1.
Introduction

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INTERPRETATION OF ENVIRONMENTAL FATE AND BEHAVIOUR DATA FOR REGULATORY PURPOSES

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ABSTRACT

The need to evaluate the risk to the environment from the use of chemicals has been a significant part of Regulations to control pesticides for many years in the UK and elsewhere. However, the data generated are not always scientifically the most useful and the fundamental principles involved with pesticide transport in soils is not sufficiently well understood to enable a more quantitative risk assessment to be undertaken. This paper presents a possible procedure for the selection of studies required and proposes a decision tree for the initial assessment of the risk of contamination of ground-water from the use of pesticides. The proposal is intended to stimulate further discussion in this important area and to encourage all interested parties to develop the necessary procedures along the most relevant lines.

INTRODUCTION

Regulatory control measures over the use of pesticides can be applied both prior to and after the marketing of a pesticidal product. Approval to market a product, however, is granted only if there is no unacceptable risk to the environment, consumer or operator and relies on the predictive capability of the regulatory scientist and the usefulness of the data generated for that purpose. Lack of understanding of the mechanisms involved in environmental fate and behaviour in the past has prevented good interpretation of data. It is clear that water and soil have become contaminated with certain pesticide residues although in almost all cases they are not toxicologically significant. In response, the UK Government and the EC have made it clear that environmental risk assessment must be given more attention than perhaps it received in the past. Clearly, there needs to be a more concerted effort to generate procedures, decision trees, mathematical models and the necessary data to validate these models and decision procedures.

The development of risk assessment procedures and fundamental research to support such assessments have not always been co-ordinated which has resulted in the generation of data not always directly relevant to that required for pesticide registration. Furthermore, the general principles involved with the assessment of the fate and behaviour of pesticides in soil and water are not universally accepted by regulatory authorities, environmental groups or the Agrochemical Industry.

To put the process of environmental fate and behaviour evaluation into context it is necessary to examine the development of procedures in other areas of the pesticide data package assessment. For example, the estimation of the exposure of operators to pesticides during application is comparable to the problems faced by regulators with the environmental data package. Such exposure estimation during mixing, loading and use of pesticides has

become accepted practice by means of the use of a predictive model (Martin, 1990). This model uses assumptions based on drift, contamination and penetration derived from studies to estimate pesticide deposition. Clearly the use of general assumptions is the weakness of many models but by evaluating the data through an iterative process the general assumptions can be replaced by more relevant data. It is envisaged that over the years those data generated to support certain uses will be fed back into the model and the assumptions will replaced by more realistic values. Furthermore, it is hoped that these would be used to predict the probability of the likely contamination. By this means the model and procedures adopted can evolve into a useful and relevant scheme rather than become a "*check list*".

There are similar schemes adopted for the assessment of consumer risk from residues in food. Both the accepted principles associated with the monitoring of data in crops and the continuously improving model used for predicting operator exposure have certain attractions which could be usefully used in estimating the fate and behaviour of pesticides and the interpretation of such data for regulatory purposes.

One complicating factor has emerged which has changed the regulatory principles adopted. This is the EC Directive on Drinking Water (80/778/EC) which has set a maximum figure of 0.1 µg/l for individual pesticides in water used for drinking. This figure is independant of any toxicological assessment and does not represent *risk*. Nevertheless it is Government policy to control the use of pesticides in such a way as to reduce the level of pesticide contamination to acceptable levels (HMSO, 1990).

It is the intention of this paper to propose a decision making procedure for the first steps in evaluating the potential to contaminate ground-water. In doing this, however, it became clear that before data could be interpreted, more relevant studies had to be selected and these had to be validated. Clearly, guidance needed to be given in three distinct area. Firstly, to generate the most relevant data a number of simple questions, to establish objectives, should be posed by both the regulators and registrants alike. The purpose of which should not be just to satisfy the regulatory requirements but to assess the safety of the pesticide in use and the need for further data. Secondly, the quality of the studies should be evaluated to determine the validity of the results. Thirdly, decision making procedures or trees should be developed to determine likely regulatory action or further data requirements.

REGULATORY OBJECTIVES

It has become clear over the years that those involved in regulatory matters, either providing the data packages or evaluating the data, have tended not to ask the most relevant questions. Therefore, the most relevant data have not been generated. Furthermore, the most relevant data requirements have not always been drafted and adopted by National and International bodies. In its simplest form, the following basic questions should be asked by the regulatory scientist or those involved in research and development, in order to select the most relevant data.

[i] Is the active ingredient (ai) or degradation product so persistent that it presents a risk to the consumers of following crops?

[ii] Is the ai or degradation product so persistent that it is likely to have an adverse effect on following crops?

[iii] Are the concentrations in soil likely to present a risk to soil organisms?

[iv] Are the predicted concentrations in water resulting from leaching or run-off likely to present a risk to aquatic organisms?

[v] Are the predicted concentrations in ground- and surface-water likely to present a toxicological risk ?

[vi] Are the predicted concentrations in ground- and surface-water likely to exceed the EC Drinking Water Directive limit of 0.1µg/l?

Examination of the objectives will highlight the obvious need to assess the data in a sequence of decision making steps. This stepwise or tiered approach has become the normal procedure employed by authorities operating under policies based on science. It is impossible to provide all of the data initially as the subsequent tiers of data generation rely on the results of previous studies. However, the data presented at each stage needs evaluating which requires an expert judgement. Clearly, computer models can be used at each stage of the evaluation but currently a such systems are not universally accepted. They are often based on broad assumptions and are often designed for different purposes or to fit a specific study. Nevertheless, almost all registration authorities carry out some sort of assessment which could be approximated by use of a single or more likely a number of models. It is hoped that those involved with modelling will see these objectives as falling within their area of interest and design systems which can be of use to regulators and not just to satisfy narrow fields of interest.

To meet the objectives presented above a basic or minimum set of studies is required on which to conduct the initial assessment and decide whether Approval is possible and to identify further data requirements. The following studies comprise the minimum data package for environmental fate and behaviour. Obviously similar minimum requirements exist for other areas of the data package such as the likely effect of the predicted level of residues on wildlife.

(i) *Basic physico-chemical properties*

M.P., V.P., P_{ow}, water solubility, pKa if appropriate;

(ii) *Laboratory degradation studies*

It is essential to conduct these laboratory studies on fresh soil, ideally the microbial activity should be measured at the start of the experiment.

(a) A soil metabolism study using radiolabelled material, in which good recovery of the radiolabel is obtained and which elucidates the stucture of all the major degradation products. It is adequate to conduct this study on one soil type.

(b) A soil dgradation study should elucidate the rate of degradation on a range of soil types including those representative of the major areas of use. Ideally this study should demonstrate the variation of rate with soil temperature and moisture. It should not be conducted over a period of greater than 100 days.

(iii) *Soil mobility studies*

Clearly if the active ingredient degrades very rapidly, this type of study may not be required, but should be performed on any stable principle degradation product. Study type (a) is the preferred option.

(a) A batch equilibrium soil sorption study on a range of soil types and active ingredient concentrations. The range of soil types should include different organic matter contents, clay contents and soil pH, including soils representative of the major areas of use. Ideally desorption measurements should be made.

(b) Soil column studies or soil thin layer chromatographs similarly covering a range of soil types.

(iv) *Fate in water*

(a) A sterile hydrolysis study elucidating the rate and route of hydrolysis of the active ingredient. The experiment must cover a range of pH values, usually 5, 7 and 9. It is acceptable for other pH values to be used, provided that a range typical of environmental values is covered. It is acceptable for a single temperature to be used.

(b) A sediment/water study demonstrating the fate, of the active ingredient in natural sediment and water. Clearly this study will not be required if hydrolysis is rapid.

(v) *Field degradation studies*

These should be performed in the UK or a region of similar climate. The concentration of active ingredient and principle degradation products at different depths should be measured to elucidate the rate of degradation under field conditions. Preferably this should be conducted on bare ground. When field studies are not conducted in the UK, arguments should be presented to demonstrate how the submitted data relates to UK conditions.

(vi) *Methods of analysis*

Appropriate methods of analysis for both soil and water are required, these should include details of the limit of determination and the recovery from fortified samples, ideally sample chromatograms should be included.

(vii) *Further studies*

To elucidate any inconsistencies in the data submitted or to further define the degradation/dissipation routes, these studies might include photolysis, leaching and volatilisation.

EVALUATION OF DATA

At a recent symposium held by the British Institute for Regulatory Affairs (in press), examples of three current pesticide products were used to illustrate the basic decisions which could be made. The regulatory objectives were outlined and data were assessed using the package of studies presented to the UK regulatory authority generated for the initial evaluation. It was clear that little thought other than to provide the National authorities with data went into the data packages. Often many more studies than were absolutely necessary were conducted or studies with little relevance were submitted.

Therefore, there is some merit in explaining the procedures involved with the actual assessment of a study report. An aerobic metabolism study has been used as an example. In assessing the quality of an acceptable report the following will need to be considered and reported.

Procedure

Test substance	-	purity/radiochemical purity
	-	position of radiolabel
Test soil	-	characterisation, eg. sand, silt, clay, pH, CEC, organic matter
	-	importance of fresh soils - assays for microbial biomass
	-	effects of mixing, drying, addition of solvents
	-	a range of soils to determine rate, one soil to determine route
	-	area of use; eg. may be glasshouse crops which may be grown on peaty soils
Study apparatus	-	open or closed
	-	air flow moist, CO_2-free air
	-	traps for organics and CO_2
Treatment rate	-	field rate and higher rates; with a soil density of 1.3 kg/l, 1g/ha is equivalent to 0.5µg/kg incorporated into the top 15 cm (not appropriate for immobile compounds)
	-	Variation one order of magnitude
Study conditions	-	timing of application
	-	temperature
	-	moisture content
	-	replication
	-	duration of study need not exceed 100 days
Sampling	-	frequency should be reported
Analysis	-	storage prior to analysis
	-	extraction procedure
	-	what was measured
	-	how it was measured

Results

Recoveries	-	95-105%
Degradation rate	-	often reported as first order kinetics
	-	sometimes biphasic
	-	sometimes has a lag period
Degradation route	-	volatile component
	-	extractable component; characterisation and quantification of parent and degradation products
	-	bound component; quantification and mechanism

INTERPRETATION OF DATA

To present on paper decision trees to satisfy all of the regulatory objectives that need answering would be too complicated for the purposes of this paper. Furthermore, many of the decisions would be based on assumptions which need further discussion and validation before they can be of general use. Of all the regulatory questions to be asked, the potential to contaminate ground-water must require the most stringent assessment (objective [vi]). Satisfying this objective will provide the basic assessment for any of the other objectives. The one problem area which has not been addressed directly is the potential to contaminate surface waters. It is assumed as a starting point that ground-water is the most vulnerable to long-term contamination and receives less treatment before consumption. Surface-waters tend to become contaminated following rainfall events resulting in episodal peaks. However, it recognised that the concentration in surface-waters will depend more directly on the quantity used in any single catchment and is, therefore, extremely difficult to predict.

Therefore, the decision making process required to satisfy objective [vi] has been used to illustrate the proposed procedures that should be adopted by regulatory authorities and registrants. Similar schemes will be necessary to assess the data to satisfy the other regulatory objectives. In addition, each decision tree will need some cross-linkage to others for the final regulatory action.

The scheme to assess potential ground-water contamination is based initially on the properties of the pesticide. Clearly, soil, climate and hydrogeology are important factors to take into consideration at later stages of the sequential assessment. Final regulatory decisions may allow the use of a product but a restriction on the area of use to reduce potential contamination may become a condition of the Approval. It is inevitable that to achieve the aims of proposing a decision tree a number of assumptions have been made. It is assumed that the levels of parent compound of 0.1µg/l do not present a toxicological risk. If they do, further regulatory action will be necessary to prevent such contamination. Also the scheme assumes that compounds with long half-lives in soil will be stable in ground-water.

The trigger points in the proposed scheme have been based broadly on the procedure of Gustafson (1989) but amended in the light of the current regulatory proposals from the US EPA and the German authorities. The format is one that has been proposed by J A Jobsen for the EPPO/Council of Europe Panel on Environmental Risk Assessment of Plant Protection Products.

PROPOSED DECISION TREE FOR THE ASSESSMENT OF THE CONTAMINATION OF GROUND-WATER

1]	What is the half-life of the compound (parent or degradation product)?	- very short <5days - otherwise	-go to 3] -go to 2]
2]	What is the mobility of the compound?	- very low, K_{oc} >4000 - otherwise	-go to 3] -go to 4]
3]	**No further work required**		-go to 11]
4]	How mobile is the compound?	- K_{oc} <500 - otherwise	-go to 5] -go to 7]
5]	How persistent is the compound?	- t_{50} >100 days - otherwise	-go to 6] -go to 7]
6]	**Unlikely to be granted a commercial level of Approval.** Extensive field data will need to be generated.		-go to 11]
7]	What is hydrolysis rate?	- t_{50} <21 days - otherwise	-go to 3] -go to 8]
8]	Application rate and timings (or predicted environmental concentrations of metabolites)?	- low risk- low rates; Spring applications; good ground cover by plants - high risk- high rates; Autumn applications; bare ground	-go to 9] -go to 10]
9]	**Commercial level of Approval could be issued on condition that lysimeter or field study be conducted to confirm expected fate and behaviour**		-go to 11]
10]	**Commercial level of Approval unlikely to be granted before results of lysimeter or field studies evaluated**		-go to 11]
11]	Are metabolites or degradation products formed in significant quantities?	- No - Yes	-go to 13] -go to 12]
12]	Is metabolite or degradation product toxicologically significant?	- No - Yes; repeat assessment on metabolite	-go to 13] -go to 1]
13]	**Assessment of fate and behaviour data complete. Make final regulatory decisions in context of Regulatory Questions**		

DISCUSSION AND CONCLUSIONS

It is hoped to develop similar schemes for other areas of risk assessment. For the exposure of aquatic organisms a scheme has already been developed based on the concentration resulting from accidental overspraying of water of 1 metre depth at the maximum field rate. Generally, exposure from drift, run-off and leaching will be very much less than from overspraying. However, registrants should take into account all possible inputs to estimate exposure when addressing the regulatory objectives. The following groups of compounds require special attention.

(i) Compounds that are extremely toxic to aquatic organisms

(ii) Very mobile compounds

(iii) Compounds and formulations prone to run-off (ie. persistent compounds and soil applied wettable powders)

The approach using simple questions to make environmental risk assessments can be readily extrapolated to other areas of the data package. Therefore, the following questions may be relevant to the overall risk assessment.

(i) Will the pesticide inputs to the aquatic environment be bound to soil particles?

(ii) Will pesticide residues be desorbed from particles into water? Adsorption/desorption studies and water/ sediment studies may provide useful information.

(iii) What effects will bound residues have on filter feeders and sediment dwelling organisms? An area for further research would be a study using a more appropriate sensitive organism

(iv) Do hydrolysis products present risks? Comparison of static and dynamic tests may be of some value.

The basic environmental exposure questions asked by regulatory authorities can be answered if the necessary data on how the concentration of a pesticide in soil, ground-water and surface-water changes with time. It is possible that a number of models could be developed to address the principle areas of concern such as persistence and leaching. These models would have to integrate pesticide properties, application rates and timings, climate data and soil types. Ultimately, such models may provide a framework for consistent regulatory decisions on at least a National basis.

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USING SOIL RESIDUE DATA TO ASSESS THE ENVIRONMENTAL SAFETY OF PESTICIDES

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ABSTRACT

The safety of a pesticide can only be assessed if one has a knowledge of both the sensitivity of organisms and their degree of exposure to the pesticide or its biologically active degradation products. Information on soil residue levels, persistence and mobility is essential in order to estimate the short and long-term exposure of organisms. Soil organisms are generally exposed to much lower residues than those above ground due to the huge dilution in soil and soil often acts as a physical barrier between the highest concentrations eg. at the soil surface and the organism. The determination of exposure levels, toxicity, safety levels and regulations is presented for plants, microorganisms, earthworms and arthropods.

INTRODUCTION

Soil residue data are essential when assessing the exposure of organisms to pesticides, but alone can not be used to assess the environmental safety of a pesticide. The safety, or conversely hazard, depends on both the sensitivity of an organism and the degree of exposure of the organism to the chemical. This is usually expressed in a simple form, such as,

Hazard = function of toxicity x exposure

and is one of the fundamental points included in any good course on human or ecological safety assessment. It is incorrect, and indeed dangerous, to ignore this fundamental principle. Those responsible for determining the safety of pesticides should not set 'safety levels' based solely on the concentration or persistence in soil without reference to the toxicity of a chemical. Otherwise there is a risk of chemicals present in low concentrations or having low persistence being classified as 'safe' when in practice they could be dangerous due to having a high toxicity. Conversely, it could result in the rejection, and thus loss of benefits, of chemicals with a lower toxicity but present in higher concentration or with longer persistence. Similarly it is ridiculous to set arbitrary limits, such as 0.1 µg/litre, for pesticides in drinking water. It is obviously equally invalid to regulate pesticides solely on their toxicological properties.

This paper considers the assessment of safety of pesticides to organisms living on or in soil, taking into account both exposure and toxicity; including plants, microorganisms, earthworms and arthropods.

PLANTS

The ability of soil to support plant growth is vital to man and most forms of terrestrial wildlife because plants provide food (either directly or indirectly) and in many cases a suitable physical environment eg. shelter.

Exposure

To estimate the initial exposure of plants to soil residues one requires a knowledge of the concentration, location and availability of the chemical as well as the important routes of uptake by the plant eg. via germinating seed, roots or emerging shoot.

The initial concentration of a pesticide is estimated from the application rate, taking into account crop interception and depth of soil containing the pesticide. For pesticides applied when there is only a sparse plant cover it is prudent to assume 100% reaches the soil; whereas if there is close to 100% plant cover it is reasonable to assume 50% reaches the soil either directly, or washed off the plants by rain. The percentage reaching the soil can be considerably less if the chemical is rapidly degraded on plant foliage. One hectare of soil 10 cm deep, weighs about one million kilograms; therefore, 100 g/ha reaching the soil is equivalent to approximately 0.1 mg/kg in the top 10 cm soil.

The change in exposure of plants to soil residues with time is controlled by both the rate of degradation and rate of movement. These can normally be reliably determined in laboratory studies. However, in cases where the safety margin is small or persistence long (half-life greater than six months), it might be necessary to measure persistence and movement under field conditions. Residue levels following multiple applications are estimated using mathematical models. It should be noted that even if a chemical has a half-life of a year the maximum amount of residue in the soil will only be twice the application rate. If safety levels to plants are marginal the result of these calculations should be checked by monitoring residue levels following repeated use under normal field conditions.

The maximum concentration of residues remains in the top few cm soil, other than for mobile chemicals (adsorption coefficient <10) with long persistence (Table 1), or of course, if the soil is cultivated. The effect of distribution on level of exposure of plants, and hence effect on plants, is difficult to predict and is best taken into account by using an appropriate distribution in the plant bioassays (see below). This also takes into account the different routes of uptake by plants.

The availability of pesticides in soil to plants is proportional to their concentration in the water and/or air phases (Riley and Morrod, 1976). The effect of adsorption on availability can be estimated from adsorption coefficients. It can also be taken into account by carrying out the plant bioassays in soil rather than solution cultures.

