Appendix I

EXPERIMENTAL GUIDELINES

1. FIELD EXPERIMENT

This experiment was carried out in maize as a common crop in all participating countries and (optionally) in one other crop chosen according to local conditions. The two crops are hereafter called maize and alternative crop respectively. If the crop is not specified in the following the comments are valid for both crops. The term pre-treatment applies only to the first treatment.

1.1. Experimental layout

1.1.1. Experimental design

Use a randomised block design with four blocks and two treatments (sprayed and unsprayed) and hence eight plots. A plot size of 25 m by 25 m. gives an experimental area of 0.5 ha. Randomise the position of sprayed and unsprayed plots within each block. Where a uniform field is not available establish the experiment so that most of the variation is between blocks and as little as possible between treatments within the same block. If there is a known gradient in the field, (e.g. soil type or slope), place the blocks perpendicular to the gradient (see Fig. 1).

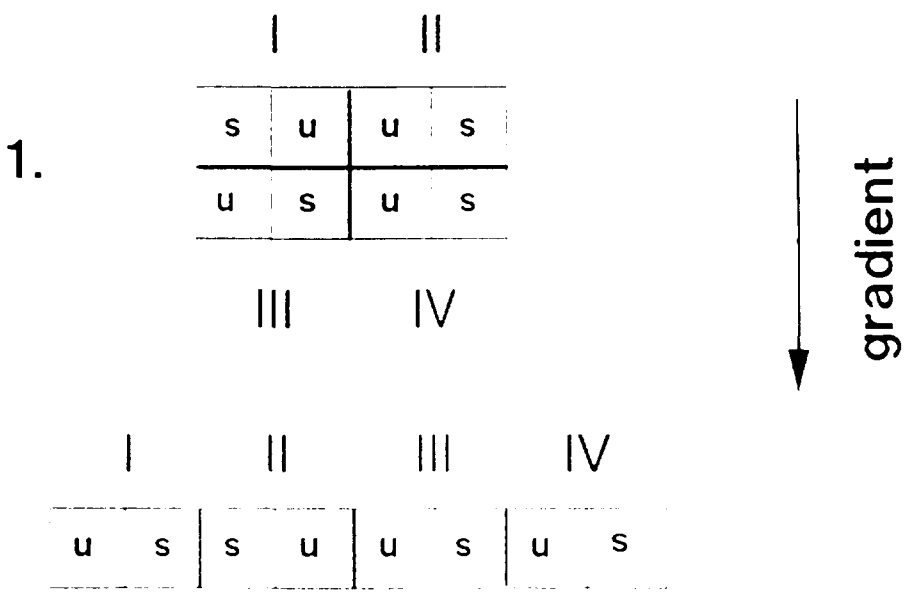

Fig. 1. Experimental design. Two examples of the layout are shown in the figure. The actual layout can be modified slightly to suit local conditions. The thick lines indicate demarcation between blocks. Blocks are marked with roman numerals. s=sprayed plot; u=unsprayed plot. The actual position of sprayed and unsprayed plots within each block is not fixed but must be randomised.

Mark out one subplot (10 m x 10 m) in the centre of each plot for sampling activities (see Fig. 2). Take samples for yield outside this subplot. Mark out other subplots for destructive sampling, e.g. plant and soil samples for residue analyses. Do not take samples within the outer 5 metres of the plot.

Analyse results by two-way analysis of variance.

1.1.2. Pesticides

1.1.2.1.Compounds

Lindane in maize and an appropriate pesticide in the alternative crop.

1.1.2.2. Rate of pesticide application

1 kg (AI) lindane divided in two applications in maize and according to local recommendations in the alternative crop.

1.1.2.3. Method of pesticide application

As a high volume (200-400 L ha^{-1}) spray in maize and according to local recommendations in the alternative crop. Choose the time of spraying to minimize spray drift (usually early in the morning) and keep the nozzle as low as possible.

