advantages and disadvantages. Thus, reactions with diazomethane are rapid (1-2 min), provide high yields and the esters derived can be used for analysis either directly or immediately after removal of the solvent (diethyl ether). In certain cases, however, the esterification with diazomethane may lead to high yields of by-products. A limited stability of diazomethane is also a weak point, as the reagents must be synthesized shortly before esterification. Reactions with methanol and HCl require 1-2 h for completion while that with BF in methanol should be heated and the resulting esters extracted from the reaction mixture. Despite some obvious shortcomings, the diazomethane method is extensively used in chromato-mass-spectrometry, since the reactant is volatile and thus does not complicate the pattern of chromatographic resolution.

Diazomethane is usually prepared by decomposing solutions of nitroso-compounds, such as N-nitroso-N-methylurea and N-nitroso-N-toluene-4-sulphomethylamide, with alkalis.

Special precautions should be taken while manipulating diazomethane. **Work with diazomethane should be done only under an aeration hood !**

For most samples it is advisable to prepare an ether solution of diazomethane.

5 g of N-nitroso-N-methylurea (0.05 mol) are added by small portions to 50 ml of diethyl ether poured over 15 ml of cooled aqueous 40% KOH solution. The reaction mixture is shaken for 10 min at 5°C. Thereafter, the yellow-dark ether solution is decanted and dried for 3 h over solid KOH. The diazomethane solution may be stored for some time in a flask plugged with a rubber stopper vented with a capillary and placed

in a dark cool place.

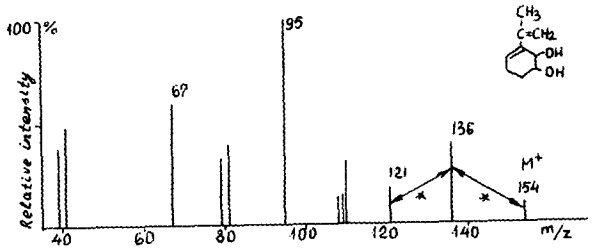
Carboxylic acids are esterified in a diethyl ether solution at room temperature. Addition of a small quantity of methanol enhances methylation. An ether solution of diazomethane is added to carboxylic acid solution until nitrogen emission stops and the reaction mixture is coloured yellowish.

$$R\text{-}COOH + CH_2N_2 \longrightarrow R\text{-}COOCH_3 + N_2$$

A variation of this method is to use three test tubes in series connected with side arms. Nitrogen is passed through. Tube 1 contains the ether, tube 2 - the generated diazomethane, and tube 3 -the acids to be esterified dissolved in the ether. Tube 2 contains 0.35 ml of 2-(2-ethoxyethoxy)ethanol, 0.35 ml of ether, and 0.5 ml of 6 g KOH in 10 ml of water. Add 5-30 mg of the fatty acids in ether in tube 3. Thereafter add 1 mole of N-nitroso-toluene-4-sulphomethylamide per milliequivalent of fatty acids into tube 2. Connect the side arms, let the nitrogen carry the diazomethane into tube 3.

The experimentalist is often faced with the necessity of providing evidence for the number of hydroxyls available in the molecule. The compound is previously analyzed by the direct input into the mass-spectrometer, and a conclusion is reached as to the availability of hydroxy groups. Thereafter, the substance is chemically modified to make possible their determination.

For example, for one of the α-methylstyrene metabolites the following mass-spectrum was recorded:

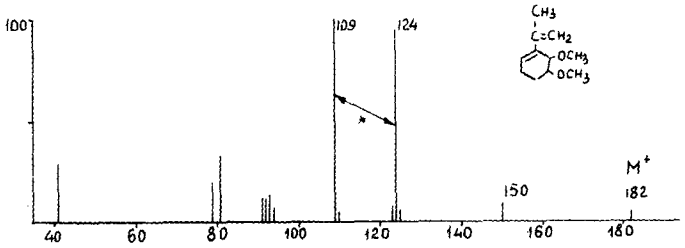


A thorough analysis of the spectrum suggested the structure of the compound as indicated above. As the compound responded positively to the

hydroxyl assay, full methylation was carried out using the standard procedure.

The compound studied (1 mmole) mixed with 0.2 ml of methanol and 0.7 g of methyliodide (or 0.3 ml and 5 mmoles) is gradually amended with 0.12 g (10 mmoles) of silver oxide and heated to 42-45°C (boiling temperature of methyliodide) for 2-3 h with reflux. Thereafter, the suspension is filtered, the filtrate being concentrated and analyzed.

The increase of 28 units in the molecular weight indicates the incorporation of 2 methyl groups.

