SESSION 7C

ENVIRONMENTAL FATE AND IMPACT OF PESTICIDES – RESULTS AND NOVEL TECHNIQUES

SESSION ORGANISER MR M. W. SKIDMORE

POSTERS 7C-1 to 7C-18

SOIL METABOLISM - AUTOMATION OF A BIOLOGICAL MATERIAL OXIDIZER

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ABSTRACT

The requirement to quantify the levels of unextracted ^{14}C-radiolabelled residues from samples, treated with radiolabelled pesticide during a metabolism study, necessitates the use of a technique known as biological material oxidation. Several commercial instruments are available.

Recent laboratory automation developments at ICI Agrochemicals Jealott's Hill Research Station, have included the successful automation of biological oxidation. Two interfaces (electronic and mechanical) were designed to allow a Harvey™ OX-300 oxidiser to be operated by a Zymark™ Laboratory Robot. The equipment is in routine unattended operation and has processed up to the present time 3000 soil samples.

INTRODUCTION

Biological material combustion is a technique used during radioactive tracer studies and is one commonly employed in pesticide metabolism chemistry. An understanding of the chemical and biochemical transformation of pesticides introduced into the environment is achieved by substituting a radiolabelled atom into the compound eg ^{3}H, ^{14}C.

Environmental matrices eg soil are treated with radiolabelled pesticide, normally ^{14}C, and are allowed to "weather" or "age". Any available remaining parent compound and its breakdown products are removed using solvent extraction techniques. The level of radioactivity in the solvent extract is then quantified by liquid scintillation counting. Any products, unextracted by the solvent extraction techniques are released by biological material oxidation or combustion, for later quantification by liquid scintillation counting. Carbon atoms within the sample to be oxidized, including radioactive carbon, are converted to carbon dioxide via high temperature oxidative conditions.

$$^{14}2C + 1\tfrac{1}{2}\ O_2 \xrightarrow{\text{heat}} {}^{14}CO_2 + {}^{14}CO$$

$$^{14}2CO + O_2 \xrightarrow{\text{heat}} {}^{14}2CO_2$$

The carbon dioxide generated is trapped by bubbling through and reacting with an adsorbent such as 2-methoxyethylamine to produce a carbamate. From the commercially available instruments, the Residue and Environmental Chemistry Section have chosen to use Harvey OX-300 Biological oxidisers because the design of the instrument is relatively simple. However, the operation of the machine is repetitive and tedious, requiring constant intervention by an operator. Therefore it represented a good target for automation, in order to release valuable manpower.

In this paper a description is given of the development of an automated biological material oxidiser, operated by a Zymark Laboratory robot.

EQUIPMENT AND METHOD

The robotic equipment supplied by Zymark Ltd is mounted on a benchtop measuring eight feet by five feet and is arranged around the cylindrical co-ordinate robotic arm. The OX-300 oxidiser is situated on the left hand side of the table, below an extracted fume hood (see Figure 1). An electronic interface allows the robot computer to interrogate and send commands to the OX-300 oxidiser. Four vial racks hold the vials which contain encapsulated soil samples (250 mg) for combustion. Vials are collected from a rack, uncapped at the gas trapping station, and the soil capsule is transferred, by pouring into a conventional quartz furnace ladle. Once the vial has been returned to the gas trapping station, upon command from the robot computer, the OX-300 oxidiser pumps scintillation cocktail to fill the vial.

From its rack position, the loaded ladle is transferred to the injector station. This is a special customised device designed jointly by ICI and Zymark, which uses five pneumatic cylinders and actuators. The interface which securely clamps a ladle, provides linear in/out travel, with rotary motion along the axis of rotation of the ladle, to seal/unseal the tapered ground glass joint of the ladle and furnace tube.

The ladle is inserted into the furnace by the injector when the OX-300 signals it is ready. The above steps are performed in reverse to complete the operation. The removal of the ladle from the furnace and its return to the rack, is followed by recapping and return of the vial to its original rack position.

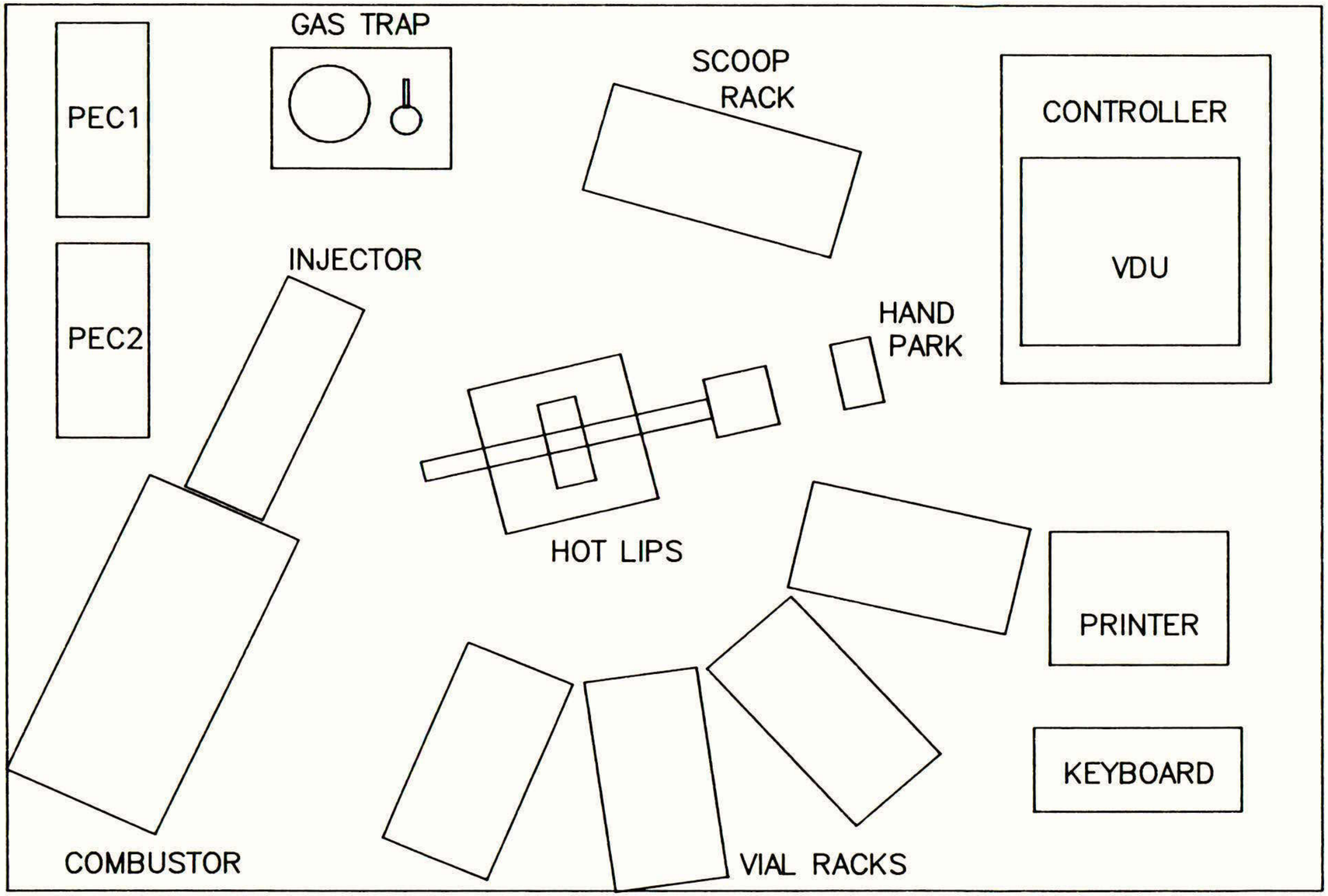

FIGURE 1 : HARVEY COMBUSTOR/ZYMARK ROBOT AUTOMATED OXIDIZER

PEC = Power and Event Controller

Vials are processed sequentially, one at a time. The programs with which the system operates were designed and written in-house, by ICI, using Zymark "Easylab" propriety software.

SAFETY CONSIDERATIONS

The automated system is fitted with an array of sensors and uses branched software routines, to make decisions, allowing continued operation of the current run. Details of these sensors are given below.

i) Three gas pressure switches monitor gas supplies to the oxidiser and compressed air to operate pneumatic actuators.

ii) Proximity switches are mounted on pneumatic actuators to confirm correct operation.

iii) A differential pressure switch monitors the status of the fume hood extraction system.

iv) An optical detector is employed to confirm the integrity of the fragile quartz ladle after each burn cycle.

The two temperature zones of the furnace are interrogated at the start of each cycle. The response to detecting any serious error, is the removal of the power supply to the OX-300 oxidiser via a solid state relay. In addition to the safety features detailed above, the robot is equipped with tactile sensing, enabling it to confirm the presence within its fingers, of an item it has attempted to pick up.

OPERATION

The system is activated using a wall mounted electrical isolator. The program to drive the system, resident in the computer, is initiated during power up and automatically prompts the user for necessary run information. Samples to be combusted are placed in vial racks, the scintillation cocktail reservoir is filled, and nitrogen and oxygen gas cylinders are checked to ensure adequate pressure for the intended run. The automated run commences when furnace temperatures have reached their normal operating range. Using a three minute burn duration, the system processes approximately ten samples per hour and will complete the maximum number of vials (128) in approximately fourteen hours (sixteen hours are available overnight). A print out is generated at the end of the run. This documents the furnace combustion and catalyst temperatures stored during each sample combustion, a useful record for good laboratory practice purposes.

CONCLUSION

The equipment described is the first successful example of a fully automated biological material oxidiser. It was commissioned in March 1988. To date, the number of combustion cycles has exceeded three thousand. The quality of data generated by the system is very good, the recovery of radioactivity introduced to the automated OX-300 is no lower than the manually operated version (approximately ninety five percent). The provision of this unattended instrument has allowed highly trained scientists to be released from a necessary but laborious task, enabling their time to be diverted towards more demanding activities.

DEVELOPMENT OF A METHOD TO INVESTIGATE THE PHOTODEGRADATION OF PESTICIDES

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ABSTRACT

A method to assess the potential photodegradation of a pesticide in the environment is described. Samples of the radiolabelled pesticide are irradiated in the laboratory, under controlled conditions, using a xenon burner to simulate sunlight. The intensity of this source is compared with natural sunlight, and a calculation is described which uses this comparison to estimate the rate of photodegradation of the pesticide in the environment, at different latitudes and seasons.

INTRODUCTION

As part of the assessment of the fate of a pesticide in the environment, the stability of the pesticide, when exposed to sunlight, must be evaluated. In normal agricultural usage a pesticide may intentionally or unintentionally become introduced into water or onto a soil surface. The possible photodegradation of a pesticide in these two different media must therefore be investigated separately.

A system has been designed to irradiate, under controlled conditions, aqueous solutions of a pesticide and soil surfaces treated with the pesticide. The system is primarily designed for use with radiolabelled pesticides, but could also be used to assess rates of photodegradation with unlabelled compounds. The results obtained with this method can be used to predict rates of photodegradation at different latitudes and for different seasons of the year.

APPARATUS AND METHODS

Light Source

An international committee, Commission Internationale de L'Eclairage has defined spectral distribution in terms of "correlated colour temperature" (CCT). This is the temperature in degrees Kelvin of a full black body radiator that most closely matches a given distribution. A CCT of c. 6500K was defined as the best representation of sunlight, designated as D65 radiation (CIE publication No 15, 1971, Judd et al. 1964).

A Hanau Suntest Accelerated Exposure Machine (Heraeus Equipment Ltd, Brentwood, Essex, UK) is used as the light source. The radiation from the xenon burner in this machine is filtered to give ultraviolet and visible light with a spectral distribution that closely approximates to D65 radiation, ie. natural sunlight.

Photolysis in water

A system was designed so that eight solutions in individual photolysis vessels could be placed beneath the xenon burner. The design of the photolysis vessels is shown in Figure 1. The vessels are made from borosilicate glass and hold a maximum sample volume of 15 ml. Each vial is capped with a quartz lid made from Vitreosil 055. This is the best optical grade vitreous silica, suitable for optical applications requiring good transmission in the near ultraviolet and visible region. Two retaining springs, attached to hooks on opposite sides of both the lid and vial, hold the lid in place. The vessels are sealed by a teflon sleeve between the lid/vial joint and a layer of teflon tape bound around the joint.

