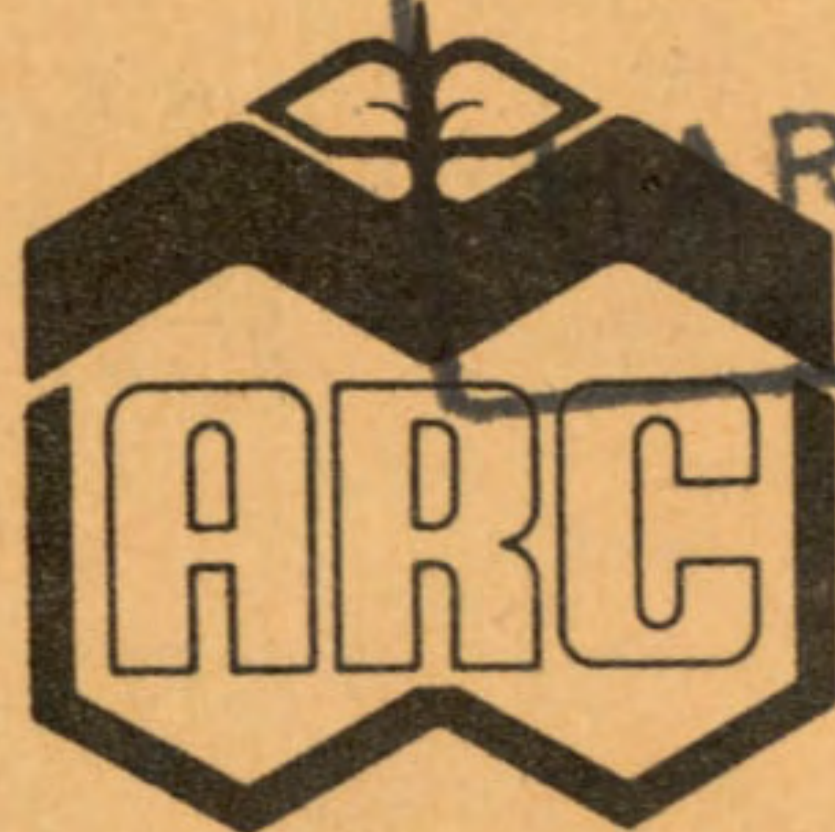


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TECHNICAL REPORT No. 59

RECOMMENDED TESTS FOR ASSESSING THE SIDE-EFFECTS OF PESTICIDES ON THE
SOIL MICROFLORA

DISPLAY UNTIL

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M P GREAVES, N J POOLE, K H DOMSCH, G JAGNOW and W VERSTRAETE

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NOTE

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RECOMMENDED TESTS FOR ASSESSING THE SIDE-EFFECTS OF
PESTICIDES ON THE SOIL MICROFLORA

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INTRODUCTION

During the period 1973-77 four symposia, dealing with pesticide side-effects on non-target soil micro-organisms, were organized jointly by the Biologische Bundesanstalt and the Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Fed. Rep. of Germany. As a result of these meetings it was decided to extend the participation at subsequent meetings. Consequently an international workshop was held at Braunschweig in 1978 and followed by a second workshop in England in 1979. The aim of these meetings was to discuss present knowledge of the means of testing side-effects of pesticides on the soil microflora and to agree on recommendations for meaningful tests, suitable for application as registration requirements.

The attached documents summarize the proceedings of the last of these workshops and present the recommendations agreed by the delegates present. A list of these delegates is appended. They represent government research institutes, universities and industry and are involved in studying side-effects of pesticides on the soil microflora, either for registration purposes or for agricultural research.

The unanimous decision of the 1979 workshop was that the recommendations should be circulated as widely as possible to persons involved with, directly or indirectly, or interested in the impact of pesticides on the soil microflora. The hope was expressed that, in this way, the recommendations will reach those responsible for framing and operating pesticide registration schemes.

The tests recommended are thought by the delegates to be those which are most appropriate at the moment, bearing in mind the present state of microbiological knowledge. It is recognized that there are many other methods with potential but it was felt that there is insufficient information to allow firm recommendation of them at the present time. The participants at the workshop wish to stress that there must be flexibility in any recommendations in order that deletion of obsolete methods and introduction of proven improved methods is easily possible. However, improvements can only come from intensive research and the delegates urge that this should be given high priority in future.

1. PRE-REGISTRATION HAZARD EVALUATION AND PREDICTION

The risks to the environment from a pesticide or its formulated product are dependent on many factors. For example, its toxic properties, persistence and mobility in the environment, the amount applied, the formulation, method and time of application and, particularly, intensity of use are all important.

Some pesticide effects on the environment may be too complex, subtle, or delayed to be detected by ordinary testing in the laboratory or the field. In any case, it is obviously impossible to cover in such trials the infinite variety of conditions under which the pesticide may be used in practice. Nevertheless, experience has shown that in many instances, predictions can be made of probable environmental effects of a compound from consideration of certain basic information.

Data have to be obtained prior to registration to allow a reasoned judgement to be made of the environmental behaviour of the product, when applied according to the recommendations for use. Such data are essentially predictive and are intended to describe those characteristics of the product relevant to the environment. They should be sufficiently comprehensive to enable the authority to make a reasonable judgement of the environmental behaviour of the product for the uses proposed. They do not seek to give data on the actual behaviour of the product in all the many environments in which it will be applied. The actual test programme has to be decided according to the characteristics and conditions of use of the product.