TABLE 1. Relationship between pesticide leaching and solid/solution soil partition coefficient K_d (Riley, 1978)

K_d	Distance surface applied pesticide leached if rain exceeds evapo-transpiration by 25 cm assuming no degradation	EXAMPLES OF PESTICIDES Name	Use	K_d (typical value in loam soil containing 3% organic matter)
0.1		Picloran	Herbicide	0.3
1	Much of pesticide leached through top 20 cm soil into subsoil	2,4-D	Herbicide	1
		Atrazine	Herbicide	3
10	Much of pesticide leached into soil but peak concentration in top 20 cm soil	Diuron	Herbicide	10
		Ethirimol	Fungicide	15
		Lindane	Insecticide	50
10^2	Only small amount of leaching and peak concentration normally in top 5 cm soil	Parathion	Insecticide	2×10^2
10^3				
10^4	No significant leaching	DDT	Insecticide	10^4
$>10^4$		Paraquat	Herbicide	$>10^5$

Plant sensitivity

Initially, bioassays are carried out on young plants in the glasshouse or CT room because young plants are more sensitive to soil residues than mature plants and the difficulty of growing plants to maturity indoors. Plants grown indoors are normally more sensitive to chemicals than in the field (Brown and Farmer, 1991).

The chemical is applied to soil to take into account the effect of soil on availability. However, the soils chosen should be representative of soils with relatively low adsorption properties eg. less than 5% organic matter content. The chemical is normally either sprayed onto the soil surface or mixed into the soil, depending on the expected distribution of the chemical following normal use. For herbicides it is useful to include rates down to 1% of the normal use rate so that results can be used to evaluate the safety from spray drift as well as in the treated area. Several species of plants should be tested, representing major groups of plants eg. monocots and dicots.

One of the areas of greatest debate in plant testing is what type of assessments should be made on the plants. Percentage emergence and plant weight (dry and wet) are frequently measured because they are quantitative and amenable to statistical analysis eg. EC50 and no significant effect levels can be calculated. However, they are not always the most sensitive indicator of effects and visual assessment of plant growth, eg. on a 0 - 10 scale is sometimes preferred (Brown and Farmer, 1991). Visual assessments can take into account effects such as plant morphology and colour.

Safety assessment

The safety of soil residues to plants, following normal use, is assessed by comparing the expected concentration in soil with the plant toxicity data. If the expected concentration is less than one tenth of the EC50 value of the most sensitive species, then it is unlikely that residues will cause appreciable damage to plants under field conditions. Similar exposure/toxicity ratios are used when assessing the safety of pesticides to birds, mammals and aquatic life. For example, the US EPA criteria is that there is presumed to be no risk to birds, mammals and aquatic life if the expected environmental concentration is <1/5, <1/5 and <1/10 of the LC50 values, respectively (Urban and Cook, 1986).

If the safety margin is low, then the need for field studies, under a range of conditions, should be considered. Obviously it is not possible to test every species of plant, therefore it is prudent to keep watch for effects on unexpectedly sensitive species during normal use of a pesticide.

Regulation

Regulatory authorities vary in their attitude to requiring plant data. Only a small number, most notably the US EPA, formally request such data. Most authorities take the view that plant safety is self regulating; ie. damage to plants is visually obvious and that pesticide companies will carry out sufficient work to ensure their products do not damage non-target plants. If they do not do that, they suffer the severe penalty of having to withdraw a product from a particular use (severe financial penalty) coupled with a risk of having to pay compensation for crop damage and/or loss of company image, both in the eyes of farmers and the general public.

MICROORGANISMS

The amount of living matter in soil is dominated by microorganisms when measured in terms of weight (Table 2) and even more so in terms of numbers. They carry out many important functions in soil. Therefore, it is necessary to consider the safety of pesticides to these organisms. A group of experts have met regularly over the last 20 years and have produced recommendations for test methods and methods of interpreting the results from the tests.

TABLE 2. Typical weights of living organisms in fertile soil

Organism	Weight (kg/ha)
Microorganisms	2000 - 20000
Earthworms	100 - 1000
Arthropods	100

Exposure

Exposure levels are estimated in the same way as for plants. The exposure levels used in the initial microbiology test are the recommended and ten times the recommended field rate (kg ai/ha) expressed as mg/kg (dry weight) soil, assuming uniform distribution in the top 10 cm of the soil and a bulk density of 1.5 (Gerber et al., 1989). Because of the general lack of appreciable effects in these tests it is not normal practice to test a wide range of dose levels to make it possible to calculate EC50 values.

Microbiological tests

The tests are based on measuring effects on the functions carried out by microorganisms in soil, rather than on the microorganisms themselves. The two main groups of tests recommended are effects on soil respiration (evolution of CO_2) and nitrogen transformations (ammonification and nitrification). Details are given by Gerber et al., 1989.

Safety assessment

If there is no effect at ten times the normal exposure level in the laboratory it is presumed there will be no effects under field conditions. Furthermore, effects due to pesticides should only trigger a need for further field testing if they are greater in magnitude and duration to those caused by natural factors, such as changes in soil moisture levels or temperature. Depressions of 50% or more frequently occur under natural conditions. The doubling time of microbial cells under field conditions is probably about ten days and recovery periods of 30 days following an approximately 90% depression are still a normal and natural phenomenon (Gerber et al., 1989; Sommerville et al., 1985). Thus these expert groups concluded that a chemical stress should be regarded as being of 'no ecological significance' if recovery occurs within 30 days and can still be 'tolerable' if recovery occurs within 60 days; delays in recovery of more than 60 days may indicate a 'critical' situation and require further investigation in field tests.

Even if an effect occurs, careful consideration is required to decide if the effect is harmful, beneficial, or of no consequence. Inhibition of conversion of nitrite to nitrate would be considered harmful because it would result in an undesirable build-up of nitrite in soil. However, reduction in rate of conversion of ammonium to nitrite/nitrate could be

considered beneficial because it would reduce nitrate leaching into groundwater, due to the much lower mobility of ammonium ions. Indeed, much effort has been spent on searching for products to inhibit the conversion of ammonium to nitrate and a small number of products, such as nitrapyrin, are marketed specifically for this purpose.

A reduction in the rate of mineralisation of organic matter in soil could also be considered beneficial if it helped to counteract the stimulation of breakdown of organic matter caused by the physical cultivation, which has resulted in a 50% decline in the organic matter content of some soils. Increased levels of organic matter would improve soil structure and water retention properties. It could also make a contribution to reducing the rate of increase of CO_2 in the atmosphere (Table 3) by increasing the amount of C 'fixed' in soils and litter, which is estimated to be of the order 1300 - 1400 x 10^{15} g C (Cannell, 1990).

TABLE 3. Estimated input of CO_2 into atmosphere (from Cannell, 1990)

Source of C	Input into atmosphere (10^{15} g C/year)
Soil organic matter/litter	60
Burning of fossil fuels	5
Deforestation	1

Regulation

Regulatory authorities in some countries request data on the safety of pesticides to soil microorganisms eg. Denmark, while others do not eg. USA EPA. Extensive testing has shown soil microorganisms to be robust and pesticides, other than extreme treatments for soil sterilisation, do not affect soil microbial processes. On the one hand such data is reassuring for the public, regulators and pesticide companies. On the other hand are such tests a sensible use of scientific manpower and resources?

EARTHWORMS

In some regions, particularly Europe, earthworms are considered to be very beneficial. They can improve the physical condition of soils and are a source of food to other wildlife. They are also considered as one of the most suitable soil animals to be used as a key bioindicator for testing for pollution of soils (Edwards, 1984).

Conversely, on some ornamental lawns and sports turf there is a desire for products to eliminate earthworms, because of the undesirability of surface soil casts.

Exposure

The same methods of assessing exposure levels are used as for plants and microorganisms.

Earthworm tests

Considerable effort has been devoted to devising methods for measuring the toxicity of chemicals to earthworms eg. by exposing them to residues on filter paper or in soil (Edwards, 1984; OECD, 1984). As with plants, soil tests are preferred because they take into account the effect of the soil on availability, and thus activity, of the chemicals. A suitable artificial loam soil has been developed (Edwards, 1984) and the most commonly used indicator species is Eisenia foetida, because of its ease of culturing in the laboratory. A range of concentrations mixed into soil are normally tested and EC50 and No Observed Effect Levels (NOEL) calculated. Test methods are described in the OECD guideline, 1984.

Safety assessment

If the NOEL is greater than the expected environmental concentration then it is unlikely the pesticide will have an appreciable effect under field conditions. However, if the safety level is marginal, then methods are available for measuring the safety of pesticides to earthworms under field conditions (eg. Edwards and Brown, 1982).

There are no guidelines on the size and duration of effects which should be considered to be ecologically significant. The ecological significance of effects can only be judged by comparing them with the effects of natural factors and other agricultural practices. Changes in soil cultivation practices (Table 4) or plant cover (Fig 1) can have major effects on earthworm populations. Most pesticides, used at normal rates, are not harmful to earthworms, and therefore present no interpretation problems.

TABLE 4. Earthworm populations in direct drilled (zero tillage) or ploughed clay soil (Ellis and Barnes, 1977)

	Number of Earthworms/m^2		
Year	Direct drilled	Ploughed	Direct drilled / Ploughed
1973	145	110	1.3
1974	345	218	1.6
1975	231	98	2.4
1976	197	50	3.9

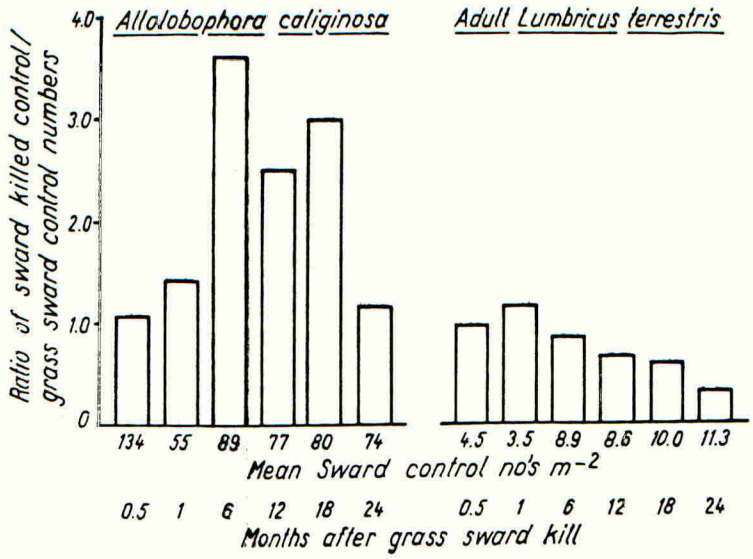

Fig. 1. Response of two common species of earthworm, A. caliginosa and L. terrestris, to grass sward removal (Edwards and Brown, 1982).

Regulation

Requests for earthworm data are mainly from regulatory authorities in Europe, but there is a growing trend to ask for such data.

SOIL ARTHROPODS

Some soil arthropods can play important roles, such as breakdown of plant remains, control pest arthropods by predation or parasitism and are a source of food to some wildlife. However, experimental methods are still under development and only briefly considered in this paper. For pesticide sprays, the highest concentration of residues will be at the soil surface immediately after spraying. Nevertheless, the effect of soil surface residues on arthropods such as carabid beetles and spiders is less than when they are sprayed directly (Brown et al., 1990).

Although the importance of some soil arthropods is recognised, most authorities, with only a small number of exceptions, such as Germany, do not routinely request data on soil arthropods because of the lack of proven methodology and ability to interpret the results of such tests.

DISCUSSION AND CONCLUSIONS

There is a wealth of methodology for determining the fate of pesticides in soil and results from these tests can be used to estimate the short and long-term exposure of organisms living on or in soil. There are also many methods available for assessing the toxicity of soil residues to plants, microorganisms and earthworms but there is a need to evaluate methods for testing their effects on soil arthropods.

Lack of effects in laboratory tests at the expected concentrations from normal field use is normally a reliable indicator of no effects under field conditions.

Soil residues of most pesticides, at levels resulting from normal use, generally have no appreciable effect on organisms living in soil, with the exception of those designed as soil insecticides or soil applied herbicides. This is perhaps not surprising considering the huge dilution of residues in soil (Table 5) and soil often acting as a physical barrier between the highest concentrations, eg. at the soil surface, and the organism. In the few cases where there are effects there is a need to improve our ability to decide if they are of any ecological significance.

TABLE 5. Typical environmental concentration immediately after application of 0.5 kg ai/ha

Material	Residue mg/kg
5% granule/bait	50,000
Cereal seed dressing	50-2000*
Grass	100
Leafy crop	50
Grain	5
Soil (10 cm deep)	0.5

* equivalent to approximately 5-100 g ai/ha.

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2.
Microbial Aspects of Persistence

Session Organiser and Chairman: MR DAVID SUETT

ENHANCED DEGRADATION OF PESTICIDES : ITS BIOCHEMICAL AND MOLECULAR BIOLOGICAL *BASIS*

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ABSTRACT

This paper reviews the biochemical and molecular biological basis of pesticide degradation with special reference to those pesticides undergoing enhanced or accelerated degradation especially in soil. It examines the importance of mobile genetic elements in the acquisition by microorganisms of novel catabolic genes encoding the breakdown of pesticides and summarises the contribution which genetic studies may make to the ammelioration of pesticide pollution and the field problem of enhanced pesticide degradation.

INTRODUCTION

The catabolic abilities of present-day micro-organisms are the result of some four thousand million years of evolution during which microorganisms have been presented with a profusion of natural organic compounds of widely-differing complexity and biological recalcitrance. One estimate of the annual turnover of biogenic material quotes a figure of 2×10^{11} tons, a large proportion of which is in the form of biopolymers and hydrocarbons. In contrast, the global annual production of synthetic organic chemicals - which has become significant only in the last 50 years - is of the order of 2×10^{8} tons, only 0.1% of the total organic matter in the biosphere (Leisinger, 1983). Virtually all molecules of biogenic origin are readily degraded by microorganisms, provided the environmental conditions are suitable; yet the sheer diversity of chemical structures available in natural products and the lack of persistence of these compounds implicates an equivalent versatility in the catabolic capabilities of competent organisms. While many synthetic organic compounds may be ultimately derived from natural precursors by the agencies of the organic chemist, such compounds frequently contain functional groups and partial structures found rarely in natural products and they are much more persistent when introduced into the environment. Such biological recalcitrance is the precursor of bioaccumulation and biomagnification. Nitro, sulphonate and halogen substituents markedly reduce the turnover of aromatic compounds and require the evolution of specific biodegradative mechanisms that eliminate or modify these substituents at an early stage in catabolism before the mineralisation of the molecule can be effected. Similarly, N- and S-heterocycles, present in many agrochemicals rarely degrade where they are required to provide sole sources of carbon and energy for micro-organisms but they are often successfully degraded as N- and S-sources (Cook and Hütter, 1981).

In the present atmosphere of the Earth, with its abundance of oxygen and in which the most stable compound of carbon is CO_2, all more-reduced organic compounds are thermodynamically unstable and, on thermodynamic grounds, cannot be excluded from serving as putative energy sources for aerobic microorganisms by which they would be oxidized to CO_2. Yet many xenobiotic compounds *are* persistent, even in the most microbially-active environments. While such synthetic compounds thus represent a potentially untapped nutrient source for those micro-organisms exhibiting the appropriate metabolic versatility, the compounds will be biodegradable only if they are substrates for the suites of enzymes acquired by micro-organisms during evolution to enable them to exploit the

multiplicity of sources of nutrients and energy normally found in nature. The study of biodegradability thus involves knowledge of both the mechanism of induction (derepression) and the modes of action of those enzymes employed in degrading natural products. In general, the catabolic pathways evolved for biogenic molecules have enzymes specific for their substrates and enzyme synthesis is efficiently regulated; cells rarely waste biochemical energy by derepressing enzymes that have no relevant physiological function (Dagley, 1984). Microbial transformations of pesticides and other xenobiotics, in contrast, are fortuitous and random; such molecules were not designed to serve as microbial substrates, so microbial enzymes attack them only if there is some structural similarity to the substrates they meet in nature. The comprehensive list, compiled by Alexander (1981), of reactions which bring about modifications of man-made compounds of environmental importance can be regarded as an indication of the degree of tolerance exhibited by microbial enzymes to alterations in the structure of their substrates. Hydrolytic enzymes which figure prominently in pesticide transformations are generally tolerant of considerable modifications in their substrates; this is particularly true for esterases, hydrolases and hydroxylases (Golovleva *et al.*, 1990) and recorded for many hydratases, aldolases and decarboxylases which show close electron-shift similarities in their respective mechanisms (Dagley, 1978). These enzymes typically modify structures but exposure of substrate analogues to one, two or even more such enzymes in a micro-organism still does not necessarily confer any benefit upon the organism unless they are acting either as detoxification agents or are co-ordinated in metabolic sequences to provide ATP for biosynthesising the cellular constituents of the organism. More typically, when they act fortuitously and in isolation upon xenobiotic analogues of their natural substrates, these enzymes result in products that frequently accumulate.

Biodegradability, then, is more likely to be achieved by incorporating into pesticide molecules, structural features encountered in their natural counterparts. The readily-degradable herbicide 2,4-D, first introduced in the 1940's, is precisely the sort of herbicide than an agrochemist would now design, based upon present knowledge of the genetics and mechanisms of microbial attack on chlorinated aromatic rings(Dagley, 1972). Similar arguments apply to many of the other agrochemicals listed in Table 1.

MICROBIAL DEGRADATION OF PESTICIDES

The dissipation and loss of biological activity of a wide range of pesticides occurs when they are mixed with soil; that this is due to a biological agency is readily demonstrated by heat sterilization of the soil, by the addition of poisons such as mercuric chloride or sodium azide, by appropriate antibiotics or by γ-irradiation, all of which bring about complete or very significant reductions in the rate of disappearance of the bioactive material. Reinoculation of fresh soil into sterilized samples rapidly re-establishes the further disappearance of the pesticide.

Such effects have been known for nearly a century since Stormer (1908) noted the marked decrease in cresol and phenol soil sterilants and Tattersfield (1928) the disappearance of naphthalene then used as an insect deterrent in greenhouse soils. These effects were attributed to microbial metabolism at Rothamstead by Gray & Thornton (1928) who isolated and described several genera of bacteria, strains of which were able to grow at the expense of such aromatic compounds. 2,4-D was, however, the first synthetic pesticide to be shown to undergo microbial degradation. Audus (1949; 1951) not only isolated from his soil percolator enrichments, a pure culture of "Bacterium globiforme" (probably an *Arthrobacter*) which utilized 2,4-D as sole C-source and produced nearly stoichiometric yields of chloride, but demonstrated what has now become a familiar pattern of removal when a novel compound is first exposed to a microbial inoculum (Cullimore, 1971). On first admission of 2,4-D to the soil percolating column, or to field soil, there was a lag of several days before disappearance of the herbicide became established but once that first application had been utilized, subsequent applications showed no lag period and the rate of utilization was usually

increased and accompanied by an increase in the numbers of specific pesticide-degrading organisms in the treated soil . The persistence of this acquired degradative capacity in a treated soil varies widely with the compound under examination but it is rarely less than several weeks after a series of successive regular applications and can extend to several years.