In order to maintain a constant temperature during irradiation the photolysis vessels are held in specially designed stainless steel tanks (Figure 3). Two of these tanks will fit side by side beneath the xenon burner. Four vessels in each tank are used to hold the test solutions. The fifth vessel in each tank is used to monitor the temperature of the solutions during the irradiation, using a Jenway 97K thermocouple and a Baird and Tatlock model 2003 thermometer. A Churchill 02/CTC HG chiller thermo circulator is used to pump water through the tanks. The temperature of the circulatory water is set so as to maintain the solutions in the irradiation vessels at 25 $\pm$ 1°C during the irradiation period.

In order to ensure that only abiotic degradation is measured the experiment is carried out under sterile conditions. The pesticide is normally irradiated in an aqueous solution buffered at a pH, in the environmentally relevant range of 5 to 9, where the chemical is hydrolytically most stable. The aqueous buffer (15 ml) is added to the photolysis vessels and eight vessels are joined in series, as shown in Figure 3, with silicone tubing. Emflon PTFE filters (microbial removal rating 0.2 μm) are inserted in the connecting tubing on either side of each vessel. This allows subsamples to be removed for analysis after suitable periods of irradiation, while still maintaining sterility in the remaining vessels.

The vessels, filters, connecting tubing and aqueous buffers are sterilised by autoclaving. After sterilisation the radiolabelled pesticide under test is added to give a suitable concentration (usually 1 - 10 mg/l). The pesticide is added in 150 μl of acetonitrile, resulting in an organic solvent concentration of 1%, using sterile techniques in a laminar flow hood. The vessels are then transferred to the cooling tank and placed under the xenon burner.

Figure 1 AQUEOUS PHOTOLYSIS VESSEL

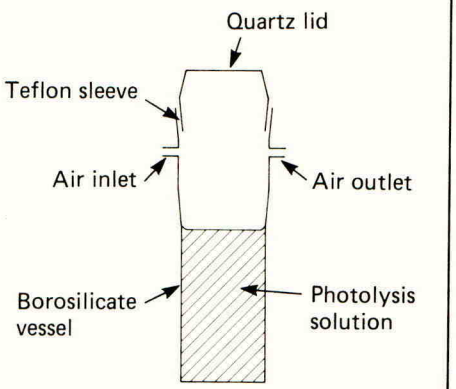


Figure 2 SOIL PHOTOLYSIS PLATE

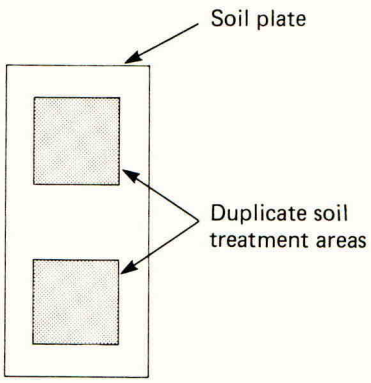


Figure 3 AQUEOUS PHOTOLYSIS SYSTEM

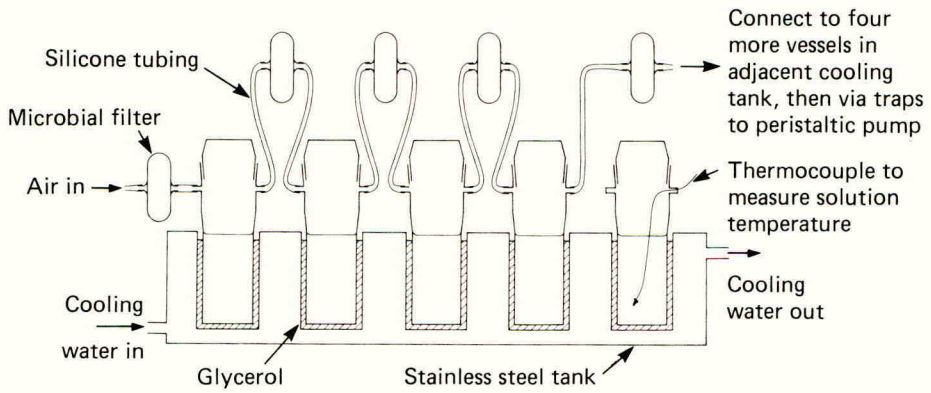
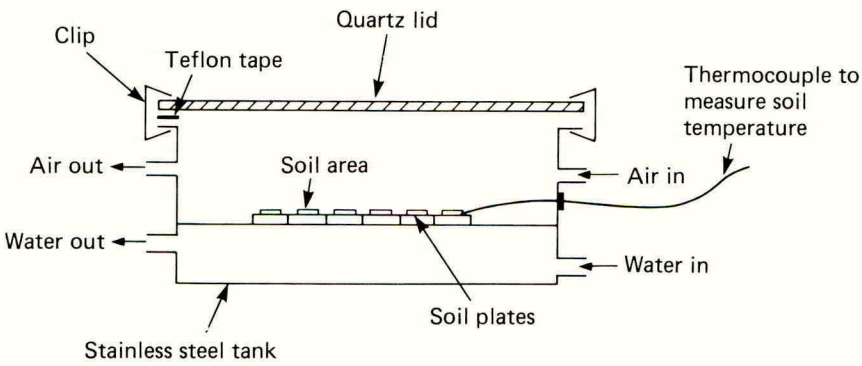


Figure 4 SOIL PHOTOLYSIS SYSTEM



In order to trap any volatile products formed during the irradiation, air is sucked through each set of vessels using a Watson Marlow 101 U/R peristaltic pump. The incoming air is bubbled through traps containing 2M potassium hydroxide solution and then through traps containing water. This ensures that moist, carbon dioxide free air enters the system. The effluent air is bubbled through a series of traps containing 1M sulphuric acid solution (to trap volatile basic compounds), methoxyethanol (to trap volatile organosoluble compounds) and ethanolamine (to trap $^{14}CO_2$ or other acidic volatile compounds). The levels of radioactivity in the traps are assessed at regular intervals by liquid scintillation counting.

After suitable periods of irradiation sub-samples (usually duplicates) are removed for analysis. By analysing samples after various periods of irradiation the rate of degradation can be measured. The use of radiolabelled pesticide will also facilitate the identification of the photodegradation products formed.

In some experiments, using this system, volatile organic compounds have distilled from the irradiated solutions and have then been absorbed by the silicone tubing before reaching the traps. To overcome this problem polyurethane foam bungs were inserted in the side arms of the photolysis vessels to absorb the volatile compounds before they reached the tubing. Any absorbed radioactivity is then recovered by extraction with a suitable organic solvent. The foam bungs (which are unstable to UV light) are protected from irradiation by covering the side arms with aluminium foil.

Photolysis on a Soil Surface

Soil surfaces are prepared by applying an aqueous slurry of soil, in a 1 mm thick layer, to a glass or stainless steel plate. After allowing the layers to dry, soil is removed from the plate to leave two duplicate treatment areas per plate (Figure 2). The required amount of ^{14}C-pesticide, dissolved in a suitable solvent, is then spotted uniformly over the soil surfaces. In order to control the temperature of the irradiated plates and to trap any volatile products formed, the plates are placed in the stainless steel cooling tank illustrated in Figure 4. The tank is then sealed with a quartz lid, using teflon tape between and bound around the lid/tank interface, and placed beneath the xenon burner. In the same way as described for photolysis in water studies, cooling water, at a pre-set temperature is pumped through the base of the tank to control the temperature of the irradiated plates. Air is also sucked through the cooling tank and any volatile products formed are trapped.

In order to monitor the temperature of the soil an additional plate is prepared and a thermocouple is inserted in the soil slurry before the slurry dries out. This plate is placed alongside the treated plates and the temperature monitored thoughhout the irradiation. Temperature control in the range 25°C ± 5°C is obtained.

Plates, ie duplicate samples, are removed at suitable intervals for analysis.

Measurement of radiation

The intensity and spectral quality of the xenon burner and sunlight are measured using an LI-1800 portable spectroradiometer. (Li-Cor Ltd, Lincoln, Nebraska, USA). This machine has a remote cosine receptor and light is transmitted from the receptor to the spectroradiometer by a fibre optic cable. The spectroradiometer measures radiation intensity in 4 nm bandwidths over a 300 - 850 nm wavelength range. Normally the LI-1800 is used to measure the total intensity for the wavelength range 300 - 400 nm, this is the high energy region of sunlight and is the region most likely to initiate photodegradation. However, if the compound under test absorbs at wavelengths higher than 400 nm the LI-1800 can be used to measure total intensity over the region where absorption occurs.

RELATING THE XENON BURNER TO SUNLIGHT

In order to relate the rate of degradation caused by the xenon burner to the rate likely to occur in sunlight, it is obviously necessary to compare the intensity of the xenon burner with sunlight. Using the LI-1800 it is possible to measure the intensity of the radiation that will impinge on each individual sample placed beneath the xenon burner. For photolysis in water studies, the remote sensor is placed in the exact position where the surface of each irradiated solution will be, a photolysis vessel with the bottom cut off is placed over the sensor and the radiation intensity incident on the sensor is recorded. Similarly for soil surface studies the incident radiation intensity, with the quartz lid held in place, is measured at each position which will be occupied by the soil plates. Midday sunlight intensity is also measured at a known latitude (latitude L) and at a given season of the year (season S). The ratio of incident light intensity underneath the xenon burner to midday sunlight at latitude L and season S can then be calculated.

$$\text{ie} \quad \frac{\text{Incident intensity underneath xenon burner}}{\text{Intensity of midday sunlight at latitude L and season S}} = A$$

Assuming a 12 h day, then 12/A h of irradiation in the Suntest is equivalent to 12 h of midday sunlight at latitude L and season S. However, sunlight intensity will vary throughout the day. Data obtained from the Meteorological Office, Bracknell, Berkshire, UK shows that total daily radiation is approximately equivalent to 0.75 times 12 h of midday radiation. Therefore, 12/A x 0.75 h irradiation in the Suntest is equivalent to one day of sunlight at latitude L and season S. Hence the rate of degradation measured in the Suntest can easily be converted to rate of degradation expected in sunlight at latitude L and season S.

The results obtained in the Suntest can also be converted to give a rate of degradation at other latitudes and seasons. This conversion is achieved using data published by T Mill et al (1982). This publication lists values for a term L_λ which is proportional to the day averaged radiation from sunlight, at specific wavelengths for each of the four seasons. Calculation of L_λ is based on one specific day for each season. This specific day is defined by the angle of declination of the sun, -20° for Winter, -10° for Autumn, +10° for Spring and +20° for Summer. Specific dates corresponding, to these angles of declination are published annually in the American Ephemeris and Nautical Almanac. Consequently midday sunlight intensity should be measured on, or close to, the date corresponding to the angle of declination on which the calculation of the relevent L_λ values is based. Values of L_λ are given to cover the wavelength region 299 - 800 nm for each of the latitudes 20, 30, 40 and 50°N. In order to convert the value for the rate of degradation at latitude L and season S to a rate at latitude X and season Y, the values for L_λ at latitude L and season S are summed for the wavelength range of interest (usually 300 - 400 nm). Similarly L_λ values are summed for latitude X and season Y and the value R is calculated.

$$\frac{L_\lambda \text{ (300 nm - 400 nm) latitude L and season S}}{L_\lambda \text{ (300 nm - 400 nm) latitude X and season Y}} = R$$

Then 12/A x 0.75 x 1/R h of irradiation in the Suntest will be equivalent to one day of sunlight at latitude X and season Y.

CONCLUSIONS

The system described in this paper provides a relatively simple and quick method of measuring the rate of photodegradation of a pesticide under controlled conditions in the laboratory. The rate of degradation obtained can then be used to estimate the rate of photodegradation which will occur at different seasons of the year and at latitudes from 20 to 50°N.

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Mill, T. et al. Laboratory Protocols for Evaluating the Fate of Organic Chemicals in Air and Water. EPS-600/3-82-022 EPA Contract 68-03-227. (NB Updated figures for L_λ were obtained from the authors in January 1986).

BIOINCUBATION SYSTEM - A NEW COMPACT AND FLEXIBLE SYSTEM FOR BIODEGRADATION STUDIES

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ABSTRACT

A compact biometer-system is presented which can be used to investigate the degradability of a pesticide in soil under a wide range of conditions. A preselected number of samples can be connected in sequence resulting in a very low detection limit for $^{14}CO_2$. The biodegradation of the experimental insecticide N-Methyl-O-2 (2-chloro, 1-methoxy) ethoxy – phenyl carbamate ("BAS 263 I") is investigated using a clay loam soil from various depths. DT_{50} values of the insecticide are determined for various soil depths to be between 1.7 - 6.9 d. Strongly time-dependent bindings of the active ingredient (a.i.) to the clay sub-soil layers are observed which cannot be correlated with K_{oc}. The data obtained are comparable with previous laboratory data and data from lysimeter studies using the same soil indicating the high potential for standardization of the system. Due to its flexibility the system may be adapted to specific needs of a test.

