Methods for Assessing the Effects of Mixtures of Chemicals
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Methods for Assessing the Effects on Non-human Biota of Mixtures of Chemicals: Tests for Pathological Effects on Insects

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ABSTRACT

Insects are organisms that quickly respond to chemical stimuli and therefore are suitable for testing the effects of mixtures of chemicals on live organisms. The study of these effects is an interdisciplinary field of research focusing on the synthesis of bioassays and chemical analyses of individual components of mixtures. The testing of biological effects consists of: (1) application (direct, indirect, combined); (2) determination of the sensitive stages and testing of the effects of different doses; and (3) evaluation of results (tissue responses, metamorphosis, diapause, reproduction, nervous system, lethal and effective doses, or 'inhibition doses'). Mixtures of chemicals generally affect insects in several ways depending on the interaction of their components: (1) individual components are active independently of each other and the effect produced by their mixture is the same as the effects of individual components; (2) mixtures consist of active and inactive compounds; (3) mixtures contain components whose action is either synergistic or antagonistic (e.g. insecticides and their synergists, etc.).

1 INTRODUCTION

Insects are organisms which quickly respond to chemical stimuli, and therefore they are very good bioindicators and test objects for the study of pathological effects of chemical compounds. Evaluation of pathological or developmental changes is generally focused on the following: (1) finding the damaged organ or organ system, and determining the extent of the damage; (2) finding which stage is most susceptible to the action of the mixtures of chemicals and perhaps discovering their effects on metamorphosis and the developmental cycle; (3) determining doses lethal or otherwise critical to all developmental stages under

various abiotic and biotic conditions; and (4) explaining the mechanism (also at the molecular level, if possible) of action of the critical or all components of a chemical mixture.

Methods should be adapted from the beginning to the initial situation as well as to the aim of research. There are two basic kinds of initial situations: (1) we know, either in detail or at least partially, the compound we are going to test without knowing its effects on a species or a group of species; or (2) we know, either in detail or partially, pathological effects on the species (changes in tissues, increased mortality, etc.) without knowing individual components of the chemical mixture. In this case, the chemical analysis of the mixture, quantitative as well as qualitative, should be made prior to testing its individual components if we want to discover the mechanisms of their action. However, this is a purely chemical question (for details see Allen, 1974; Shepard, 1958, 1960); commonly used methods are thin-layer chromatography, gas chromatography and high-performance liquid chromatography (e.g. Sherma and Zweig, 1974). However, chemical analysis (i.e. knowledge of the components of a mixture) by itself is merely an auxiliary step in investigating the effects of chemical mixtures. If only the pathological effects of a mixture are known, the question of which chemical or group of chemicals is responsible for specific effects is not purely a chemical question. The study of these questions is then a complex interdisciplinary field of research requiring the combination of analytical methods with bioassays. In the following text we shall focus on the methods for testing mixtures of chemicals using insects, and present a survey of the currently known types of interaction of their individual components.

2 TESTING OF THE BIOLOGICAL EFFECTS OF MIXTURES OF CHEMICALS

Biological effects are usually tested according to the following scheme: (a) application; (b) determination of the susceptible stage and effects of different doses; (c) evaluation of results, including the assessment of toxicological and morphological effects as well as those on metamorphosis, ovarian, and embryonic development, evaluation of effects manifested at the population level, and the study of the metabolism of individual components of the mixture.

2.1 Application

Methods of application can be roughly divided into three categories: direct, indirect, and combined. Direct application is one of the basic laboratory methods, being the only one that enables the determination of acute toxicity of a chemical mixture or its components, which is an indispensable starting point for bioassays. However, if the ecological background of the effects of individual chemical compounds and their mixtures is to be determined, indirect application (combination of oral and contact applications) is necessary.