This phenomenon has been termed **enhanced degradation** and has now been observed with a very wide range of insecticides, herbicides and fungicides. The immediate benefits of enhanced degradation for the current products of an industry which gave us such persistent compounds as DDT, dieldrin and BHC are at first sight all positive. This is particularly true of compounds which are foliar-acting or systemic, because they do not exert their biological activity through the soil; any soil residues are potential contaminants of both surface and ground water. Enhanced degradation of these compounds was thus largely an academic curiosity which has been used to explore the evolution of novel metabolic activities in microorganisms (Pemberton *et al.*, 1979). In contrast, soil-incorporated pesticides must, for economic as well as biological reasons, persist for sufficient time in the soil *milieux* to be effective in controlling their target pest even though they are exposed to a biologically-active microflora. Enhanced degradation of such compounds rapidly reduces their concentration below an active threshhold and leads to potential loss of control of the target organism. While the demonstration of enhanced degradation has not always been paralleled, at least in the U.K.,by a related failure of field performance of the compound on test, the list of chemicals showing evidence of enhanced degradation in field soils has grown rapidly and now spans several classes of compounds currently used to protect major crops (Table 1). For most of these compounds, pure cultures or mixed cultures or consortia of micro-organisms with the appropriate catabolic potential have been isolated with the aim of determining the catabolic route by which the compound is degraded. It is perhaps significant that organisms effecting pesticide degradation are nearly all bacteria. To some extent this may be the result of experimental enrichment techniques biased towards bacterial isolations but more likely it is due to the propensity of fungi, like most other eucaryotes, to detoxify xenobiotic substrates by one or two biotransformation reactions which do involve substantial structural degradations (Dagley, 1981). One pertinent hydrolysis or oxidative step is all that is required to remove the biologically-active properties of carbamate or phosphorothioate insecticides, or of the dicarboximide fungicides, while leaving the molecular structure otherwise largely intact; enhanced degradation of a pesticide, therefore, does not necessarily require its complete mineralization (Lee, 1984; Bailey & Coffey, 1986; Saxena *et al.*, 1987; Arunachalam & Lakshman, 1988). The fact that bacteria often effect mineralisation stems from the fact that they have evolved or acquired the genes coding for the enzymes of peripheral catabolic pathways that channel these molecules into central metabolism. The source of this genetic material is, in many instances, plasmids.

THE MOLECULAR BIOLOGY OF PESTICIDE DEGRADATION

A plethora of functions are now known to be plasmid-encoded (Hardy, 1981). Plasmids, extrachromosomal pieces of covalently-closed, circular DNA (cccDNA) which reproduce independently of cell division, have been found naturally in almost every genus of prokaryotic, and in some eucaryotic, micro-organisms so far examined; their ubiquity itself implicates their evolutionary importance. Plasmids may represent parasitic DNA which exploits its host, or simply accessory bacterial DNA which allows an organism to experiment with mutations and genetic rearrangements independent of its chromosome, but it is now recognised that plasmids have been seminal in producing the diversity of reactions which have evolved in bacterial catabolism. The fact that plasmid-encoded pathways exist for the degradation of many naturally-occuring compounds (Table 2) would seem to indicate that plasmids have a long evolutionary history and involvement in the development of new metabolic traits. It would thus be reasonable to expect that when microorganisms are challenged with novel synthetic structures it would be the evolution of genes borne on plasmids which would meet that challenge. This hypothesis is well supported by the steadily-increasing number of pathways for the degradation of novel synthetic compounds, many of

TABLE 1. **Micro-organisms effecting degradation or biotransformation of pesticides**

Pesticide	Organism
Benomyl	unidentified
Carbofuran	*Pseudomonas* *Bacillus* *Arthrobacter* *Azospirillum* *Streptomyces* *Flavobacterium*
Diazinon	*Flavobacterium*
Dalapon	*Rhizobium* *Ps. putida* *Moraxella*
DDT	*Hydrogenomonas* *Mucor* 7 bacteria, 9 fungi
EPTC	29 fungi, 9 bacteria *Flavobacterium* and *Methylomonas*
Fenitrothion	*Flavobacterium* *Alcaligenes*
Isofenphos [and related compounds]	*Pseudomonas* sp. *Arthrobacter*
Linuron	*Bacillus sphaericus*
Malathion	*Arthrobacter*
Parathion	*Flavobacterium* *Ps. diminuta* *Arthrobacter*
Mephosfolan	unidentified
Methylparathion	*Pseudomonas*
Pentachlorophenol	*Arthrobacter* *Flavobacterium*
Propanil	*Fusarium solani*
2,4-D and MCPA	*Arthrobacter* *Pseudomonas* 18 bacteria
2,4,5-T	*Ps. cepacia*
Triazines	bacteria and fungi *Pseudomonas* sp. and *Klebsiella*
Iprodione	Soil enrichment cultures Gram-negative rod
Vinclozalin	Gram-negative rod

them pesticides, which have been located on plasmid genes (the list in Table 2 is by no means exhaustive).

Nearly twenty years ago, Waid (1972) suggested that the persistence, sometimes for several months, of pesticide degradation in soils following a regime of repetitive pesticide usage, was due to the maintenance of the pesticide-degrading genes on plasmids in small numbers of the soil microflora from which they could be disseminated when subjected to the selective pressure of a later addition of the pesticide to the same plot. The involvement of plasmids in bacterial evolution is strongly suggested by DNA sequence homologies found on many degradative plasmids, even those which exhibit distinct restriction digest profiles (Heinaru *et al.*, 1978; Mulbry *et al.*, 1986; Mulbry *et al.*, 1987; Ghosal *et al.*,1985b; Tsuchiya *et al.*, 1989). Evidence has also accumulated that many degradative pathways and resistance determinants are encoded on plasmid-borne transposons (de la Cruz & Grinstead, 1982; Negoro *et al.*, 1984; Tsuda & Iino, 1987), which facilitates their rapid exchange between plasmids and from plasmids to chromosome or *vice versa*. Other genetic rearrangements which may be mediated by plasmid-borne transposons include gene duplications, deletions and inversions. Transfer of such plasmids and transposons may further lead to novel combinations of degradative genes being brought together in a single organism giving it, for instance, increased resistance to toxicants or an expanded catabolic diversity (Reinecke & Knackmuss, 1979; Lehrbach *et al.*, 1984; Rojo *et al.*, 1987). Gene duplication followed by evolutionary divergence of the duplicate gene permits the evolution of new enzymes or improves the substrate specificity of enzymes perhaps catalysing a slow rate-limiting reaction on a xenobiotic analogue of its natural substrate (Ghosal *et al.*, 1985a).

The instability of gene duplications sometimes leads to deletions arising from homologous recombination between the duplicate genes; this happens particularly under non-selective conditions. Such deletions probably serve to relieve the organism of the need to replicate unnecessary DNA sequences, but they may also have the effect of derepressing genes, the normal expression of which has been regulated by the presence of novel genes on an introduced plasmid (de Smet *et al.*, 1989). The enzyme products of some genes produce "suicide metabolites" from xenobiotic substrates analogues; for example the acyl chloride and chloroquinone products of chlorocatechol oxidations. Deletions, or at least mutations, in these genes would be an obvious advantage to an organism subject to such toxic intermediate products (Jeenes *et al.*, 1982; Haugland *et al.*, 1990). The likelihood of gene duplications is strongly indicated by extensive nucleotide homology between the genes coding for essential steps in pesticide catabolism and the ancestral genes coding for analogous steps in the degradation of the corresponding natural product. Values of 50-80% homology are by no means uncommon (Aldrich *et al.*, 1987; Ghosal and You, 1989).

Even single point mutations which cause a single amino acid substitution in the protein product of the gene have, on occasion, been shown to alter the phenotype of an organism. Such a mutation was sufficient to increase the substrate range of the acetamide amidohydrolase of *Ps. aeruginosa* to encompass acylanilides (Clarke, 1980). Pemberton *et al.*, (1979) found a similar point mutation in the gene for 2,4-D monooxygenase was sufficient to permit *Alcaligenes eutrophus* JMP134 to degrade phenoxyacetic acid, not normally a substrate, as well as 2,4-D itself.

PLASMID-CARRYING MICRO-ORGANISMS IN THE NATURAL ENVIRONMENT

Examination of bacteria isolated from the environment soon reveals the ubiquity of plasmids. Organisms isolated for their novel biodegradative properties are particularly replete with plasmid molecules. Kanagawa *et al.* (1989) isolated a *Pseudomonas* sp. which grew on 6-aminohexanoate cyclic dimer as sole source of carbon and energy and found this strain to carry at least six plasmid species. A similar situation was found with a parathion-hydrolysing *Flavobacterium* sp. which harboured at least four plasmids (Karns *et al.*, 1986a). Multiple

TABLE 2. **Organic compounds degraded by plasmid-encoded catabolic sequences**

Compound	Plasmid	Organism
Naturally-occurring compounds		
Resorcinol	pBSW13	*Acinetobacter* sp.
Toluene	pWWO	*Pseudomonas* sp.
Phenol	-	*Alcaligenes* sp.
Nicotine	NIC	*Arthrobacter* sp.
Benzene	pWW174	*Alcaligenes* sp.
Polyoromatic hydrocarbons	pKG2	*Beijerinckia* sp.
Octane	OCT	*Pseudomonas* sp.
Salicylate	SAL	*Pseudomonas* sp.
Naphthalene	NAH	*Pseudomonas* sp.
Camphor	CAM	*Pseudomonas* sp.
Catechin	pAMB1	*Pseudomonas* sp.
Cinnamate	pCINN5	*Pseudomonas* sp.
Ferulate	pFER2	*Pseudomonas* sp.
Ethylbenzene	pEB	*Pseudomonas* sp.
Cresol	pND50	*Pseudomonas* sp.
Aniline	pCIT1	*Pseudomonas* sp.
	pLA1	*Rhodococcus* sp.
Isopropylbenzene	pRB4	*Pseudomonas* sp.
Trimethylbenzene	pTMB	*Pseudomonas* sp.
Phenylacetate	pWW17	*Pseudomonas* sp.
Lignin	-	*Lignobacter* sp.
Opines	Ti plasmids	*Agrobacterium* sp.
Sugars	-	*E. coli*
Synthetic organic compounds		
3-Chlorobenzoate	pWR1	*Pseudomonas* sp.
2,4-D: MCPA	pJP4	*Alcaligenes* sp.
Parathion	pCS1	*Pseudomonas* sp.
Styrene	pEG	*Pseudomonas* sp.
2,6-Dichlorotoluene	-	*Pseudomonas* sp.
Nylon dimers	pOAD2	*Flavobacterium* sp.
p-Chlorobiphenyl	pKF1	*Acinetobacter* sp.
	pSS50	
Haloacetates	pUO1	*Moraxella* sp.
2-Haloalkanoic acids	pUU204	*Pseudomonas* sp.
Alkylbenzenesulphonates	ASL	*Pseudomonas* sp.
EPTC	-	*Arthrobacter* sp.
Carbaryl	pPDL111	*Achromobacter* WMIII
Carbofuran		*Achromobacter* sp.
Phenylcarbamates	-	*Pseudomonas* sp.
Chloridazon	-	Soil Bacterium
Triazines	-	*Pseudomonas* sp.

plasmids have been reported in phenylcarbamate-degrading pseudomonads (Vega *et al.*, 1988) and in carbofuran- (Head *et al.*, 1989) and iprodione-degrading bacteria (Staines, unpublished observations).

Bacteria isolated from polluted environments in general carry larger plasmids than do organisms from pristine environments and more frequently harbour multiple plasmids (Glassman & McNicol, 1981). A correlation between the proportion of plasmid-bearing strains and the pollution status of aquatic environments was also noted by Burton *et al.* (1982) and Day *et al.* (1988). Mercury-resistance plasmids, some of which are associated with resistance to the organomercurics used in seed dressings, are virtually ubiquitous in many mercury-tolerant bacteria isolated from the environment (Bale *et al.*, 1988; Jobling *et al.*, 1988a). One important consideration in assessing the role of plasmid genes in pesticide degradation is the determination of whether or not plasmid transfer occurs between micro-organisms in their natural environment. The circumstantial evidence available suggests that it does. The mercury resistance plasmids isolated by Bale *et al.* (1988) and Jobling *et al.* (1988b) from quite different organisms showed similar endonuclease restriction patterns. Restriction analysis of the plasmids pJP3, pJP4, pJP5 and pJP7 which code for 2,4-D catabolism, each obtained from independently-isolated *Alcaligenes eutrophus* and *Alcaligenes paradoxus* strains, also revealed that the plasmids were all but identical (Don & Pemberton, 1981) and were almost certainly recruited from a common source. Identical TOL plasmids were also identified by Duggleby *et al.* (1977), in independently-isolated *Pseudomonas* strains obtained as far apart as Japan, Wales and the USA. That plasmid transfer can occur in the aquatic environment has now been demonstrated directly by the work of Bale and his colleagues (1988) who have proved elegantly that plasmid-encoded mercury resistance determinants are transferred in the river epilithon. O'Morchoe *et al.* (1988) found that the natural microflora of a lake reduced the frequency of plasmid transfer among introduced laboratory strains but also resulted in the isolation of transconjugants bearing plasmids which had incurred deletions and rearrangements. Plasmid transfer has now recently been demonstrated in soil (Van Elsas *et al.*, 1987). This area of research has been extensively reviewed by Trevors *et al.* (1987). Specific genes, either on the chromosome or on plasmids, of bacteria in their natural environment may be detected *in situ* with great sensitivity by gene probes. Direct DNA-DNA colony hybridization (Sayler *et al.*, 1985) and probe hybridization following amplification of the target DNA by the polymerase chain reaction (Chaudhry *et al.*, 1989) have both been successful. Mercury-resistance operons were readily detected in estuarine environmental samples by DNA probes indicating the presence of mercury-resistance plasmids. Pettigrew & Sayler (1986) also reported that 0.3% of the culturable heterotrophs isolated from chlorobiphenyl-contaminated sediments carried DNA sequences homologous to the chlorobiphenyl degradative plasmid pSS50.

GENETICS OF THE ENHANCED DEGRADATION OF PESTICIDES

Although pesticide metabolism has been investigated extensively over the last twenty years (see Table 1), for only a few compounds has the full suite of responsible microbial enzymes been examined. The instances where the genetic basis of pesticide breakdown has been investigated are fewer still and those few instances have usually had a practical basis such as: (i) to devise methods for the detoxification of pesticide wastes or for bioremediation of persistent pesticides in contaminated soils and natural waters, or (ii) to understand how pesticide biodegradation arises, evolves and is disseminated among the soil microflora. This is particularly pertinent for the 15 or so pesticides showing enhanced degradation because it may lead to means whereby the phenomenon can be controlled or minimised. Fortunately, the microorganisms showing enhanced degradation abilities have proved reasonably easy to charactize genetically using modern recombinant-DNA techniques.

THE CHLOROPHENYLALKANOATE HERBICIDES. The best-known of these herbicides is 2,4-D which was one of the first synthetic herbicides to be introduced and was

historically the first to show enhanced biodegradation by a variety of bacterial genera (Rochkind *et al.*, 1986). The pathways of 2,4-D and MCPA catabolism were elucidated some twenty years ago (Tiedje & Alexander, 1969; Evans *et al.*, 1971) and facilitated the subsequent identification of the enzymes catalysing each of the seven steps in the pathway from 2,4-D to 3-oxoadipate, the genes for the first six of which (*tfd*A to *tfd*F) were plasmid-encoded (Don & Pemberton, 1981) while the seventh, which codes for chloromaleyl reductase (Don *et al.*, 1985) was chromosomal. Thus, although the best known of the plasmids pJP4 is readily transmissable to other bacteria and its 2,4-D genes are expressed in these hosts, transconjugants lacking a chromosomally-encoded chloromaleyl reductase are still unable to utilize 2,4-D as a sole source of carbon for growth (Kukor *et al.*, 1989). The entire nucleotide sequences of the structural genes *tfd*A to *tfd*F (Perkins *et al.*, 1990) are now known and the regulatory gene *tfd*R has also been cloned (Harker *et al.*, 1989). The structural genes are clustered in two operons, one of which contains *tfd*A and the other *tfd*CDEF, beteween which *tfd*R lies. The product of *tfd*R renders expression of the two operons inducible by 2,4-D (or 3-chlorobenzoate) (Harker *et al.*, 1989). Gene *tfd*B, however, is independently regulated but not by *tfd*R.

Phenoxyalkanoate herbicides with longer aliphatic side chains appear to undergo chromosomally encoded β-oxidation of the alkyl chain after initial cleavage at the ether linkage (Macrae *et al.*, 1963) both in the target plant and in most micro-organisms degrading these products, but *Nocardia coeliaca* operates an additional α-oxidation in the metabolism of homologues with C_{10} or C_{11} side-chain carbons (Taylor & Wain, 1962).

Because the chlorophenoxyalkanoate herbicides are foliar-applied, their enhanced biodegradation by soil organisms has little economic importance; interest in the genetics of their catabolism has thus largely focussed on the evidence which this system has offered to explain dissemination and evolution of novel catabolic genes. Firstly, Southern hybridisation analysis revealed substantial DNA homology between 2,4-D degradation genes on plasmids obtained from quite independently-isolated *Alcaligenes eutrophus* and A. *paradoxus* strains (Don & Pemberton, 1981) and from a *Flavobacterium* isolated several years after the former two (Chaudhry & Huang, 1988),implying that 2,4-D genes may be disseminated through soil microbial populations on conjugal plasmids. Secondly, comparisons between both DNA and protein sequences in the gene and gene product of *tfd*CDEF (encoded by plasmid pJP4) and *clc*ABC (chlorobenzoate catabolism) found on plasmid pAC27, revealed 62% sequence homology, strongly suggesting their origin in a common ancestral gene (Ghosal & You, 1989; Perkins *et al.*, 1990). More pertinent was the observation that in the DNA sequences of the genes for catechol metabolism in the degradation of naturally-occurring aromatic compounds and those for chlorocatechol metabolism, there was a comparable degree of homology to that linking 2,4-D and chlorobenzoate catabolism (Frantz *et al.*, 1987). More compelling evidence for the evolution of genes for the degradation of the chloroaromatic herbicides from those involved in the catabolism of their biogenic analogues would be difficult to find.

2,4,5-T degrades with much more difficulty than other chlorophenoxyalkanoates but this herbicide is converted to 2,4,5-trichlorophenol by *Pseud. fluorescens* while *P. cepacia* AC1100 can utilize 2,4,5-T as sole carbon and energy source (Kilbane *et al.*, 1982) with 97% degradation and stoichiometric chloride release within 6 days. When cells of *P. cepacia* AC1100 and *A.eutrophus* JMP134 (which contains plasmid pJP4) were combined, they were able to degrade either 2,4,5-T or 2,4-D as single substrates but only very poorly as a mixture. This was traced to the inhibition of strain JMP134 by the 2,4,5-T component of the mixture and the auto-inhibition of strain AC1100 by chloroquinol which it produces as a dead-end metabolite of 2,4-D. If pJP4 is conjugated into strain AC1100, however, the transconjugant can simultaneously metabolise both substrates in a mixture of 2,4,5-T and 2,4-D (Haugland *et al.*, 1990) which the authors attribute to the maintenance of low intracellular concentrations of the inhibitory compounds when the combined enzymic suites occur in the same cell.

SOIL-ADMINISTERED PESTICIDES

Although by no means all organophosphate and carbamate pesticides are soil-administered, representatives of these two groups offer the best examples of pesticides undergoing enhanced degradation in the soil and the practical problems arising from the ensuing lack of pest control; further, the responsible bacteria have proved amenable to genetic analysis. Although several carbamates and phosphorothioates are extensively biodegraded, only a single hydrolytic step is required to inactivate these insecticides, the biological activity of which is due exclusively to the phosphorothioate and carbamate moieties. The products of the hydrolase are usually more than 100-fold less toxic than the parent molecule. Investigations have thus tended to concentrate on these hydrolases as likely detoxification agents for agricultural wastes, and for studying the distribution of the hydrolase genes in the environment. Some phosphorothioate ester and carbamate ester hydrolases show considerable lack of substrate specificity, which may account for instances of cross-enhanced degradation by structurally-related compounds (Read 1986; Suett & Jukes, 1988) yet there are few instances of such cross-adaptation to breakdown among the organophosphates in the field.