2. PRIMARY DATA NEEDED FOR PREDICTING ENVIRONMENTAL HAZARDS

Properties of the pesticide

Physico-chemical properties of pesticide
Biological activity
Metabolism + residue studies incl. persistence and mobility
Mammalian toxicology
Toxicological data on other species

Use pattern

Formulations
Methods of application
Site of application
Time of application
The amount of pesticide applied
Scale of use
Climate and geographical locality

The above information on the use pattern is an important element for the estimation of the expected environmental concentration and the probable sites of deposition. If a pesticide is likely to reach the soil, and if a potential hazard is predicted from the primary data, the relevant studies selected from those described below should be carried out.

3. RECOMMENDED TESTS

3.1 TEST CONDITIONS

Selection of soils

Two generally occurring agricultural soils, on which the pesticide is likely to be applied, should be chosen to represent conditions where the soil microflora may be under a) relatively high stress from the pesticide, b) relatively low stress from the pesticide. (In many cases these will be a sandy soil low in humus and a loamy soil). This information can be predicted from the physical and chemical properties of the compound and from the degradation rate of the pesticide and its metabolites in various soils. The soil should have followed a usual cropping pattern and preferably have received no pesticide, or only pesticides which are known not to affect microbial processes, for five years. Soil contents of total carbon, clay, silt, sand, pH and time of sampling should be stated.

Soil collection and treatment

The soil should, wherever possible, be at a moisture content suitable for sieving. The top 10cm only should be used and the vegetation, soil animals (macro-fauna) and stones should be removed. The soil should be passed through a 2mm sieve. If too wet to sieve, it should be dried by spreading but must never be air dry (above 6 bar) as this adversely affects the microbial biomass. The soil should be thoroughly mixed before use.

Soil storage

The object of the subsequent tests is to investigate the effects of a pesticide on the soil microflora. It is therefore desirable that the soil used should be as fresh as possible. If storage is necessary, loss of microbial biomass can be minimized by keeping the soils at 2° - 4°C in loosely tied plastic bags which allow free access of air. Drying or water-logging of the soil must be avoided at all stages of storage and/or treatment. The soil should not be kept for more than 10 weeks and before use should be kept at 20 ± 2°C for 2 days to allow for equilibration.

Pesticide dosage

1. Recommended field rate (kg ai/ha) expressed as mg/kg soil, assuming uniform distribution in the top 5cm of the soil.
Fumigants should be used at a dose corresponding to the recommended concentration, taking the actual time of exposure into consideration.
2. Ten times the recommended field rate. Fumigants at 5 times the recommended concentration, taking the actual time of exposure into consideration. For foliar-applied herbicides the upper dose may be determined by crop tolerance.

3.2 SOIL RESPIRATION TESTS

The most suitable method is the determination by continuous or semi-continuous monitoring, of the evolution of CO₂ from soil aliquots (100g) kept in the dark at 20 ± 2°C. The soil moisture content should be pF 2.5. The tests should run for a minimum period of 30 days, the actual duration being dependent on the rate of degradation of the compound.

The recommended experimental schemes are as follows:-

1. Untreated soil
2. Soil plus pesticide
3. Soil amended with 0.5% lucerne meal (C:N ratio 16 ± 2 , milled to pass a 0.5mm sieve)
4. Soil amended with 0.5% lucerne meal plus pesticide.

3.3 LITTERBAG TEST

This test should only be imposed when side-effects are detected with the lucerne-respiration test.

Wheat straw (haulms cut in c. 2cm internodal pieces) is recommended, though other plant materials may be substituted depending on the intended use of the pesticide (e.g. apple leaves for pesticides generally used in orchards). Nylon bags (10 x 10cm, 2mm mesh) are filled with 2g (dry matter) of straw. The straw should have been treated (by spraying or dipping) with a pesticide dosage equivalent to that normally applied to 100cm² soil area. To reduce variability and to prevent plant roots penetrating the bags, PVC tubes (20cm diam., 20cm length) are sunk into the soil around the bags, which are buried at a depth of 5cm. There should be at least 6 replicate bags for each sample date. The experimental field site should have the main crops for which the pesticide is intended. Samples should be taken at the middle and at the end of the vegetative growth period. Decomposition is measured gravimetrically and, in soils with high organic content, losses in soil weight during ashing must be accounted for.

3.4 AMMONIFICATION AND NITRIFICATION

Ammonification and nitrification, as parts of the N-mineralization process, are useful criteria to study potential side-effects of pesticides. Ammonification is the indicator for the release of nitrogen bound in organic matter and its availability for plant nutrition. Nitrification, i.e. the oxidation of ammonium to nitrite and nitrate, is carried out by sensitive bacteria. Thus, impairment of nitrification may be indicative of harmful influences of pesticides.

The release of ammonium from organic matter can be studied in the same way as described for the mineralization of carbon, i.e. addition of 0.5% lucerne-meal to soil. If ammonification is not affected, the two steps of nitrification can be studied in the same experiment. If ammonification is inhibited, a separate experiment is required to study nitrification independently. This is achieved by adding ammonium sulphate (100 ppm N) to the soil and following the disappearance of NH_4^+ and the appearance of NO_2^- and NO_3^- .

The study of ammonification needs to be carried out until equilibrium is reached between ammonification and nitrification, but in any case for not less than 4 weeks.

The study of nitrification of ammonium sulphate has to be continued until either the added substrate has been converted or until an equilibrium has been reached between ammonification from soil organic matter and nitrification.

Care must be taken, especially with clay soils, to avoid anoxia which might cause denitrification.