INTRODUCTION

Studies to investigate the biodegradation of pesticides in soil are a prerequisite to the registrability of the compound by many authorities.

However, each authority seems to have adopted different guidelines for the studies. There is therefore a need for a compact, space saving system which will house a large number of individual soil samples, and which can be used within a wide range of different temperatures under both aerobic and anaerobic conditions.

These requirements can be fullfilled by the bioincubation system presented in this paper using the temperature controlled incubation chambers which contain separate incubation-units. In such an incubation-unit a preselected number of up to 14 samples can be connected in sequence resulting in a corresponding improvement in the detection limit for $^{14}CO_2$.

The advantages of the system, as well as its potential for standardization, are demonstrated by an investigation of the biodegradation of the experimental insecticide N-Methyl-O-2 (2-chloro, 1-methoxy) ethoxy phenyl carbamate ("BAS 263 I") in various soil depths under aerobic and anaerobic conditions. The results are compared with the data obtained by the respective lysimeter studies using the same soil and by previous similar laboratory studies.

MATERIALS AND METHODS

Biometer-system

The entire biometer-system is shown in figure 1. For these systems incubation-chambers of various sizes have been choosen (type BKE 50, 60 and 80, Memmert, Schwabach, FRG). Temperatures between 3 °C - 60 °C can either be kept constant over the period of the incubation or be changed according to a preselected temperature program. Up to 36 cylindrical separately removable incubation units (inner diameter: 8.2 - 10 cm, length: 37 - 42 cm) can be set up horizontally in an incubation chamber. Up to 14 individual soil samples (20 - 100 g each) can be set up in sequence in one incubation unit. Using the above mentioned incubation-chambers 224 - 504 samples of 25 g soil, and 80 - 196 samples of 100 g soil can be incubated. In order to preserve either aerobic or anaerobic conditions a constant stream of water

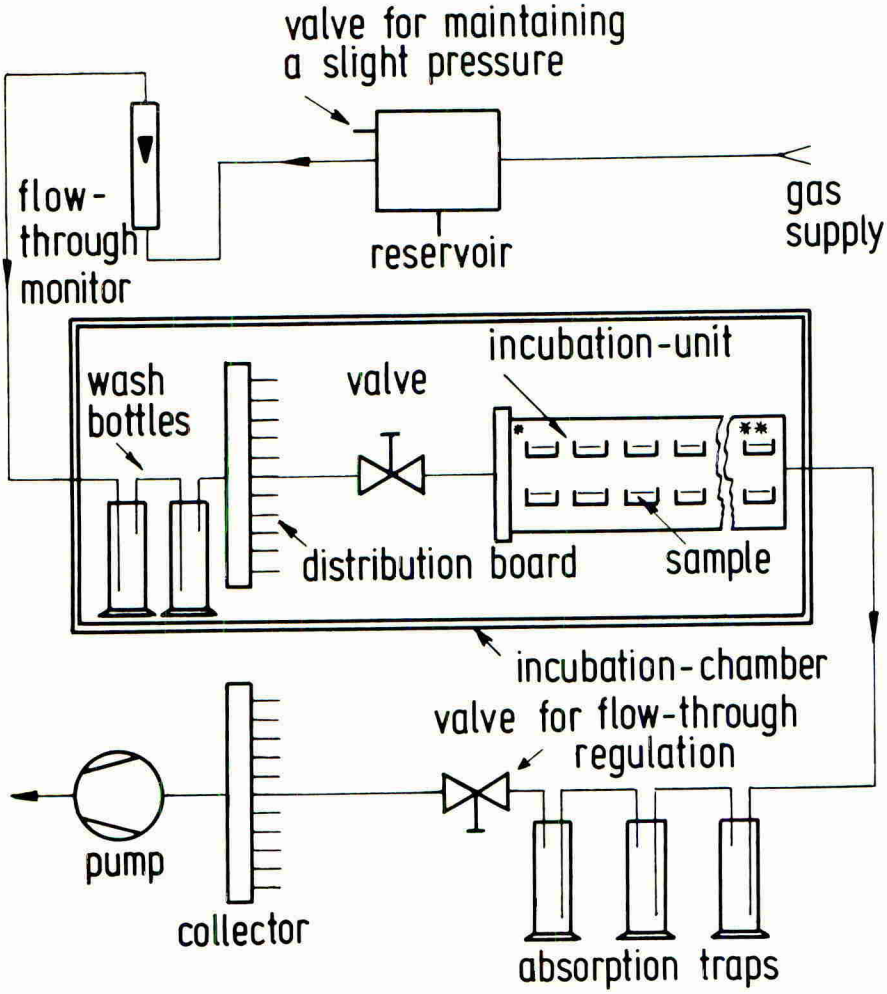

Figure 1: Biometer-system

saturated air or nitrogen is pulled through each incubation unit separately using a reciprotor pump. The outgoing gas is bubbled through three absorption traps in sequence in order to collect volatile metabolites as well as evolved $^{14}CO_2$, for subsequent quantification. The flow rate of the gas (c. 100 ml/h - 10 l/h) is adjusted by a valve and controlled using a flow-through monitor. The gas supply is connected via a reservoir and safety valve under slight pressure.

Soil

The soil used for the biodegradation studies was freshly taken from a field in the "Hildesheimer Börde", FRG. The soil was collected in depths of 0 - 20 cm, 40 - 60 cm, and 60 - 100 cm. The different soil layers were characterized both physicochemically and microbiologically.

Application of the a.i.

The a.i. with a specific radioactivity of 0.281 MBq/ mg was applied to the individual soil samples to give a concentration of 3 mg/ kg soil. The water content of the soil was adjusted to 40 % of its maximum water holding capacity.

Incubation

The fortified soil samples of all layers were each incubated aerobically. Additionally, fortified soil of the 60 - 100 cm layer was incubated anaerobically by flooding with oxygen free water and passing nitrogen over the samples.

Sampling

All samples were incubated in duplicate. At respective sampling times one sample was taken from the front (= *, refer figure 1) a second was taken from the back (= **, refer figure 1) of the incubation unit. The samples were extracted and analyzed separately. Using this method, a possible sorption of volatile metabolites into soil in the back of the unit should be detectable.

Extraction procedure and analyses

The soil samples were extracted successively with a mixture of soil-methylenechloride-water buffered at pH = 7, 2, 10. The organic layers as well as the aqueous layers were combined and radioassayed. The residues remaining in the soil were quantified by combustion and liquid scintillation counting. The organic layers were analyzed for the active ingredient by tlc on silica gel plates using the system hexane/acetone/methylenechloride (45/10/45; V/V/V).

RESULTS AND DISCUSSION

The soil used in this study was a clay loam with a high clay amount in all the various soil layers and a high microbial biomass in the sub soil layers. The characteristics of the various layers of soil are shown in table 1.

TABLE 1

Physicochemical and microbiological characteristics of soil

Parameters	soil layer (cm) 0 - 20	40 - 60	60 - 100
org. C-content (%)	1.75	0.5	0.18
pH-value	7.5	7.7	7.6
CEC (mVal/100 g)	23.0	17.0	9.5
MWC (g H_2/100 g dry soil)	42.0	56.0	55.0
particle size (%)			
0.002 mm	25.0	23.0	17.0
0.002 - 0.02 mm	25.0	26.0	29.0
0.02 - 0.2 mm	47.0	50.0	54.0
0.2 mm	5.0	1.0	1.0
microbial biomass (mg C/100 g dry soil)	42.8	6.5	7.5

The fate of the a.i. on the respective soil layers under aerobic and anaerobic conditions was followed over a period of 30 d analysing for non-extractable residues, mineralization and disappearance time (DT_{50}) (tables 2 - 5). Furthermore, the results obtained from a sample in front of the incubation unit (f) were compared with a sample in the back of the unit (b).

TABLE 2

Amount of non-extractable residues (% initially applied material, i.TRR)

incubation time (d)	sample 0 - 20 cm		40 - 60 cm		60 - 100 cm		60 - 100 cm anaerobic	
	f	b	f	b	f	b	f	b
0	1.9	2.0	1.4	1.3	1.2	1.2	1.4	1.5
2	12.4	13.7	34.1	32.3	32.6	27.2	-	-
4	28.0	17.8	59.7	--	48.6	52.1	52.9	55.0
8	45.2	63.7	90.2	84.7	76.8	63.7	67.7	67.0
16	58.7	58.5	86.9	92.0	87.5	83.9	73.3	67.2
30	69.3	68.4	91.5	93.1	89.7	90.9	81.4	82.1

TABLE 3

CO_2 formation (% i.TRR)

incubation time (d)	sample 0 - 20 cm	40 - 60 cm	60 - 100 cm	60 - 100 cm anaerobic
0	n.d.	n.d.	n.d.	n.d.
2	0.3	n.d.	n.d.	n.d.
4	1.6	0.02	n.d	n.d.
8	5.6	0.2	0.6	0.07
16	15.4	0.4	3.1	0.2
30	21.3	0.9	4.2	0.3

n.d. = not detectable, detection limit = 0.01 % TRR

TABLE 4

Recoveries after different times of incubation (% i.TRR)

incubation time (d)	sample 0 - 20 cm		40 - 60 cm		60 - 100 cm		60 - 100 cm anaerobic	
	f	b	f	b	f	b	f	b
0	100.6	100.7	102.4	102.2	101.7	98.6	101.9	101.8
2	97.2	95.6	91.5	94.5	93.5	89.6	--	--
4	98.3	92.2	92.5	--	90.0	89.2	94.8	97.4
8	92.1	91.3	97.1	92.9	93.0	79.7	91.7	93.9
16	94.7	90.5	89.4	95.2	94.9	90.5	92.5	88.6
30	90.3	91.4	94.3	89.7	92.3	91.4	94.0	92.9

TABLE 5

Disappearance time DT_{50} of the a.i. (d)

	sample 0 - 20 cm		40 - 60 cm		60 - 100 cm		60 - 100 cm anaerobic	
	f	b	f	b	f	b	f	b
DT_{50}	6.9	6.9	2.17	2.24	2.8	3.0	1.9	1.7

Comparison of all values for the samples taken in front and in the back of the incubation-units does not show any significant differences which might originate from resorption of volatile metabolites. In order to avoid any interferences samples should be worked up successively from the back of the unit. Recoveries between 90 - 100 % of the initially applied material indicate that the system is free from leaks. For all soil layers - especially for the sub soil layers (40 - 60 cm, 60 - 100 cm, incubated aerobically) a time dependent formation of soil non extractable residues can be observed. Parallel with formation of non extractable residues the DT_{50} values for the a.i. - wich could be characterized by tlc - decrease with depth of the soil layers. Though the microbial biomass in the sub soil-layers is high no significant mineralization occurs apart from the 0 - 20 cm layer. However, for the anaerobically incubated sub-soil layer (60 - 100 cm) formation of extractable metabolites can be observed as indicated by a relativly higher extractability and a low DT_{50} value for the a.i. This was confirmed by tlc analysis where relatively higher amounts of metabolites were found for the anaerobically incubated samples.

Conclusions

In summary, the advantages of our biometer system are as follows: The compact system can be used to investigate the degradability of pesticides in soil under a wide range of various conditions. Especially, degradation studies under both aerobic and anaerobic conditions as well as combinations of both can be carried out in an incubation unit. The units themselves, all connections, and tubings are made of stainless steel in order to avoid any sorption and loss of radioactive material. The detection limit for evolved $^{14}CO_2$ is 0,01 % TRR. The system gives reproducible results for the disappearance of the parent compound: Previous laboratory studies resulted in DT50 values of DT50 = 20 d (Huber, (18)) and DT50 = 29 d (Keller, 1987) using comparable soils. Interpretation of the behaviour of the compound might be a fast sorption of the a.i. to the organic carbon in the upper soil layer which can be extracted with the solvent. In the sub-soil layers with a decreasing amount of organic carbon some form of slower but strong binding of the a.i. seems to occur possibly to the clay minerals. These results indicate the presence and importance of sorption processes in the sub soil layers which cannot be correlated with K_{oc}. The leaching behaviour of the compound has been investigated using lysimeter studies (Kördel & Herrchen, 1988). When analyzing the different soil layers of the lysimeters 1 year after application down to 100 cm for non-extractable residues as well as the a.i., non extractable residues only could be found down to 40 cm soil depth. No parent compound was detectable in one of the soil layers. These findings confirm the results of the biometer studies, where also formation of non-extractable residues and a fast dissapearence of the parent compound could be observed. Under field conditions these sorption processes might be important when the pesticide and/or metabolites persist for some time in the respective soil layers. These processes also have to be taken into consideration when simulating the environmental leaching behaviour of pesticides into the groundwater by computer models.