The Phosphorothioate Insectides. Enhanced degradation in soil carrying maize crops of an organophosphate pesticide was first reported for diazinon . From soil enrichments, a *Flavobacterium* sp. (ATCC 27551) was isolated in the Phillipines by Sethunathan & Yoshida (1972) which contained a constitutive membrane-bound hydrolase of mol. wt 35000, now known to comprise 336 amino acids but translated as a pro-enzyme with a 29-residue leader peptide that was eventually cleaved (Mulbry & Karns, 1989b). When partially-purified, this enzyme (termed phosphotriesterase or parathion hydrolase) was shown by Brown (1980) to be active against a wide range of phosphorothioate esters with electron-withdrawing aromatic or heterocyclic leaving groups. Several other bacteria possessing parathion hydrolases have subsequently been isolated (reviewed by Munnecke *et al.*, 1982) but interest has focussed on the *Flavobacterium* enzyme and on that from *Pseudomonas diminuta* MG, isolated some 10 years later in the USA by Serdar *et al.*, (1982), which were biochemically identical (Mulbry & Karns, 1989a). Parathion hydrolase activity in both strains was associated with distinct plasmids present in the respective bacteria but restriction mapping of the two plasmids showed that they were totally unrelated except for the presence of homologous hydrolase (*opd)* genes (Mulbry *et al.*, 1987). When these two independent genes from different genera were later sequenced, they were almost completely homologous, differing in only one base pair over the 975 base pair open reading frame of the gene. Such identity indicates their almost certain common source. In a further independently-isolated *Pseudomonas* sp., this time degrading methyl-parathion, DNA closely homologous to the *opd* gene was again detected (Chaudhry *et al.*, 1988) prompting suggestions that the *Flavobacterium opd* gene had begun to spread globally in a manner analogous to that of antibiotic-resistance genes. Shelton & Somich (1988) initiated a deliberate study of the suspected pervasive gene by isolating many new bacterial strains with parathion hydrolase activity from a variety of sources but little similarity between the enzymes in native or sub-unit size, in substrate affinities, in cellular locations or in divalent ion activation was found by Mulbry & Karns (1989a).

The stability and broad substrate specificity of parathion hydrolases has made them attractive as agents for detoxification of organophosphate wastes in used containers or accidentally contaminated areas (Munnecke, 1976) or in the large volumes of aqueous residues from sheep and cattle-dips (Karns *et al.*, 1987), but their economic use for such activities requires their production in much larger quantities than that obtainable from natural isolates. When introduced into *E. coli*, the *opd* gene is very poorly expressed with its own promoter but expression has been improved in plasmid constructs by using the *lac* promoter, by replacing the DNA sequences coding for the *opd* leader protein with *E. coli* translational

signals, and by the addition of Co^{2+} or Zn^{2+} to the cell culture medium (Serdar *et al.*, 1989). When introduced into *Streptomyces lividans*, the *opd* gene is not only expressed but the hydrolase product is secreted into the culture medium (Steierte *et al.*, 1989), thus making down-stream recovery potentially both convenient and cheap. Whether such biological detoxification agents become commercially viable will depend upon whether their substrates are superceded or continue to be used in pest management programmes.

The Carbamate Pesticides. Carbamates, in spite of their large scale production, have the attraction of limited persistence in the environment even under abiotic conditions, because the stability of the carbamate residue is particularly pH dependent. Bacteria have nevertheless been isolated which, in pure culture, can degrade phenylcarbamates (Marty & Vouges, 1987; Vega *et al.*, 1988) thiocarbamates (Mueller *et al.*, 1988; Tam *et al.*, 1987) and methylcarbamates (Helweg, 1972; Karns *et al.*, 1986b; Head *et al.*, 1988). Carbamates find use as herbicides (chlorpropham, EPTC, butylate, barban), fungicides (maneb, thiram, benomyl) and foliar- and soil-administered insecticides (pirimicarb, methiocarb, oxamyl; carbaryl, carbofuran, aldicarb) in which either the amino or carboxy groups are derivatized. Consequently, esterases or amidohydrolases may be involved in their bacterial degradation. The development of accelerated biodegradation of carbamates, resulting in reduced performance of the pesticide, is of particular concern for carbamate soil insecticides such as carbaryl, aldicarb and carbofuran which are used against the pests of very large acreage cereals and against several soil-borne pests of root crops (Suett & Walker, 1988) and has prompted both enzyme and genetic studies in an effort to provide the biological basis of the phenomenon. Carbamates such as carbofuran and carbaryl lose their pesticidal activity upon hydrolysis of the carbamate moiety so soil bacteria need to produce only a carbamate hydrolase to render the molecule biologically inactive. Some isolates e.g., the *Flavobacterium* MS2d of Head *et al.*, (1989), completely mineralise the carbofuran molecule while others like the *Achromobacter* of Karns *et al.*, (1986b) hydrolyse only the methylcarbamate ester and utilize the methylamine generated as a source of nitrogen. Only a few of these carbamate hydrolases have been characterised (Marty & Vouges, 1987; Derbyshire *et al.*, 1987; Head 1990) but their substrate specificities have little in common so their genes would seem likely to be equally unrelated.

Plasmids have been associated with carbamate degradation in several bacteria (Table 3). The loss of the characteristic carbamate-degrading phenotype by plasmid-curing has usually been assumed to correlate with the loss of the carbamate hydrolase function on that plasmid but inability of plasmid-cured strains to grow on a carbamate could equally result from the loss of any one subsequent catabolic step, or several sequential steps, if they are plasmid-encoded.

TABLE 3. **Plasmids associated with carbamate utilization in bacteria**

Organism	Curing agent	Plasmid	Size(kbp)	Reference
Flavobacterium TE1	acridines, heat	pSMB2	100	Mueller *et al.*, 1988
Arthrobacter TE1	acridines, heat	-	67	Tam *et al.*, 1987
Achromobacter WM111	not cured	pPDL111	>100	Tomasek & Karns, 1989
Flavobacterium MS2d	growth in rich medium	pIH3	>199	Head 1990
	transposon mutagenesis	pIH4	79	Head, 1990

The wild-type *Flavobacterium* MS2d isolated by Head *et al.*, (1988) showed a more complex pattern; it contained many plasmids (Fig. 1) but growth through several subcultures in nutrient broth supplemented with yeast extract and free from carbofuran, yielded cultures in which up to 67% of the final population had lost the ability to grow with carbofuran. These cells retained their ability to hydrolyse carbofuran to carbofuran phenol (cfh) but proved unable to utilize the phenol

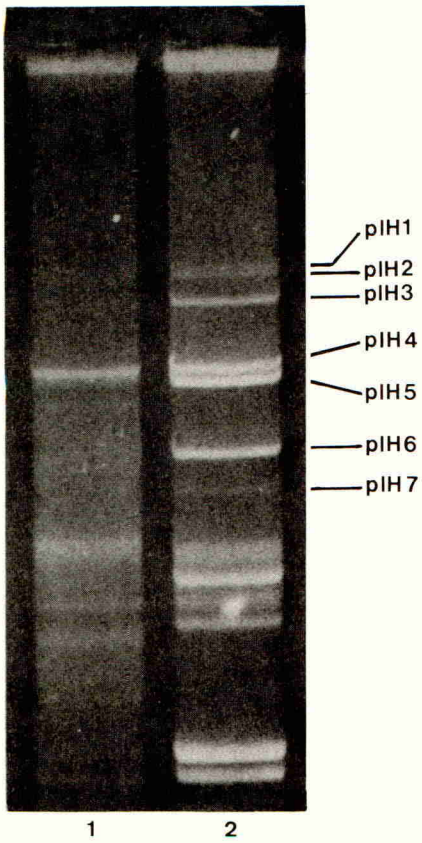

Figure 1. *Plasmid profile of wild-type and rifampicin-cured strains of* Flavobacterium *MS2d.*

Lane 1, a cured derivative of MS2d after 30 subcultures on rich media containing rifampicin; lane 2, wild-type strain with no treatment. Plasmid pIH4 is the only large plasmid remaining in the cured strain; it carried the *cfh* gene. The cfp phenotype is carried on pIH3.

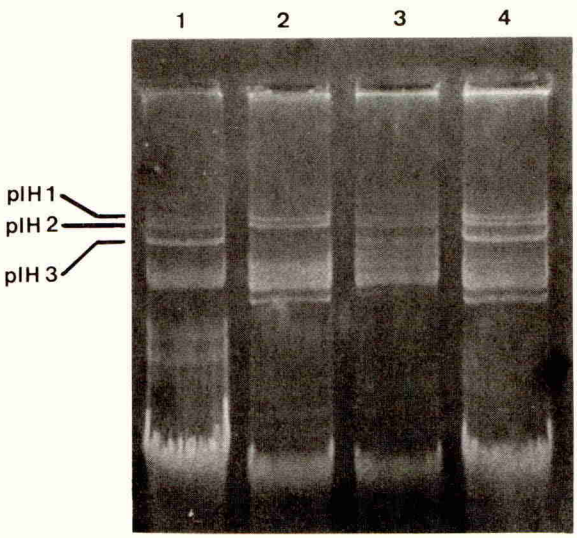

Figure 2. *Plasmid profile of wild-type and derivative strains of MS2d*

Lane 1, wild-type (cfh^+, cfp^+); lane 2, a cured strain that has lost pIH3 and can no longer grow with carbofuran or carbofuran phenol although it retains the ability to hydrolyse carbofuran (cfh^+,cfp^-); lanes 3 and 4, transconjugants which have simultaneously regained pIH3 by conjugation and the cfh^+,cfp^+ phenotype.

for growth, (cfp) i.e., they had the cfh^+, cfp^- phenotype. Such cured strains, while hydrolysing carbofuran at a rate identical to that of the wild-type strain, accumulated the phenol stoichiometrically from carbofuran; all isolates with this phenotype had lost plasmid pIH3 (Fig 2). The association of pIH3 with carbofuran phenol catabolism was subsequently confirmed by conjugating it back into a cfh^+, cfp^- cured strain which regained the capacity to grow with carbofuran phenol (cfh^+,cfp^+) (Fig. 2).

Extended attempts to cure the carbofuran hydrolase activity in MS2d were unsuccessful so Head (1990) used transposon mutagenesis with *E. coli* Sm10 (carrying plasmid pSUP5011, the Tn5-*mob* donor) which gave frequences of mutation of the order of 10^{-5} per recipient. Southern hybridisation with a probe of pSUP5011 DNA, radiolabelled by nick translation, identified the site of the inactivated *cfh* gene which was located on plasmid pIH4. It is worth emphasising that multiplasmid-determined degradation of compounds by *natural* isolates is quite rare. The separate maintenance of the *cfh* gene on a plasmid distinct from that carrying genes for the further catabolism of its carbofuran phenol product suggests that the detoxification role of carbofuran hydrolase may have been acquired by the bacterium independently of the (perhaps rarer) ability to use it as a carbon and energy source.

The gene *mcd* (methylcarbamate degradation) coding for the carbofuran hydrolase on *Achromobacter* plasmid pPDL111 (Table 3) has recently been localized to a *ScaI-ClaI* region of approx. 3kbp after first cloning a 14kbp *Eco* RI fragment of plasmid DNA from *Achromobacter* WM111 into *P.putida* using the vector pLAFRI. Western blots of the protein products of the 3kbp fragment, expressed at low level in *P.putida*, revealed the two 70kD protein subunits seen on SDS-PAGE of purified carbofuran hydrolase (Tomasek & Karns, 1989). This remains the only gene coding for carbamate degradation that has so far been isolated .

The Dicarboximide Fungicides. Enhanced degradation of vinclozalin and iprodione was described by Walker *et al.*, (1986) and Walker (1987). It may be induced in certain soils by as little as a single pre-treatment yet may persist for several years (Suett & Walker, 1988). The accelerated degradation may also be easily transferred from one fresh soil to another by as little as a 0.5% (w/w) inoculum but not if the donor soil is sterilized first (Mitchell, unpublished results). Head (1990), Mitchell (unpubl.) and Staines (unpubl.) working at Newcastle and Finkelstein (pers., comm.) in the USSR, have all isolated mixed bacterial cultures that degrade iprodione or vinclozolin in mineral salts media; in these, as in treated soils, 3,5-dichloroaniline (3,5-DCA) accumulates as an intermediate and persists if the cultures are using the fungicides only as a nitrogen source. When using them as both carbon and nitrogen sources, however, the accumulated 3,5-DCA disappears in cultures within five days and cannot be accounted for as polymerised or condensed products; it is mineralised. The isolation of single bacterial strains from the Newcastle enrichments has been difficult but was recently achieved by Miss Staines who has found iprodione-degrading isolates with one, and some others with two, large plasmids; the smaller of these is of about 120 kbp and the other much larger. Because of the stability of these plasmids, cured strains have not yet been achieved so we cannot, at present, unequivocally associate either of these plasmids with iprodione breakdown.

RELEVANCE OF MOLECULAR BIOLOGY TO ENHANCED DEGRADATION

Although the demonstration of enhanced pesticide degradation in a soil is not necessarily reflected in the loss of pest control in the field, failures of performance of a number of soil-applied pesticides since the 1970's, now attributed to enhanced degradation, and the apparent ease with which the phenomenon can be disseminated, are leading to more careful crop management techniques on the basis that to prevent the establishment of enhanced degradation is much easier than to eradicate, or control, it. Further, with the limited range of pesticides currently available for soil application, their injudicious use may well lead to loss of efficacy of even these. The facts that for many of these pesticides, the degrading ability is now known to be plasmid-encoded and that (particularly "promiscuous") plasmids can spread naturally among microbial populations, is one relevant contribution that molecular biology has already made to

understanding the phenomenon. There are several others which can be summarised as follows:

(a) Prediction of situations in which enhanced degradation may occur requires in-depth studies of soil microbial ecology, particularly the distribution of degrading organisms *in situ* and whether they possess genes for degradation. The development of **gene probes** to detect in such bacteria the specific gene sequences coding for particular pesticide-degrading enzymes is well-advanced.

(b) purification of such enzymes and the study of their substrate specificity, their regulation and their inhibitors has particular pertinence for practical agronomy;

(i) **substrate specificities** indicate the range of analogous structures that are attacked and thus the limit to the range of comparable structures that need to be avoided for subsequent application at the same field site,

(ii) studies of the **regulation** of degrading enzymes are important in establishing whether analogues are inducers as well as substrates and thus whether there might be significant cross-enhancement of degradation by using pesticide analogues. The recognition of **anti-inducers** to a degrading enzyme (Clarke, 1980) would enable such compounds to be incorporated into formulations to minimise onset of pesticide degradation by an inducible enzyme. This approach is one step further back from the use of potent **specific enzyme inhibitors**, such as the use of methylcarbamate inhibitors of the amidase that degrades propanil (Blake and Kaufman, 1975), or **"extenders"** such as the organophosphorus dietholate which increases the persistence of EPTC in problem soils, and the fungicide fentin which reduces the rates of diphenamid degradation in soils. Extenders may be case-specific but only fundamental studies over a much wider range of examples will bring this to light.

(iii) Poor or inefficient expression of enzyme proteins in their native bacteria only come to light when the appropriate structural and regulatory genes are cloned into other species. Appropriate manipulations of the promoter regions of such genes in recipient strains may lead to hyper-production and to efficient extracellular excretion of pesticide degrading enzymes that make them available cheaply as **detoxification agents** for container or accidental spill clean-up.

The role of plasmids in pesticide degradation has provided several opportunities for examining their role as "the crucible of bacterial evolution" (Richmond and Wiedeman, 1974). The evidence of remarkably-conserved nucleotide sequences coding for pesticide-degrading enzymes on plasmids in taxonomically-distinct bacteria isolated from geographically remote sites strongly hints at the potential global spread of these plasmids; it has already happened for antibiotic-resistance factors under the selection pressure of current medical and agricultural practices. The widespread loss of pest control by enhanced degradation may be presently remote but that is no reason to avoid the adoption of appropriate precautions.

[The list of organisms and plasmids in Tables 1 and 2 were fully referenced but these have been omitted because of space restrictions. A full list of these references is available from the authors.]

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ENHANCED BIODEGRADATION OF SOIL INSECTICIDES IN THE USA--SIGNIFICANCE AND MANAGEMENT

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ABSTRACT

In the U.S. the greatest proportion of insecticides are applied to corn; most of the treatments are directed to control corn rootworm larvae in the soil. Enhanced biodegradation of pesticides has had at least a qualitative impact on pest control in corn agroecosystems. Pesticide use has been narrowed to essentially two compounds, chlorpyrifos and terbufos. Manufacturers now test new compounds for the ability to condition soils for enhanced biodegradation. The importance of enhanced biodegradation has diminished over the last several years because corn rootworm populations have generally been low. When pest populations resurge, the efficacy of soil insecticides will be uncertain. Proposed management strategies for long-term use of soil insecticides are discussed with regard to maintaining and improving efficacy.

INTRODUCTION

Because prophylactic treatment is the strategy most frequently used to control soil pests, the efficacy of the pesticide depends on its persistence in the soil before the appearance of the pest. Paradoxically, biodegradation of pesticides, which has been viewed as a positive process for reducing environmental hazards, has become a "double-edged sword" to management of crop damage by soil-borne pests. A growing body of literature has shown that some environmentally labile pesticides are degraded by physiologically adapted or conditioned microorganisms at accelerated rates in soils that are retreated with the same chemical. In several cases, this enhancement of biodegradation has been associated with ineffective pest control.

Nearly 10 years after the first report of enhanced biodegradation of corn soil insecticides (Felsot *et al.*, 1981), which form the largest insecticide market in the U.S. (Adkisson, 1986), it would be useful to review the current status of the phenomenon. This paper examines the significance of enhanced biodegradation of insecticides to crop protection in the Corn Belt and the management options for either preventing or coping with the development of enhanced biodegradation.

SIGNIFICANCE OF ENHANCED BIODEGRADATION IN CORN AGROECOSYSTEMS

At first an empirical curiosity observed in the laboratory (Audus, 1949) and in the field (Kirkland and Fryer, 1966), enhanced biodegradation of pesticides (specifically chlorinated phenoxyacetate herbicides) was not linked to efficacy of pest control. The recognition of its adverse effect on pest control was first reported in rice culture, where diazinon's failure to control brown planthopper could not be explained by the development of insect resistance (Sethunathan, 1971; Sethunathan and Pathak, 1972). Several years later, extension agents in the U.S. Corn

Belt reported carbofuran failed to control corn rootworms (Felsot, 1989). Links between accelerated degradation of carbofuran and repeated use in fields with efficacy problems were initially made during the late 1970's (Felsot, 1989), and further studies during the 1980's showed that lack of efficacy was better explained by accelerated degradation of carbofuran rather than altered insect susceptibility (Felsot *et al.*, 1985; Felsot *et al.*, 1988)

The relationship between carbofuran efficacy and persistence in soil was bolstered by the nearly simultaneous discovery of the adverse consequences of repeatedly using thiocarbamate herbicides, which was first reported from New Zealand in 1979 (Rahman *et al.*, 1979). EPTC was especially needed in the northern sector of the Corn Belt where it was particularly effective against wild proso millet and shattercane (Harvey *et al.*, 1987). In terms of economic impact, however, enhanced biodegradation of insecticides potentially affected the entire Corn Belt. Of the >32 million ha of corn grown annually in the U.S., >12 million ha are treated with insecticides, the majority of which are applied to the soil.

To estimate the potential economic impact of enhanced biodegradation on corn production in the U.S, a knowledge of the currently used products and the extent of complaints about crop protection failures is needed. Unfortunately, records of pesticide use are not currently required in the U.S., and therefore, estimates for specific insecticides used in corn must be gathered state-by-state from university extension specialists. In a recent interview of 8 field crop entomologists representing the Corn Belt states of Illinois, Indiana, Iowa, Missouri, Nebraska, S. Dakota and Wisconsin, several trends were noted (Felsot, unpublished). Although state pesticide use records were somewhat dated, current estimates indicated approximately 30-35% of the total corn acreage was treated with a soil insecticide. Terbufos and chlorpyrifos had major market shares and were followed by fonofos. Use of carbofuran was considered minor.