3.5 NITROGEN FIXATION

Tests should be restricted to situations where symbiotic nitrogen-fixation may be affected by the pesticide, i.e. where pesticides are used directly on legumes, where legumes are a normal constituent of the crop rotation system, or where mixed leguminous/non-leguminous cropping is practised. The choice of the test legume should depend on the 'use pattern' of that particular pesticide.

The assessment method should recognise the unique symbiotic relationship between the host plant and Rhizobium, and hence should determine in one test both plant and bacterial response to the pesticide. The following information therefore will be required: measurement of plant growth over time, plant yield and an estimate of healthy nodulation. These experiments will be conducted in pots containing a soil type suitable for growth of the plant. The plant seeds would be inoculated with Rhizobium only if no suitable bacteria are present naturally in the soil. When required, the plants will receive a mineral fertilizer which excludes nitrogen.

If the pesticide causes a critical effect in these tests, then further data should be provided on the direct effects of the pesticide on the Rhizobium organism, the nodulation process and the nitrogen-fixation (e.g. acetylene reduction) ability of the whole plant or intact root system.

For pesticides to be used in rice culture, the effects of the pesticide on the growth and nitrogen-fixing (e.g. acetylene reduction) ability of a Cyanobacterium (e.g. Anabaena) and on the Azolla/Anabaena symbiosis should be determined.

Results of acetylene reduction tests should be interpreted with caution. Conditions of the test should be rigorously standardized and isolated nodules should not be used.

Present knowledge indicates that nitrogen fixation in the rhizosphere of agricultural crops, and that due to free-living bacteria, does not warrant regulatory studies at this stage. We would, however, recommend this area for further research funding.

4. INTERPRETATION AND EVALUATION OF DATA

(Klaus H Domsch)

The problem of deciding the relative importance of each observed effect a chemical may have is fundamental to side-effect testing. It has yet to be decided how to interpret or evaluate the importance of the sum of effects, both inhibitory and stimulatory, which could occur when investigating multiple microbial activities.