2.1.1 Direct Application

One of the direct methods of application is injection of a known solution at a known concentration with a syringe (e.g. Hamilton, etc.). Another frequently used kind of direct application is topical (FAO, 1970; Needham and Devonshire, 1973), the active compound plus vehicle, or solution of the compound, being applied to the body of the chosen developmental stage with calibrated capillaries or microcapillaries, micropipettes, or dispensers of various other kinds. It is also possible to apply the active compounds by spraying experimental animals in a sedimentation (Potter) tower (Potter, 1952). This method is successfully used with relatively immotile insects and also with large sample populations. A similar method is the spraying of leaves of a host plant; test organisms are then placed on the leaves (spray-residue test) (Hrdý and Kuldová, 1981). A dip test is a quick preliminary method developed in its present form by Sawicki *et al.* (1978). The test is suitable for small experimental animals, samples of which are dipped in a suspension of insecticides or other active compounds. Topical application enables quick work, excludes contamination, and narcosis is not necessary in most cases (with the exception of very motile forms). However, there is one disadvantage: many compounds do not penetrate the insect cuticle (Treherne, 1957), or they substantially change passing through the cuticle. A considerable loss due to evaporation from the body surface has been ascertained with some chemicals (Devonshire, 1973). For direct application, places where the cuticle is thin are usually chosen, or places covered by resilin cuticle (intersegmental membranes, etc.).

A physiological solution (Ringer, Pringle, and others) is used as a solvent for polar substances, and oily vehicles (e.g. neutral natural oils that are quickly resorbed by haemolymph) are used for lipoid substances. Examples of other suitable solvents and vehicles are acetone, ethanol, dioxan, heptane, octane, ethylcellosolve, dimethyl sulphoxide, 0.1–1 % Tween 80, and others. These substances are biologically active, however negligibly, by themselves. The solvent activity differences are also manifested in combination with an active agent of the same kind (Sláma *et al.*, 1974) and should be taken into account.

Water (boiled in order to remove dissolved oxygen) is commonly used as a narcotic; experimental insects are submerged for 5–20 minutes before injection. This method cannot be used with stages whose gas exchange at respiration is minimal (some larvae or pupae). Low temperatures (usually 2–10°C) or carbon dioxide are also used for immobilization. Insects can also be narcotized by anaesthetics commonly used for vertebrates.

2.1.2 Indirect and Combined Application

Indirect methods of application are used mainly for the testing of effects on insect development in environments containing known concentrations of the investigated compounds. Administration of the active components in food is often

more effective than direct application. Vapours of some compounds are also used, and chemicals dissolved in water are used in experiments with aquatic insects (inactive emulgators must be used with dissoluble substances, etc.). Since laboratory and field conditions vary greatly for the rearing and study of different species, the methods should be individually modified.

Combined application of chemical compounds is preferable in field tests, contamination by direct application usually not being as important as re-contamination through environment. Also the field population of a species always is far more diverse than a laboratory one, as different susceptible stages occur simultaneously. The combined contact and oral application of active substances can occur in the laboratory such as in cases when a substance has been mixed with the medium in which larvae live.

2.2 Determination of the Sensitive Stages and Testing of the Effects of Different Doses

There are only limited general rules for determining stages that are most susceptible to individual components of a mixture. In most cases it is impossible to avoid various routine toxicological tests. Also the great variability of tolerance to toxic or biologically active compounds requires different approaches to individual stages of the experimental organism. There is usually not much difference in the effects of pesticides on larvae and adults; sometimes the adults, and especially eggs, are more resistant. However, adults are more susceptible to other compounds. Substances with sterilizing effects (some alkylating agents and antimetabolites) affect most of the stages in which the development of gonads is completed. Biologically active compounds (e.g. juvenoids, precocenes, antibiotics, etc.) are effective with many species (*Pyrrhocoris*, *Dysdercus*, *Rhodnius*, *Locusta*, *Tenebrio*, *Galleria*) only at the beginning of larval instars and immediately after the larval–pupal transformation; eggs are affected only during approximately the first third of embryonic development or, for flies and aphids, only before oviposition. As for compounds occurring in the environment, it is far more important to concentrate testing on the link of the trophic chain affected by their residues rather than on their immediate toxicity.

Bioassays aimed at the effects of chemicals or their combinations on entire populations are of great practical importance. Doses for LD_{50}, ID_{50}, etc., are generally somewhat different at this level for reasons of the usually combined application. Moreover, there are many pathological effects revealed only at the population level. Mortality and some of the other characteristics of pesticides are commonly evaluated by simple tests (FAO, 1970; Hrdý and Kuldová, 1981; Sawicki *et al.*, 1978). Besides, there are specific tests for biologically active compounds (e.g. *Tribolium* or *Drosophila* population assay, and others). The choice of methods is difficult to establish because eggs are hard to find in the field. Populations are mostly heterogeneous (different developmental stages), and

individuals are exposed to active compounds in different degrees of intensity. The usual procedure is statistical evaluation of a sample of the same size either prior to contamination or some time after it. Such experiments are long-term, requiring careful planning and choice of methods.