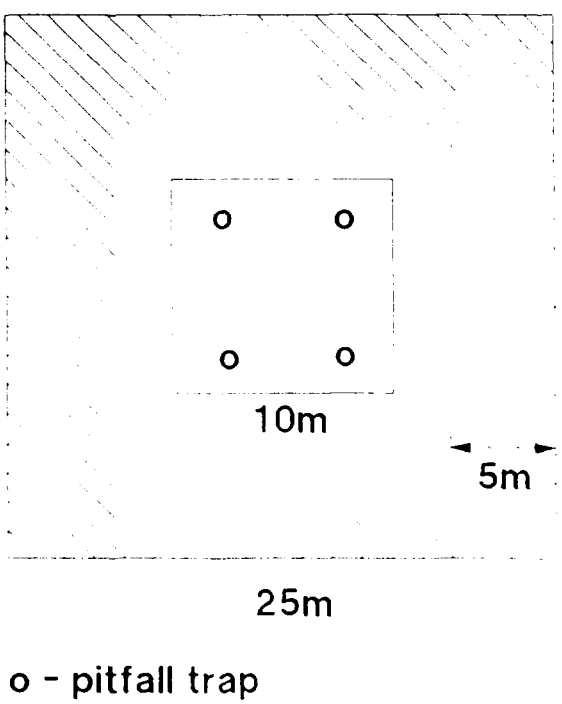

Fig. 2. Plot layout. Do not sample in the hatched outer area. The 2.5 metres surrounding the 10m *by 10 m subplot can be used for destructive sampling, e.g. soil samples and yield.*

1.1.2.4.Timing

Maize: The first application of lindane at the three leaf stage and the second two weeks later at a rate of 0.5 kg AI ha^{-1} on each occasion.

Alternative crop: according to local recommendations.

1.1.3. Field maintenance

1.1.3.1. Weeding

Mechanical

1.1.3.2. Seed dressing:

Accepted if necessary to secure the crop.

1.1.4. Background information to be recorded

1.1.4.1. Pesticide:	Formulation Manufacturer (including trade name) Dose rate Application details (type of sprayer nozzle type and identification letters/numbers, concentration of spray solution, volume rate of application)
1.1.4.2. Soil:	Mechanical composition (% sand, silt & clay) organic carbon content pH (specify soil/water ratio of slurry)
1.1.4.3. Plot:	Size Method of cultivation Previous cropping Neighbouring crops Previous pesticide history (if known) Fertilizers applied
1.1.4.4. Crop:	Variety If seed dressed specify dressing Seed rate Sowing date Density of established crop Spraying date Harvest date
1.1.4.5. Weather:	As much as is available regarding rainfall and temperature. Ideally on a daily basis. At least monthly rainfall and mean, maximum and minimum temperatures.

1.2. Monitoring pests

1.2.1. Species

Stem borers in maize and one chosen key pest in the alternative crop.

1.2.2. Sampling

(i) Maize crop: search all plants in the subplots for plant damage and record the observations as proportion of damaged plants in each subplot. An estimate of the population of stem borers in maize is optional, made 2-4 weeks after the last spray by dissecting 50 stalks, chosen randomly outside the subplots, and counting all larvae and tunnnels.

(ii) Alternative crop: according to the standard method for crop and pest.

1.2.3. Sampling intervals

One immediately pre-treatment and thereafter every two weeks for two months. Dissection of stalks to be done only once.

1.3.Monitoring non target arthropods

The objectives are to get good estimates of both ground living and foliar living predators. It is, therefore important that appropriate methods for these categories are used. The ground living fauna can be assessed with pitfall trapping or ground search and the foliar living fauna with sweep netting or D-vac sampling. None of these methods is perfect. Pitfall traps are rather easy to use but the catches are dependent on both number of insects and their activity. Ground searches are more tedious but give a more reliable estimate of predator density if performed correctly. Pitfall traps, because they are relatively easy to use, are suggested as the standard method for ground living predators.

The best method for foliar living arthropods is D-vac sampling. However, the apparatus is expensive and not readily available. Sweep netting is not an exact method but should give a reasonably accurate estimate of predator density. Sweep netting is suggested as the standard method for this study. However, those institutes that can use a D-vac sampler can substitute sweep netting with D-vac sampling.

It is very important that time and place of catch of each sample is identified. Each sample must, for statistical reasons, be counted and stored separately. It is not necessary to identify the catches beyond the indicated categories. However, it is recommended that all the listed categories are identified. If possible, store the samples in 50 - 70 % alcohol in airtight containers for future identification of important species.

1.3.1. Soil living insects

The following taxa should be identified and quantified where possible:

Order Collembola (spring tails)
Class Aranae (spiders)
Fam. Coccinellidae (lady bird beetles)

Fam. Formicidae (ants)
Fam. Carabidae (ground beetles).

Other groups (e.g. parasitoids, crickets, etc.) could be added if they are considered important natural enemies and/or are caught in substantial numbers.

1.3.1.1. Pitfall traps

Populations of ground living arthropods can be assessed periodically using simple pitfall traps. Plastic pots or beakers (7~10 cm in diameter; 10- 5 cm deep) buried with the upper rim at soil surface level can be used. It is important that the rim does not protrude above the soil surface (see Fig 3). Four traps are placed in each subplot (see Fig 3). The traps should normally be kept covered with lids or boards. The traps are uncovered and filled to half volume with water with a few drops of detergent (e.g. washing up liquid) at each sampling occasion. The traps are emptied after 48 hours.

Do not use coloured traps (they may attract or repel certain species). Use white or grey traps.