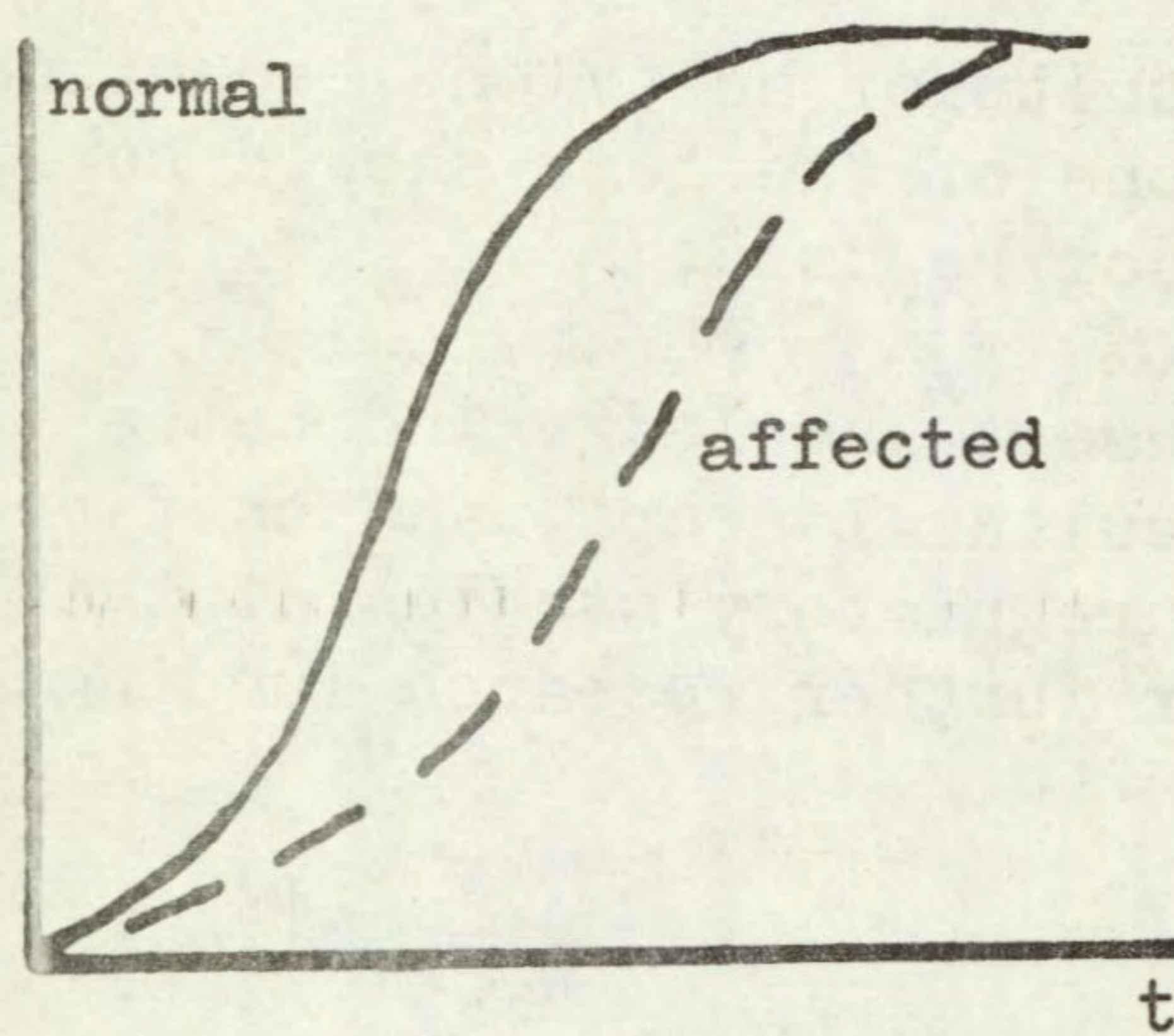
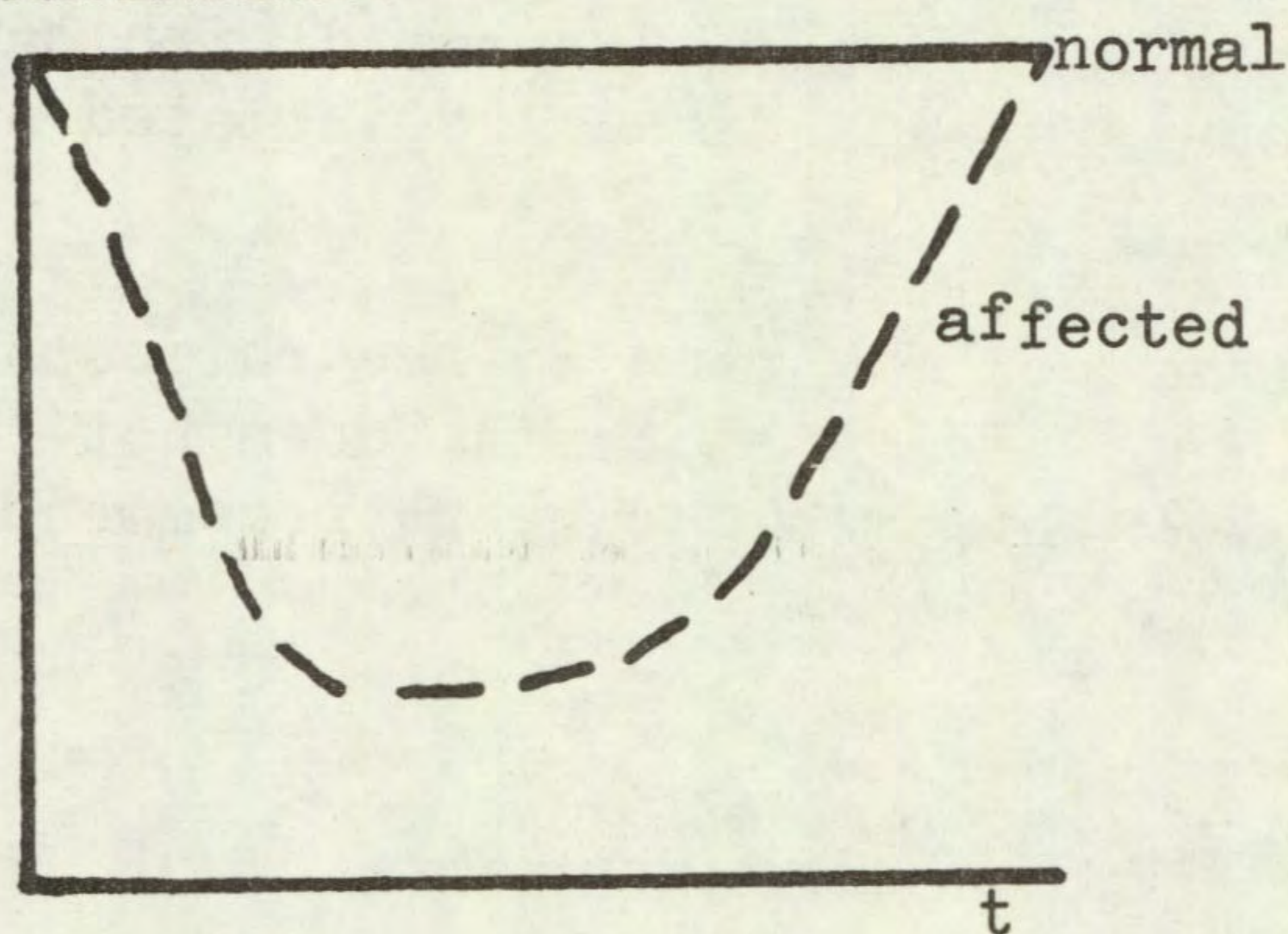
The meeting agreed that there is an urgent need to develop means of objectively assessing the data obtained from recommended tests of pesticide side-effects. An outline of one system being developed by K H Domsch is given here.