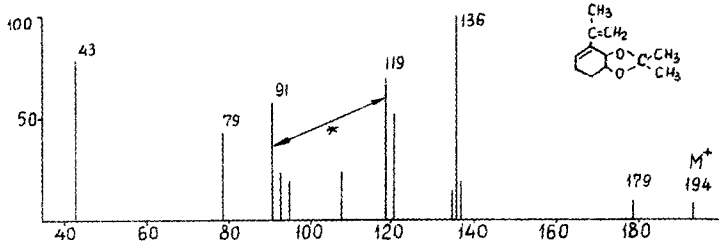


In some cases, mass-spectrometric analysis permits determination of the spacious orientation of hydroxy groups in the molecule. Thus, to elucidate whether the hydroxyls are in the trans- or cis- configuration, an isopropyldienic derivative of the compound under study was synthesized.

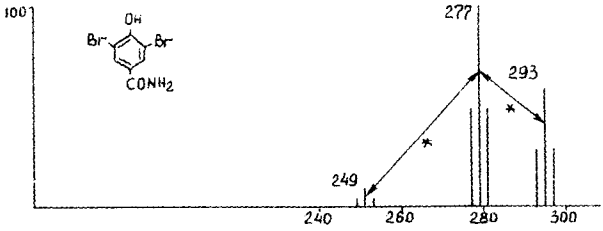
The compound (1 mg) was dissolved in 0.2 ml of acetone and added with 1.0 μl of conc. H_2SO_4 and left to stand overnight. The solution was dissolved in ether and $NaHCO_3$ to remove sulphuric acid, dried over anhydrous sodium sulphate, and analyzed.

Hence, the compound under study is cis-2,3-dihydroxy-1-isopropenyl-6-cyclohexene.

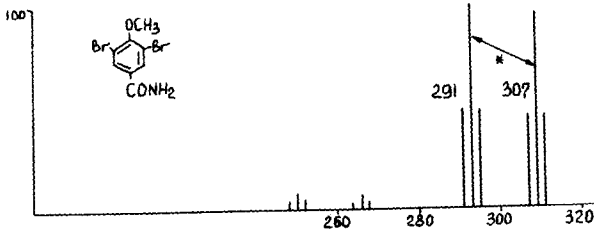
Similar examples of methylation aimed at establishing the structure of studied compounds may be shown for microbial degradation of the herbicide bromoxynil. For one of its metabolites the following mass-spectrum was recorded. It suggests the structure shown below.



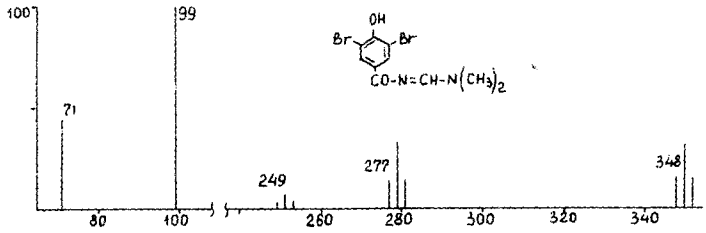
To prove the presence of a hydroxy group, a methylated derivative was synthesized,



whereas the NH group was suggested by the formation of azomethine (Schiff bases) readily obtainable by heating primary amines with the appropriate reagents, e.g., the earlier-mentioned Methyl-8.



Place 0-50 mg of the acids in a flask, add 1 ml of Methyl-8 for freely soluble samples. As a uniform procedure to ensure the complete dissolution and reaction, it is suggested that the sample mixture be heated 10-15 min at 60°C. Inject aliquots of the reaction mixture directly into the gas chromatograph.



MICROBIAL TECHNOLOGIES FOR PURIFICATION OF INDUSTRIAL WASTE WATERS

R.M. Alieva, V.A. Stukanov, and D.B. Dzhusupova

I. Immobilization of Active Microbial Strains for Purification of Industrial Effluents

Intensive development of various industries has resulted in an increased environmental discharge of numerous noxious organic compounds and toxic ions of heavy metals. Many of these substances are recalcitrant and persist in the environment for long periods.

In some instances the existing physico-chemical methods are no longer efficient for the adequate degradation of pollutants, while the technology is cumbersome and expensive. Microbial technologies based on the use of active strains, able to degrade toxicants, appear to be more promising.

Degradative microorganisms become effective technologically, only when fixed on inert supports. In other words, there is a need for immobilized microbial cultures capable of degrading high concentrations of pollutants in industrial waste waters.

In the present work, laboratory models intended for purification of industrial effluents make use of two microbial cultures: *Pseudomonas aeruginosa* - an active degrader of α-methylstyrene (AMS) and *Aeromonas*

dechromatica able to reduce the toxic form of chromium (VI) to a less toxic Cr (III) precipitated as its hydroxide.

The waste water purification process is preceded by the biomass production step.