The best way to collect the catches from pitfall traps is to flush the whole contents of the trap trough a sieve (ca 1 mm mesh). The catch is then washed down with 70% alcohol (use a wash bottle) through a funnel into a container (this can be done in the field with some training). The arthropods are kept in 70% alcohol for later identification.

1.3.1.2. Ground search

Half metre square, wooden or metal quadrats are used for sampling. The quadrat is placed at random locations in the subplot. The vegetation and soil surface is carefully inspected

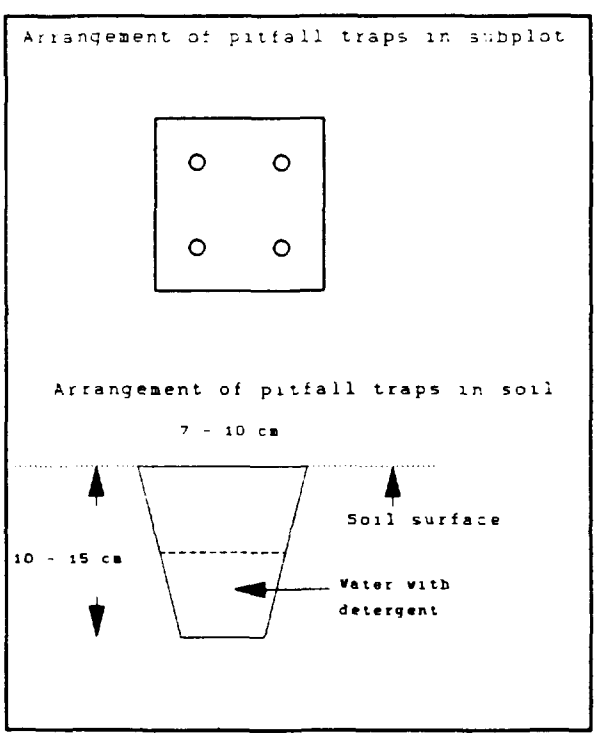

Fig 3. Arrangement of pitfall traps.

and any arthropods are caught (use a soft forceps or a pooter) and stored in 70% alcohol for later identification. Ground search should be done in the morning a few hours after sunrise, and always at the same time of the day.

NOTE! Ground search should ideally involve removal of the vegetation and inspection of the soil down to 5 cm to be of real value. However, in view of the small subplots, this would be too destructive to other sampling activities.

1.3.1.3. Pooter

The pooter or aspirator is convenient for the rapid collection of small insects in sweep nets, during ground search, etc. (Fig. 4). The catching end is placed above the insect and, by sucking the mouthpiece, the operator can catch the insects inside the bigger glass tube. By removing one of the corks the pooter can be emptied into a container.

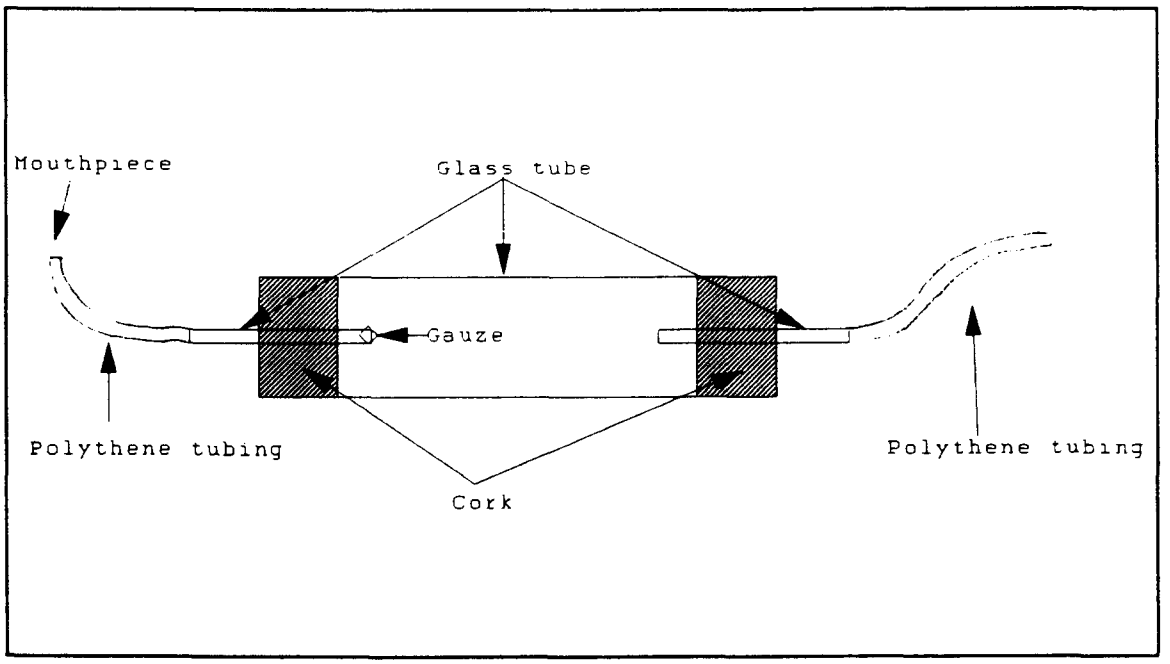

Fig. 4. The pooter.