4.1 TYPES OF MICROBIAL RESPONSES

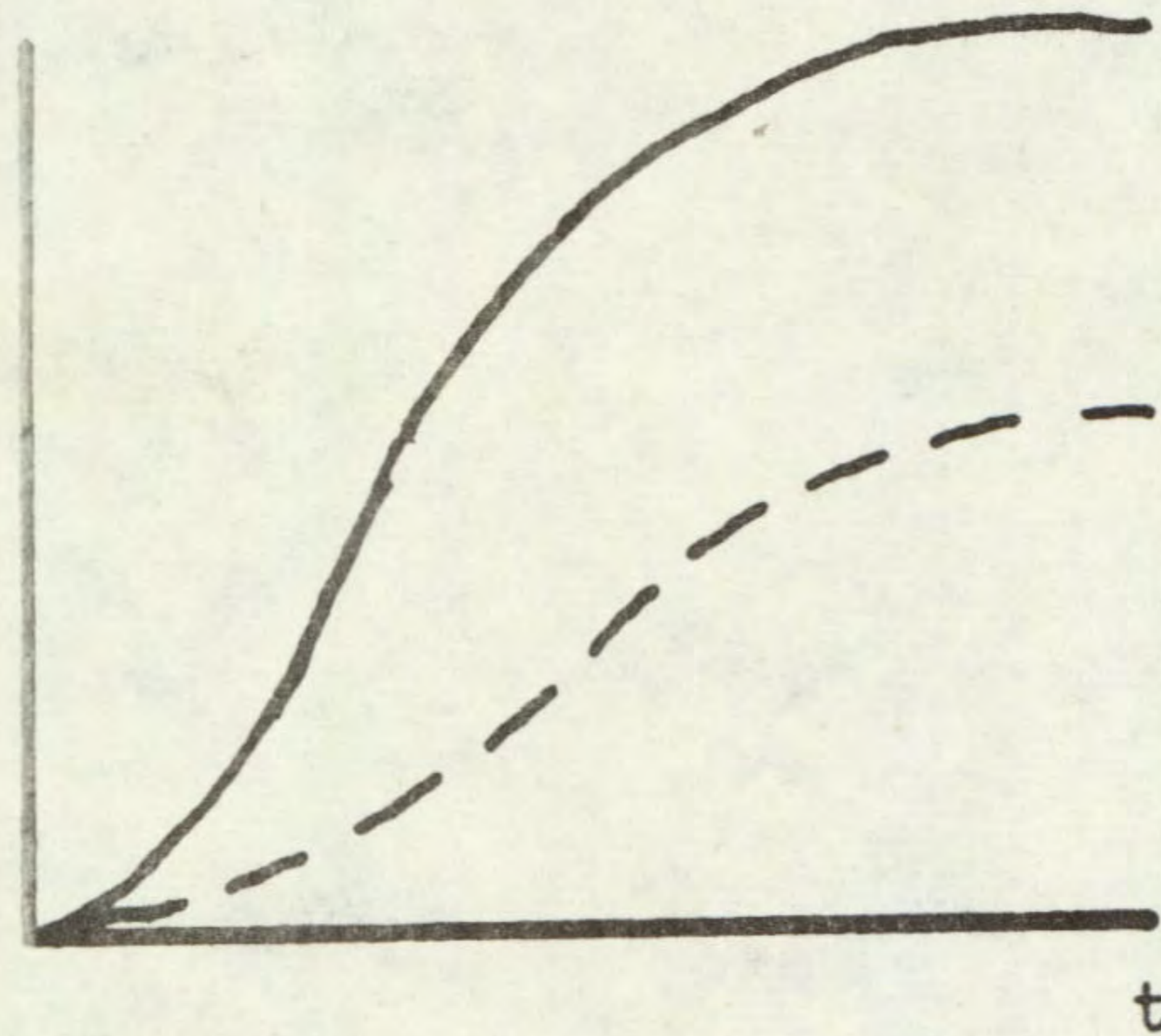
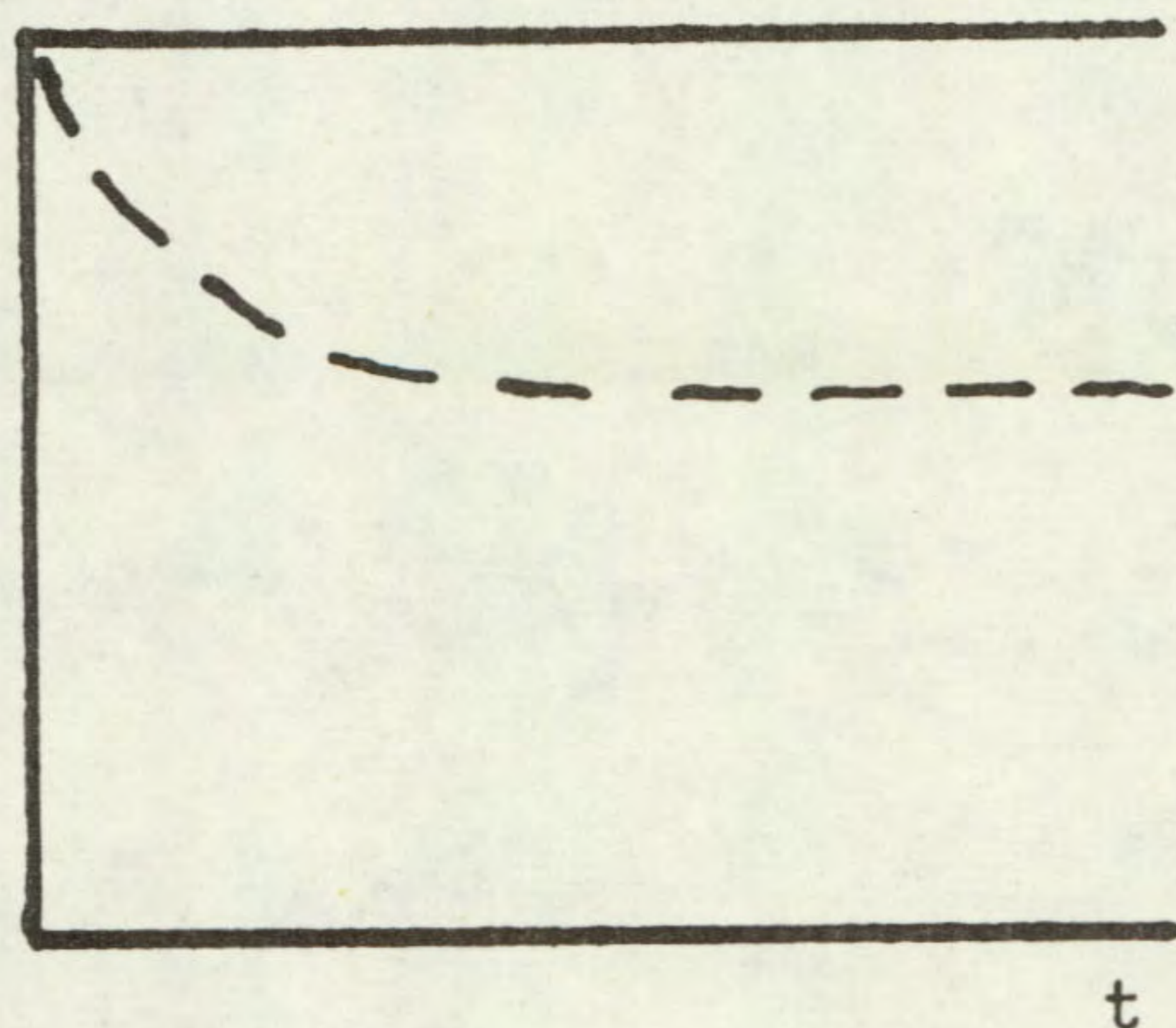
Chemicals with biocidal properties can produce a limited number of negative responses in reactive biological systems (Fig. 1). Microbial processes or populations may be established at a high level and they may normally continuously maintain this level, or may start at a low level and increase during the monitoring period. The effects themselves may be reversible or irreversible within this period. The 4 resulting types cover most of the responses thus far observed.