2.3 Evaluation of Results

The most complex phase of the testing of the effects of chemicals or their mixtures is the evaluation of results. The usual histological, histochemical, and toxicological data are used.

Histochemical methods are employed for the solving of certain problems on the basis of previously acquired facts. They are designed for, and directed toward, solving a molecular problem on a cellular basis. The result should give a more detailed picture of the response of a tissue to a certain stimulus. Histochemical and cytochemical methods are therefore excellent tools for testing a specific hypothesis about the mechanism of action of a toxic chemical(s), but they are poor for generalized screening of tissue injury (Wachsmuth, 1981a,b). Toxicological tests commonly used for investigations of pesticides (Finney, 1971) suffice in some cases, but more complex methods are necessary for the study of sublethal doses or biologically active preparations. Experiments should be planned in accordance with the fact that some phases in insect ontogeny (e.g. larval–adult or pupal–adult metamorphosis) and some tissues which undergo substantial changes during ontogeny (e.g. reproductive system) are eminently suitable for testing the effects of compounds because of their high sensitivity.

As concerns metamorphosis, the effects of some substances or their combinations are manifested mainly by prolonging some stages (e.g. the prepupal stage is prolonged by applying a mixture of bacterial toxins), and in some cases by the occurrence of forms intermediate between the pupa and adult in Exopterygota, or larva–pupa or pupa–adult in Endopterygota. The effects of these compounds are expressed as the relation of dose to the percentage of morphologically affected individuals (e.g. with juvenoids, metabolic inhibitors, and antibiotics), and there are various special tests enabling evaluation of the intermediate forms. All these changes are connected with changes in integument, usually tested by the Galleria wax wound test (Gilbert and Schneidermann, 1960; Schneidermann and Gilbert, 1959; Wilde *et al.*, 1968, 1971). Also there are more specialized methods, such as the Galleria metamorphosis test and *Hemiptera–Rhodnius*, *Pyrrhocoris* (e.g. Sláma *et al.*, 1974).

Diapause is another critical phase. However, it is affected by a complex of several factors including physical-abiotic (photoperiod, temperature, etc.) and biotic ones, so that it is very difficult to do separate studies on the effects of some chemicals, and so far there have been few data on the effects of chemicals on diapause. More data have been published on some biologically active compounds.

Pathological effects are usually manifested in inner organs. External teratological effects on integument are rare; they have been induced by, for example, a mixture of toxins of *Bacillus thuringiensis* (Burgerjon, 1972, 1974). Reproductive organs and the nervous system are most susceptible to substances produced by pathogens. Haemolymph (transportation and metabolism of active substances), gut epithelium and fat body (substances produced by parasites and pathogens), and the humoral system (biologically active compounds, e.g. juvenoids and precocenes) are also intensively studied, but the reproductive system is central in the response to sublethal and trace treatments.

Landa *et al.* (1983) described in detail methods suitable for studies on the reproductive system, especially the histological and morphological examination of gonads and research of fecundity and fertility. Ovarian studies are economical in this respect, as disorders in oogenesis can easily be observed macroscopically as degenerating follicles. Histological processing of gonads (staining of semithin sections) will certainly result in much more detailed information on the morphological manifestations of histopathological effects (degree of damage to follicular epithelium, vacuolation of ooplasm, inhibited division of germ cells in the germarium, etc.; for details see Landa *et al.*, 1983). Unfortunately, no generally applicable test evaluating the degree of histopathological damage to gonads has yet been developed. Such changes can be assessed indirectly through investigations of the fecundity of treated females mated by healthy males. Histopathological effects are also known in males (namely degeneration of germ cells in the stage of spermatogonia or spermatocytes, fully developed sperm being unaffected by most compounds). The reproductive ability of males has not been studied in detail; it can only be assessed indirectly through the hatchability of eggs laid by healthy females. For some kinds of compounds of gonadotropic activity, there are certain specific tests (e.g. a highly specific bioassay of juvenoids). Females with inactive ovaries are usually used (allatectomized or diapausing) and results are evaluated according to the intensity of yolk deposition and development of mature eggs.