NOTE! It is important to place a piece of gauze at the end of the glass tube leading into the mouth in order to avoid the risk of inhaling small insects or other particles.

1.3.1.4. D-Vac

The D-vac can be used for both plant and ground sampling. The cone with the biggest opening (1 sq. ft.) is for ground sampling and the smaller (1/3 sq. ft. opening) is for plant sampling.

Take ground samples at 4 different places in each subplot. A sample consists of five subsamples. Each subsample consists of a 5 second suck with the sampling cone lightly pressed to the ground. Take subsamples at intervals of two or three metres until 5 subsamples have been taken without emptying the collecting bag or switching off the

engine in between. After the 5th subsample, remove the collecting cone and empty the collecting bag into a plastic bag. Leave the engine running until the catch is secured in the plastic bag to prevent the insects flying away. Two persons are needed and prior training is necessary. It is recommended to carry a screw driver so that the air duct can be loosened from the engine to recover the bag in the event it is dropped. Repeat the whole procedure of 5 subsamples at 4 different places (e.g. near each pitfall trap) in each subplot.

1.3.1.5. Soil cores

A soil corer 5 cm in diameter and at least 20 cm long is recommended. Ideally it should be possible to divide the sample into sections, for example using a design like that of Tanton [1] (Fig. 5) but this is not essential. Four soil cores (5 cm in diameter 15 cm deep) should be taken from each subplot prior to treatment and again after two months.

The arthropods in the soil sample can be extracted in a Berelese-Tulgren funnel (see Fig. 6). The diameter of the funnel can be between 15-40 cm but the angle should be steep. Laboratory glass funnels are suitable but plastic should be avoided because of potential problems caused by static electricity. The mesh size of the metal gauze should be about 3mm. Some kind of heat source (e.g. a light bulb) should be placed above the sample or the extraction should be done in a warm room. The purpose is to obtain a temperature gradient of 10-20°C from top to bottom of the sample. The sample should be broken into

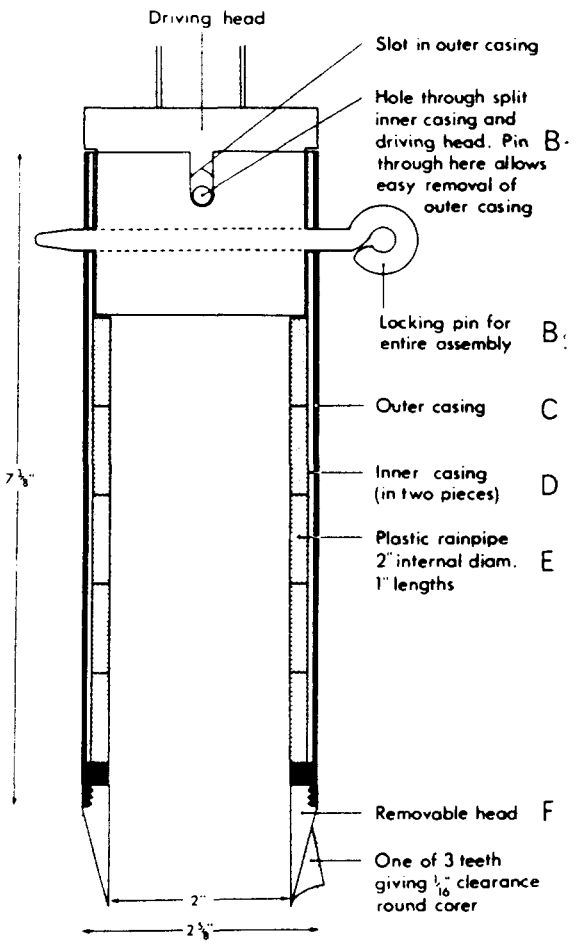

Fig. 5. Soil corer with split liner.