The cell biomass of active cultures capable of oxidizing AMS and reducing Cr (VI) and intended for purification purposes is grown in matrices with beef-peptone agar (BPA). The BPA medium is inoculated with a suspension of microorganisms in sterile tap water. In 18-24 hours the cell biomass cultured in matrices is washed off with a sterile liquid medium E-8 for preparing a thick suspension of cells to be further used in a laboratory installation for the waste water treatment.

The bulk of the cell biomass is produced in the 30 l fermenters. Inoculi for fermenters are prepared in 0.5 l Erlenmeyer flasks with 100 ml of nutrient broth (NB) amended with 2-3 loops of the culture grown in test tubes on slant BPA (1:10). Inoculated flasks are cultured on a shaker (220 r/min) for 16-18 h at 29°C. One 30 l fermenter requires 6 flasks with the inoculation material. Microorganisms are cultivated in fermenters with a NB for 18 h at 29°C. Thereafter the liquid culture is centrifuged at 10,000 *g* for 10-15 min. The paste-like biomass is packed and stored at 2 to 4°C.

An important step in the development of technologies for waste water purification is the fixation or immobilization of microorganisms on inert supports to create a stable technological system capable of processing high concentrations of pollutants.

Materials assayed as inert supports in the present work were glass fibres, porolon and glass cloth. Immobilization of degradative strains on inert supports is rather a simple and inexpensive technique.

Of all tested materials the glass cloth was found to be best suited for the task, as it offered a developed surface for immobilization and produced no deleterious effect on microorganisms. Immobilization of microbial cultures capable of degrading AMS was carried out by direct contact of the thick cell suspension with the glass cloth.

For this, 5-7 cm wide, hirsute pieces of glass cloth prepared previously are immersed in a tub filled with the thick cell suspension of microbial degraders containing 10^{12} to 10^{14} cells/ml. After the 18-24

hour contact the glass cloth with the immobilized cells is taken out of the tub and packed into the aerotank and aerofilter of the installation. The recommended content of microorganisms immobilized on the support is 10^{10} to 10^{12} cells per 1 m^2 of glass cloth. To attain the recommended cell density on the glass cloth loaded into the installation, additional quantities of microbial biomass are spread over loaded layers of glass cloth. For this purpose the 18-day bacterial culture grown in matrices on BPA and suspended in the liquid E-8 medium is used. For 2 days portions of a thick cell suspension (10^{12}-10^{14} cells/ml) are fed through the lid of the tank and uniformly spread over layers of glass cloth.

Cell suspensions of microorganisms prepared for reducing Cr (VI) to Cr (III) in waste water are immobilized on glass cloth in the same manner.

II. Operation Principles of an Installation for Microbial Purification of Industrial Waste Waters

The previous section described basic steps of the biomass production and its immobilization on glass cloth. The present part is devoted to getting acquainted with the basic principles of functioning of the installation for biological removal from waste water of volatile organic compounds and substances containing ions of heavy metals.

1. Installation for the Waste Water Microbial Purification of Volatile Organic Compounds

The installation is a combination of an aerotank and an aerofilter. A laboratory model of such installation is represented in Fig. 1.

It is a column-shaped reservoir functioning as a biofilter composed of an aerotank (1) topped with an aerofilter. The size of the aerofilter may vary depending on the quantity and volatility of pollutants. The waste water is fed through an inlet located below the upper packing level of the aerofilter. In such a way volatile pollutants contained in waste water do not escape to the atmosphere but start to be degraded by microorganisms in the aerofilter. Secondary purification of the effluent is effected in the aerotank which occupies the bottom part of the column

and contains the same type of microflora. A nutrient solution required to maintain microbial population on the glass cloth is fed through the top of the installation (5), while air is pumped in counter current via the bottom (4). The purified water is removed via the sump (6).

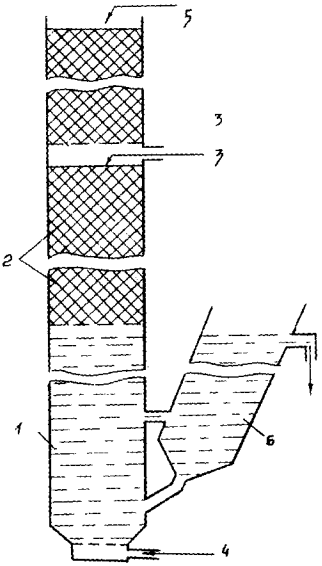


FIGURE 1. Installation for microbial waste water purification of volatile organic compounds.

1, aeration tank; *2*, aerofilter; *3*, input of waste water; *4*, air supply; *5*, input of nutrient solution; *6*, settling basin.

The installation makes use of microbial cultures immobilized on glass cloth.

The waste water under cleaning is polluted with α-methylstyrene (AMS), a homologue of benzene. It is a colourless liquid with a strong odour and slightly soluble in water. *Pseudomonas aeruginosa* is used as a degradation agent.