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METABOLIC PATHWAYS OF PROPICONAZOLE IN RATS AND MICE

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ABSTRACT

The numerous metabolites isolated from rat urine and faeces and identified by ^{1}H-nmr and/or mass spectroscopy revealed a wide variety of biotransformations being operative in the degradation of propiconazole.
Major sites for enzymatic attack are the propyl side chain and the cleavage of the dioxolane ring.
The 2,4-dichlorophenyl ring is attacked in various ways including the formation of a cyclohexadiene ring system, hydroxylation, replacement of the chlorine substituent by a hydroxy group and introduction of a methylthio group.
Also the 1,2,4-triazole ring is oxidatively attacked leading to hydroxy derivatives.
The vast majority of the alcoholic and phenolic metabolites are renally excreted as sulfuric acid and glucuronic acid conjugates.
Comparative tlc and hplc studies on the metabolite profiles in the excreta of propiconazole treated rats and mice revealed that the major metabolic pathway in mice is via cleavage of the dioxolane ring.

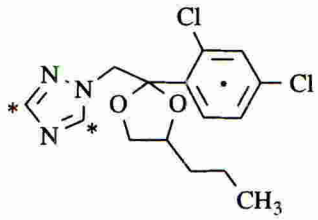
INTRODUCTION

The metabolism of 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolane-2-yl]methyl]-1H-1,2,4-triazole, the active ingredient of the fungicide TILT®, was investigated in male rats and in male and female mice.

Single oral doses of triazole or phenyl [^{14}C]-propiconazole applied to rats and mice were readily absorbed from the intestinal tract into the systemic circulation. Independent of the dose (0.5 - 31.4 mg/kg) 75 - 80% of the triazole and more than 90% of the phenyl label (32.5 mg/kg) were excreted within 24 hours by rats via urine and faeces. Prior to a single oral dose (0.8 - 475 mg/kg) of phenyl labelled propiconazole mice had been pretreated for 21 days with unlabelled material at the respective dose level. Within 24 hours the mice eliminated 64 to 94% of the dose mainly via urine. The excretion pattern was slightly influenced by the sex of the animals and the dose level.

The excreta obtained in these experiments were used for the investigation on the nature of the metabolites.

MATERIAL AND METHODS



propiconazole

• [U-^{14}C]

* ^{14}C

[U-^{14}C]phenyl or [3,5-^{14}C]triazole labelled propiconazole was orally administered to young adult rats (strain: Tif: RAI f (SPF)) at a dose level of 31.4 mg/kg and mice (CD-1) at dose levels of 0.8, 19, and 475 mg/kg, respectively. The labelled test material was dissolved in a mixture of water/ethanol/polyethylene glycol 200 and the unlabelled material (pretreatment of mice) was admixed to the diet. The animals were housed in metabolism cages to allow separate collection of urine and faeces. The urinary metabolites were first partitioned between ether and water, the faecal metabolites were extracted with a mixture of methanol/water. Further separation and purification was carried out by hplc using various stationary and mobile phases. The spectroscopic analyses were carried out using a Varian mass spectrometer and a Brucker nmr spectrometer (360 MHz).

RESULTS AND DISCUSSION

The structures of the metabolites excreted by rats and mice revealed a wide array of biotransformations. From the 22 phase I metabolites identified in urine and faeces the metabolic pathways of propiconazole in rats and mice are proposed (Figure 1).

In both species the majority of the alcoholic and phenolic compounds were renally excreted as sulfuric acid and glucuronic acid conjugates.

The tricyclic metabolites are present in two or more stereoisomeric forms which were separated by chromatographic means. In the discussion and Figure 1 (pathways) stereoisomeric differences of the metabolites are not considered.

Metabolic pathways in the rat

A small portion of the applied propiconazole (I) is excreted unchanged with the faeces. It is assumed that this amount passed the intestinal tract of the animal without being resorbed. The first metabolic reaction occurs at the n-propyl side chain, leading to the α- and β-hydroxy derivatives IV and III. The corresponding γ-hydroxy derivative (II) was not found, presumably because the further oxidation proceeds readily to the carboxy derivative (VII). The secondary alcohols, i.e. Met. IV and III, are further oxidized resulting in the α,β-diol (VI) and the β,γ-diol (V), respectively. All mono- and dihydroxy derivatives were found only in the faeces. Further oxidation of the diols lead to α-hydroxy carboxylic acid (VIII), representing the major metabolite identified. Whether or not VII is a substrate for the formation of VIII remains questionable.

The derivatives with an oxidized n-propyl side chain, i.e. the alcohols and acids are further subjected to side chain shortening reactions yielding acetic acid and formic acid derivatives (IX and X). Corresponding pathways

via α-hydroxy acids and 1,2-diols have been reported in the literature (Delbressine et al. 1980, Vickers et al. 1980, Sporstol et al. 1982).

It is assumed that oxidative attack on the dioxolane ring occurs independently of the actual status of the original n-propyl side chain and consequently all tricyclic metabolites identified lead via dioxolane ring cleavage to XI. During this cleavage the dioxolane ring C-atoms are proposedly released as CO_2.

Once XI, a rather lipophilic compound has been formed, a wide variety of metabolic reactions occurs.

By far the major portion of XI is reduced to the corresponding alcohol (XIII) and only a small amount is directly hydroxylated at the phenyl ring (XII). This hydroxylation of the phenyl ring at C_5' is one of the prominent metabolic pathways (XIV and IXX). To an even higher extent one of the two chlorine atoms is replaced by a hydroxy group (XVII, XVIII, XXII).

A small amount of the triazole moiety is hydroxylated at C_5 (XV, IXX, XX).

The sulfur containing metabolites identified (XVI, XX, XXI, XXII) require special consideration.

The methylthiolation, i.e. the metabolic process leading to the formation of methylthio metabolites, of aromatic systems, is known for several xenobiotics including paracetamol in rats and mice (Hart et al. 1982), bromazepam in rats (Tateishi et al. 1976) and 2,5,2',5'-tetrachlorobiphenyl in mice (Mio et al. 1976).

The most likely metabolic origin of these methylthio metabolites is through the reaction of an arenoxide with a sulfur nucleophile, presumably glutathione. The isolation of XVI, being a dihydro derivative , supports that assumption for propiconazole. Metabolites, analogous to XVI, have been reported in the literature for several compounds, including naphthalene in rats and mice (Stillwell et al. 1978, Stillwell et al. 1982) bromobenzene in rats (Mizutani et al. 1978) and phenanthrene in rats and guinea pig (Lertratanangkon et al. 1982).

Reactions on the arenoxide with glutathione yields primarily a glutathionyl conjugate of the hydroxy cyclohexadiene derivative and that of a phenyl derivative. The rearomatization process is obviously accompanied with a shift of the S-substituent. In the cyclohexadiene derivative (XVI) the methylthio group is located at C_6', whereas in the phenyl derivatives (XX, XXI, XXII) this substituent was found at C_5'. The glutathione derivatives are degraded to the cysteinates, cleaved by a C-S-lyase and ultimately methylated to form the methylthio Metabolites (XVI, XX, XXI, XXII).

Metabolic pathways in the mice

The urinary metabolite pattern of mice - independent of sex, dose level and pretreatment - is dominated by one metabolite, i.e. the glucuronic acid conjugate of XIII. This implies, that the major metabolic pathways in mice proceeds via elimination of the dioxolane ring leading, via ketone formation (XI) to the corresponding alcohol (XIII), which is ultimately conjugated with glucuronic acid to yield the major metabolite.

However, there is a striking sex difference as this metabolite represents 30% of the dose in males, but only 15% of the dose in females.

Additionally, another metabolite characterized as the α-hydroxy-carboxy acid (VIII), where the dioxolane ring is still intact, represents only a minor metabolite in males (3%), but a major one in females (16% of dose).

It appears, that the extent of dioxolane ring cleavage is significantly higher in males compared to females, as about 70% and 40% of the urinary radioactivity can be attributed to this pathway, respectively. The corresponding figure for the male rat is about 30%.

Based on the elucidated structure of the dominating metabolite present in the urine (XIII) and the characterization of further metabolites in urine and faeces, it can be concluded that the extent of the dioxolane ring cleavage was the most striking difference between male mice and rats regarding the disposition of propiconazole.

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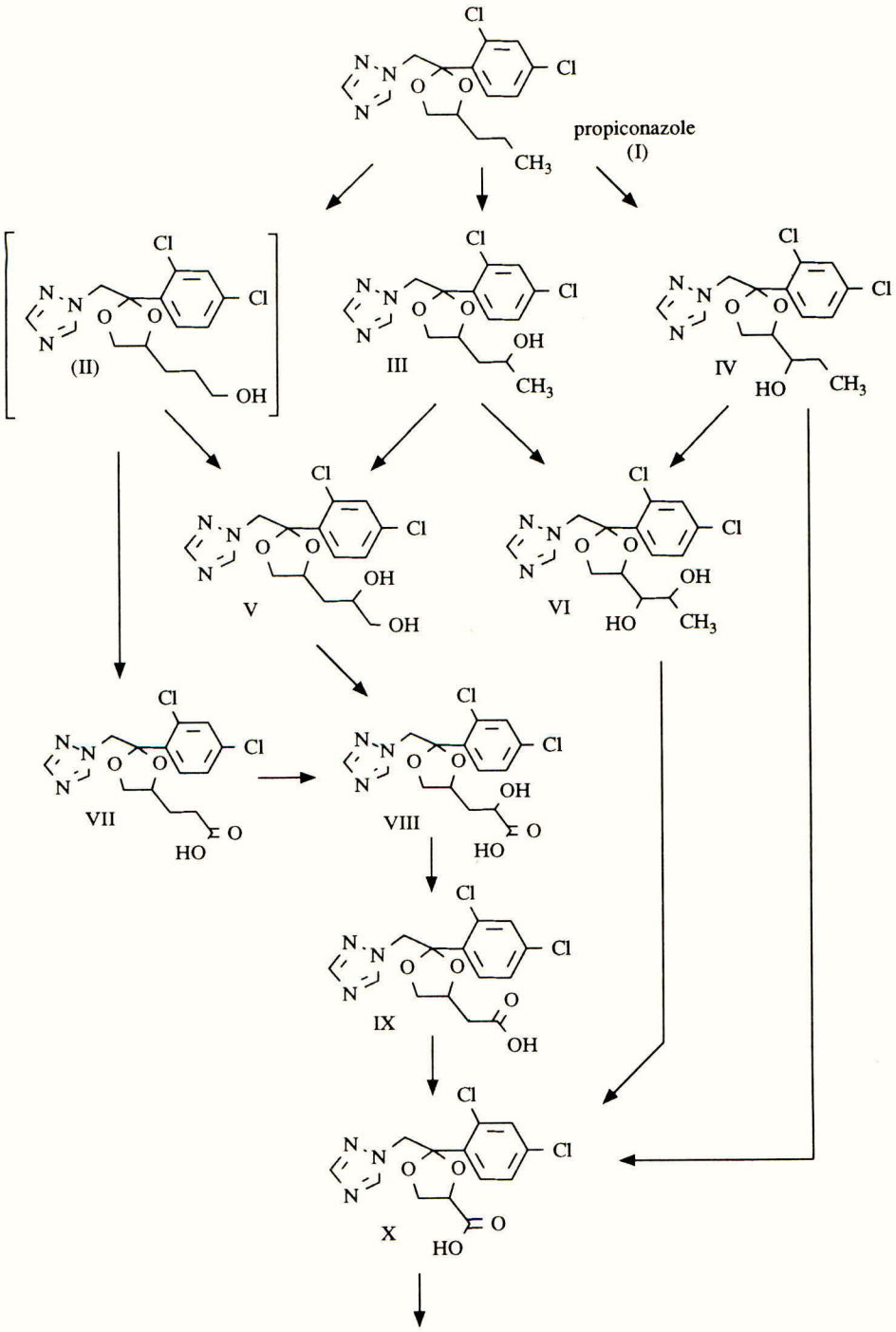
FIGURE 1: Proposed Metabolic Pathways of Propiconazole in Rats and Mice