FIG. 1. Principal types of reversible and irreversible negative responses

REVERSIBLE



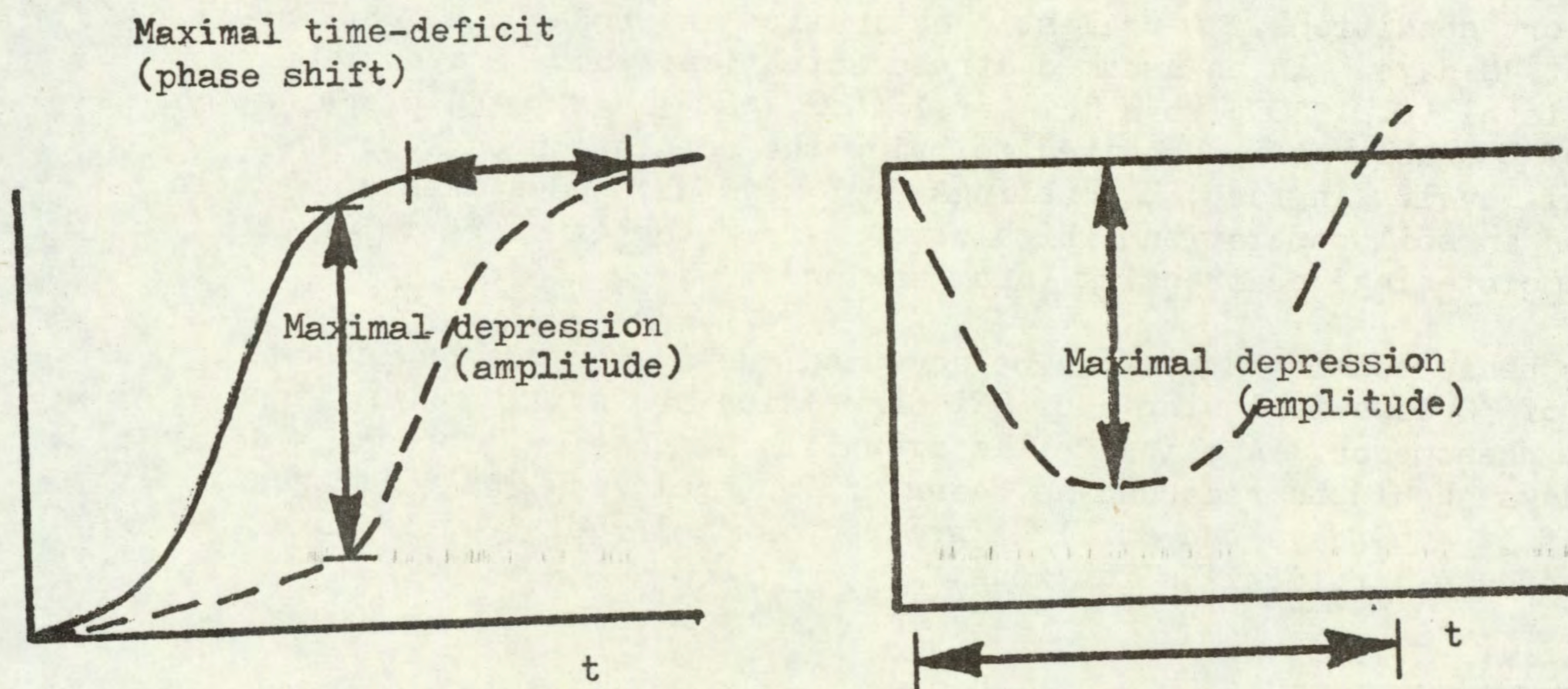
IRREVERSIBLE



It is evident that any monitoring system which would allow such an uncomplicated recognition of reaction types requires high quality data. Since a chemical which is incorporated into the soil reacts in a dynamic system which has a multitude of ways and means for repair and restitution, the time factor has a key position. Side-effect data, without information on the time-dependent changes of the microbial response, are only of limited use for evaluation purposes.