Upon immobilization of the *P. aeruginosa* culture on glass cloth and its packing into the aerofilter, a nutrient solution containing mineral salts is fed with a metering pump in the upper part of the installation. At the same time, the waste water with AMS in a concentration of up to 2 g/l is introduced on to the glass cloth with the immobilized microbial culture via the inlet below the upper level of the glass cloth packing. The major portion of AMS is degraded in the aerotank but another part, due to its volatility, is oxidized in the aerofilter. The purified water

containing suspended microbial cells is directed to a sump where the cells settle. Air is pumped into the tank through the water with microorganisms at a constant flow rate of 1.5-2.0 l/min in a counter-flow mode, the polluted water descending by infiltration through the glass cloth with the immobilized microbial culture. In such a way microorganisms in the tank and filter are continuously supplied with oxygen. Simultaneously, the ascending flow of air brings some quantity of AMS from the waste water into the filter. In the filter the toxicant is assimilated by immobilized microorganisms. Thus the degradation of AMS occurs over the total volume including the tank and aerofilter.

To control the stability of the working parameters of the installation, the waste water with AMS is continuously fed into the filter and the tank of a 5-6 l working volume for 5-7 days at a flow rate of 3 l/24 h. The waste water with AMS concentration of 1 to 2 g/l is introduced at a flow rate of 2.3 ml/min.

After this, the basic work parameters are measured: AMS concentration, pH and chemical oxygen demand (COD) in the influent and effluent water, amount of microorganisms in 1 ml of liquid from the tank.

AMS concentrations are measured spectrophotometrically using the Specord UV-VIS model, COD - with the bichromate technique and medium acidity - with a pH-meter.

Microbial counts per 1 ml of liquid from the tank are performed by sample inoculation on Petri dishes with BPA. A sample of 0.1 ml volume is inoculated on BPA and spread over its surface with a spatula. Inoculated dishes are incubated in a thermostat at 29°C for 24-48 h. Thereafter, microbial populations are counted, and AMS-degrading microorganisms are assessed as percentage of the total microflora.

The results characterizing the extent of waste water purification are presented in Table 1.

It can be seen that in this installation microorganisms totally remove the AMS in concentrations up to 2 g/l. Microbial degradation of AMS is accompanied by a slight pH drop and a drastic decrease in the COD as shown by the effluent waste water analysis. The waste water purification results in higher amounts of microorganisms with increasing AMS concentrations, microbial degraders of AMS accounting for 80% of the

total microflora in the aerotank.

TABLE 1. Parameters of microbial degradation of AMS in waste water in conditions of continuous culture.

AMS conc., mg/l		pH		COD		Microbial
incoming water	outcoming water	incom. water	outcom. water	incom. water	outcom. water	counts per 1 ml tank liquid
1000	0	7.2	6.9	2720	90	$29 \cdot 10^6$
1500	0	7.2	6.9	4460	130	$620 \cdot 10^6$
2000	0	7.2	6.8	5970	180	$2560 \cdot 10^6$

2. Installation for the Waste Water Microbial Purification of Toxic Ions of Heavy Metals

Heavy metal ions, including those of chromium, are rated amongst most toxic components of industrial sewage. Certain microorganisms are capable of reducing compounds of Cr (VI) to Cr (III) and precipitating the latter in the form of its hydroxide.

Reduction of chromate takes place in the presence of organic compounds used by the microorganisms as sources of carbon and energy, the chromate being the source of oxygen. Under such conditions the reduction of Cr (VI) is brought about by *Aeromonas dechromatica, Pseudomonas fluorescens*, and other microorganisms. They can utilize organic matter in the form of peptone, cisteine, glucose, components of activated sludge.

These reductive characters of microorganisms may be put to good use in purification of industrial waste waters. A laboratory model of the installation for microbial removal of chromate from industrial waste waters is composed of the following units (Fig.2): bioreduction unit (1), sump or settling basin (2) and aeration tank (3).

The upper part of a 5 l reduction unit is packed with strips of glass cloth containing immobilized cells of *Aeromonas dechromatica*. This vessel is covered with a lid to isolate it from the atmosphere and has a taper bottom for removing reduced and precipitated chromium. Waste water polluted with Cr(VI) and treated with peptone is continuously fed at the

flow rate of 12 l/24 h into the bioreduction unit. The waste water is introduced at the lower level of the glass cloth packing. The bioreduction unit is the site of total Cr (VI) reduction by cells of *A. dechromatica*, the initial yellow-pink coloration turns grey-blue.