A recent survey in Illinois (Pike *et al.*, 1990), which may serve as a paradigm for the Corn Belt, showed that soil insecticide use had declined by half since 1978. Extensive educational efforts had been made to convince farmers to rotate annually corn and soybeans, which would lower corn rootworm infestations to sub-economic levels when corn is planted again. Despite this fool-proof pest control technique, Pike *et al.* (1990) estimated soil insecticide treatments on approximately 14% of the 2.7 million ha planted to corn following a soybean crop. An estimated 83% of the 1.1 million ha of nonrotated corn were treated in 1988.

Complaints about the efficacy of nearly every product have been made to extension personnel throughout the Corn Belt. The greatest number of complaints occurred in the late 1970's and the early 1980's when carbofuran was used on the largest percentage of ha. Terbufos and chlorpyrifos were not commercialized for rootworm control until the mid 1970's; prior to that time the market had been dominated by the chlorinated cyclodienes and carbofuran. In the aforementioned interview with Corn Belt entomologists (Felsot, unpublished) the estimated number of corn acres affected by diminished insecticide efficacy ranged from <1 to 5% of the total treated.

Extension personnel felt that problems of soil insecticide efficacy may have arisen from a combination of factors, including improper calibration or application procedures as well as enhanced biodegradation. Among registered corn rootworm soil insecticides, only fonofos and carbofuran have definitively been shown from field studies in a corn crop to undergo enhanced biodegradation (Chapman and Harris, 1990; Felsot and

Tollefson, 1990). Ethoprop, which is used on a small proportion of the corn acreage, has been shown to undergo enhanced biodegradation in potato fields in the Netherlands (Smelt *et al.*, 1987). Convincing evidence has been presented to show that chlorpyrifos does not cause microbial adaptations for biodegradation (Racke *et al.*, 1990), and only the toxic metabolites of terbufos, t. sulfoxide and t. sulfone, are known to undergo enhanced biodegradation (Chapman and Harris, 1990). The rampant publicity surrounding enhanced biodegradation during the 1980's has forced corn farmers toward the use of only two insecticides, chlorpyrifos and terbufos. It is pertinent that these products are also the most insecticidally active compounds in soil (Sutter, 1982).

Interviews with Corn Belt entomologists confirmed that the number of complaints about insecticide efficacy dwindled to insignificant levels after 1986 but still involved nearly every product (Felsot, unpublished). The feeling that enhanced biodegradation was no longer a major phenomenon may have been prevalent because corn rootworm populations have been low to moderate since approximately 1986. When enhanced biodegradation was perceived as a major problem affecting efficacy, adult corn rootworm populations reached high levels during the years 1975-77, 1980-81, and 1984-85 (Colwell, 1985). It follows that the more widespread and intensive a pest infestation, then the greater probability of crop protection failures, especially when tens of millions of acres are involved. Indeed, high corn rootworm populations have been shown to cause significant root damage despite the use of insecticides, although the damage is less than in unprotected roots (Sutter *et al.*, 1981). Futhermore, corn lodging (where the corn has fallen over) and yields, the only two parameters upon which the farmer judges product efficacy, are not significantly lowered until populations are very high (Sutter *et al.*, 1990). Thus, lack of corn rootworm pressure makes all products seem to perform well. The significance of this observation is that enhanced biodegradation may be more widespread than many extension agents, farmers, and manufacturers believe, but lack of economically damaging insect populations masks the potential impact.

Because most Corn Belt states have relied exclusively on complaints relayed to Extension offices instead of compiling well designed surveys of pesticide efficacy problems, estimating the total economic impact of enhanced biodegradation has been difficult. Nevertheless, there has been a qualitative effect. First, manufacturers may have been skeptical of the importance of enhanced biodegradation across the total corn market, but they had to divert funds to research and development protocols that studied the potential of their products to condition soils for enhanced biodegradation. Second, all players in the corn market have realized that any product can fail for a myriad of reasons, but there are few products left to choose from. Even experimental soil insecticides have been shown under laboratory conditions to be susceptible to enhanced biodegradation (Chapman and Harris, 1990). The lack of insecticide choices and new products causes tremendous uncertainty about what will happen with the next population explosion of corn rootworms.

The phenomenon of enhanced biodegradation has now been studied from ecological, biochemical, and genetic perspectives (e.g., Chapman *et al.* 1986a; Derbyshire et al., 1987; Chaudhry and Ali, 1988; Head *et al.*, 1990; Turco and Knopka, 1990). Ironically, just as great progress is being reported in understanding the molecular biology of carbofuran and EPTC biodegradation, other unrelated pesticides have been reported to undergo enhanced biodegradation. One of the most notable and perhaps costliest cases involved isofenphos; within two years of its commercial introduction in the Corn Belt, its manufacturer halted sales because of widespread complaints about its pest control performance. Even though initial

research proclaimed that isofenphos was one of the longer lived organophosphorus insecticides in soil (Chapman and Harris, 1982; Felsot, 1984), later research left little doubt about the rapidity with which soil could be conditioned for enhanced biodegradation of isofenphos (Chapman et al., 1986b; Abou-Assaf *et al.*, 1986). This story not only underscores the potential economic impact of enhanced biodegradation, but it also emphasizes that there is still much to be learned by long-term studies of biodegradation under field conditions. More importantly, all aspects of biodegradation must continue to be studied to develop appropriate management practices for using soil pesticides in general.

LONG-TERM MANAGEMENT OF SOIL INSECTICIDES IN CORN AGROECOSYSTEMS

Because chemical control of corn rootworm feeding damage will remain the predominant management strategy in corn monocultures into the next century, it is important to develop best management practices for the use of soil insecticides. The basic premise from which to begin developing management strategies is that microbial adaptations for metabolism are natural processes which cannot be eliminated completely. If the development of enhanced biodegradation is not avoided altogether, then we can only cope with it once it appears. The techniques available and proposed for avoiding or coping with the adverse effects of enhanced biodegradation have been classified as either operational or technological strategies (Felsot, 1989). Some of these have been recently assessed from an experimental perspective (Felsot and Tollefson, 1990). These same strategies should be useful for longterm management of soil insecticides to avoid efficacy problems of all kinds.

Operational strategies

Operational strategies for coping with enhanced biodegradation rely on management techniques based on biological principles. They do not require any alterations in chemistry or formulation of the pesticide. These strategies include conservation of pesticides, crop rotation, proper calibration of equipment, altered timing of applications, and chemical rotations.

Conservation of pesticides is accomplished by adhering to the philosophy of integrated pest management: thorough understanding of the agroecosystem, monitoring of pest populations through scouting, and application of pesticides only when economic thresholds are reached. The goal is elimination of prophylactic pesticide treatments, which predominate in the Corn Belt. Strong arguments have indicated that many of the insecticide treatments against CRW are not needed (Turpin and York 1981). When a pesticide is needed, research with aldicarb in the U.K. has shown that use of the lower rates commercially recommended for controlling root maggots inhibited the development of enhanced biodegradation even if the soil was conditioned by previous treatments (Suett and Jukes, 1988). Similarly, field studies in Illinois have shown that corn rootworm control was not diminished by using 50-75% of recommended application rates (Gray *et al.*, 1991).

Appropriate agronomic practices can greatly reduce the need for pesticides. Because corn rootworms cannot complete their development on soybeans, corn-soybean rotations have long been known to be the panacea for controlling corn rootworm infestations. Prevalence of crop rotational systems in the UK has been suggested as the reason for only a few isolated instances of pest control problems associated with enhanced biodegradation of pesticides (Suett, 1987).

Proper calibration of insecticide application equipment is critical for CRW control. Soil attenuates bioactivity owing to sorptive processes as well as a rapidly changing insecticide concentration even in the absence of microbial adaptation (Felsot and Lew, 1989). Eighty-five percent of farmers tested in a Canadian study applied less than the recommended amount of insecticide to corn fields (Ellis, 1982). Given current concerns about pesticide runoff and leaching, overapplication can create an environmental hazard as well as the economic dilemma of wasteful inputs.

In adhering to principles of IPM, application of corn rootworm insecticides closer to the time when larvae are actively feeding can be beneficial. Application of carbofuran during cultivation time rather than at planting time can circumvent enhanced biodegradation by providing a higher dose of chemical when the larvae are actively feeding. The tenability of this hypothesis has been illustrated by successful cultivation-time treatments of carbofuran in Illinois and Iowa (Felsot and Tollefson, 1990). The success of cultivation-time applications, however, depends on favorable soil conditions during a relatively short time in early June. Of course, this practice would circumvent enhanced biodegradation rather than prevent its development.

A derivative of better timing of applications is the control of adult corn rootworms to prevent egg laying and thus reduce the larval population for the subsequent crop. Such a practice, which used carbaryl as an adulticide, was first tried in Iowa during the mid-1970's with some success (Felsot and Tollefson, 1990); control of root damage by adult suppression was as effective as the use of soil insecticides. Because the insecticide would not be applied directly to the ground and much of it would be intercepted by a well-developed crop canopy, development of enhanced biodegradation could be avoided. The dosage per ha could probably be reduced by using an insecticide bait with feeding arrestants or attractants (Metcalf *et al.*, 1987). Indeed, several Corn Belt researchers are currently studying the use of adulticiding for control of larval populations by using tiny amounts of insecticide mixed with cucubitacin feeding arrestants.

Initially proposed to counter the development of insect resistance (Georghiou, 1980), rotations of insecticides of different chemical classes has been proposed as a strategy for coping with enhanced biodegradation of carbamates. Rotations of fensulfothion and carbofuran, however, failed to prevent the development of enhanced biodegradation of either compound in rutabaga fields (Read, 1983). In contrast, rotations of fenamiphos with other organophosphorus insecticides after enhanced biodegradation had developed in banana plantations overcame rapid degradation and restored nematicidal activity (Anderson and Lafuerza, 1990). Efficacy of the thiocarbamate herbicides EPTC and butylate were improved by use only in alternate years (Rudyanski *et al.*, 1987).

A long-term study using field plots arranged in a randomized block design with multi-year and rotational treatments of carbofuran, fonofos, and terbufos has shown that persistence and efficacy of carbofuran was not improved by rotations with either fonofos or terbufos (Felsot and Tollefson, 1990; Felsot, 1990). Fonofos dissipation, however, was significantly slower in plots alternately treated with carbofuran or terbufos than in plots successively treated with fonofos. The degradation of parent terbufos was not affected by multiyear treatments, but less sulfoxide metabolite was recovered following five years of terbufos applications. Persistence of t. sulfoxide was prolonged when terbufos was rotated with fonofos or carbofuran (Felsot, 1990).

Technological strategies

Technological strategies require alterations in formulation chemistry or structural chemistry of the insecticide. The alternatives include extenders and inhibitors, new formulation technology, and directed chemistry.

Extenders and inhibitors are additives to pesticide formulations that improve residual bioactivity by preventing or at least slowing down biodegradation. Inhibitors are well known from enzymology, and they increase chemical persistence by inhibition of specific degradative enzymes. Extenders increase chemical persistence by generalized adverse effects on soil microbial populations. These compounds are not used in currently registered soil insecticide formulations, but one commercial formulation of EPTC herbicide contains dietholate, a parathion analog. This compound slows the biodegradation rate of EPTC in soils adapted for enhanced biodegradation of thiocarbamates (Harvey *et al.*, 1987; Obrigawitch *et al.*, 1982). Unfortunately, repeated applications of the formulation containing the inhibitor eventually caused a loss of efficacy (Harvey *et al.* 1986, Rudyanski *et al.*, 1987). The inhibitory effects of methyl carbamate insecticides on chlorpropham herbicide degradation are well known (Kaufman *et al.*, 1970), and extended dodder control was observed when chlorpropham was mixed with carbaryl (Dawson, 1984). One twist on the inhibitor/extender strategy would be the synthesis of a pesticide whose metabolites are toxic to microorganisms. Such activity has been ascribed to trichloropyridinol, the hydrolysis product of chlorpyrifos in soil (Somasundaram *et al.*, 1989).

Several fungicides and fumigants extended insecticide persistence presumably by producing adverse effects on normal microbial communities (Ferris and Lichtenstein, 1980; Suett, 1987). Analogous to the use of chemical extenders is soil solarization. Accelerated degradation of the fungicide MBC was delayed by mulching soil with polyethylene for four weeks during the summer in Israel (Yarden *et al.*, 1987). In Canada, similar results were obtained with aldicarb, carbofuran, and isofenphos (Chapman and Harris, 1990). Soil solarization would be feasible for small acreages of high value crops but not for large acreages of field crops.

If the availability of an insecticide to microorganisms was reduced without affecting uptake by the insect, than a longer effective residual life would result. Alternative formulation technologies, such as controlled-release, may be feasible for extending the biological activity of soil insecticides (Stokes et al., 1973). Controlled release formulations of carbofuran persisted unchanged for 2 months in soils adapted for enhanced biodegradation of carbofuran, but field applications did not result in improved corn rootworm control (Felsot and Tollefson, 1990).

Insecticide chemistry needs to be directed toward specific soil pests so that the minimal amount of chemical is needed for control. At recommended rates of application, insecticide concentrations approximate 6 ppm in the upper 10 cm of soil. If concentrations of soil insecticides below 0.1 ppm were highly active against root-feeding insect pests, when 98% of the insecticide had degraded, bioactive amounts would be left. Accelerated degradation of the insecticide would become irrelevant. Also, biodegradation slows down significantly at low insecticide concentrations. The concept of a threshold concentration necessary to stimulate microbial enrichment (Alexander, 1985) suggests that initial rates of highly active insecticides can be lowered to help alleviate degradation problems. The inability of low carbofuran concentrations (Chapman *et al.*, 1986a) and low

rates of field applications of aldicarb (Suett and Jukes, 1988) to induce microbial enrichment supports this hypothesis.

Directed syntheses may yield chemical structures that are resistant to mineralizing activity of soil microflora, but are still cometabolized so that environmental pollution is minimized. For example, the commercially used thiocarbamate herbicides, which are readily susceptible to enhanced biodegradation, have alkyl chains attached to a thiocarbamyl functional group. Cycloate, however, has a cyclohexane ring and does not seem to condition soil for its accelerated biodegradation (Harvey *et al.*, 1986). Synthetic pyrethroid insecticides are susceptible to rapid hydrolysis, but new structures have been developed with comparatively long persistence in soil (Bewick *et al.*, 1986). In field studies, the new soil insecticide tefluthrin was not susceptible to enhanced biodegradation after repeated use (Chapman and Harris, 1990). Tefluthrin combines the desirable properties of high insecticidal actvity (application rates are only 0.1-0.2 kg/ha), adequate residual bioactivity in soil, and no apparent conditioning for enhanced biodegradation.

In conclusion, the operational techniques are the most desirable management strategies in the long-term because they are more compatible with environmental goals and require less research and development than the technological alternatives. Evidence has shown that chemical rotations may help alleviate enhanced biodegradation of organophosphorus insecticides but not carbamates. Rotations do not have to be between insecticide classes. The most promising technological alternatives may be new formulation technologies and directed chemistry, but unfortunately, these will be the most expensive.

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ISOLATION OF A BACTERIAL CULTURE CAPABLE OF DEGRADING LINURON

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ABSTRACT

Enhanced degradation of linuron occurred in soils at Wellesbourne following repeated applications of the herbicide. These effects were more pronounced in laboratory incubations than in field persistence studies. Direct isolation of bacteria from soil failed to identify bacteria with the ability to degrade the herbicide. Sequential enrichment culture in a mineral base medium led to the isolation of an apparently stable mixed culture of bacteria which would degrade the herbicide.

INTRODUCTION

Evidence for the enhanced biodegradation in soil of an increasing number of soil-applied pesticides has been published in recent years, and the topic reviewed in detail by Racke & Coats (1990). Although the phenomenon can lead to severe performance problems in terms of pest, disease or weed control, there is increasing evidence that this may be the exception rather than the rule in that changes in degradation rate can occur which do not affect biological activity (Cotterill & Owen, 1989). In recent experiments, Walker & Welch (1991) demonstrated that enhanced degradation of several soil-applied herbicides is possible following repeated application at the same site. The compounds included linuron, propyzamide and alachlor, all of which are well-established residual herbicides whose performance is generally considered to be unaffected by repeated application. The present paper summarises the evidence for enhanced biodegradation of one of these compounds, linuron, and describes initial attempts to isolate bacteria that may be responsible for its degradation in soil.

EXPERIMENTAL METHODS

Evidence for enhanced degradation in the field

Full details of the field experiment were given by Walker & Welch (1991). It was prepared in Wharf Ground field at Horticulture Research International (HRI), Wellesbourne (Table 1), and comprised duplicate main plots (20 x 1.5 m), each divided into four subplots, 5 m long. Appropriate subplots were sprayed with linuron at 1.0 kg AI/ha on 13 May, 4 July and 2 September 1987 to give duplicate subplots sprayed once, twice or three times, and duplicate untreated control subplots. On 10 May 1988 the duplicate main plots, including the previously untreated areas, were sprayed again with 1.0 kg AI/ha linuron. All plots were sampled at intervals during the subsequent 99 d and linuron residues were measured by HPLC after extraction of soil samples with methanol (Walker, 1987).

Evidence for enhanced degradation in laboratory incubations

After completion of the field experiment in August 1988, samples of soil were taken from the 0-5 cm layer of the subplots that had received four doses of linuron and from an adjacent area that had received no herbicide. Linuron was incubated in these soils under standard conditions (Walker & Welch, 1991) and the linuron content of sub-samples was measured at intervals over the subsequent 49 d.

Further laboratory studies were made at HRI (Wellesbourne) with six different soils (Table 1) which had been treated previously with linuron at 8 mg/kg on 22 September 1988, 7 February, 21 April and 9 August 1989 and incubated moist at 20°C as before (Walker & Welch, 1991). Untreated samples of the different soils were obtained from the field and all soils were incubated with a fresh addition of 8.0 mg/kg linuron. Residues were measured at intervals over the subsequent 36 d.

TABLE 1. Soil properties.

Soil	Organic matter (%)	Clay (%)	Sand (%)	pH	Water content (% at 33kPa)
Long Close	2.20	15	80	6.3	17.7
Pump Ground	2.60	17	70	6.6	12.9
Plum Orchard	2.62	18	70	6.7	14.7
Cottage Field	4.01	25	62	6.8	15.2
Hunts Mill	2.17	13	76	6.5	8.5
Wharf Ground	2.49	18	68	6.2	12.8

Direct isolation of degrading bacteria

Duplicate 1 g samples of linuron-treated soil from Plum Orchard which had been used in the laboratory incubation experiments were suspended in 9 ml of sterile tap water and shaken for 30 min. Aliquots (0.1ml) of serial ten-fold dilutions of these suspensions were then used to prepare spread plates of four different media (Table 2) with and without linuron (10 mg/l). The linuron was added to the molten media as a solution in ethanol. Plates were incubated at 15°C for 4 d.

Colonies from the linuron-supplemented plates were sub-cultured to 10 ml of the corresponding liquid medium and incubated at 15°C to test for their ability to degrade linuron in pure culture. Linuron concentration was monitored by HPLC.

Enrichment culture

Further attempts were made to obtain linuron degrading bacteria by enrichment culture. Soil from Plum Orchard was given a further treatment with linuron (10 mg/kg) and incubated at 20°C. After three days (when the rate of

TABLE 2. Materials used to supplememt mineral base in media used for direct isolation of linuron degrading bacteria from soil. All media contained in g/l: KH_2PO_4, 2.27; $Na_2HPO_4.12H_2O$, 5.97; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$ 0.01; $MnSO_4H_2O$, 0.02; ferric amm. citrate, 0.01; agar 15.0.