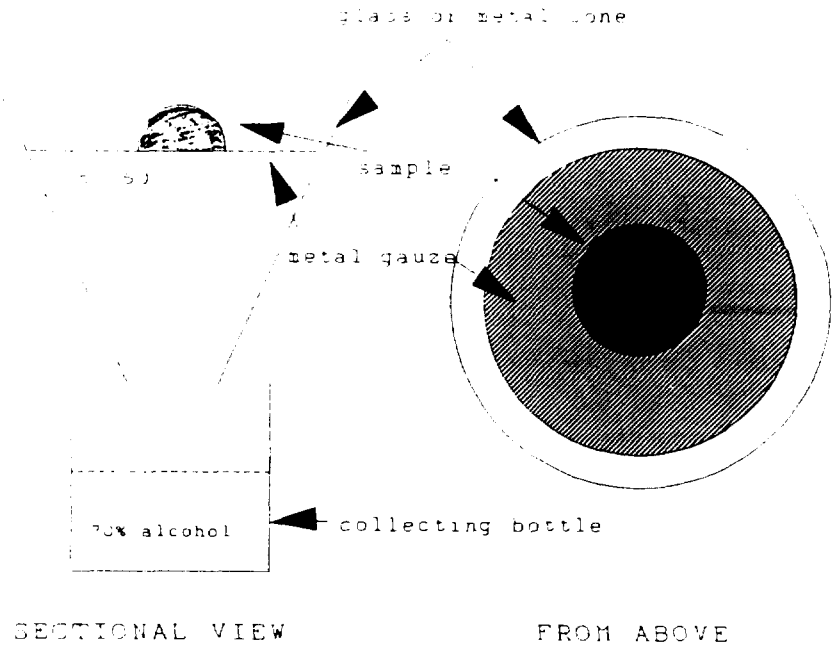

Fig. 6. The Berlese-Tullgren funnel.

smaller sections before extraction. The sample should, however, be at least 1 cm thick to avoid it drying out. Leave the sample in the funnel for 72 h. Extraction should be done as soon as possible after sampling but it is still possible for up to 4 months if the sample is stored at 4-6°C.

1.3.2. Foliar living insects

1.3.2.1. Sweep net

A standard sweep net consists of a cloth bag fitted into a wire loop (ca 40 cm in diameter) and attached to a 50-60 cm long handle. The net bag is cone shaped and approximately 60 cm deep. As you sweep, tilt the lower rim of the net so it is ca. five cm in advance of the upper rim to catch insects which drop from the plant. Be careful to keep the net below the tops of the plants until the end of the sweep. A single sweep consists of one 180° arc as you step forward. Raise the net at the end of each sweep and reverse the direction of your swing alternately. Take five sweeps for each diagonal. Two diagonals (= two samples) is taken per subplot (see Fig. 7). The catch can be collected with a pooter or put directly in a container with 70% alcohol.

1.3.2.2. D-Vac

As with the ground search, plant samples consist of 5 subsamples. In this case each subsample consists of one plant. Take a total of 4 samples in each plot. To make the sampling as exhaustive as possible place. the cone over the whole plants when they are small; when larger, sweep the plants with the cone from all sides. Since the method is impractical on large plants, sample plants only in the first 6 weeks.