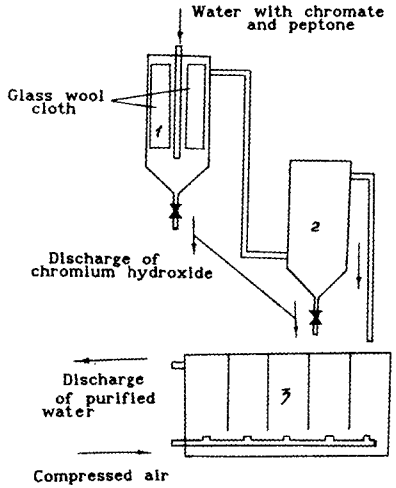


FIGURE 2. Installation for microbial purification of Cr-containing water.

1, bioreduction unit; *2*, settling basin; *3*, aeration tank.

The waste water with reduced Cr (III) is let from the bioreduction unit via the upper tap into a 5 l settling basin also with a taper base for removal of chromium. In the settling basin the chromium (III) hydroxide formed is totally removed, and the solution becomes colourless. The accumulating chromium hydroxide is removed at regular intervals. The colourless waste water containing some organic matter is directed into the aeration tank.

The aeration tank is a rectangular 10 l basin with partitions which provide for a uniform water flow. Compressed air is pumped into the tank through valves installed in its bottom. The excessive organic matter is oxidized in the aeration tank by air oxygen with the help of activated sludge. The purified waste water is either recycled or discharged.

The process of microbial chromate reduction depends on the concentration of organic matter, availability of biogenic elements, and the temperature. The rate and the degree of chromate reduction is proportional to concentration of organic matter. Therefore, the

bioconversion unit is supplied with an excessive organic matter compared to chromate. That is why only part of the supplied organic matter is oxidized in the bioreduction unit. The reduced chromium compounds are mostly amorphous and readily form colloidal solutions and stable suspensions which do not settle in the bioreducer. They can deleteriously affect the performance of the aeration tank and therefore should be completely removed in the settling basin. The excessive organic matter is readily removed in the aeration tank by the activated sludge from communal sewage treatment plants, since the organics used for chromate reduction are of natural origin.

The following sequence of operations should be performed in order to bring about a model waste water purification of heavy metals (exemplified by Cr removal).

A solution is prepared containing chromate (0.1 g/l) and peptone (5 g/l). It is poured into the bioreducer, then the glass cloth with the immobilized cells of *A. dechromatica* is introduced.

After 6-8-hour contact a variation of coloration and precipitation are observed thereby pointing to microbial reduction of chromate. Soon after, the chromate and peptone solution is fed to the bioreduction unit at a rate of 0.5 l/h. Peptone is utilized as a source of carbon and biogenic elements. The solution with excessive organic matter and suspension of Cr (III) is let into the settling basin where the chromium is totally removed from water. The Cr-free solution is let into the aeration tank and treated with 0.5 l of activated sludge from an urban sewage disposal plant and aerated with compressed air. After oxidation and settling of organic matter the purified water is either recycled or discharged. All purification operations are carried out at 20-25°C .

Thereafter, the installation may be used for a continuous purification with a preset rate of polluted water flow amended with peptone while discharging periodically (once a day) the deposited chromium hydroxide. The efficiency of purification is controlled by periodical analyses for the presence of chromium and determinations of COD.

Similar procedure may be used for removing other heavy metals from industrial waste waters.

EVALUATION OF THE PESTICIDE TOXICITY TO LEGUMINOUS-RHIZOBIAL SYMBIOSIS

L.N. Paromenskaya

Introduction

An indispensable condition for the pesticide application in agriculture is the safety of natural biological systems which provide for high productivity and quality of crops. Of special significance are the leguminous plants that develop in symbiosis with nodule bacteria. In conditions of symbiosis, the leguminous crops fix molecular nitrogen thus covering totally or partially their requirements for this nutritive element.

Pesticides, usually endowed with physiologically active characters,

produce certain effects on the processes of symbiotic nitrogen fixation.

Thorough studies, carried out in recent years, revealed that many of these toxicants applied in concentrations close to those required for high productivity are able to arrest, entirely or at least partially, the nitrogen fixation by symbiotic systems thus decreasing the importance of leguminous crops as nitrogen accumulators.

All this points to a need for a preliminary evaluation of the chemicals recommended for use on pulse plantations with due regard to possible implications for leguminous-rhizobial symbiosis. The necessity for such an assessment becomes more obvious, if one bears in mind that in order to enhance the efficiency of symbiosis, the current farming practices the world over make a wide use of seed dressing with preparations of rhizobia prior to their sowing. This permits production of additional millions of tons of grain and forage crops as well as sparing large quantities of nitrogen fertilizers. Unfortunate errors committed while selecting chemicals for crop protection may bring to zero the efficiency of the bacterial preparations employed.

Leguminous-bacterial symbiosis is known to involve two organisms which under certain conditions can develop independently and differ by their resistance to chemicals. In this context, the studies of the pesticidal effect on the interaction of rhizobia with leguminous plants are subdivided into several steps. The basic steps and the sequence of their realization are shown in Fig. 1.