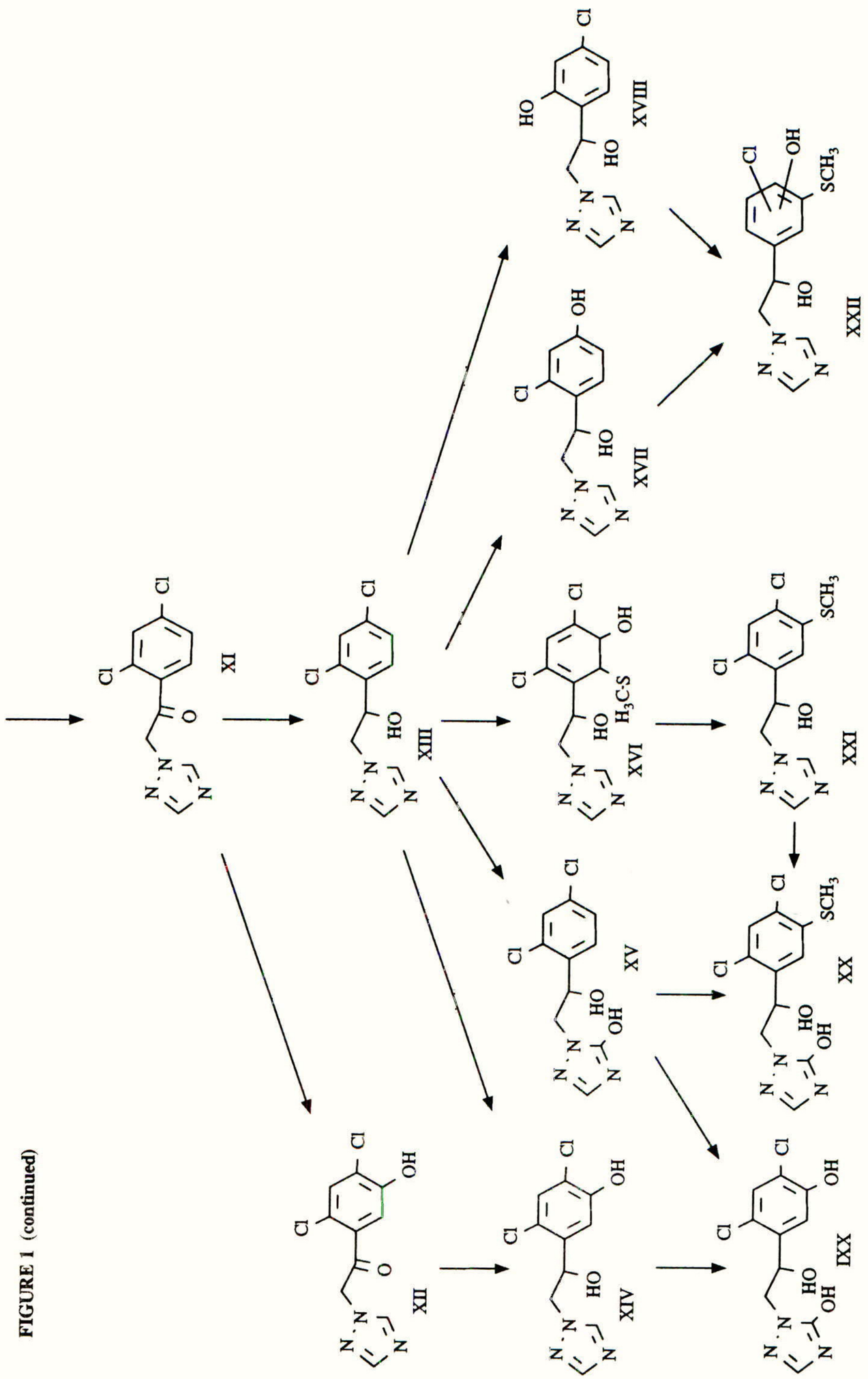


FIGURE 1 (continued)

IDENTIFICATION OF METABOLITES OF TERBACIL IN RAT URINE

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ABSTRACT

As part of a study on the metabolism of ^{14}C-terbacil in rats after administration of a 6.5 mg/kg oral dose, the major radioactive components in urine and faeces were separated and quantified. The structures of these isolated metabolites were investigated by fast atom bombardment (FAB) ms and in some cases proton nmr. For the major sulphate conjugate metabolites, confirmation of structural assignments was obtained by synthesis of reference compounds.

The primary biotransformation products of terbacil in 0-48 hour urine were derived from hydroxylation of the 6-methyl group. This metabolite was present as the aglycone (A, 2% dose), glucuronide (B, 33% dose) and sulphate (C, 9% dose). A further metabolite was identified in which an N-acetylcysteine substituent was attached to the 6-methyl group (D, 3% dose). Minor metabolites (each 1-3% dose) were isolated which exhibited mass spectra indicative of methylsulphinyl (F and G) and methylsulphonyl (H) substituents derived by degradation of N-acetylcysteine metabolites.

An alternative biotransformation pathway involved replacement of the 5-chloro substituent in terbacil with a hydroxy group which was conjugated with sulphate (E, 17% dose).

INTRODUCTION

Terbacil, 3-tert-butyl-5-chloro-6-methyluracil is a selective herbicide for control of many annual and some perennial weeds in crops. In order to provide information to assist in safety evaluation, the identity of the major biotransformation products in the rat were investigated.

MATERIALS AND METHODS

Authentic terbacil, [2-^{14}C] terbacil (97% radiochemical purity), 6-hydroxymethyl-terbacil, 5-hydroxy-deschloro-terbacil were supplied by Du Pont de Nemours & Co., Wilmington, Delaware, U.S.A.

Groups of adult CD rats (5 male and 5 female) of c. 200 g bodyweight were given single oral doses of ^{14}C-terbacil at nominal dose levels of either 6.5 (low) or 500 (high) mg/kg. Urine and faeces were collected at suitable intervals for 5 days after dosing.

Aliquots of representative samples of urine were applied directly to tlc plates for chromatography. Samples of urine or metabolites were also incubated for 18 hours at 37°C with either β-glucuronidase/sulphatase (Helix Pomatia, Type H1), or sulphatase (Abalone Entrails, Type VIII) obtained from Sigma Chemical Co.,U.K. Metabolites were isolated from 0-48 hour urine (after the 500 mg/kg doses) by the following sequence; freeze-drying and extraction with methanol, isolation by preparative tlc and purification by preparative

hplc. Tlc was on silica gel plates using the solvent systems 1) ethyl acetate, 2) chloroform : ethyl acetate : propan-2-ol : glacial acetic acid (30 : 30 : 20 : 10 by vol) and 3) ethyl acetate : propan-2-ol : water (65 : 25 : 15 by vol). Hplc was carried out using Waters 6000A pumps, UK6 universal injector and 660 Solvent Programmer (Millipore, U.K.) with a Spherisorb S10 ODS 25 cm (5 mm i.d.) column (Hichrome U.K.). Hplc was performed using gradient elution from 5-30% methanol in water or in some cases 0.01 M ammonium formate pH4 buffer to 100% methanol in 25 minutes at 2 ml/min.

Mass spectra were obtained using a VG 7070E double-focusing instrument linked to a VG 11-250 data system (VG Analytical Ltd., Manchester, U.K.). For FAB spectra samples were applied to the probe with thioglycerol in some cases enhanced with 1 M sodium or potassium chloride. Some samples were trimethylsilylated by treatment with BSTFA/TMCS (Pierce & Warriner, Chester, U.K.). Proton nmr of metabolites in methanol-d were obtained using Bruker WP80, 80MH instrument with Fourier Transform (Polytechnic of North London, School of Chemistry, London, U.K.).

The supplied 6-hydroxymethyl- and 5-hydroxy-deschloroterbacil compounds were sulphated by stirring at room temperature with pyridine-sulphur trioxide in pyridine for 24 hours and heating to reflux with trimethylamine-sulphur trioxide in DMF for 1 hour respectively. The reaction mixtures were extracted with diethyl ether after adding 1 M sodium hydroxide solution. The sulphated products were isolated from the aqueous solution as the sodium salts by freeze-drying and by preparative tlc using solvent system 3.

RESULTS

After single 6.5 mg/kg oral doses of ^{14}C-terbacil, means of 81% and 20% dose were excreted in the 0-120 hour urine and faeces respectively mostly during the first 48 hours. After single 500 mg/kg oral doses, means of 77% and 16% dose were excreted in 0-120 hour urine and faeces respectively. Radioactivity in urine was separated into several components by tlc using solvent system 2. Additional components were separated by either tlc system 1 or 3 and/or by hplc. There was no qualitative difference in the radioactive components in urine after the 6.5 mg/kg (Table 1) and 500 mg/kg doses.

Metabolite A was shown to co-chromatograph with the 6-hydroxymethyl-terbacil reference compound by both tlc and hplc. The EI and CI mass spectra of metabolite A both before and after trimethylsilylation were virtually identical to those from this reference compound. These showed peaks corresponding to the molecular ion (M;EI) and (M+H;CI) and the major fragment (M-But+H) where But is $CH_2=C(CH_3)2$. The trimethylsilylated compounds gave corresponding ions consistent with a mono-TMS derivative. In addition to the assigned peaks in the FAB mass spectra of metabolite A (Table 2) further peaks at m/z 177 (base peak) and 301 corresponded to [M-But+H] and [M+3Na] respectively.

TABLE 1

Proportions (% dose) of radioactive components in urine after single 6.5 mg/kg doses of ^{14}C-terbacil. Components separated by the tlc with solvent system 2.

Component	0-24 hour		24-48 hour	
	Male	Female	Male	Female
B	23	32	3	7
C/D	11	7	3	3
E	15	17	2	0.9
F/G	4	1	0.2	0.1
A/H	3	2	1	1
Unknowns*	6	8	1	1

Components were separated in additional chromatographic systems; the major components C and D accounted for overall means of 9 and 3% dose respectively in 0-48 hour urine. *Mixture of components with each < 5% dose.

Metabolite B was hydrolysed by β-glucuronidase/sulphatase to a component with the same RF values as 6-hydroxymethyl-terbacil. After trimethylsilylation the CI mass spectrum showed chlorine-containing peaks at m/z 697, 641, 625 corresponding to [M+H], [M-But+H] and [M-But-CH_3] respectively for a tetra-(TMS) derivative of the glucuronide of metabolite A. In addition, peaks at m/z 465 and 375 were also present typical of tetra- and tri-(TMS) glucuronide fragments respectively. In addition to the major peaks in the FAB spectra (Figure 1, Table 2) a base peak at m/z 215 was present corresponding to M-glucuronic acid. The KCl enhanced FAB mass spectrum showed characteristic shifts in peaks due to replacement of Na with K (Figure 1).

Metabolite C was of similar polarity to metabolite B but was unaffected by treatment with either β-glucuronidase or sulphatase. In addition to the assigned peaks in the FAB spectrum (Table 2), there were peaks at m/z 215 (base) and 691 corresponding to [M-HSO_4] and [2M-2H+3Na] respectively. The proton nmr spectrum showed no singlet in the region for the 6-methyl group (1.9-2.2 ppm) confirming that this was the site of biotransformation. These results indicated that metabolite C was the sulphate of 6-hydroxymethyl-terbacil which was resistant to hydrolysis by sulphatase. Confirmation of the assignment was obtained by comparison with the synthetic sulphate prepared from 6-hydroxymethyl-terbacil.

Metabolite D was similar in polarity to metabolite C. Assigned peaks in the FAB mass spectrum are given in Table 2. The proton nmr spectrum showed no singlet for a 6-methyl group but a singlet at 1.98 ppm (3H) assigned to an acetyl group. The ms and nmr data were consistent with a compound containing an N-acetylcysteine substituent on the 6-methyl group of terbacil.

Metabolite E was hydrolysed after incubation with sulphatase to a component with a similar Rf value to 5-hydroxy-deschloroterbacil. In addition to the assigned peaks in the FAB spectrum a peak at m/z 215 was present which corresponded to [M-HSO_4]. The proton nmr spectrum was essentially

identical to that of terbacil. These results indicated that metabolite E was the sulphate of 5-hydroxy-deschloroterbacil and this was confirmed by comparison with the synthetic sulphate.

Metabolite F gave a CI mass spectrum with chlorine-containing peaks at m/z 279 and 223 (base peak) corresponding to [M+H] and [M-But+H]. Peaks in the FAB spectrum supported these assignments (Table 2) and a structure was assigned with a methylsulphinyl substituent on the 6-methyl group. With metabolite G after trimethylsilylation, peaks in the CI spectra (not containing chlorine) at m/z 405 and 349 corresponded to [M+1] and [M-But+H] for a di-TMS derivative of a compound with m.wt 260. Assigned peaks in the FAB spectrum of metabolite G (not derivatised) (Table 2) were consistent with a structure containing a methylsulphinyl substituent on the 6-methyl group and a hydroxy group in place of the 5-chloro group. Metabolite H had chlorine-containing peaks in the CI spectra at m/z 295 and 239 corresponding to [M+1] and [M-But+H]. The FAB mass spectrum supported these assignments (Table 2) and the proposed structure with a methylsulphonyl substituent on the 6-methyl group of terbacil.