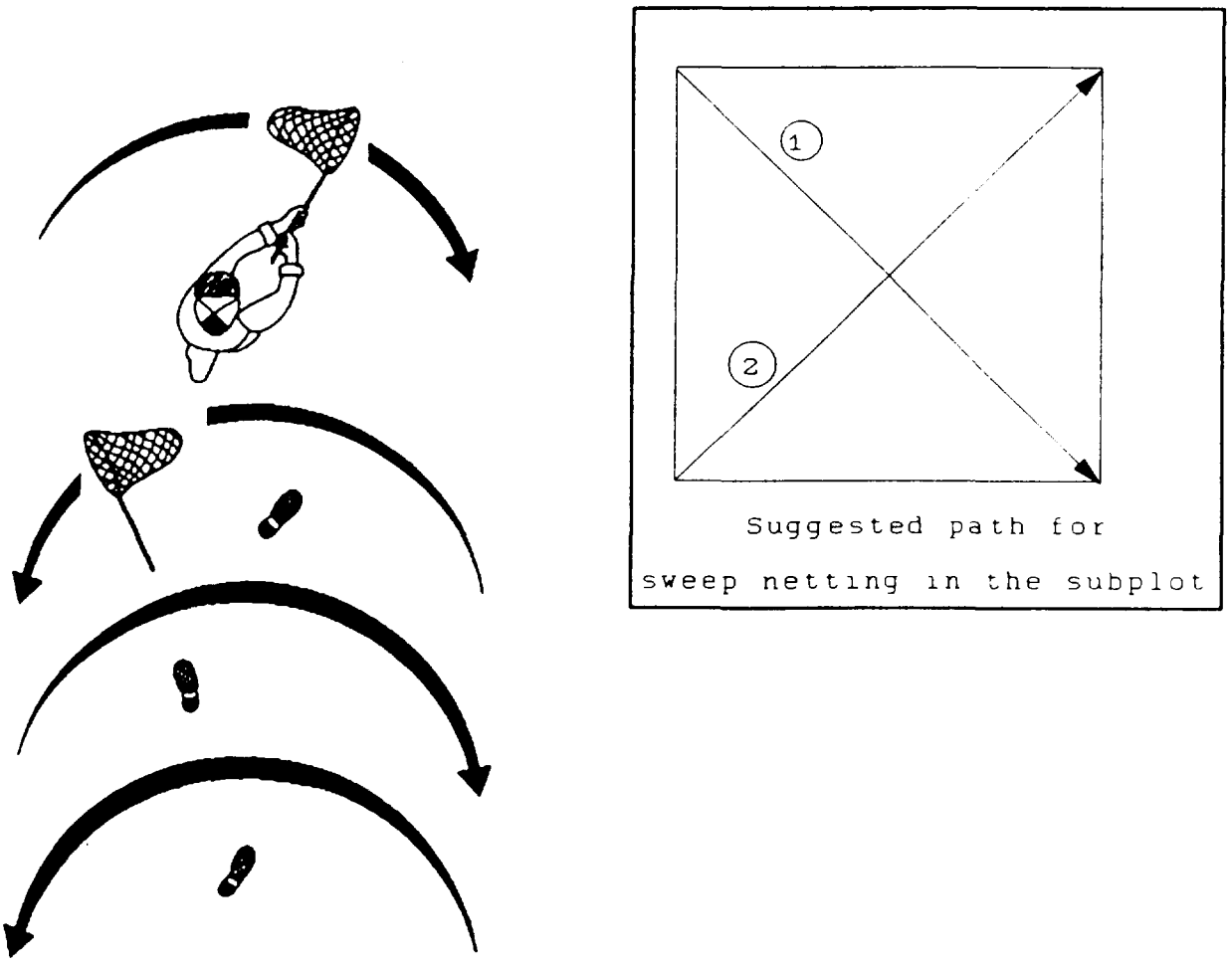

Fig. 7. Use of the sweep net.

1.3.3. Soil biology

1.3.3.1. Categories

Breakdown of organic matter (compulsory), earthworms and soil fauna (optional).

1.3.3.2. Sampling methods

(i) Breakdown of organic material: Cut disks (2.5 cm diameter), using a cork borer or similar tool, from suitable leaves collected from trees of a single, identified spp growing nearby the experimental plots. The leaves should be rather tough, uniform and growing on the same side of the tree. Place batches of fifty disks in nylon mesh (7-10 mm aperture) bags (ca. 12 cm square), closed with staples and attached to a marker peg with thread. Bury four bags per subplot at random to a depth of ca. 5 cm prior to treatment. Dig up the bags after 2-5 months and wash the contents, then dry in an oven (ca. 600 C for 24 hours) and weigh. Dry and weigh two additional batches of fifty leaves prior to treatment. Present the results in two ways: (a) percent loss in weight (the freshly dried disks are 100%) and (b) the actual weight of the content.

Note! It is important that the bags are left in the soil for an "appropriate", time that is when approximately 50% of the organic matter has been broken down. It is therefore recommended to bury two extra bags in two control plots to be used to decide when to dig up the rest of the bags.Thus one extra bag can be dug up after 1-2 months and the second after 2-3 months.

(ii) Earthworms: Use half metre square, wooden or metal quadrats for sampling. Lay them on the soil surface and apply nine litres of dilute formalin (50 mL of 40% in 9 litre water) gradually with a fine spray watering can so that the soil does not become flooded. Most of the worms come to the surface within 15 minutes, and can be picked off and collected into jars containing 5% formalin for storage and eventual identification to species. Take two samples per subplot prior to treatment and again after two months.

1 3.4. Yield

1.3.4.1. Sampling

Estimate yield from 4 subplots (2 m × 2 m) in each plot. Because sampling activities may have an effect on the yield, locate the subplots for yield outside, but close to, the subplots for sampling. Note the moisture content in the crop at harvest.

1.3.5. Residues

1.3.5.1. Categories

Crop (compulsory), soil, earthworms and insects (optional).

(i) Crop sampling: For basic analysis a sample of 12 plants per plot is suggested. Collect the leaves only 1 day pre-treatment, 1 day post-treatment, after 2 weeks and after 4 weeks. Weigh, chop, preferably mechanically, and thoroughly mix. Take duplicate subsamples for dry weight and residue analyses.