Inclusion in this figure of particular steps or selection of specific techniques are determined by the targets on which the effect of a pesticide should be focused, the manner of its application and the state of our knowledge of the toxicant's environmental behaviour and fate.

Thus, if the preliminary screening and subsequent tests established the concentrations of the chemical which are toxic for leguminous crops, then the first step of this work may be omitted.

Tests with the seed dressing agents may apparently be confined to the evaluation of their toxicity to nodular bacteria.

The final step of preliminary tests through laboratory and vegetative experiments should consist in elaborating a technological

approach for the use of chemicals to protect crops and plant growth regulators with due regard to their application in combination with preparations of rhizobia.

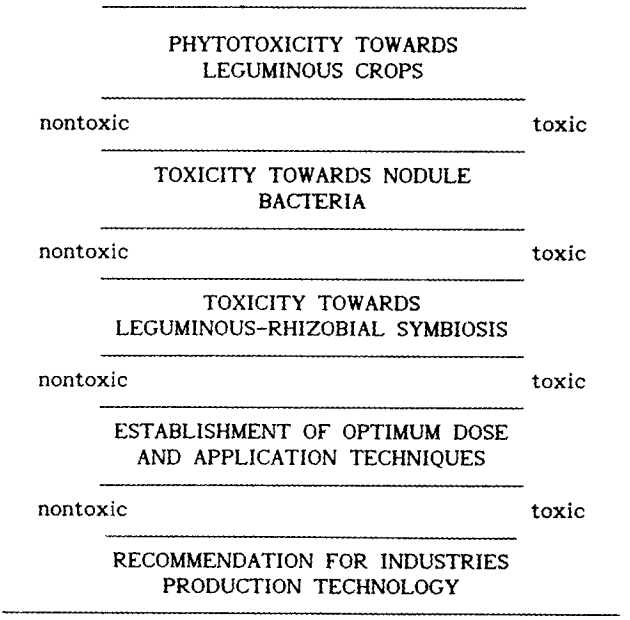


FIGURE 1. Evaluation of the toxic effect of chemicals.

1. Evaluation of the Toxicity of Chemical Compounds to Nodule Bacteria

Such an assessment is performed in order to select the most resistant strains or to establish the variability of this character within a rhizobial population.

It is advisable to conduct studies of the toxic effect of chemicals on rhizobial bacteria using agarized nutrient media.

As the dehydrogenase activity is one of the most important indices of the efficiency of rhizobia, it is recommended to amend the nutrient medium with 2,3,5-triphenyltetrazonium chloride (TTC) in a concentration of 0.01% to fulfill the role of indicators of dehydrogenase activity in bacteria. In this case colonies of active cultures are coloured red

due to reduction of TTC to triphenyl formazan.

For a rapid evaluation of the pesticide toxicity use may be made of various modifications of the diffusion method.

1.1. Technique of 'Slant Agar'

A melted agarized medium of 10 ml volume is added with 10 mg of the pesticide under study, thoroughly mixed in a test tube and transferred on a Petri dish placed in an inclined plane, as shown in Fig. 2.

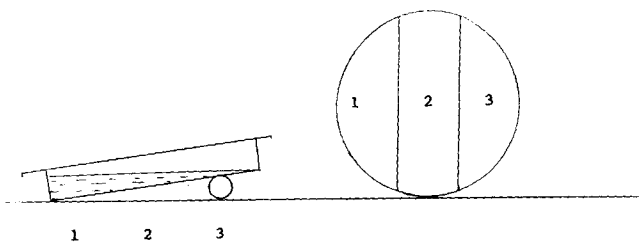
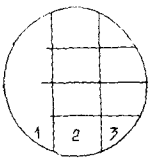


FIGURE 2. Preparation of the 'slant agar' for evaluating the bactericidal effect of pesticides.

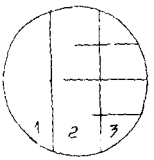
Upon the agar setting, the circle of the dish bottom is divided into three equal cuts marking them with China ink or a graphitic pencil. After this the dish is placed into a horizontal position and supplemented with 10 ml of agarized medium free of bacterial preparation. A 2-3 day old bacterial culture is inoculated by three parallel streaks which are perpendicular to the bottom-dissecting lines. Observations and microbial counts are performed in 3 days for the fast- and in 5 days for the slow growing strains of *Rhizobia*. Microbial growth is assessed from characters of the streaks and by their coloration with triphenyl formazan.

The absence of bacterial growth along the streaks in particular zones is indicative of the pesticide toxicity which is expressed as 1, 2 or 3 (Fig. 3). Based on such a 3-point scale evaluation, a conclusion is drawn on the character and advisability of further pesticide tests.



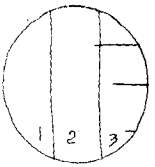
Inhibition of bacterial growth along Streak *1* (1 point).
Restricted use allowed for seed dressing.