The other methods for investigating fecundity and fertility are quite simple: recording total numbers of eggs laid, intervals between batches in females ovipositing for a long time, numbers of eggs in individual batches, percentage of hatching eggs, and also the viability or mortality of the first larval instars.

An important test of pathological effects is the determination of egg mortality and the effects of chemicals on embryogenesis. Treatment is usually either topical or combined. The ovicidal effects must be related to actual hatchability in the control (Sláma, 1971). Histological processing of material is needed in most cases where effects on embryogenesis are to be identified and the stage detected in which embryogenesis is usually blocked. It is advisable in all these cases to use species with a short development time, high fecundity, and a relatively long oviposition period (e.g. bugs or houseflies) enabling observations of possible gradual withdrawal of ovicidal effects. With insects, the kind of embryogenesis

should be taken into account. Eggs of the regulation type generally show greater plasticity if exposed to chemicals (morphological defects easily develop) than eggs of the mosaic type.

The effects of toxic compounds on the nervous system can be investigated in two principal ways. One is histochemistry and cytochemistry which enable the detection of changes not immediately in nerve cells but in enzymes and other molecules and ions closely connected with the function of the nervous system. These methods help to locate the site and manner of action of the active agent (Wachsmuth, 1981b). Another method is investigation of the action potential of nerves; this is used in cases where the active agent immediately affects excitable membranes (Vijverberg *et al.*, 1982). This method can also be employed for detection of the effects of various compounds on excitable membranes of muscle tissues (Huddart, 1977; Osborne and Hart, 1979).

Investigations of the mechanism of action of individual active components of mixtures are very complicated. The following characteristics are usually examined: penetration of cuticle, stability within the body, rate of passage through haemolymph, distribution of substances in different insect tissues, effects on the most important enzymatic systems, etc. Biochemical methods are used that are also employed for other purposes in biology. Determination of a compound's ability to penetrate cuticle is of particular importance where contact compounds are concerned (e.g. insecticides; for description and evaluation of methods see, for example, Shepard, 1958, 1960). Also used are analyses of tissue homogenates, compounds labelled by radioactive isotopes, autoradiography, determination of enzymatic activity, etc.

A common-probit system with LD_{50} based on determination of lethal doses (Bliss, 1935; Finney, 1971) has been worked out for pesticides as well as for 'biological' insecticides; actually, it can be used for all toxic substances. A sample population's response to the action of a substance or substances is alternatively yes/no, depending on the concentration of the substance or substances. Less often used characteristics are LD_{90}, LD_{100}, etc. As regards the study of biologically active compounds, so-called 'effective doses' (ED_{20}, ED_{50}, ED_{90}, ED_{100}, etc.) have been determined for diverse, often unrelated species. The effective doses are specifically related to a given species and stage. Some authors therefore recommend the use of 'inhibition doses' (ID_{50}) in these cases. Inhibition doses would be universal and data obtained by applying one substance to different developmental stages, or to different species, could be compared with different substances and different developmental stages. It has been found that with some kinds of compounds (e.g. juvenoids) there are clear correlations between dose and degree of inhibition of progressive changes in certain structures and functions if treatment has been done under standard conditions (Sláma *et al.*, 1974).

Another attempt at a certain unification of the effects of toxic substances has been the introduction of the pT concept which is the reciprocal of the logarithm

of the molar concentration of harmful compounds. It allows a simple, quantitative expression of the potential for toxicity of a wide variety of compounds on a molar basis (Luckey and Venugopal, 1977).

3 SYNTHESIS OF INFORMATION

This stage is the climax of experimental investigation of pathological effects of chemical compounds. We should realize that very little is known about the effects of chemicals on insects. It seems so far that chemical mixtures, with the exception of the synergism of insecticides, are not of such key importance as with vertebrates. Of course, there are various kinds of compounds on which synthesis of partial information can be based. There are four particular kinds of interaction among individual components of mixtures, as follows.

3.1 Independent Components

All components are active by themselves, acting independently, and the effects of the mixture are the same as those of its individually applied components. We may say that the effects summate, depending on the number of components. This is the case with some combinations of natural substances, but usually it is an ideal theoretical possibility. Insecticides without apparent synergic effects are sometimes used as examples of this kind of mixture, but possible interactions of their components are little known.