(ii) Soil sampling: Take 12 cores at random from each subplot. Cores 2.5 diameter and 15 cm deep are advised. Weigh the cores before and after removal of any stones and grind to pass a 0.25 cm sieve. Mix the ground cores thoroughly to provide one bulk sample for each plot by successive quartering of the soil on a plastic sheet or in a mechanical mixing device. Take subsamples in duplicate for measurement of moisture content and for residue analysis pre-treatment, one day post- treatment and after 2 and 6 months from both treated and untreated plots.

(iii) Earthworms: Worms can be obtained for residue analyses either by the formalin method or by digging. If formalin is used, drop the earthworms into a bucket of clean water immediately they emerge on to the soil surface. Then leave for one day on a damp filter paper in covered petri dishes to allow them to empty their intestines. Then wash, remove surplus moisture with clean tissue paper, and place in clean petri dishes in a deep-freeze and leave until brittle. Take samples pre-treatment and after two months.

1.4. Higher fauna

It is not possible to make up detailed protocols for this area at present. The methods must be adjusted to local conditions and to the range of species that are chosen.

Basically two different approaches can be adopted:

1. Field trial
2. Extensive survey.

In both cases there must be a choice of suitable "indicator" species to study. The criteria could be:

species that are particularly likely to be exposed to the chemical;
species thought to be particularly sensitive to exposure;
species for which an adverse effect would be especially damaging;
abundant species permitting large samples for data collection;
relatively sedentary species;
species whose ecology and behaviour provide easy opportunities to measure effects.

It is unlikely that a single species will fulfill all these roles, and it may be desirable to study a range of species from several categories, at least initially.

1.4.1. Field trial

This type of study requires rather large areas of land (several hectares) where the use of pesticides can be supervised. There must also be some kind of control data to enable comparison. This could be done in two ways: to look for changes before and after treatment on the same site or to compare the treated area with an untreated. A combination of both approaches is recommended.

The measurement should include:

estimation of population numbers (e.g. trapping for mammals and nest counts for birds);
reproductive success;
corpse counts;
residue analyses (corpses, blood, eggs);
biomarkers (if applicable).

Although dead animals give the clearest evidence of a damaging effect this may not always be the most useful approach to detect adverse effects from pesticide use. Carcass searching should, therefore, be complemented with a measure of other effects, e.g. estimation on population changes and reproductive success. If possible a biomarker should be used such as egg shell thinning or repression of enzymatic processes.

1.4.2. Extensive survey

This survey should be done as a comparative study between an area with regular use of organochlorine pesticides (contaminated area) and an area with no such use (control area).

This should include:

background survey, i.e. information on pesticide use, information on residues in higher fauna and identification of indicator species;
identification of contaminated area and control area;
collection of samples for residue analyses.

One crucial step is the choice of indicator species (see above). For this type of study it is important that relatively sedentary species are chosen. For birds, it is recommended to chose one or a few predatory birds e.g. falcons and one or a few insect eating birds, e.g. shrikes.

1.4.3. Sampling for residue analyses

1.4.3.1. Carcasses

Carcasses may have been shot or simply found dead in the field. They should be put in plastic bags, labelled and taken to the laboratory (preferably at 0-4°C) where they should be dissected as soon as possible. Carcass weights and fresh organ and/or tissue weights should be recorded. Liver samples should always be analysed and where appropriate brain, breast muscle and fat should also be sampled. Whole tissues or organs should be homogenised and subsamples taken as needed. If storage is necessary samples or subsamples should be kept at -20°C (or lower) in glass tubes or vessels fitted with tight sealing caps.

1.4.3.2. Eggs

After weighing, eggs should be carefully blown to remove all the contents. The content should be weighed and stored, if necessary, at -20°C. The shells should be retained to assess shell thickness either by the weight/volume ratio method or by direct measurement of sections.

A very useful publication for this area is Sommerville, L. & Walker, C.H. [2].

2. RESIDUE ANALYSES

2.1. Residue analyses (unlabelled pesticides)

If chlorinated hydrocarbons are analysed routinely, follow routine procedures. If not, the following notes can be used as a basis to develop a procedure. No extraction and cleanup procedure can be guaranteed to work first time. Always check the recovery through the procedure by 'spiking' samples (at least 3) of untreated substrate with a known quantity of analyte. The easiest way to do this is to use ^{14}C labelled pesticides, 3.7 mBq (0.1 g Ci) per sample is enough.

Always run control (untreated) samples of substrate through the procedure to establish thechromatographic background. This is necessary to determine the limit of determination. There are a number of statistical procedures that have been proposed but for most purposes the 'rule of thumb' approach of setting the limit of determination at twice the blank value is satisfactory.

2.1.1. Extraction

2.1.1.1. Plant and non-fatty animal tissue

Homogenise 10-20 g tissue in 200 mL methanol. Filter if necessary. Take a known volume of filtrate (up to 142 mL) and add 2.5 times that volume of water to give a solution containing not more than 40% methanol. The final volume should not exceed 500 mL.