Compound	g/l	Medium			
		1	2	3	4
Yeast extract	0.5		+		
Glucose	1.0		+	+	
Casamino acids	0.5		+	+	+

linuron degradation would be expected to be greatest) 0.5 g of the soil was used to inoculate flasks containing 20 ml of nine different liquid media (Table 3). These media all had the same mineral base and contained linuron (10 mg/l) added as a solution in ethanol, but varied in their nitrogen and carbon sources.

The flasks were incubated at 25°C on a shaking platform and the change in linuron concentration with time was monitored. When the linuron concentration in any flask approached zero, 0.5 ml of culture was transferred to a fresh flask containing 20 ml of the same medium which was then incubated and the linuron concentration monitored as before.

Studies on the enriched culture

The culture obtained by enrichment in A3 medium was used for further studies, since in this medium the herbicide was the main source of carbon and nitrogen. This linuron-degrading culture (LDC) was taken through five passages (sub-cultures) before being frozen at -76°C with 15% glycerol as a cryoprotectant. Following freezing the culture was thawed and used as inoculum for linuron-containing liquid A3 medium. Spread plates of dilutions of LDC were prepared on several occasions. Different colony types were sub-cultured from these plates and assayed for their linuron-degrading ability.

RESULTS AND DISCUSSION

Evidence for enhanced degradation in the field

The results from the field persistence study (Figure 1a) demonstrate that linuron degraded least rapidly in the soil treated for the first time, but differences in rates of residue decline between the other treatments were small. The times to 50% initial loss were approximately 60, 35, 35 and 30 d in soil treated for the first, second, third and fourth time respectively.

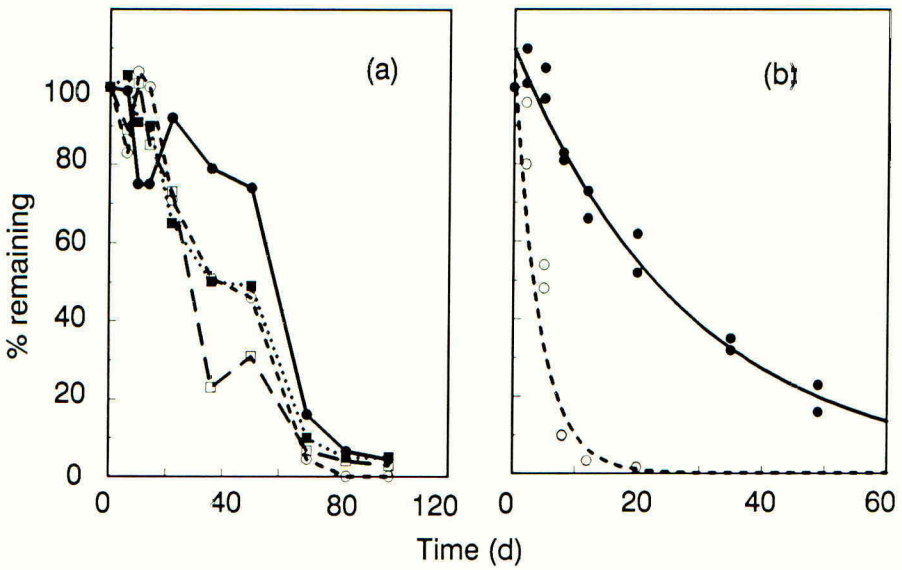

Figure 1. Degradation of linuron in soil (a) under field conditions in 1988 following 0 (●), 1 (○), 2 (■), or 3 (□) previous treatments in 1987; (b) in laboratory incubations, pre-treated (○) and control (●).

TABLE 3. Materials used to supplement mineral base in media used for enrichment of linuron degrading organisms. All media contained in g/l: KH_2PO_4, 2.27; $Na_2HPO_4.12H_2O$, 5.97; MgCl, 0.5; $CaCl_2.2H_2O$ 0.01; $MnSO_4H_2O$, 0.02; $FeSO_4$, 0.005; agar 15.0.

Compound	g/l	Medium								
		A1	A2	A3	B1	B2	B3	C1	C2	C3
Succinate	1.0	+			+			+		
Glucose	1.0		+			+			+	
NaCl	1.0	+	+	+						
NH_4Cl	1.0				+	+	+	+	+	+
Yeast extract	0.5							+	+	+
Casamino acids	0.5							+	+	+

Evidence for enhanced degradation in laboratory incubations

The results using soil from the field experiment are shown in Figure 1b and indicate considerable enhancement of linuron degradation rate. The time to 50% initial loss was reduced from about 20 d in the control soil sample to approximately 3 d in the soil that had received four previous applications of linuron in the field. Residues were not detected after 20 d in the pre-treated soil but approximately 20% of the initial amount remained in the control soil after 49 d.

Measurements of linuron degradation in the six laboratory pre-treated soils and their controls are shown in Figure 2. The data demonstrate that considerable enhancement of degradation rate was induced in all six soils. The herbicide disappeared most rapidly from the pre-treated samples of soil from the Plum Orchard and Cottage Field sites in which less than 10% of the amount applied remained after incubation for 4 d. No detectable residues were present in the pre-treated Plum Orchard soil after incubation for periods greater than 11 d.

Direct isolation of degrading bacteria

Total colony counts from the media containing linuron were 5 to 10 times lower than on the media which contained no linuron, suggesting that linuron has an inhibitory effect on some soil bacteria. None of the 62 isolates sub-cultured from the linuron-containing isolation plates showed any linuron-degrading ability during three weeks incubation.

These results highlighted several problems. (i) The time of sampling relative to the last herbicide treatment may be critical for successful isolation. Hence, as complete linuron degradation occurred over a period of five days or less in the enhanced soil, the numbers of the bacteria responsible for its degradation may have considerably declined or may have become quiescent during the month between the attempted isolations and the most recent linuron treatment. (ii) The growth of bacteria on isolation plates does not necessarily mean that they were active in the soil. (iii) The degradation may be done by several strains acting together.

Enrichment culture

Degradation of linuron occurred in all of the media inoculated with soil but was initially most rapid in the most heavily-supplemented media (type C). The degradative ability of the soil cultures could be transferred by repeated sub-culturing to further flasks of liquid media, but the ability to transfer it was influenced by the medium, so that the degradative ability was most stable in the least supplemented or most minimal media (A3) (Figure 3). In some of the more heavily supplemented media the degradative ability declined or even disappeared after two sub-cultures.

This apparent contradiction demonstrates an important aspect of media selection; for although degradation rates may initially be greater in relatively rich media with adequate supplies of carbon and nitrogen, culturing in such media will tend to select for organisms which can utilise the available carbohydrates and amino acids rather than those which have the ability to degrade the linuron, which is present in much lower concentration. On the other hand, although degradation rates may initially be lower in nutritionally-poorer media, the selection pressure for linuron-degrading

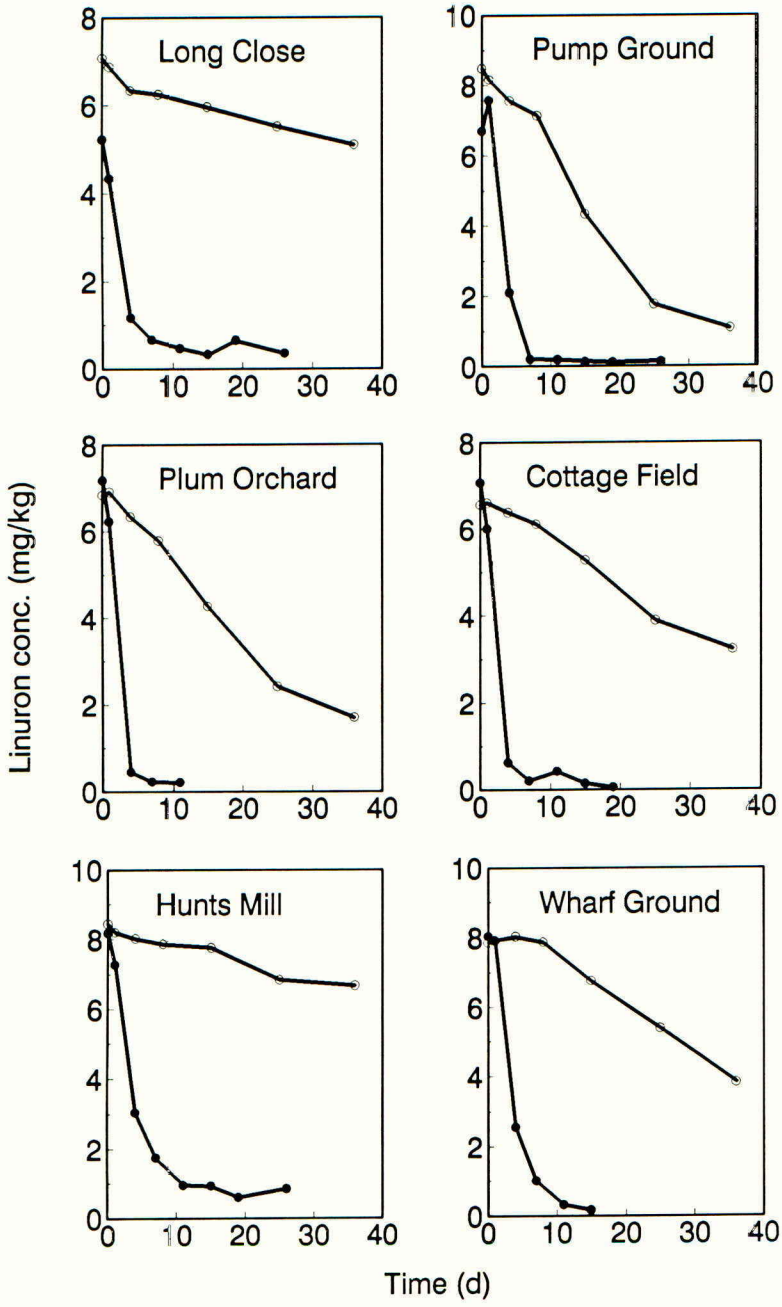

Figure 2. Degradation of freshly-applied linuron in laboratory incubations at 20°C in six soils either previously untreated (○) or following four previous treatments (●).

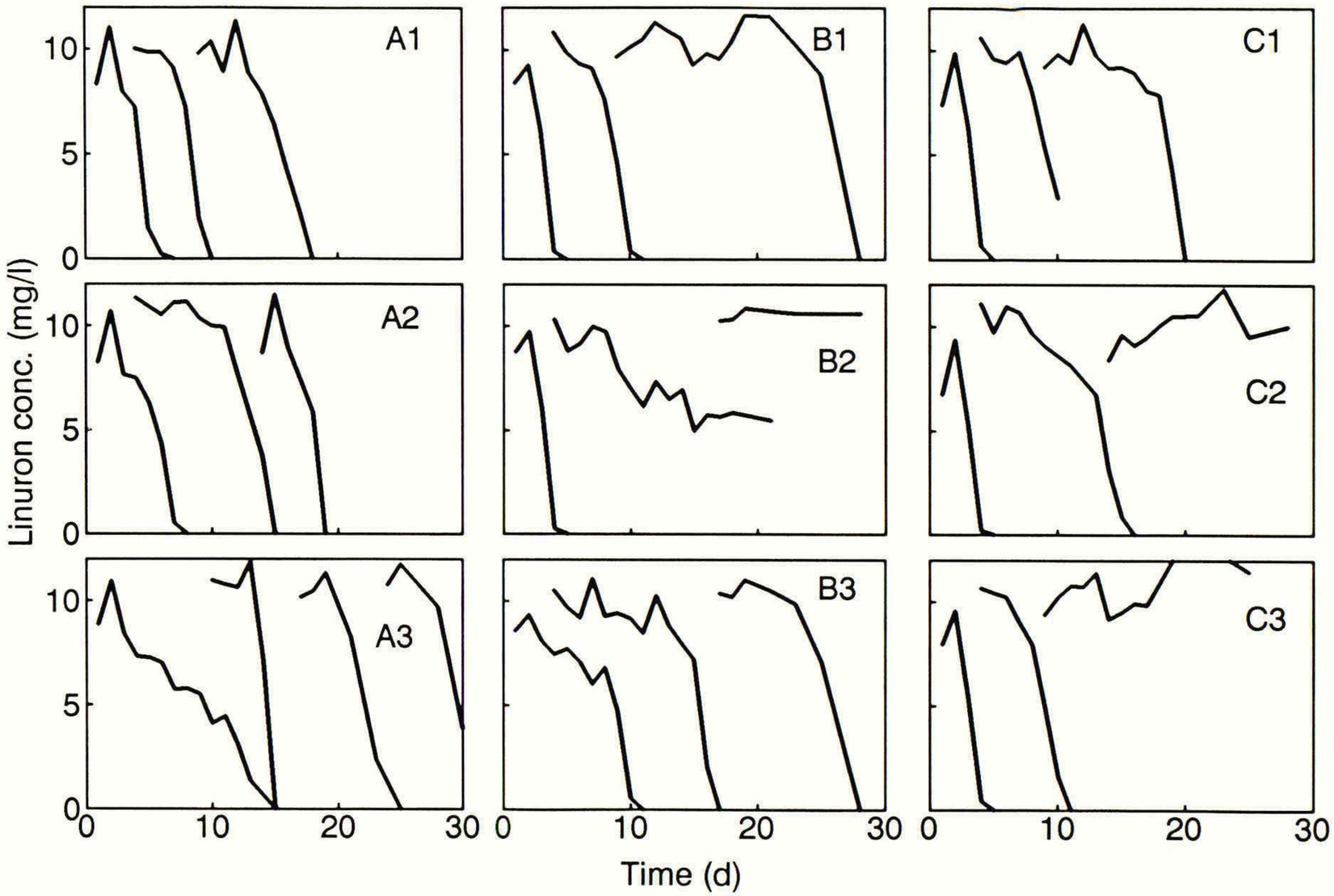

Figure 3. Linuron degradation in shake flask cultures of nine different liquid media, during repeated sub-culturing (each line represents a sub-culture of the previous line). Key to media: A, no nitrogen source; B, NH_4^+ nitrogen; C, complex (amino) nitrogen; 1, succinate C-source; 2, glucose C-source; 3, no C-source.

ability is much greater as it represents a greater proportion of the available carbon and nitrogen. Hence the degradative ability of organisms enriched in the minimal medium was much more stable upon repeated sub-culturing. Another important aspect is that the linuron was added as a solution in ethanol and this may represent a significant source of carbon to the culture and hence may have had an effect on the components of the final culture.

As it appeared to be one of the most stable in repeated sub-culturing, the degrading culture obtained with A3 medium was chosen for use in further investigations.

Studies on the enriched culture

The LDC (obtained with A3 medium) retained its linuron-degrading ability following freezing and thawing and through nine passages in liquid media. Based on observations of spread plates prepared from dilutions of the LDC, there were initially thought to be four components (colony types) in the mixture. These four colony types were each sub-cultured and assayed for their ability to degrade linuron. None of the four showed any degradative ability in pure culture. More recent evidence, however, has indicated that the LDC is predominately composed of six different (based on colony morphology) bacterial types, plus at least 13 other morphologically-distinct strains present in smaller numbers. Assessment of the ability of each of these strains to degrade linuron in pure culture and their characterisation is currently in progress.

CONCLUSIONS

The results of the field studies and soil incubations show that enhanced degradation of the herbicide linuron can occur following repeated applications and that this degradation can be relatively rapid. The failure of the attempt at direct isolations illustrates some of the drawbacks of this approach. The direct isolations showed that linuron may have a significant inhibitory effect on soil bacteria. Experiments with enrichment culturing demonstrated the importance of the choice of media for successful isolation of degrading bacteria. The absence of a single bacterial type in the enriched culture suggests that degradation of linuron in these soils may be a complex process.

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SOME FACTORS AFFECTING THE ACCELERATED BIODEGRADATION OF CARBOFURAN IN SUGAR BEET CULTIVATIONS OF CENTRAL BELGIUM

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ABSTRACT

Soil samples were taken on 8 sugar beet farms (5 with a history of carbofuran treatment, 3 without) of central Belgium between February and June 1990. The 26 fields sampled had been cropped with sugar beet (and thus, in some cases, carbofuran-treated) 1, 2, 3 or even 4 years before sampling. For each sample, a biodegradation index (BDI) was determined using carbofuran degradation measurements obtained after two successive insecticide treatments in the laboratory. The carbofuran treatment history, soil pH and the time elapsed since the last carbofuran application were shown to influence the BDI of the sampled soils.

INTRODUCTION

The accelerated biodegradation of soil insecticides is particularly well documented for some N-methyl carbamates such as carbofuran (Racke and Coats, 1988). This phenomenon has been reported in North America and in Western Europe where carbofuran is used under continuous crop conditions (monoculture) or at a high application rate (horticulture) (Felsot *et al.*, 1982 ; Walker and Suett, 1986 ; Suett, 1989).

In Belgium, enhanced biodegradation of carbofuran has also been reported (Van De Steene *et al.*, 1990) for soils from regions with intensive horticulture and insecticide utilization. One of the most important uses of carbofuran is, however, in the sugar beet crop where the compound is applied in the sowing row at the rate of 750 g a.i./ha. De Proft (1989) reported that the control of pygmy mangold beetle (*Atomaria linearis*) and nematodes (*Heterodera schachtii*) was unsatisfactory in sites of repeated use of carbofuran (sugar beet monoculture).

The aim of the present study was to further evaluate the risks of unsatisfactory control by carbofuran in sugar beet crops under various crop rotations. For this purpose, the biodegradation properties were evaluated using a biodegradation index that was determined on 26 soils taken in 8 different locations in central Belgium. The influence of soil pH on the biodegradation properties of the soils was also determined.

EXPERIMENTAL

The soils were sampled from February to June 1990 on 8 sugar beet farms. Five of them had a carbofuran history (Table 3) ; they were situated in Vlijtingen (Vl-1 to Vl-4), Buzet (Bz-1 to Bz-4), Ophain (Op-1 to Op-4) and Spy (Sp-1 to Sp-4). Three other farms where carbofuran had never been

used were also included, i.e Emines (Em-1 to Em-3), Sint Truiden (St-1 to St-2) and Meldert (Ml-1). An additional soil, which had not been treated with pesticides for the last 10 years (organic farming), was sampled in Vlijtingen (Vl-5). Whenever possible, the soils collected covered the full range of crops included in the farm's rotation. For the farms where a classical triennial rotation was in use, this meant that samples were taken in fields where sugar beet, wheat and winter barley had been or were to be sown. Some soils were also taken under sugar beet monoculture or biennial rotation conditions (Vl-1 and Op-2, respectively). Some farmers have recently abandoned carbofuran for other treatments, so it was also possible, in two farms, to take soils for which the last carbofuran treatment took place 4 years before sampling (Bz-4, Sp-4).

For each plot, 10 replicate samples weighing c. 50 g were taken from the 1-15 cm horizon. The pH (water:soil = 5:1) and moisture contents were determined on subsamples within 1 to 3 days of sampling. Ten g of each soil sample were then treated with 1 ml of an aqueous carbofuran solution at 50 mg/l and incubated at room temperature in open vials that were stored in a closed cabinet. Under these conditions, the moisture content of the samples could be maintained close to 80 % of their field capacity. After 6 days, 1 g was removed and extracted by shaking with 10 ml of water for 1 h and, after centrifugating, the supernatant was kept frozen until analysis of the carbofuran content. After 6 more days, a second 1 g sample was removed and extracted. A further 1 ml carbofuran solution (50 mg/l) was then added and a final extraction was done after a further 6 days, i.e. 18 days after the initial carbofuran treatment.