3.2 Mixtures of Active and Inactive Components

A mixture consists of active and inactive components, the inactive ones being mostly vehicles or dispensers of the active agents (the inactive components may be somewhat active, but mostly to a negligible extent, e.g. polar solvents, acetone, etc.). Activity may sometimes depend only on the experimental species: the 'inactive' component may be very toxic to another organism (e.g. the endotoxin of *B. thuringiensis*).

3.3 Cases of Synergism and Antagonism

The activity of one component of a mixture can be substantially affected by another which may have no toxic effect by itself. Such cases are due to synergism or antagonism. Synergism occurs when the two inhibitors together produce an inhibition greater than expected from independence (see section 3.1). Antagonism is the reverse of synergism.

Methylenedioxyphenyl compounds (MDP) rank among the most important synergists from the standpoint of their history, common use, and number of active compounds. Piperonyl butoxide, sulphoxide, propyl isome, and Tropital are

the most widespread commercial products. Other important compounds are sesamex, myristicin, sesamin, and sesamolin. Substances other than MDP derivatives also act as synergists, for example compounds containing *N*-alkyl groupings, such as SKF 525A, some insecticides, 1,2,3-benzothiadiazoles, some thiocyanates, phthalates, phthaleins, and fluoresceins, etc. (Al-Badry and Knowles, 1980; Casida, 1970; Jordan and Smith, 1981).

Most insecticides are synergized by piperonyl butoxide (PB) or sesamex, at least in houseflies. As for synergism of PB or sesamex with pyrethrum, the degree of synergism is higher for chrysanthemic esters than for pyrethric esters and for cinerolone esters than for pyrethrolone esters. As regards pyrethrin analogues, the alcohol side-chain and the *trans*-methyl group of the isobutenyl moiety are of the greatest importance for the synergistic effect.

Some chlorinated hydrocarbon insecticides are synergized by certain MDP compounds and other synergists. DDT is synergized in the housefly by sesamex, PB, piperonyl cyclonen, *S*, *S*, *S*-tributylphosphorothioate (DEF), SKF 525A, and sulphoxide. Methoxychlor is synergized by sesamex, PB, DEF, and piperonyl cyclonen in various strains of the housefly.

As concerns organophosphorus insecticides, MDP compounds, in particular sesamex, are effective synergists of diazinone, coumaphos, disulphotone, and dicrotophos. PB acts as a synergist with malathion when applied to malathion-resistant strains of the housefly, but it does not affect the action of parathion, methyl parathion, and malathion in susceptible housefly strains. Attia *et al.* (1980) described similar effects of PB when applied with malathion, dichlorvos, pirimiphos ethyl, diazinon, and fenitrothion to the larvae of susceptible and resistant strains of *Plodia interpunctella*.

Many methyl- and dimethylcarbamates have proved to be very sensitive to the synergizing effects of synergists. The ratio of synergist to insecticide, usually about 10 : 1, is lower with these carbamates. The toxicity of carbaryl and propoxur to flies, cockroaches, lepidopteran caterpillars, aphids, and several other insect groups is substantially enhanced by PB or aryl 2-propynyl ethers. Chlordimeform and demethylchlordimeform, used against mite adults and eggs, are effectively synergized by sesamex, PB, and SKF 525A (Kuwahara, 1978).

The mechanism of action of synergists is based on their ability to competitively inhibit an enzyme or enzymatic system. Microsomal monooxygenases (MFO), a system of microsomal enzymes, are inhibited in most cases. This complex of enzymes causes oxidative detoxication of pesticides (e.g. hydroxylation, dealkylation, etc.) and other xenobiotics in an organism. MDP compounds are most frequently used for tests done on insects, with the aim to determine whether the given compound is metabolically unstable owing to its degradation by oxidation. Synergists mostly act as alternative substrates for MFO, so that it is necessary that there should be an excess of the synergist compared with the pesticide if its effect is to be distinct.

Most insecticides and drugs are not metabolized by oxidation alone but also,

in various degrees, by hydrolytic cleavage, conjugation, dehydrochlorination, and/or other processes. Hydrolysis is mainly caused by esterases which may be inhibited by synergists such as triphenylphosphate, triorthocresylphosphate, some organophosphorus insecticides, etc. Different esterases are differently sensitive to certain synergists (see, for example, Kuwahara *et al.*, 1981). Synergists affect the rate of detoxication or, in some cases, activation responses and often transfer the most important part of detoxication to non-oxidative metabolism.