TABLE 2

Major fragments in positive ion FAB mass spectra of isolated metabolites (But; iso-butylene). *Spectra contained characteristic chlorine-containing peaks.

Metabolite (proposed m.wt.)	Major fragments (m/z)					
	M-But+Na	M-But-H+2Na	M-But-2H+3Na	M+Na	M-H+2Na	M-2H+3Na
A* (232)	199	-	-	255	277	-
B* (408)	-	397	419	-	453	475
C* (312)	279	301	323	-	357	379
D (359)	326	348	370	-	404	426
E (278)	245	267	289	-	323	345
F* (278)	245	267	-	301	323	-
G (260)	227	-	-	283	305	327
H* (294)	261	283	-	317	339	-

Figure 1. FAB mass spectra of metabolite B : (6-hydroxymethylterbacil glucuronide) Glucuronyl (Gl); $C_6H_9O_7$

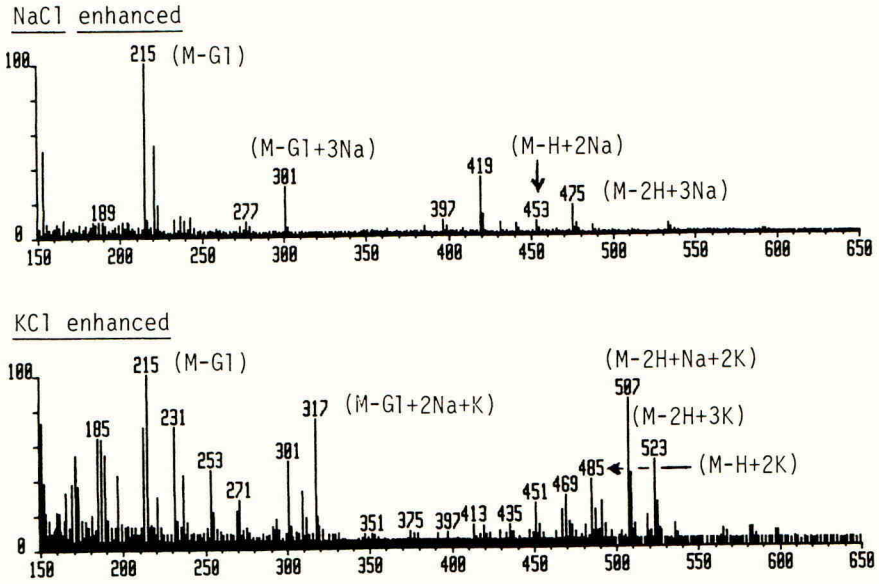
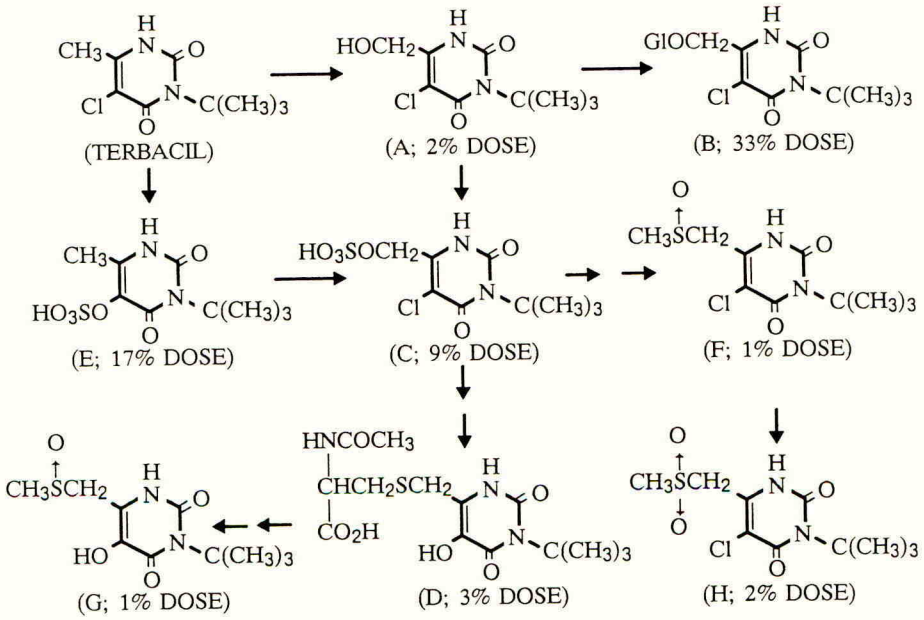


Figure 2. Proposed biotransformation pathway for terbacil; Glucuronyl (Gl)



DISCUSSION

The primary biotransformation pathways for terbacil are hydroxylation of the 6-methyl group and replacement of the 5-chloro group with a hydroxy group (Figure 2). This latter biotransformation has been shown to occur in acluracil (Kaul et al., 1982). The sulphate metabolite C is a suitable intermediate for reaction with glutathione (Hawkins, 1981) which in turn would be a precursor of the N-acetylcysteine metabolite D. The alternative structure for D with a 6-hydroxymethyl group and the chlorine substituted by the N-acetylcysteine is less likely in view of the structures of metabolites F and H. The proposed structures for the minor metabolites F, G and H are likely products of an established pathway for the catabolism of N-acetylcysteine metabolites (Bakke and Gustafsson, 1986). Degradation to intermediate thiols may occur in the intestine after elimination of the precursor metabolites in the bile. Subsequent S-methylation and oxidation would produce sulphoxides and sulphones (Hanzlik, 1983). The biotransformation of terbacil in dogs has been reported to be mostly via hydroxylation of the 6-methyl group (Rhodes et al., 1969). An appreciable proportion of this metabolite was excreted as glucuronide and/or sulphate conjugates. A similar biotransformation of bromacil occurs in rats (Gardiner et al., 1969).

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LYSIMETER EXPERIMENTS AND SIMULATION MODELS TO EVALUATE THE POTENTIAL OF PESTICIDES TO LEACHE INTO GROUNDWATER

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ABSTRACT

It is of essential importance in the assesment of a pesticide's fate to investigate its mobility including its potential to reach groundwater.

The handling of lysimeters, which represent experimental field simulation models, practicability of leachate sampling and detection limits are highlighted using the experimental insecticide N-Methyl-O-2 (2-chloro, 1-methoxy) ethoxy phenyl carbamate ("BAS 263 I") as a model compound. In this study two lysimeters were treated with the insecticide and one of the lysimeters was planted with rape. During the observation period of one year concentration levels of the insecticide itself or its metabolites did not exceed 0.1 µg/l of the leachate. After one year the remaining radioactivity was detected as bound residues in the 0 - 40 cm soil layer (detection limit 1.8 µg/kg soil).

Total residues in the cultivated crop are below $1.3 \cdot 10^{-4}$ µg/kg. Detailed data on soil characteristics as well as depth-dependent DT-50 values of the compound in various soil layers are used in computer-simulating systems to assess leaching behaviour. By critical comparison of the results obtained the respective models can be validated. Additionally, modified environmental situations as compared to those investigated experimentally.

INTRODUCTION

Besides metabolism and stability the leaching behaviour of a pesticide is of essential importance for its potential to reach groundwater (Herzel, 1987).

According to the EEC-Directive permissible levels for one pesticide in drinking water is 0.1 µg/l and 0.5 µg/l for total. The lysimeter technique, which is a compromise between laboratory testing and field studies, provides a means to determine the influence of external parameters (physical, chemical and microbial soil properties, climate, quantities of leachate/time, vegetation) on the fate of chemical in the total system under environmentally relevant conditions. The data obtained from these studies should enable a prediction of the chemical's behaviour under field conditions to be developed.

A radioactively labelled compound allows bound residues to be-quantified thus establishing a mass-balance of the compound under investigation. Detection limits of the EEC-Directive, below 0.1 µg/l, are also easily achieved.

Results of the lysimeter studies have to be interpretated as follows: In cases where less than 0.1 µg/l of a pesticide or its biologically active metabolites leach through a lysimeter of 1.5 m depth, i.e. below the root zone, it can be assumed that these chemicals do not represent an undue hazard to groundwater, for the soil and climate investigated. If the concentration in the leachate below the root zone exceeds this limit, further processes influencing disappearance in deeper sub soil, comparison of groundwater flow with groundwater formation have to be considered. Following the concept of sequential approach the first step may be to use mathematical simulation models.

A modified SESOIL model (Bonazountas and Wagner, 1984) is used to estimate the concentrations of the pesticide in the soil. This model and code documentation are available from the Office of Toxic Substances (OTS) of the US Environmental Protection Agency.

Processes simulated in SESOIL can be categorized in three cycles (the hydrologic cycle, sediment cycle, and pollutant cycle): The hydrologic cycle the theoretical basis of which was developed and validated by Eagleson (Eagleson, 1978), includes rainfall, surface and groundwater runoff, infiltration, soil moisture, and evapotranspiration. The sediment cycle includes sediment resuspension due to wind and sediment washload as a result of rain storms. Within the pollutant cycle volatilization, adsorption, decay, and biological transformation are simulated.

Data requirements of SESOIL are not extensive: A minimum of soil and chemical data as well as monthly meteorological values are needed for a simulation.

In our simulations the disappearence of pollutant in the experiment is defined as degradation in soil. Effects caused by erosion (sediment cycle) are neglected.

This mathematical simulation model has to be validated with input data generated in field experiments, lysimeter studies, and laboratory investigations, and comparison of the respective results. After validation and/or modification of the models a modified, environmental situation (e.g. worst case) may be simulated and predicted using the respective realistic basic data.

MATERIALS AND METHODS

Soil monoliths

In September 1986 two undisturbed soil monoliths (1 m in depth and diameter) were taken from a field in the 'Hildesheimer Börde', FRG, which had been planted with wheat in the same year (by means of a method developed by the Fraunhofer Institute). Tanks to collect the leachate were connected with the outer container at a height of 10 cm from the bottom in order to simulate a groundwater level at 90 cm depth.

Application of the active ingredient (a.i.) and sowing

Two lysimeters were used in parallel, one planted with winter-rape seed and an unplanted control. 400 mg rape seed (species 'Lindora') were sown in drills at 15 cm distance. 4 g formulation, each containing 40 mg a.i., were topically applied to both lysimeters leaving 5 cm from the edges of the lysimeter. (Date of application: September 23, 1986.)

Climate-data recording

The following climate data were recorded continuously: Air temperature, air humidity, soil temperature in 10 and 30 cm depth, amount of precipitation.

Leachate clean-up and analyses

a) Quantification of dissolved $^{14}CO_2$: 250 ml of the respective leachate were acidified (pH = 1), CO_2-free air was passed over the solution for 24 hours. The outgoing gas was bubbled through two absorption traps placed in sequence and containing Carbosorb/Permafluor (1:1, V/V, United Technologies Packard, Frankfurt, FRG). After 24 h the trapping solutions were radiocounted.

b) Concentration of $^{14}CO_2$-free leachate: Prior to quantitation of radioactive material by LSC, 1 - 2 l $^{14}CO_2$-free leachate were concentrated (1:100 - 1:200) by freezedrying.

Harvest and analyses of plant material

At harvest (August 13, 1987), the total plant material, i.e. pods, grain (872 g air dried material in total) and further plant material (432 g air dried material in total), was collected, dried, homogenized, combusted, and assayed for total radioactive residues.

Clean-up of soil monoliths

At termination of the study the total soil was divided into 10 cm segments. Aliquots of the respective segments were air dried, combusted and radioassayed for total radioactive residues sorbed to the soil. Aliquots of the upper layers (0 - 40 cm) were acidified and analyzed for evolving $^{14}CO_2$. 20 ml acetone/chloroform (1:1, V/V) were added to 30 g of the system soil/1 M H_2SO_4. The mixture was shaken for 30 min, then the layers were separated by centrifugation and removal of the organic layer. The organic layer was concentrated and analyzed by tlc using the system hexane/acetone (60:40, V/V).

Supplementary investigations on depth-depending disappearance of the a.i.

The disappearance of the a.i. was investigated additionally under controlled laboratory conditions for the 0 - 20 cm, 40 - 60 cm and 60 - 100 cm soil layers (Herrchen, M. et al., 1988).

RESULTS AND DISCUSSION

Analysis of the leachate

During the whole investigation period (October 10, 1986-September 1, 1987) 867 l of the leachate were collected for the lysimeter planted with rape, 920 l for the one without vegetation. The difference in volume effects the different watering regimes used for the lysimeters.

The following analysis scheme for the leachate samples is proposed:

- Quantification of dissolved $^{14}CO_2$
- Concentration of a $^{14}CO_2$-free sample and quantification of radioactive material by LSC, if necessary after preceding clean-up
- Quantification of the active ingredient and, if necessary, of the main metabolites.