The analyses of carbofuran were done using a simplified method described elsewhere (Pussemier *et al.*, 1990). Briefly, 4 x 50 µl of each solution to be analysed were transferred to separate wells of a 96-well microtitration plate. Standard carbofuran solution (125 µg/l) and appropriate blanks were added on the same plate. A buffered mixture (50 µl) containing acetylcholinesterase and Ellman's reagent (DTNB) was then added to all the wells and after incubating at room temperature for 2.5 h, acetylthiocholine (50µl) was added. After hydrolysis of this substrate, the thiocholine released reacted with Ellman's reagent to give a yellow colour, the absorbance of which was measured at 405 nm using a microcolorimeter. In the presence of carbofuran, the enzymatic hydrolysis was inhibited, so that the absorbance measured was proportional to the carbofuran concentration in the aqueous solution. It was possible, in this way, to determine quickly and on a large number of water extracts the samples where more than 75 % of the introduced carbofuran had been degraded. This "yes or no" answer, ("yes" meaning more than 75 % degradation) was very well fitted for the determination of a biodegradation index (BDI), which was calculated by summing the number of positive answers obtained for the 3 successive degradation tests.

RESULTS AND DISCUSSION

Determination of the biodegradation index (BDI)

Three examples of BDI determination are presented in Table 1. They were selected because they give a good illustration of the results obtained with soils with contrasted behaviours.

TABLE 1. Examples of the determination of the biodegradation index (BDI) in three contrasted soils (- and + indicate less or more than 75 % degradation, respectively).

Sample replicate	carbofuran degradation after			BDI	pH
	6 days	12 days	18 days		
Example 1 : soil Em-2 (no carbofuran history)					
1	-	+	-	1	7.4
2	-	-	-	0	7.7
3	-	-	-	0	7.0
4	-	-	-	0	7.1
5	-	+	-	1	6.7
6	-	-	-	0	6.6
7	-	-	-	0	6.2
8	-	-	-	0	6.3
9	-	-	-	0	6.6
10	-	-	-	0	7.3
				mean 0.2	
Example 2 : soil Op-4 (carbofuran 3 years before)					
1	-	+	+	2	7.5
2	-	+	+	2	7.2
3	-	+	+	2	7.3
4	-	-	-	0	6.5
5	-	+	+	2	7.7
6	-	-	+	1	7.3
7	-	+	+	2	7.7
8	-	+	+	2	7.5
9	-	+	+	2	7.9
10	-	+	+	2	7.4
				mean 1.7	
Example 3 : soil Bz-1 (carbofuran 1 year before)					
1	-	+	+	2	7.8
2	-	+	+	2	8.0
3	+	+	+	3	8.1
4	+	+	+	3	7.8
5	+	+	+	3	7.6
6	+	+	+	3	7.9
7	+	+	+	3	8.0
8	+	+	+	3	7.9
9	+	+	+	3	8.0
10	+	+	+	3	8.0
				mean 2.8	

Example 1 (soil Em-2) is a soil with no carbofuran treatment history. The analyses revealed that for all samples there was < 75 % carbofuran degradation after the first 6 days of incubation. After 12 days, two samples were characterized by high carbofuran degradation but, after 6 more days and a second addition of carbofuran, the degradation again was < 75 %. Most of the degradation ratings were negative and the mean BDI value was only 0.2, indicating that there was no enhancement of the degradation during the 18-day incubation period.

The second example is given for a soil treated 3 years before with carbofuran. With 8 of the 10 samples, there was extensive degradation after 12 days but not after 6 days. Six days after the second carbofuran addition, degradation was again extensive in 9 of the 10 samples, indicating that it was enhanced. The mean BDI for this soil was 1.7.

In the third example, which is obtained with a soil treated one year previously with carbofuran, most samples showed rapid degradation 6 days after the first addition of carbofuran. The mean BDI of 2.8 was very close to the maximum in the scale adopted for this study, indicating that, from the beginning of the incubation, degradation was enhanced.

Effect of previous insecticide treatments.

Carbofuran treatment history clearly influenced the BDI. In the 7 fields sampled in farms where carbofuran had never been used, the BDI was always < 1 (Table 2). In the 19 other fields where at least one carbofuran treatment had been applied during the previous 4 years, the BDI exceeded 1.7, with the exception of one field (Vl-4) that had been treated 3 years before (Table 3). Treatments with other insecticides such as aldicarb, thiofanox and terbufos had no significant influence on the BDI.

The time elapsed since the last carbofuran treatment also influenced the BDI. There were only small differences when carbofuran had been applied one or two years before but, after 3 or 4 years, a decrease in the BDI was observed in most cases. Also, when the treatments had been applied at shorter intervals (monoculture as for Vl-1 or biennial crop rotation as in Op-2) the BDI values were larger than under classical crop rotation conditions.

Effect of pH.

For the 7 soils with no carbofuran treatment history, a satisfactory correlation was found between the BDI and pH averaged for the 10 samples of each soil (r_{soils} = 0.89). This is in good agreement with other studies (Getzin, 1973 ; Pussemier *et al.*, 1980) that have shown an increase in the chemical and biological degradation rate of carbofuran with increasing soil pH.

For the soils with a history of carbofuran treatment, such correlations are more difficult because, as shown above, the time elapsed since the last treatment as well as differences in treatments intervals can also influence the BDI. Nevertheless, although no significant correlation is expected to be obtained from the comparison between soils with different histories, it is possible to study possible pH effects by comparing values for individual samples from the same plot ($r_{samples}$). This is illustrated,

TABLE 2. Insecticide history, biodegradation index (BDI) and pH for soils with no carbofuran treatment history. SD = standard deviation.

Soil	Soil insecticide history	BDI (SD)	pH (SD)
Vl-5	pesticides never used	0.1 (0.4)	7.1 (0.2)
Ml-1	aldicarb 1 year before	0.9 (0.9)	7.4 (0.6)
St-1	thiofanox 1 year before	0 (0)	6.3 (0.5)
St-2	thiofanox 1 year before	0 (0)	6.5 (0.4)
Em-1	terbufos 1 year before	0.9 (1.3)	8.1 (0.1)
Em-2	terbufos 2 years before	0.2 (0.4)	6.9 (0.5)
Em-3	terbufos 3 years before	0.8 (1.0)	8.0 (0.1)

TABLE 3. Carbofuran treatments, biodegradation index (BDI), pH and correlation coefficient (r) between BDI and pH for various soils with carbofuran treatment history. SD = standard deviation.

Soil	Previous carbofuran treatment	BDI (SD)	pH (SD)	$r_{samples}$
Vl-1	each year during the last 7 years	2.7 (0.5)	8.3 (0.3)	-0.36
Vl-2	1 year before	2.3 (0.5)	8.0 (0.3)	-0.29
Vl-3	2 years before	2.1 (0.3)	8.0 (0.2)	-0.08
Vl-4	3 years before	0.2 (0.4)	8.3 (0.3)	-0.35
Bz-1	1 year before	2.8 (0.1)	7.9 (0.1)	0.25
Bz-2	2 years before	2.9 (0.3)	8.1 (0.2)	0.12
Bz-3	3 years before	1.9 (0.3)	7.5 (0.4)	0.23
Bz-4	4 years before	1.9 (0.3)	7.3 (0.4)	0.55*
Rx-1	1 year before	2.2 (1.0)	6.2 (0.3)	0.68*
Rx-2	2 years before	1.9 (0.3)	6.3 (0.2)	0.59*
Rx-3	3 years before	2.0 (0.5)	7.2 (0.4)	0.27
Op-1	1 year before	2.2 (0.4)	7.6 (0.2)	0.24
Op-2	2 years before (biennial rotation)	2.5 (0.5)	7.1 (0.3)	0.13
Op-3	2 years before (triennial rotation)	2.1 (0.3)	8.0 (0.1)	-0.39
Op-4	3 years before	1.7 (0.7)	7.4 (0.4)	0.82**
Sp-1	1 year before	2.6 (0.5)	8.0 (0.2)	0.09
Sp-2	2 years before	2.6 (0.5)	8.0 (0.1)	-0.30
Sp-3	3 years before	2.2 (0.4)	8.0 (0.2)	0.47
Sp-4	4 years before	2.1 (0.3)	7.6 (0.3)	0.62*

* significant at p = 0.95
** significant at p = 0.99

for example, using the data given for the soil Op-4 in Table 1, for which there is a good correlation ($r_{samples}$ = 0,82) between the BDI and the pH values of the individual samples. In this plot, the pH effect was detected because the samples were not homogenous, with the result that some variations in the BDI and pH values were indicated by the high standard deviation (SD) values of 0.7 and 0.4 respectively (Table 3). In contrast samples from the Bz-1 plot were characterized by very similar BDI and pH values (SD = 0.1), so that effects of the pH on the acceleration of the biodegradation were not evident.

The $r_{samples}$ values calculated between BDI and pH are listed in Table 3 for all the soils previously treated with carbofuran. The most significative correlations ($r_{samples}$ = 0.82 and 0.68) are found with the soils Op-4 and Rx-1, which are also characterized by the highest SD values for BDI (0.7 and 1.0, respectively). Similar but less marked correlations were observed with soils Sp-4, Rx-2 and Bz-4. Within these 5 soils, the pH of which ranged from 6.2 to 7.6, it was evident that the ability to degrade carbofuran rapidly increased with increasing soil pH.

For the soils characterized by higher pH values, the correlation coefficients decreased and became negative when the soil pH exceeded 8.0 suggesting that, at higher pH levels, there was no correlation with accelerated degradation. This could be a result of rapid chemical and biological degradation in alkaline soils reducing the persistence of carbofuran and thus, by limiting the extent to which soil microcrganisms are exposed to the insecticide, minimising microbial adaptation.

According to a recent study published by Suett and Jukes (1990), the accelerated biodegradation of the organophosphorus insecticide mephosfolan is also favoured by an increase of the soil pH. In this latter study, the soils were taken in hop farms and were characterized by pH values below 7.5. Thus carbofuran and mephosfolan show some similarity in behaviour with accelerated biodegradation properties seeming to be favoured by increasing the soil pH, at least in the 5.8 - 8.0 range.

CONCLUSIONS

- It is possible to determine a biodegradation index (BDI) for soil based on 2 successive carbofuran applications to soil samples and 3 carbofuran determinations performed at successive intervals of 6 days.

- Since the determination of the BDI requires only a "yes" or "no" type of information (more than 75 % degradation or not), the carbofuran analyses can be performed using simplified, low cost analytical techniques and equipment.

- The BDI values obtained allow good discrimination between soil samples with or without carbofuran history.

- The soil pH seems to be an important factor influencing the development of accelerated biodegradation properties, at least in the acid and neutral range.

- The accelerated biodegradation, once acquired by a soil, persists at least 2 years. There are thus potential risks for a lack of adequate carbofuran persistence on sugar beet crop, even if the product is used only once in a triennial rotation.

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DEGRADATION OF FLUAZIFOP-BUTYL BY SOIL MICROORGANISMS

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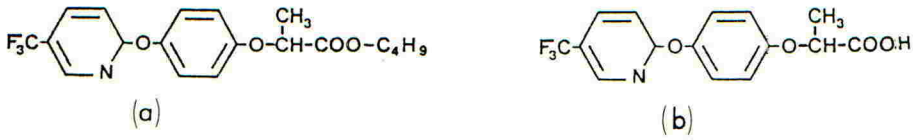
ABSTRACT

Laboratory studies were conducted to determine the biodegradability of the herbicide fluazifop-butyl. The compound was rapidly hydrolyzed by a mixed culture enriched from a landfill leacheate. The hydrolysis product, fluazifop, was further degraded by the same mixed culture to 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol. The metabolite accumulated when fluazifop was in the medium as a sole source of carbon and energy whereas it was further degraded when sodium acetate or sodium succinate was added to fluazifop as cometabolites. Results indicate a cometabolic degradative pathway.

INTRODUCTION

Fluazifop-butyl (butyl 2 - (4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenoxy) propionate) (Fig. 1) is a herbicide for the selective postemergence control of annual grassy weeds in broadleaf crops (Horellou et al., 1982). The pathway of fluazifop-butyl degradation in soil involves both chemical and microbial processes (Nègre et al., 1988). The major product of degradation has been identified as the hydrolysis product fluazifop: (2 - (4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenoxy) propionic acid) (Fig. 1) (Bewick, 1986; Smith, 1987). Fluazifop is further degraded with a half life generally less than 12 weeks (Arnold et al., 1982). Fusi et al. (1988), working on the adsorption of fluazifop-butyl and fluazifop by two homoionic smectites observed that these compounds were adsorbed on smectites by both direct and indirect coordination between the C=O group of the herbicide and the exchangeable cation. Moreover, they observed that fluazifop-butyl but not fluazifop undergoes abiotic degradation to give 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol.



(a) (b)

Figure 1. Fluazifop-butyl (a) and fluazifop (b).

Data on the involvement of microorganisms in the degradation of fluazifop-butyl and fluazifop are limited. Nègre et al. (1988) reported a slower dissipation of fluazifop-butyl and fluazifop in sterile soil than in non-sterile soil, indicating that microorganisms play a role in the degradation of this herbicide.

The purpose of this study was to examine the capability of microbial cultures to degrade fluazifop-butyl, and to determine the rate of degradation.

MATERIALS AND METHODS

Enrichment cultures

For enrichment, 500 ml Erlenmeyer flasks containing 100 ml liquid mineral medium M9 were used. The medium composition was (per litre): Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; pH adjusted to 7, autoclaved, then 2.5 ml of 1 M $MgSO_4$, 2.5 ml of 36 mM $FeSO_4.7H_2O$ and 2.5 ml of a trace element solution containing (per litre): MgO, 10.75 g; $CaCO_3$, 2 g; $FeSO_4.7H_2O$, 4.5 g; $ZnSO_4.7H_2O$, 1.44 g; $MnSO_4.4H_2O$, 1.12 g; $CuSO_4.5H_2O$, 0.25 g; $CoSO_4.7H_2O$, 0.28 g; H_3BO_3, 0.06 g; 1 M HCl, 51.3 ml was added. Fluazifop-butyl (10 mg) either as a sole carbon and energy source or supplemented with sodium acetate (0.1% wt/V) or sodium succinate (0.1% wt/V) was added directly to the medium. Samples of leacheate from a landfill, activated sludges from a waste water treatment plant, soils and freshwaters were used as inocula. The flasks were incubated at 30°C on a rotary shaker. After a week, 50 ml of each culture was used to inoculate fresh medium. This process was repeated three times. After the third transfer, the cultures which were able to grow in the presence of fluazifop-butyl were tested for their ability to grow in the presence of fluazifop. The enriched cultures (50 ml) were tranferred to 500 ml Erlenmeyer flasks containing 100 ml liquid mineral medium M9 and either fluazifop (10 mg) as a sole source of carbon or fluazifop (10 mg) supplemented with sodium acetate (0.1% wt/V) or sodium succinate (0.1% wt/V) were added. The cultures were incubated at 30°C and 50 ml samples were transferred to fresh medium every week. After three transfers, samples

from the enrichment cultures were used to determine the degradation of fluazifop by the mixed cultures.

Degradation studies

The ability of the enriched cultures to degrade fluazifop was studied by inoculating, with 10 ml of preculture, three series of Erlenmeyer flasks each containing 60 ml of mineral medium M9 added with: a) 20 ug fluazifop as a sole source of carbon (FA); b) 20 ug fluazifop and 0.1% sodium acetate (FA+AC); c) 20 ug fluazifop and 0.1% sodium succinate (FA+S). All treatments were in duplicate. The flasks were incubated at 30°C on a rotary shaker. At 0, 4, 8, 15 and 28 days after inoculation, 5 ml portions were aseptically removed from each flask to determine the concentration of fluazifop. Residues of fluazifop in the medium were extracted three times with 5 ml dichloromethane. The extracts pooled from the three extractions were evaporated to dryness at 35°C on a rotary evaporator, the residues redissolved in 5 ml acetonitrile then analyzed by HPLC. HPLC analyses were performed with a Varian HPLC system using a LiChrospher C18 column and a UV/VIS detector operating at a wavelength of 270 nm. The mobile phase (1 ml/min) comprised water acidified to pH 3.00 with othophosphoric acid added with sodium laurylsulphate 0.1% + acetonitrile (45+55 V/V).

Measurement of microbial growth

Growth of mixed cultures in the flasks where degradation studies were performed was determined by the plate count technique. One ml was removed from the cultures at 0, 4, 8, 15 and 28 days after inoculation, serially diluted X 10 and plated into PCA agar (Difco). The plates were incubated at 30°C for 5 days before counting.

Isolation and identification of degradation products

In order to produce sufficient quantities of metabolites for identification 100 ml portions of the mixed cultures were tranferred to 1 litre Erlenmeyer flasks containing 300 ml mineral medium M9 and 6 mg of fluazifop as sole source of carbon or supplemented with sodium acetate or succinate (0.1% wt/V). When the concentration of fluazifop had declined to approximately 50% of the initial dose, the cultures were pooled in a separatory funnel and extracted with dichloromethane (3 X 400 ml). The combined extracts were evaporated to dryness at 35°C on a rotary evaporator, the residue was dissolved in 1 ml acetone and subjected to thin layer chromatoraphy (TLC). TLC was carried out on precoated Silica Gel 60 F254 with a layer thickness of 0.25 mm (E. Merck, Damstadt, Germany). Authentic standard of fluazifop was cochromatographed. The plates were developed in a solvent system of chloroform:methanol:water (65:25:1 by volume). Dark areas, visualized using a UV lamp (254 nm),

were scraped from the plates and extracted with acetone for analysis by GC-MS and GC-IR techniques. The electron impact mass spectra were obtained on a Hewlet Packard Model 5970. The GC-IR spectra were obtained on a Hewlett Packard GC-IRD Model 5975.

RESULTS

Enrichment cultures

A mixed culture able to grow on fluazifop-butyl supplemented with sodium acetate was enriched from a landfill leacheate. The culture was also able to grow in FA, FA+AC and FA+S, indicating that the compound have been biodegraded.

Degradation studies

Biodegradation of fluazifop occurred in the three cultures tested but stopped after about 14 days of incubation by which time the concentration of fluazifop had declined to about 50% of the initial dose (Figs. 2-4). Degradation during the first 8 days was slowest in culture FA+S (Fig. 4). Degradation was accompanied by microbial growth, which stopped after 4 days of incubation (Figs. 2-4).

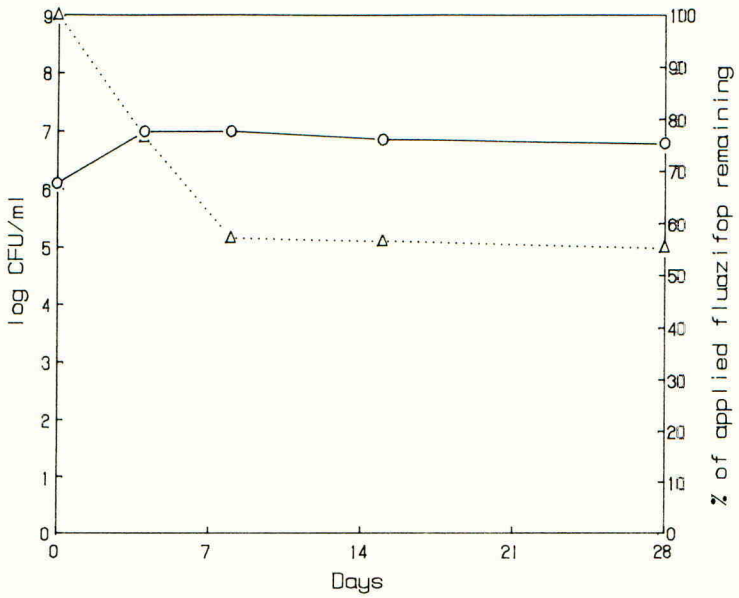


FIGURE 2. Growth of mixed culture (o) and degradation of fluazifop (△) in medium M9 containing fluazifop as sole source of C (culture FA). CFU = colony forming unit.