2.1.1.2. Fatty animal tissue and fish

Homogenise 10 g tissue, add 20 g Na_2S0_4 and mix thoroughly. Transfer to a 250 mL separating funnel and add 50 mL hexane or petroleum ether followed by 100 mL acetonitrile saturated with hexane or petroleum ether and shake 1 minute. Drain the acetonitrile into a 1 L separating funnel. Repeat with 3 further portions of 50 mL acetonitrile and combine the extracts. (For reasons of economy you could check the recovery using smaller quantities of acetonitrile.) Add 500 mL water, 40 mL of saturated NaCl solution and 50 mL petroleum ether or hexane. Shake 30 seconds, allow layers to separate and drain aqueous layer into a 2nd 1 L separating funnel. Shake aqueous layer with 50 mL petroleum ether or hexane. Discard aqueous layer and combine the petroleum ether/hexane extracts. Drain through a plug of 10 g Na_2SO (held in a funnel for example) into an evaporating basin or a rotary evaporator. Evaporate just to dryness and dissolve residue in 10 mL methanol. Dilute with 25 mL water.

2.1.1.3. Soil

Extract 50 g soil with methanol in a Soxhlet apparatus for 10 cycles (2-4 hours). Dilute the methanol extract with 2.5 volumes of water as above.

2.1.2. Cleanup using solid phase extraction (SPE) columns

A number of suppliers produce SPE columns. They vary slightly and different batches from the same manufacturer may also vary, so the procedure needs to be checked. Recovery of standard from the column should be at least 90%. For this analysis 6 mL C-18 SPE columns are needed.

2.1.2.1. Column conditioning

Pass two column volumes of methanol, followed by two column volumes of distilled water through the column.

2.1.2.2. Sample addition

Add 200 mL of water diluted methanol extract to the extraction column(s). The flow rate through the column(s) should be 30-40 mL/min. Residual particulates can be removed by placing a 6 mL filtration column and adaptor between the extraction column and reservoir.

2.1.2.1. Column wash

Remove reservoir(s) and wash with one column volume of distilled water. Air dry column under vacuum for 10 minutes.

2.1.2.3. Sample elution

Elute with three 500 µL aliquots of hexane and make up eluate to 2 mL with hexane.

2.1.3.Gas chromatography

There are many sets of GLC conditions cited in the literature. Examples include:

Glass column 5 feet long, 1/4 in diameter packed with 5% QF I on 60-80 mesh Chromosorb G. Column temp. 220°C, detector temp 235°C carrier gas 20 mL/min [3].

Glass column 165 cm × 2 mm packed with 1: 1 mixture of 10% DC-200 and 15% QF-1 on Gas Chrom G. Injector temp 235°C, column 220°C, detector 275°C. Carrier gas 70 mL/min [4].

Glass column 1 m × 3 mm packed with 5% SE30 on Chromosorb W. Column temperature 200°C [5].

2.2. Analysis of (^{14}C) organochlorine pesticides in maize

(i) Homogenize the maize sample with methanol as in 2.1.1.1.

(ii) Take three replicate samples of not more than 300 mg dry weight and combust in a biological material oxidizer, trapping evolved $^{14}CO_2$ in Carbosorb. Analyze the trapped $^{14}CO_2$ in LSC to give total radioactivity in the sample.

(iii) Weigh a 5g sample of maize homogenate into a flask and add 20mL methanol. Shake for 30 min. then sonicate for 5 min. in an ultra sonic bath. Transfer the sample to a centrifuge tube, centrifuge for 10 min. at 3000 RPM and remove supernatant. Determine total volume and count 2 × 1 aliquots in LSC for calculation of total extractable radioactivity. Dry the residue and combust to determine unextractable radioactivity. Determine total recovery.

SUMMARY OF SAMPLING PROGRAMME

Type of sampling	Compulsory/Optional	No. of pre-treatment samples	Intervals of post-treatment samples
Plant damage	Comp	1	2, 4, 6, 8, 10 weeks
Dissection of stalks	Opt	0	ca. 8 weeks
Pitfall traps	Comp	1	0*, 2, 4, 6, 8, 10 weeks
D-vac (ground)	Comp	1	1 day, 2, 4, 6, 8, 10 weeks
D-vac (plant)	Comp	1	1 day, 2, 4, 6 weeks
Breakdown organic matter	Comp	-	2-5 months
Earthworms	Opt	1	ca. 2 months
Soil fauna	Opt	1	ca. 2 months
Yield	Comp	-	-
Residues in crop	Comp	1	1 day, 2, 4 weeks
Residues in soil	Opt	11	1 day, 2, 6 months
Residues in earthworms	Opt	1	ca. 2 months

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