The pesticide is studied for its effect on leguminous-rhizobial symbiosis in assays on vegetating plants.



Inhibition of bacterial growth along Streak *2* (2 points).
Not allowed for seed dressing.

The pesticide is studied for its effect on leguminous-rhizobial symbiosis by treatments of soil and vegetating plants.



Inhibition of bacterial growth along Streak 3 (3 points)
Not recommended for use on leguminous crop plantations.

FIGURE 3. Evaluation of the pesticide toxicity to *Rhizobium*

1.2. Method of 'Paper Discs'

One ml of a suspension of the 2-3 day old *Rhizobium* culture is inoculated on a Petri dish followed by addition of 15-20 ml of melted agarized nutrient medium cooled to 40°C. The content of the dish is agitated with circular movements and a slight balancing of the dish.

Discs of dense filter paper (10 mm in diameter) saturated with a solution (suspension) of a pesticide are placed on the surface of solidified agar.

Saturation is done by dipping them into 1% solution (suspension or emulsion) of the pesticide under test, then air-drying. The dishes are incubated in a thermostat at 27°C. In 3-5 days the diameters of sterile zones around the discs are measured (total diameter minus that of the disc).

Pesticidal toxicity is evaluated in the following manner.

1. Diameter of the sterile zone is greater than 10 mm: the pesticide is highly toxic and not allowed for use on leguminous crops.
2. Diameter of the sterile zone is within 5 to 10 mm: the pesticide is

moderately toxic and not allowed for seed dressing.

3. Diameter of the sterile zone is less than 5 mm: the pesticide displays a low toxic effect; allowed for restricted use on seeds and leguminous crop plantations by application to soil and to the vegetating plants.

1.3. Seed 'Dressing' Method

The chemical compounds aimed at dressing the pulse seed may be applied directly to seed. For this, the seeds are dressed with a toxicant in the dose and form required for production needs (water suspension, emulsion, powder etc.).

The seeds treated with the pesticide are evenly spread over, and slightly pressed in, the surface of agarized nutritive medium inoculated with selected strains of tubercular bacteria.

The inhibiting effect is evaluated from the size of the sterile zone formed around the seeds.

When seeds are polluted with an alien microflora, it is advisable to sterilize them for 2-3 days in the chloroform vapour or in some other manner.

The appearance of a sterile zone around seeds treated with a pesticide points to its inapplicability for dressing the pulse seed.

2. Assessment of the Pesticide Toxic Effect on the Efficiency of Leguminous-Rhizobial Symbiosis

Initial steps of studying the pesticide effect on the symbiosis of *Rhizobium* and leguminous crops are brought about by staging either vegetation experiments with sandy soil-based cultures or microbial growth tests on agarized media.

2.1. Microbial Growth Tests

For a rapid analysis of the effect of pesticides on the leguminous-rhizobial symbiosis of fine-seed pulses (clover, alfalfa, esparcet) investigation may be based on microvegetative tests. For this, 60 ml test tubes are filled with 30 ml of agarized nitrogen-free nutrient medium. The cotton-stoppered test tubes are sterilized in an autoclave.

Sowing is effected with the previously sterilized and germinated seeds followed by their inoculation with the strains of *Rhizobium* under study. A 2-3 days old bacterial culture containing about 10^8 cells/ml is used for inoculation. The seed without bacterial inoculation is used for control tests.

The pesticide under study is introduced into a melted agar nutrient medium cooled to 40°C.

2.2. Evaluation of the Nitrogenase Activity of Nodules

Intact roots with nodules or individual nodules separated from the plant rhizome are used for analysis of nitrogenase activity. In the first case, roots of an individual plant overgrown with nodules are placed into 20-100 ml flasks and plugged with rubber stoppers. To render the flasks air-tight, the rubber stoppers are pressed down by screwing the flasks with plastic lids with a perforation at the centre. 2-10 ml of acetylene (10% of the flask volume) are introduced into the flask a with a syringe through rubber stoppers and a one-hour incubation at 27°C follows. Then 2-10 ml of Nessler reagent are injected into the flask, and the ethylene produced is analyzed by gas chromatography using the flame ionization detector. Gas samples (0.1-1.0 ml depending on nitrogenase activity) from flasks are injected into the injector on a stainless steel column (4mm in inner diameter and 1 m long) packed with Chromaton containing 10% of β-oxydipropionitrile. Carrier gas - nitrogen, flow rate - 50 cm /min. The ethylene peak appears after 12 s. The nitrogenase activity is expressed in micromoles (μM) or nanomoles (nM) of ethylene produced per one plant per hour and derived from the following relation:

$$C_2H_4 = \frac{H_s C_{st} V}{H_{st} V_s}$$

where, H_s is the height of the sample's ethylene peak, mm; H_{st} is the height of chromatographed ethylene standard, mm; V_{st} is the volume of ethylene standard, ml; V_s is the volume of gas sample, ml; V is the flask volume, ml.