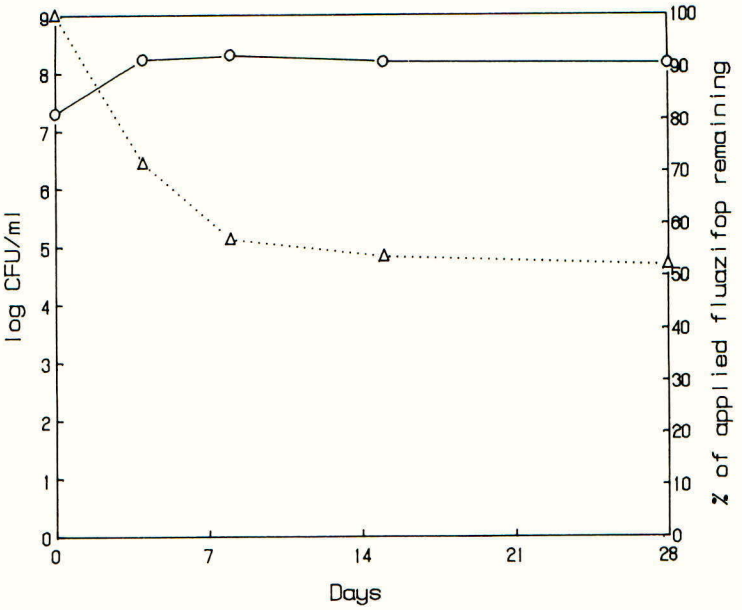


FIGURE 3. Growth of mixed culture (o) and degradation of fluazifop (△) in medium M9 containing fluazifop and 0.1% wt/V sodium acetate (culture FA+AC). CFU = colony forming unit.

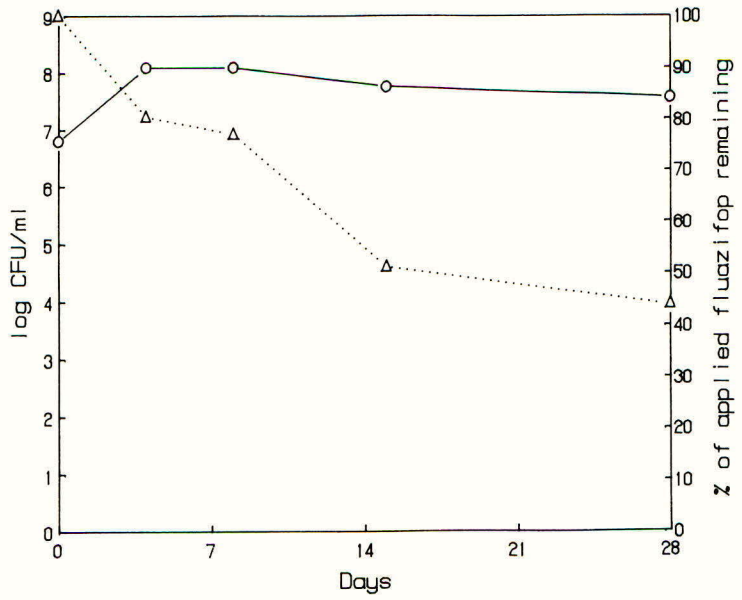


FIGURE 4. Growth of mixed culture (o) and degradation of fluazifop (△) in medium M9 containing fluazifop and 0.1% wt/V sodium succinate (culture FA+S). CFU = colony forming unit.

HPLC analysis of the extracts showed that incubation in culture FA yielded a second major peak in addition to the fluazifop. The retention time of this peak was identical to that obtained with an analytical standard of 4 - (5 - trifluoromethyl - pyridyloxy) phenol (metabolite I). The amount of metabolite I detected in these extracts was stoichiometrically equivalent to the amount of fluazifop that had disappeared presumably due to the absence of further metabolism under these conditions. Trace amounts of metabolite I were detected initially in the extracts of cultures FA+AC and FA+S but were not detectable after 14 days.

There was no evidence of fluazifop volatilization, sorption by glass or chemical degradation during the test period when the same concentration of the compound was added to uninoculated flasks.

Identification of metabolites

Under the TLC test conditions, the Rf value of fluazifop was 0.66. Two unknown products appeared at Rf 0.23 and 0.80 in the extracts from all the cultures. In the extract from culture FA a third compound was detected at Rf 0.90. The mass spectrum of the compound with Rf 0.90 eluted from the TLC showed a molecular ion at m/e 255 and the same fragmentation pattern as the mass spectrum of the standard of fluazifop-butyl for m/e<255. The ion m/e 255 agreed with the mass of the compound 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol (Fig. 5).

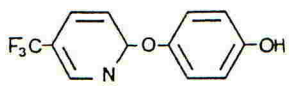


FIGURE 5. 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol (metabolite I)

The major fragment peaks observed at m/e 227 and m/e 146 corresponded respectively to the elimination of carbon monoxide which is a typical rearrangement of phenols (Dyke et al., 1971) and to the cleavage of the ether bond. The molecular structure of the compound was confirmed by the IR spectrum which showed a band characteristic of free OH (3660 cm) and the bands of CF3 (1327 cm) and of aromatic groups (1612, 1505, 1490 cm) identical with those of fluazifop, while the COOH and aliphatic CH absorptions were absent.

Concentration of the metabolites with Rf values of 0.23 and 0.80 were too small to allow structural investigation.

DISCUSSION

Partial biodegradation of fluazifop occurred under the three cultural

conditions tested but was slowest in culture FA+S. Biodegradation was associated with the formation of 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol. This intermediate accumulates in culture FA but was degraded further in cultures FA+AC and FA+S. These observations suggest that acetate and succinate are cosubstrates for the degradation of the metabolite.

The limited degradation of fluazifop in all three cultures cannot, as yet, be explained. However, growth in batch cultures may have limited the availability of some essential nutrients, thus hindering the continued degradation of the compound. It is also possible that, in culture FA, the continued accumulation of metabolite I may have limited the degradation of the parent compound. Further research is being done to study the fate of fluazifop in a medium with a higher content of cosubstrate and to identify the breakdown products of 4 - (5 - trifluoromethyl - 2 - pyridyloxy) phenol.

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EFFECTS OF SOIL TREATMENTS WITH ALDICARB, CARBOFURAN AND CHLORFENVINPHOS ON THE SIZE AND COMPOSITION OF MICROBIAL BIOMASS

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ABSTRACT

The effects of different concentrations of the soil-applied insecticides aldicarb, carbofuran, and chlorfenvinphos on the size and composition of soil biomass were monitored in field-based experiments. Whereas chlorfenvinphos produced no significant changes in any of the microbiological parameters monitored, carbofuran application affected numbers of plateable prokaryotes (without modifying gross biomass size) and aldicarb produced significant, dose-dependent changes in both biomass size and composition. Total fungal biomass followed the same trends as overall biomass in aldicarb-treated soils. Accelerated degradation of all the insecticides was observed, and the relationship of this phenomenon to biomass changes is discussed.

INTRODUCTION

The interactions between pesticides and microorganisms in soil ecosystems are highly complex. On the one hand, pesticides may serve as nutrient sources for a proportion of soil biomass, and indeed microbial decomposition of pesticides in soils has generally been considered a good thing, resulting, as it does, in their breakdown and inactivation (though this does not preclude the formation of toxic metabolites). However, microbiological breakdown may develop to the extent that the biological efficacy of soil-applied pesticides may be seriously reduced, a phenomenon often referred to as 'accelerated degradation' (e.g. Suett & Jukes, 1988). On the other hand, pesticides may have toxic side effects on non-target soil microorganisms, and induce changes in the size, composition and activity of soil biomass (Somerville & Greaves, 1987). This paper reports on some effects on soil biomass of three soil-applied insecticides that have, in the past, been associated with accelerated degradation.

MATERIALS AND METHODS

Soils

Soil samples were obtained in 1989 and 1990 from six field sites at Horticulture Research International, Wellesbourne, where experiments had been established to study the biological performance of freshly-applied insecticides in soils with different histories of insecticide treatment. Granular formulations of the insecticides aldicarb (10% a.i., Embetec Crop Protection) and carbofuran (5% a.i., Bayer U.K. Ltd) had been applied at sites 1-4 by the bow-wave method (Makepeace, 1965) as continuous, exponentially-increasing doses (Thompson, 1984). Chlorfenvinphos (10% a.i., Shell Chemicals UK Ltd) had been applied similarly at sites 5 and 6. The treatment histories and properties of the six soils studied are summarised in Table 1.

TABLE 1. Treatment histories and properties of soils

Site	Insecticide treatment history	Log-dose applied	pH (H_2O)	organic carbon %	total nitrogen %
1	carbofuran 1987	April 1989	6.1	1.1	0.11
2	" 1988	"	6.5	1.0	0.11
3	" 1986	"	5.6	1.1	0.11
4	" 1986	"	6.4	1.4	0.13
5	no prev. treatment	April 1990	6.7	0.9	0.10
6	chlorfenvinphos 1986 & 1989	"	6.5	1.0	0.09

Samples for microbiological analysis were taken 100(±2) days after the log-dose applications. At each site, duplicate samples of c.1kg were taken from three sub-plots within each row at doses equivalent to 0.65, 1.4 & 4.0kg aldicarb/ha, 0.48, 1.0 & 2.3kg carbofuran/ha and 0.45, 0.82 & 1.52kg chlorfenvinphos/ha. Samples were sealed in polyethylene bags and transferred to cold storage (4°C) within 4 hr, where they were retained prior to microbiological analysis.

Microbiological analyses

Total biomass in soil samples was determined by the fumigation-extraction method (Vance *et al.*, 1987). Total bacterial numbers in soils were determined by counting numbers in dispersed soil extracts using scanning electron microscopy (Williamson & Johnson, 1990), and numbers of viable bacteria using plate counts. In the latter, oligotrophic bacteria were differentiated from copiotrophic bacteria by using different solid media; the oligotrophic medium consisted of chloroform-fumigated soil extract (Vance *et al.*, 1987) diluted with water to produce a 10μg soluble C/ml solution, gelled with 0.7% agarose (Sigma, Type 1), and the copiotrophic medium was R2 agar (Reasoner & Geldreich, 1985), which contains *c.*1,250μg C/ml. Total fungal biomass was estimated by measuring biovolumes of methylene blue-stained hyphae, and vital fungi by using fluorescein diacetate (Williamson & Johnson, 1990). Fungal and actinomycete propagules were counted using selective solid media (Johnson *et al.*, 1991).

Other soil analyses

Soil samples were analysed for pH, moisture contents, total nitrogen, organic carbon and texture; the methods used have been described in detail elsewhere (Johnson *et al.*, 1991).

RESULTS

All significant differences between sample means were established at the 95% confidence limit.

Soil properties

The soils were sandy loams with similar textures of, approximately, 80% sand, 8% silt and 12% clay. There were some variations in the properties of the soils (Table 1). Soil from site 3 was the most acidic and soil from site 4 contained most organic carbon.

Assessment of soil biomass

Figure 1 summarises the results of the biomass measurements. None of the carbofuran- or chlorfenvinphos-treated soils showed significant dose-related changes in biomass. However, in aldicarb-treated soils from sites 1, 2 and 4, biomass was greatest in the soils treated with 1.4kg a.i./ha. Levels in these mid-dose soils were significantly larger than in the corresponding untreated soils, and, in soils from sites 1 and 4, were significantly greater than in soils treated with the largest dose (4.0 kg/ha).

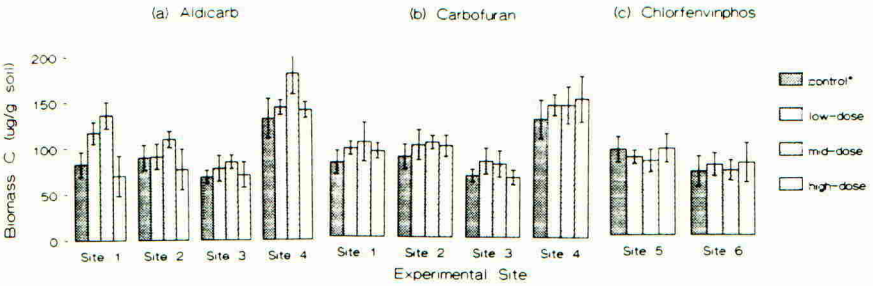

FIGURE 1. Changes in soil biomass following log-dose applications of insecticides to previously-treated soils. Control*soils did not receive log-dose treatments. Vertical bars represent standard deviations.

Fungal populations of treated soils showed similar trends to total biomass, i.e. no patterns or significant differences with carbofuran or chlorfenvinphos treatments, whilst modifications were evident in aldicarb-treated soils. However, whilst this was the case with total hyphal biomass, and to a lesser extent with plateable propagules, vital fungal biomass was not affected by aldicarb treatment (Table 2).

TABLE 2. Changes in soil fungi as a result of aldicarb application (± standard deviation).

Dosage rate	Total fungal biomass (μg C/g soil)	Vital fungal biomass (μg C/g soil)	Plateable propagules (no./g soil)
zero	35.8±13.4	4.5±1.2	10.1±2.2
low	48.7±10.5	4.0±1.2	10.1±0.6
mid	74.6±12.6	4.5±0.9	10.3±2.3
high	30.7±6.2	4.5±1.3	6.5±1.1

Total numbers of bacteria were not affected by any of the pesticides at any level of treatment. However, plate counts of copiotrophic soil bacteria were considerably (though not always significantly) greater in aldicarb and carbofuran-treated soils than in controls, and oligotrophs were greater in carbofuran-treated soils (Fig.

2). Numbers of plateable anaerobic bacteria were reduced by 50% under high carbofuran dosage. Actinomycete propagules were also frequently more abundant in aldicarb soils (Fig. 2).

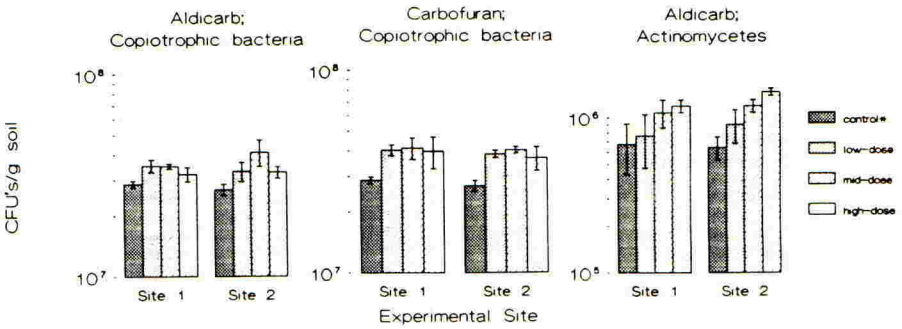

FIGURE 2. Changes in numbers of soil bacteria and actinomycetes following log-dose applications of insecticides to previously-treated soils. Control* soils did not receive log-dose treatments. Vertical bars represent standard deviations; CFU's = colony forming units.

Laboratory studies with soils sampled immediately before application of the log-dose treatments showed marked differences in the stabilities of freshly-applied insecticides (Suett, unpublished data). The initial half-life of carbofuran was correlated directly with the time elapsed since the previous carbofuran treatment and, with chlorfenvinphos, was significantly shorter in the previously-treated soil from site 6 than in soil from site 5. Although between-site differences with aldicarb were relatively small, the insecticide degraded most rapidly in soil from site 4, which also had the largest biomass.

DISCUSSION

It appears that different soil-applied insecticides can produce variable responses in soil microbial populations. Whilst the organophosphate chlorfenvinphos failed to induce detectable changes in either the size or composition of soil biomass, these were noted with the carbamates carbofuran and, especially, aldicarb.

Duah-Yentumi & Johnson (1976) reported that neither a single nor a multiple (5X) application of carbofuran induced major changes in soil biomass, except that colony-forming units (CFU's) of aerobic bacteria increased and those of anaerobic bacteria decreased, following single and multiple applications, respectively. Turco & Konopka (1990), however, reported that, in a laboratory-based experiment, application of carbofuran at 100ppm caused a significant short-term decrease in the size of soil biomass. This effect was not observed at 10ppm dosage rate, and only noted with enhanced (or aggressive) soils. Our data suggests

than any overall biomass effects produced by carbofuran treatment, over a wide dosage range, are not apparent 100 days following application: the only exception to this was the stimulation of aerobic bacteria. The scale of this increase, *c*.1.5 x 10^7/g soil, corresponds to an increase of <1μg biomass C/g soil (assuming an average biomass of 5 x 10^{-13} g/soil bacterium, and a carbon content of 10% of wet biomass), and is insufficient to significantly modify total biomass figures, and affect numbers of total bacteria (*c*.10^9/g) which include non-viable as well as viable cells. Although it is possible that this increase resulted directly from bacterial breakdown of carbofuran, this seems unlikely as: (a) there was no correlation with dosage rate, (b) the bacteria isolated from treated and control soils showed a similar diversity in colony and cellular morphologies (which may not have been the case if, as frequently cited, the breakdown of carbofuran is restricted to a small proportion of the whole bacterial population), and (c) although the carbonyl side chain of carbofuran is readily mineralised, the ring moiety is more recalcitrant (Turco & Konopka, 1990).

The increased biomass noted in aldicarb-treated soils (20-50μg C/g soil in the mid-dosage areas) could not be directly attributable, in total, to degradation of aldicarb (applied at 1.4kg/ha in these areas) nor to the inorganic carrier material. Total fungal biomass followed the same trends as total biomass in these soils, implying that the observed changes were due, in the main, to soil fungal populations. Read (1986) had earlier reported growth of soil fungi on corn grit granules containing aldicarb, and concluded that the fungi were using the carbamate as a source of nutrition. However, whereas fungal growth was stimulated at aldicarb concentrations between 250 and 1000 ppm, at 5000 ppm it was inhibited. This is similar to the results found in the present study where the was a stimulation of fungal biomass in the low-mid dose range, but inhibition (relative to mid-dose areas) in soils receiving high applications. Although the overall concentrations of pesticide applied was very different in the present field study and in Read's laboratory experiments, enhanced localised concentration around the vicinity of granules in the former may be the reason for the similar observations. It was interesting to note that there was no evidence of aldicarb toxicity to bacterial and actinomycete populations over the concentration range tested in the field.

Changes in the size and composition of soil biomass will be time-dependent, and it may be argued that the 'snapshot' data presented herein may not reveal the full extent of such changes. In order to ascertain whether or not there were chlorfenvinphos-induced short-term effects, which were not apparent at 100 days, soil samples which had been collected 10 days following application were subjected to the same range of microbiological analyses. Once again, no significant differences were found between treated and control soils. However, the differences between total and vital fungal biomass in aldicarb-treated soils implies that the stimulation (and possibly inhibition) effects were relatively transient and that, although 100 days after application total fungal biomass remained greater in low-mid zones than elsewhere,

overall fungal activity had equalised.

The effects of soil physicochemical parameters on modifying any changes produced in soil biomass by pesticide application has received relatively little attention. It was interesting, therefore, to note that the aldicarb effects on total biomass varied with soil pH, and were not evident in the most acidic soil. This ties in with the observation by Read (1986) that aldicarb-degrading microorganisms were essentially inactive below pH 5.8.

The relationship between soil biomass and enhanced degradation of pesticides is complex. Whilst there was some evidence in the present study to support the view that degradation can be related to biomass size, this may not necessarily be the case, particularly if degradation is limited to a small proportion of total biomass. Thus the major difference in chlorfenvinphos degradation rates in sites 5 and 6 could not be correlated with significant changes in biomass. Reduced biomass in soils treated with some fungicides has been reported, even though these fungicides underwent enhanced degradation (Duah-Yentumi & Johnson, 1986). Clearly, whether pesticides stimulate (directly or indirectly) or inhibit components of soil biomass may not always relate directly to their accelerated breakdown.

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