The action of synergists depends, apart from their interaction with the detoxication enzyme, on several other important factors. Other than the kind of insecticide, the kind of synergist, and the species (or strain) of insects they are applied to, the effectiveness of a synergist depends on the speed with which it penetrates the organism and reaches the place of action. Other factors are the affinity of the synergist to the target enzyme and the rate at which the synergist disintegrates in the organism. The rate of the synergist's penetration into the organism should be higher than that of the given insecticide, and the synergist's affinity to the detoxication enzyme should not be lower than that of the insecticide.

Synergism is utilized in practice, often enabling a considerable reduction of the doses of insecticides if suitable synergists are applied. Most of the commercial pyrethroids contain an eight- to ten-fold excess of a synergist, usually PB, that enhances their toxicity approximately ten times (Elliott and Jones, 1978).

Other than the MDP derivatives mentioned above, various combinations of insecticides can produce synergistic effects. An example of this is the effective use of profenofos, sulprofos, and DEF as synergists of *cis*-permethrin against *Trichoplusia ni* larvae and adult houseflies. However, their synergistic effect was small with *trans*-permethrin (Gaughan *et al.*, 1980).

Synergism has also been described after applications of insecticides combined with 'biological' insecticides (bacterial preparations), and occurred between substances produced by two kinds of pathogens (e.g. viruses × bacteria, or bacteria × microsporidia). These were cases of non-specific synergism, the insecticide acting more intensively in organisms weakened by infection. Well known is the synergism of chlorinated hydrocarbon insecticides and microsporidial infections in *Otiorhynchus* weevils. Intoxicated and infected individuals died within nine days, while 10 % of the 'uncontaminated' beetles still were in a 'knock-down' state 14 days later (Weiser, 1951). Very little is known about synergism and the potentiation of biologically active compounds. Bowers (1968, 1971) pointed out the possible potentiation of the effects of juvenoids by some insecticidal synergists.

The titre of the juvenile hormone in peripheral tissues of dipterans is regulated by the MFO system. A joint action of PB and analogues of the juvenile hormone is utilized for affecting the metabolism of certain insect hormones (Fisher and Mayer, 1982).

Fales *et al.* (1970) successfully synergized effects of pyrethrins using some hormonal analogues, but did not achieve as good results as with common synergists (PB). Cruickshank (1971) increased the activity of two juvenoids 1000-fold by mixing them, as compared with separate application of the components. Apparently, it was a case of competition between the compounds during their metabolic degradation. Corey (1971) and Riddiford *et al.* (1971) reported potentiation of some juvenoids by their synthetic imino analogues.

Any of the quantitative methods such as the spray-residue test, topical application, or a dip test, is suitable for testing insects and the effects of synergists. Different ratios of the amount of insecticide and synergist ought to be tested. Compounds which penetrate an organism slowly should be administered earlier than fast-acting ones (e.g. PB is applied 0.5–24 hours earlier than an insecticide). The synergistic effect can be assessed using probit analysis (Finney, 1971) as soon as the synergist's toxicity, the most suitable time of pretreatment with it, and the most effective synergist-to-insecticide ratio are known.

It should be stated that we have very few data on the direct effects of non-specific chemicals on insects, and fewer still on the effects of the mixtures of chemicals occurring in the environment, with the exception of combinations of pesticides. Also our knowledge of indirect (secondary) effects of mixtures of chemicals is very scarce (see Alstad *et al.*, 1982). These effects are manifested mainly by behavioural changes. For instance, sulphur trioxide and sulphuric acid reduced fruit harvest in orchards near an industrial centre because of a reduced efficiency of pollinating bees (Przybylski, 1968).

However, as summarized in this paper, we have sufficient data on the effects of insecticides, biologically active compounds and pathogens on target and non-target organisms, and also enough methods for testing them.

We believe that a broader utilization of these methods will enable the testing of a wider range of the individual chemicals and their mixtures occurring in the environment, including chemicals not specifically used against insects. Data thus obtained will serve not only for determining their adverse effects on insects, which are an important part of ecosystems, but also, in view of the usefulness of insects as bioindicators, for assessing harmful effects on other animals and man.

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