The reaction types indicating reversible effects may be analysed more specifically in order to recognise their quantitative aspects. There are two main criteria which can be applied to all reversible negative responses: a) the amplitude of the maximal depression, and b) the phase-shift due to a retarded process expressed as maximal time deficit (Fig. 2). It should be mentioned here that, from an ecological point of view, irrespective of the magnitude of depression, the delay in the re-establishment of a negatively influenced microbial structure or function ranks higher in the hierarchy of evaluation criteria.

FIG. 2. Criteria used for the evaluation of reversible side-effects



4.2 INTERPRETATION OF RESULTS

Reversible effects

The recognition of response types is a pre-requisite for further evaluation, but does not allow a decision to be made on which effects might be ecologically insignificant, tolerable or even critical. In order to approach this problem, it is proposed that in situations where the application of pesticides is justified from an agricultural point of view, the magnitude of response to the man-made chemical stress should be compared with that of naturally occurring stress situations. From studies in microbial ecology a wealth of information is available on the consequences of naturally occurring 'catastrophes' in soil microhabitats: water availability is frequently reduced to critical levels or soil is flooded and gas exchange is limited; the temperature often deviates from optimum conditions or nutrients are lacking and cells are eliminated by soil animal grazing, micro-parasites or other adverse conditions.

The magnitude of depressions in soil microbial systems under perfectly normal conditions may be illustrated by the following examples:

When water potentials decrease between 1 and 5000 kPa, microbial functions like ammonification, nitrification or respiration are reduced by 10 - 90% with bacteria being more sensitive than fungi. Changes in soil temperature frequently reduce biomass and cell densities by more than 70%; limited gas exchange due to compaction or flooding may reduce cell densities or microbial functions by 50 - 95%.

From ecological data of this type, it can be concluded that depressions of 50% or more, frequently occur under natural conditions. Soil meteorological data prove that wide variations in microenvironmental conditions are normal events. If these statements can be accepted, depressions due to man-made chemical stress must not be overestimated, as long as they are of a magnitude comparable to those found in natural situations.

Likewise, the determination of the time required for the restitution of normal microbial populations or functions (maximal time deficit) after the end of natural stress conditions is required as an ecological 'yardstick'. A key parameter is the doubling-time of microbial cells. Under field conditions doubling-times are considerably longer than under optimum laboratory conditions. A realistic estimation is probably a doubling-time of about 10 days. In an assumed stress situation, where a reduction from a high level (e.g. 100) to a low level (e.g. 12.5) has taken place, a total of 3 doublings is required to bring the population back to the original level. In fact, 3 doublings or 3 x 10 days have frequently been observed in soil populations which recover from the exposure to drying (and remoistening) or freezing (and thawing).

If this basic consideration can be accepted, delay periods or phase-shifts of 30 days following a c. 90% depression are still a normal and natural phenomenon. Applied to the situation of chemical stress, a delay of 30 days should be regarded as being of 'no ecological significance'. A delay of 31 - 60 days can still be grouped as 'tolerable' while more than 60 days indicate 'critical' situations.

Irreversible effects

The side-effects which are apparently irreversible require separate consideration from the two above-mentioned response types. These side-effects are characterised by deficits of populations or functions when pesticide-treated soils are compared with non-treated soils at the end of the monitoring period. The deficits may be due to a) the persistence of the toxic agent or b) the inability of a population to recover from a depression. The general potential hazard can be recognised by high deficits (> 50%) or long-lasting deficits (> 60 days) at the end of the monitoring period, or both.

If pesticides should cause side-effects of this irreversible type, the first indication of such cases must be followed in two steps: a) confirmation by repeated tests under a set of different environmental conditions, and b) field testing.

5. UNSUITABLE TESTS

5.1 PURE CULTURES

The meeting considered that, though useful in certain research studies, they are unsuitable for regulatory studies because:

Isolated organisms may be metabolically atypical of their form in soil. Furthermore, they can change progressively during storage.

They are normally stimulated to artificially high metabolic rates by growth in normal laboratory media.

They are removed from their normal ecological associations.

Interpretation of results is difficult and extrapolation to field situations impossible.

Thus, they are not able to reflect, in any meaningful way, the side-effects pesticides may have. Side-effects are best tested for by observing microbial processes in soil.

5.2 SOIL ENZYMES

The consensus of the meeting was that, with the present state of the art, soil enzyme activities would be of little value when monitoring side-effects of pesticides on the microflora. The principal reasons are as follows:

1. The total enzymic capacity of a soil is made up of various fractions (i.e. proliferating microbes, substrate turnover in dead cells and debris, enzymes associated with colloidal humic-matter, etc) and it is extremely difficult to quantify the contribution of each to the catalysis of a particular substrate.
2. Current research specifically identifies the term 'soil enzymes' with the humic-matter and, although this is believed to play an important role in substrate turnover, there is no universally-agreed methodology. Almost any result can be achieved by varying assay conditions (temperature, pH, substrate concentrations).

Phosphatase and dehydrogenase have been suggested as useful enzymes for monitoring pesticide side-effects. Even if the previously stated drawbacks were resolved, it is believed that these two enzymes are unsuitable. Phosphatase is a collection of enzymes, usually measured by using an artificial substrate (e.g. p-nitrophenyl phosphate), and whose activity bears little relation to total phosphate availability in soils. Dehydrogenases reflect a broad range of microbial oxidative activities, do not accumulate to any extent (based on the definition above, they are not soil enzymes) and yet they do not consistently correlate to microbial numbers, CO₂ evolution or O₂ consumption. Additionally, activity may be dependent upon the nature and concentration of amended C-substrates and alternative electron acceptors. Moreover, we have already agreed upon a method for assessing total microbial activity, viz. CO₂ evolution.