Testing of the samples for dissolved $^{14}CO_2$ did not provide significant results. Therefore it is assumed that the leachate does not contain significant amounts of dissolved carbonate.

After the quantification of dissolved $^{14}CO_2$ the tested sample normally is concentrated by a factor of 100. The detection limit requested by the EEC-Directive is 0.1 µg/l of a.i. If we assume a specific radioactivity of the a.i. of 1 x 10^9 Bq/g and count 1 ml of solution a count of 10 Bq/ml will be needed to achive this limit of detection. The limit could be decreased by a factor of 10 to 20 if a control lysimeter is used. For this lysimeter comparable leachates are obtained without any ^{14}C-materials added. So background levels and interferences caused by the treatment of the samples and by soil matrix are recorded separately and exact values can be calculated by difference.

The values obtained for the investigated active ingredient itself or for potential metabolites upon analyses of different leachate samples were below the critical limit of 0.1 µg/l leachate.

Analysis of the harvested crops

The rape plants harvested in August 1987 were analyzed for the uptake of radioactive labelled material. Total residues in the cultivated crop especially in the edible parts of the plants were below 1.3 x 10^{-4} µg/kg.

Analysis of the soil monoliths to estimate migration and metabolism of the a.i.

The majority of radioactive residue was recovered in the soil layers above 40 cm depth and could be characterized as bound residues. The detection limit of this part of the experiment was 1.8 µg/kg soil.

In the soil segments of the lysimeter which had been planted with rape, 97.1 % of the initially applied rarioactive was recovered between 0 and 40 cm depth; for the lysimeter without vegetation 51.4 % of the initially applied radioactivity was recovered above 30 cm depth. These considerable differences may be explained by different degradation rates caused by different temperatures of the soil surface: Due to higher temperatures of the upper soil layer of the unplanted lysimeter the active ingredient mineralized to a considerable extent; the $^{14}CO_2$ produced was released to the environment.

Summarizing the results of leachate and soil profile analyses it can be concluded that the active ingredient and its metabolites do not leach. About 1 year after application the persisting radioactive material is distributed in the soil layers between 0 - 40 cm depth and were characterized as bound residues.

Use of mathematical simulation models

Basic data and information obtained by the lysimeter studies were compared with the results of the simulation model SESOIL. The following data from the lysimeter studies were used: detailed soil characteristics, amount if applied a.i./area, and data based on monthly meteorological values of the "Aberg, Sauerland, FRG", where the studies were carried out. Additionally calculated physicochemical parameters of BAS 263 I as well as laboratory test data on the disappearance of the pesticide in the various soil layers were used.

The three-dimensional figure 1 shows that BAS 263 I is not detected in the groundwater within 10 years even if 50 kg/ha are applied in October. The results fit with the results obtained from the 1 year lysimeter study where concentrations exceeding 0.1 µg/l of the insecticide were not found in the leachate.

This agreement was to be expected since low disappearance-time values (DT 50) were measured between 6.9 and 1.8 days for the soil layers.

The simulation model only considers the disappearance of the initial compound due to degradation but excludes specifics of its fate and metabolism, as well as the fate of the metabolites formed. Persistence in soil due to formation of bound residues is not considered. However, additional lysimeter studies as well as laboratory investigations might provide the required additional information e.g. on various processes and the pesticide's fate in sub soil layers. This information is of interest for soil protection and for the estimation of leaching of the pesticide and/or its biologically active metabolites.

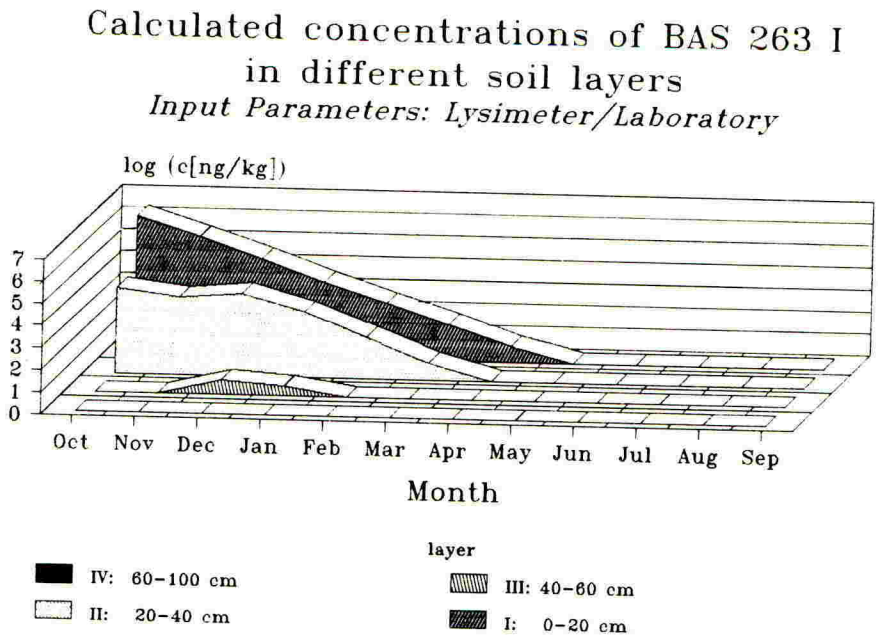

Figure 1

Additionally, a modified environmental situation was simulated using a different, experimentally found disappearance time (20 d for the covering soil layer), temperate meteorological data (climate date of Hamburg, FRG) a typical German soil (Parabrownearth, Triftern, FRG), and an amount of 1.5 kg/ha of applied a.i. For this environmental situation 0.01 µg/l of BAS 263 I would be found in the groundwater (figure 2). These data show a good agreement between experimental results and the output of the Sesoil simulation for the non-leaching test chemical.

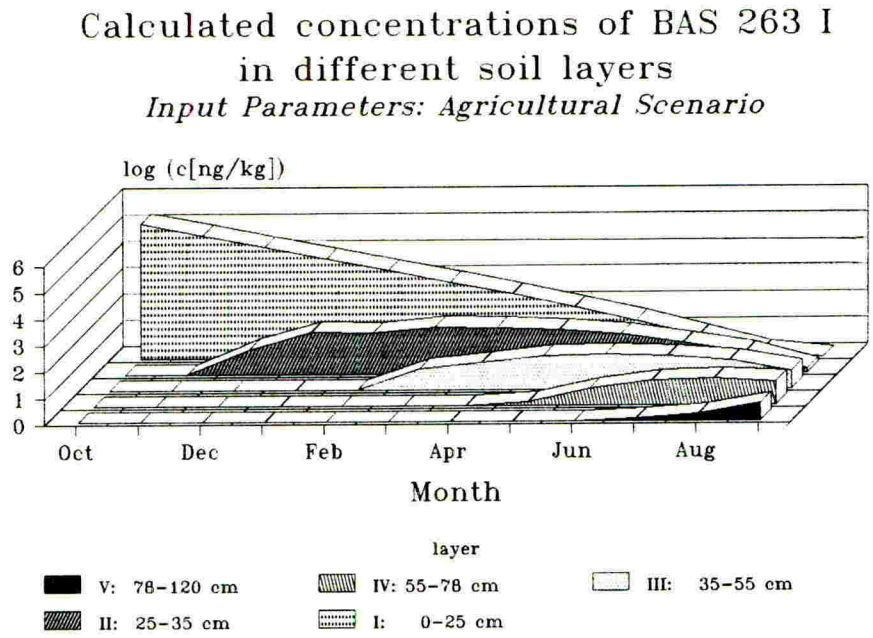

Figure 2

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IDENTIFICATION OF METABOLITES OF VINCLOZOLIN IN HEN LIVER

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ABSTRACT

As part of a study of the metabolism of ^{14}C-vinclozolin in laying hens, the nature of major metabolites in edible tissues was investigated. *In vitro* incubation of ^{14}C-vinclozolin in hen liver 9000g supernatant was used to generate sufficient quantities of tissue metabolites for gc-ms analysis. Confirmation of structural assignments was obtained by chromatographic comparison with authentic reference compounds.

The primary biotransformation products of vinclozolin in hen tissues were derived from the parent compound by epoxidation of the vinyl group, followed by hydration of the intermediate epoxide (which was not detected), and by hydrolytic cleavage of the heterocyclic ring. The major metabolite in tissues resulted from a combination of all these processes. An intermediate, dihydroxylated metabolite, in which the heterocyclic ring remained intact, was present in only low concentrations in tissues from the *in vivo* study, but was formed in substantial amounts in the *in vitro* incubation.

INTRODUCTION

Since plant material containing residues of crop protection agents may be fed to food-producing animals, it is necessary as part of the safety evaluation of a compound, to investigate the nature of residues which may ultimately occur in edible tissues. Major unknown metabolites of a compound in tissues may need to be isolated, purified and analysed by spectroscopic techniques and there can be practical difficulties in obtaining sufficient material for analysis from an *in vivo* feeding study. This problem was encountered during a study of the metabolism of the fungicide vinclozolin in hens. The work described in this paper was conducted with the objective of using *in vitro* methods to generate identifiable quantities of unknown vinclozolin metabolites which had already been detected in the livers of birds from an *in vivo* feeding study.

MATERIALS

Non-radiolabelled vinclozolin, [U-^{14}C-phenyl]vinclozolin (Figure 1), and potential metabolite reference compounds were supplied by BASF AG, 6703 Limbergerhof, W. Germany. The radiochemical purity of ^{14}C-vinclozolin was greater than 98%.

METHODS

In vivo feeding study

Ten laying hens (brown hybrid strain) were dosed once daily for five days with ^{14}C-vinclozolin at a nominal rate of 20 mg/day. Doses were administered orally in gelatin capsules. The birds were sacrificed four hours after the final dose. Samples of liver, muscle and fat from these birds, were homogenised and extracted sequentially with methanol and methanol: water (1:1, vol/vol). Following clean-up by sorbent extraction, the pooled extracts from each tissue were analysed by tlc.

In vitro study

Two hens of the same strain as used in the in vivo study were sacrificed by cervical dislocation and the livers removed immediately. The following procedures were performed at a temperature of 4°C. Portions of fresh liver (3g) were homogenised with aliquots (10ml) of pH 7.4 sodium/potassium phosphate buffer solution (100mM) containing 1.15% w/v potassium chloride. The homogenates were centrifuged at 9000g for 20 minutes and the supernatants decanted and pooled into a single sample. Aliquots (5ml) of 9000g supernatant were dispensed into glass conical flasks. To each flask was added a solution of ^{14}C-vinclozolin (0.5 mg) in methanol (0.1ml). One flask was taken for analysis immediately. The other flasks were shaken at 37°C for periods from 0.5 to 3 hours after addition of ^{14}C-vinclozolin. For analysis, aliquots of supernatant (0.5ml) were processed by sorbent extraction.

The methanolic eluates from the sorbent, which contained more than 93% of the original supernatant radioactivity, were analysed by tlc. Solutions obtained from in vivo and in vitro studies were co-chromatographed in order to compare the metabolite profiles in the two systems. Solutions were also co-chromatographed with reference compounds.

Chromatography

Tlc was on silica gel pre-layered plates, using the solvent system chloroform: methanol: acetic acid (90: 10: 1, v/v/v). Radioscans were obtained using a Berthold LB2842 linear analyser (Laboratory Impex Ltd., Twickenham U.K.). Hplc was carried out using Waters 6000A pumps and U6K universal injector (Millipore Ltd., Harrow, U.K.) linked to a Berthold LB 504 radioactivity monitor fitted with a solid scintillant flow-through cell. A spherisorb 5µ ODS 1 column, 25cm long by 8mm internal diameter was used at ambient temperature. The mobile phase was acetonitrile: water (1: 1, v/v) at a flow rate of 2 ml/minute.

Isolation of metabolites

Metabolites were isolated from the incubated 9000g supernatant by the following sequence: sorbent extraction, isolation by preparative tlc and purification by hplc. Isolated metabolites were re-analysed by tlc and also subjected to gc-ms.

Mass spectrometry

Mass spectra were obtained using a VG 7070E double-focusing instrument linked to a VG 11-250 data system (VG Analytical Ltd., Manchester, U.K.). Isolated metabolites were trimethylsilylated by treatment with BSTFA/TMCS (Pierce and Warriner Ltd., Chester, U.K.) prior to introduction into the mass spectrometer *via* an HP 5712A gas chromatograph (Hewlett Packard Ltd., Wokingham, U.K.). Gc separations were accomplished on a 12.5m long by 0.2mm internal diameter fused silica column coated with dimethylsilicone bonded phase at a film thickness of 0.33 μm, using helium as carrier gas and a temperature programme from 65°C to 300°C. Mass spectra were recorded repetitively under alternative chemical/electron impact ionisation conditions. Isobutane was used as the reagent gas for chemical ionisation.