Assessment of the Effect of Chemicals on Nitrogenase Activity

Nodules isolated from plant rootlets are used in experiments aimed at evaluation of the direct effect of chemicals on a set of enzymes bringing about fixation of molecular nitrogen. For this purpose, plants of leguminous crops inoculated with the strains of the *Rhizobium* under study are selected during the bud-blooming phase. The plant rhizome is cleared of soil subsrate and washed with water. Nodules are separated from rootlets and immediately placed on Petri dishes filled with a solution of the studied pesticide. Production-required concentrations of toxicants are used, in some cases 2-10-fold and 50-fold dilutions are preferred. Water-immersed nodules are used as controls.

Both the control and experimental nodule-containing solutions are incubated in a chamber for one hour at 2 to 5°C. After incubation the solution is poured out and the nodules spread on filter paper are slightly dried. Nodule weights of 200 mg are placed in 20 ml flasks and the activity of nitrogenase evaluated from the rate of acetylene reduction, as described above.

To ensure reproducibility of results, 8 to 10 parallel experimental runs are carried out. The nitrogenase activity is expressed in nanomoles (M) of ethylene per 0.2 g nodule weight and assessed as percentage of he control.

3. Nutrition Media

3.1. Pea broth (for fast-growing nodule bacteria):

Pea water	- 1 litre
Sucrose	20 g
Agar	15 g

Preparation of pea broth: a 100 g weight of hulled peas is covered with tap water and boiled for 40 min, then cooled and filtered through a multilayer gauze. The pea water filtrate is diluted to 1 litre and further used for the medium preparation.

Nutrition media are sterilized for 20 min at 1 atm.

3.2. Lupine flour broth (for bacteria exhib

Lupine flour water	-	1.0 litre
KH_2PO_4	-	0.5 g
K_2HPO_4	-	0.5 g
$MgSO_4 \cdot 7H_2O$	-	0.2 g
NaCl	-	0.2 g
$CaSO_4 \cdot 2H_2O$	-	0.1 g
$(NH_4)_2MoO_4$	-	trace
Mannitol	-	20.0 g
Agar	-	15.0 g

Preparation of lupine flour broth: lupine seeds are germinated in a cuvette, dried at 60°C and ground. A sample (10 g) of lupine flour is covered with tap water (1 l), boiled for 40 min, cooled and passed through a cotton-gauze filter. The filtrate is diluted to 1 l volume and used for preparation of nutrient media.

Lupine flour-based media are sterilized for 20 min at 0.5 atm.

3.3. Mannitol-yeast medium (for nodule bacteria exhibiting slow grow

Mannitol	-	10.0 g
KH_2PO_4	-	0.5 g
$MgSO_4 \cdot 7H_2O$	-	0.2 g
NaCl	-	0.1 g
$CaCO_3$	-	1.0 g
Yeast extract	-	10.0 g
Tap water	-	1.0 litre
Agar	-	15.0 g

Sterilization is effected in an autoclave for 20 min at 0.5 at

3.4. Nutrient media for plants (in vegetation experiments)

Gelrigel's medium:

Solution 1.

$Ca(NO_3)_2$ (anhydrous)	-	0.049 g
$FeCl_3 \cdot 6H_2O$	-	0.025 g
KCl	-	0.075 g
H_2O	-	1 litre

Solution 2.

KH_2PO_4	-	0.126 g
$MgSO_4 \cdot 7H_2O$	-	0.123 g
H_2O	-	1 litre

2.2 ... of microelements: prepared as for 1 litre ... but only one ... for the medium composition)

$ZnSO_4 \cdot 7H_2O$	- 0.022 g
$MnSO_4$	- 1.810 g
$Na_2B_4O_7 \cdot 4H_2O$	- 2.630 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	- 1.630 g
$Co(NO_3)_2 \cdot H_2O$	- 0.62 g
H_2O	- 1 litre

ACKNOWLEDGMENTS

The authors wish to thank the members of the Scientific Advisory ...mittee for the UNEP project "Microbial Technologies for Degradation ... Persistent Pollutants" and the participants in the 1988 training ...se "Microbial Degradation of Xenobiotics" for comments and valuable ...ussions. Special thanks go to Prof. Roy Greenhalgh who kindly ...epted to read and edit the English version of the manuscript.

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2,10.90 г. Тираж 515 экз. Зак. 2901Р. Уч.-изд.л. 4,9.
Усл.печ.л. 6,75. Изд. № 242. Цена 1 р.

Отпечатано с оригинала-макета в Отделе научно-технической информации Научного центра биологических исследований АН СССР в Пущине

1p.