6. FUTURE DEVELOPMENTS

Applied research should be directed at ways of improving our ability to identify, quantify and evaluate potential harmful effects of pesticides to soil microbial activities, which are essential to the maintenance of soil fertility. World agriculture is dependent on the continuing supply of effective, safe chemicals for pest control. Any requirement which adds significantly to development costs, without making a valuable contribution to environmental safety, must be evaluated most critically.

Basic research is the only way in which the information essential to the applied scientists can be obtained. As such it must take considerable priority in competition for funding. It is essential that we understand microbial behaviour in soils more thoroughly. In particular, the interactions between pesticide, soil, crop plant and micro-organisms require continued exhaustive research.

Obviously, the choice of important specific areas of research depends, to a great extent, on individual bias. However, the following topics seem to warrant special attention with regard to the effect of individual pesticides and of combinations and sequences of pesticides.

1. Rhizosphere studies - in particular nodulation of economically important legumes and mycorrhizal associations.
2. Soil-borne plant pathogens including microbial antagonism.
3. Microbial aspects of soil structure.
4. Microbial aspects of nutrient cycling.

These subjects can be studied effectively only if appropriate valid methods are developed and applied.

In all research particular attention should be paid to co-ordination with other specialists, wherever possible.

There is a pressing need for the development of means of interpreting data obtained in experiments. Such interpretation should pay heed to the normal behaviour of microbial communities under what may be termed acceptable stress.

It is vital that those responsible for the introduction and imposition of regulatory requirements take full note of the advice of microbiologists and other specialists.

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ABBREVIATIONS

ångström	Å	freezing point	f.p.
Abstract	Abs.	from summary	F.s.
acid equivalent*	a.e.	gallon	gal
acre	ac	gallons per hour	gal/h
active ingredient*	a.i.	gallons per acre	gal/ac
approximately equal to*	≈	gas liquid chromatography	GLC
aqueous concentrate	a.c.	gramme	g
bibliography	bibl.	hectare	ha
boiling point	b.p.	hectokilogram	hkg
bushel	bu	high volume	HV
centigrade	C	horse power	hp
centimetre*	cm	hour	h
concentrated	concd	hundredweight*	cwt
concentration	concn	hydrogen ion concentration*	pH
concentration x time product	ct	inch	in.
concentration required to kill 50% test animals	LC50	infra red	i.r.
cubic centimetre*	cm ³	kilogramme	kg
cubic foot*	ft ³	kilo (x10 ³)	k
cubic inch*	in ³	less than	<
cubic metre*	m ³	litre	l.
cubic yard*	yd ³	low volume	LV
cultivar(s)	cv.	maximum	max.
curie*	Ci	median lethal dose	LD50
degree Celsius*	°C	medium volume	MV
degree centigrade	°C	melting point	m.p.
degree Fahrenheit*	°F	metre	m
diameter	diam.	micro (x10 ⁻⁶)	μ
diameter at breast height	d.b.h.	microgramme*	μg
divided by*	÷ or /	micromicro (pico: x10 ⁻¹²)*	μμ
dry matter	d.m.	micrometre (micron)*	μm (or μ)
emulsifiable concentrate	e.c.	micron (micrometre)*†	μm (or μ)
equal to*	=	miles per hour*	mile/h
fluid	fl.	milli (x10 ⁻³)	m
foot	ft	milliequivalent*	m.equiv.
		milligramme	mg
		millilitre	ml

† The name micrometre is preferred to micron and μm is preferred to μ.

millimetre*	mm	pre-emergence	pre-em.
millimicro* (nano: $\times 10^{-9}$)	n or mp	quart	quart
minimum	min.	relative humidity	r.h.
minus	-	revolution per minute*	rev/min
minute	min	second	s
molar concentration*	M (small cap)	soluble concentrate	s.c.
molecule, molecular	mol.	soluble powder	s.p.
more than	>	solution	soln
multiplied by*	x	species (singular)	sp.
normal concentration*	N (small cap)	species (plural)	spp.
not dated	n.d.	specific gravity	sp. gr.
oil miscible concentrate	o.m.c. (tables only)	square foot*	ft ²
organic matter	o.m.	square inch	in ²
ounce	oz	square metre*	m ²
ounces per gallon	oz/gal	square root of*	√
page	p.	sub-species*	ssp.
pages	pp.	summary	s.
parts per million	ppm	temperature	temp.
parts per million by volume	ppmv	ton	ton
parts per million by weight	ppmw	tonne	t
percent(age)	%	ultra-low volume	ULV
pico (micromicro: $\times 10^{-12}$)	p or pp	ultra violet	u.v.
pint	pint	vapour density	v.d.
pints per acre	pints/ac	vapour pressure	v.p.
plus or minus*	+ -	<u>varietas</u>	var.
post-emergence	post-em	volt	V
pound	lb	volume	vol.
pound per acre*	lb/ac	volume per volume	v/v
pounds per minute	lb/min	water soluble powder	w.s.p. (tables only)
pound per square inch*	lb/in ²	watt	W
powder for dry application	p. (tables only)	weight	wt
power take off	p.t.o.	weight per volume*	w/v
precipitate (noun)	ppt.	weight per weight*	w/w
		wettable powder	w.p.
		yard	yd
		yards per minute	yd/min

* Those marked * should normally be used in the text as well as in tables etc.

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