RESULTS

All tissues from the *in vivo* feeding study contained qualitatively similar metabolite profiles (Figure 2). Fat was the only tissue which contained significant quantities of unchanged vinclozolin but muscle, fat and liver all contained two major metabolites (I and II). Liver contained a higher proportion of polar material than the other tissues, which was not further investigated.

The identity of metabolite I (Figure 1) was intially established by co-chromatography with a reference standard. This metabolite is the product of extensive metabolism of vinclozolin involving dihydroxylation of the vinyl group, hydrolytic opening of the heterocyclic ring and decarboxylation. Metabolite I has previously been reported as the major metabolite of vinclozolin in the rat (Huber et al, 1986). It was also possible to establish by tlc that pairs of diastereomers of metabolite I were formed. Metabolite II did not correspond to any reference standard available at the time. It was present in tissues at relatively lower concentrations and generation of sufficient quantities for isolation would have required dosing additional birds. Metabolite II was also not present in hen excreta in significant amounts (results not shown).

Following incubation of vinclozolin with hen liver 9000g supernatant tlc showed that the supernatant extract contained a qualitatively similar profile of mobile metabolites to an *in vivo* liver extract (Figure 2). The presence of metabolites I and II in the *in vitro* preparations was confirmed by co-chromatography. These metabolites were isolated from the *in vitro* preparation and analysed by gc-ms. The structure of metabolite I and the presence of pairs of diastereomers were confirmed (results not shown). Some degradation of metabolite II occurred during hplc purification (shown by Tlc) and also probably during derivatisation, and the reconstructed ion current profile obtained during gc-ms analysis presented a complicated picture (Figure 3). The peak at scan 710 could be assigned to a tris-TMS derivative of metabolite I while the spectra from the region of scan 680 could be interpreted in terms of a bis-TMS derivative of the epoxide resulting from dehydration of the terminal carbon atoms of metabolite I. This compound was available as a reference standard and did not co-chromatograph with metabolite II. Ei and ci spectra obtained from the regions of scan 762 and 780 were essentially identical.

The ci spectra (scans 762, 780, Figure 3) showed chlorine-containing peaks at m/z 392 corresponding to [M+H] for a mono-TMS derivative of the dihydroxyvinclozolin structure shown for metabolite II in Figure 1. The ci spectra from scans 718 and 726 were also very similar and showed major chlorine-containing ions at m/z 464 corresponding to [M+H] for a bis-TMS derivative of the proposed metabolite II structure.

The mass spectra obtained from the metabolite II isolate allowed a likely structure to be proposed. Following this proposal, the authentic reference standard was obtained and the structure of metabolite II as shown in Figure 1 was confirmed. The separation during gc-ms analysis, of components giving identical mass spectra, was indicative of the presence of pairs of diastereomers.

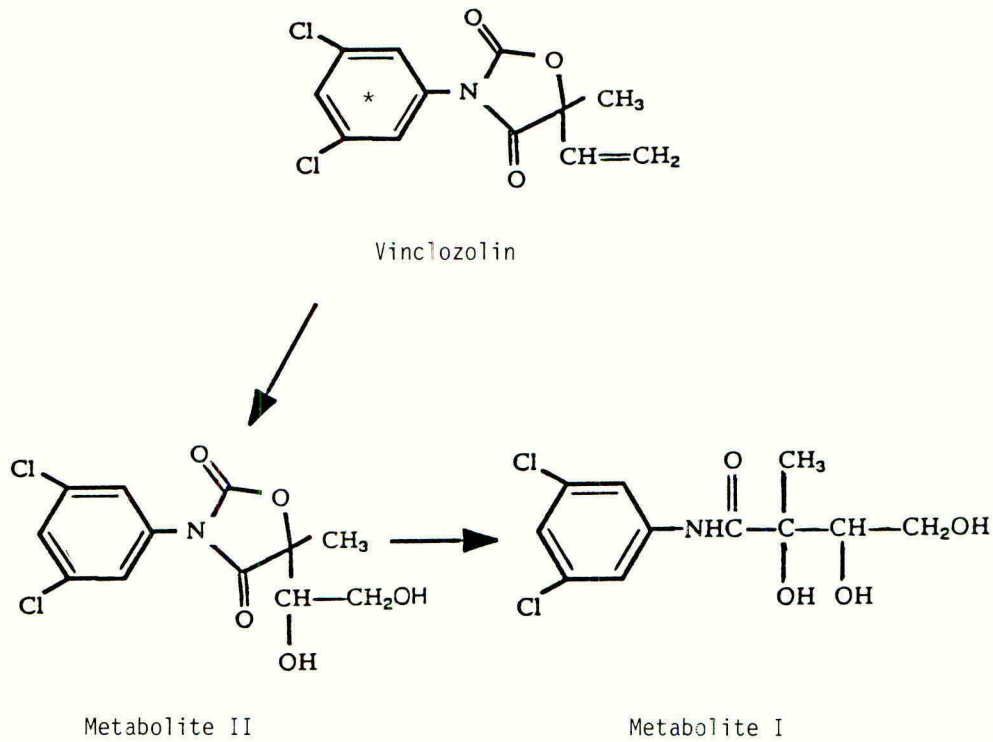

Fig. 1. Proposed biotransformation pathway for ^{14}C-vinclozolin in the laying hen (* indicates position of ^{14}C-label)

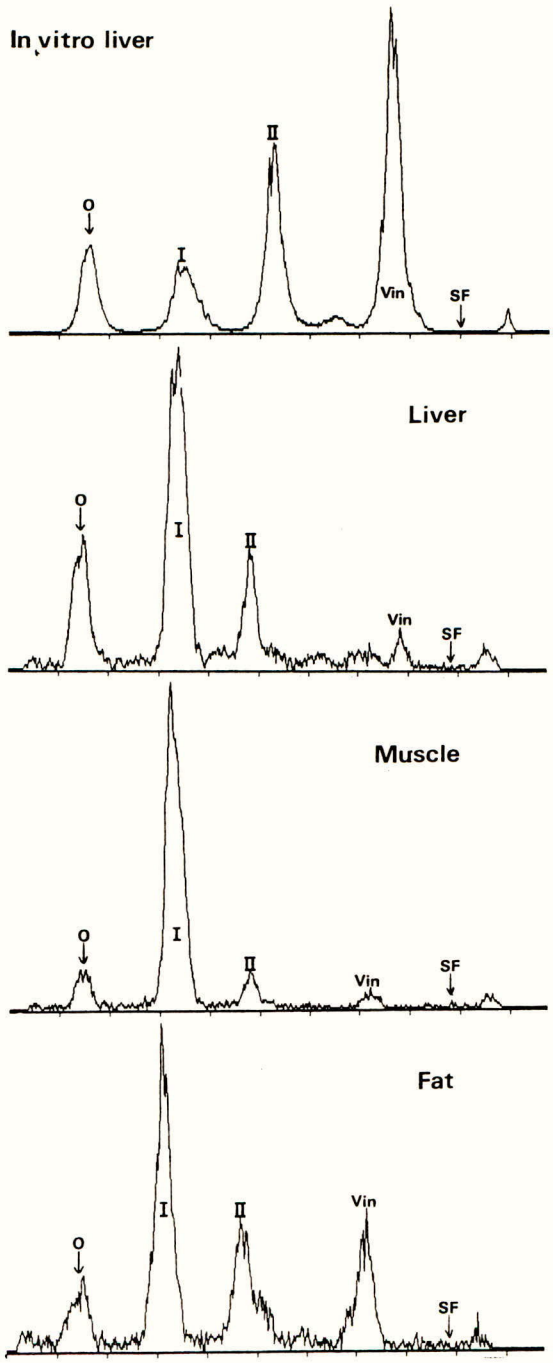

Fig. 2. Thin-layer radiochromatograms of extracts obtained from in vitro incubation of ^{14}C-vinclozolin and from tissues of laying hens after oral administration of ^{14}C-vinclozolin.
O = origin, SF = solvent front, Vin = vinclozolin

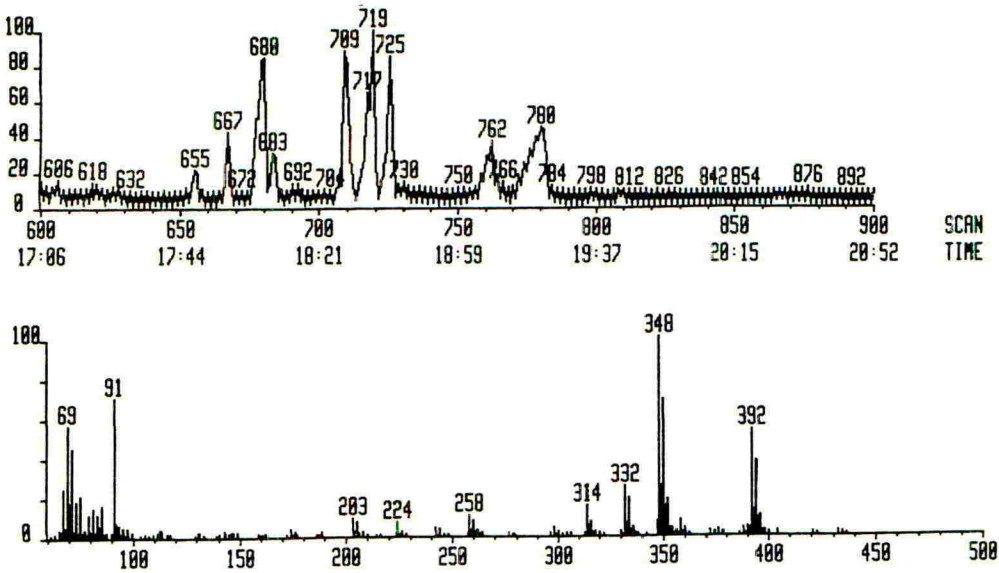

Fig. 3 Total ion current profile and chemical ionisation mass spectrum (scan 780) obtained during gc-ms analysis of metabolite II + BSTFA/TMCS

CONCLUSIONS

In the study of the metabolism of pesticides in food-producing animals, for safety evaluation purposes, studies with whole animals cannot be replaced by in vitro experiments. However for the investigation of the nature of metabolites in tissues, the use of in vitro methods may in certain circumstances be preferable to the dosing of additional animals. The advantages of in vitro methods include:

(1) Economy in the use of animals.
(2) Economy in the use of radiolabelled compound.
(3) Results may be obtained relatively quickly.
(4) Sample extraction and clean-up is easier than with whole tissue samples.
(5) May enable isolation of larger amounts of metabolites to facilitate identification.

The principal disadvantage is that the in vitro biotransformation pathways may be different from those observed in vivo, particularly in the case of compounds where metabolism by gut microflora is important. In the case of vinclozolin the major phase I biotransformations were the same in vivo and in vitro and application of the in vitro method resulted in successful identification of an important intermediary metabolite which was not a major excretory product.

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DEGRADATION OF CARBOFURAN, IPRODIONE AND VINCLOZOLIN BY SOIL BACTERIA AND INITIAL EVIDENCE FOR PLASMID INVOLVEMENT IN THEIR METABOLISM

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ABSTRACT

Bacterial cultures with the ability to degrade the insecticide carbofuran and the fungicides iprodione and vinclozolin were isolated from soils exhibiting enhanced degradation of these chemicals. Mixed enrichment cultures and pure isolates of carbofuran-degrading bacteria utilising the appropriate compound as sole source of carbon and energy effected total loss of the parent molecule within 70h. Fungicide-degrading mixed enrichments showed similarly high activity against iprodione and vinclozolin. Iprodione was converted to 3,5 dichloroaniline stoichiometrically within 50h and vinclozolin within 30h. Pure strains capable of metabolising the fungicides, however, have been refractory to isolation attempts. Plasmid screening has consistently revealed high molecular weight extrachromosomal DNA molecules in carbofuran-degrading organisms. Plasmids have also been identified in fungicide-degrading cultures though with significantly less success than in carbofuran cultures.

INTRODUCTION

The phenomenon of enhanced degradation of pesticides in certain soils has been known since the late 1940's. It was first demonstrated with the phenoxyalkanoate herbicides 2,4D and MCPA (Audus, 1949; Audus, 1951) and since this discovery a number of pesticides have been found to degrade more rapidly in soils treated previously with that compound than in identical untreated soils. The range of chemicals which have proved to be subject to enhanced degradation is extensive and now includes chloridazon (Engvilt and Jensen, 1969), TCA (Torstensson, 1976), EPTC (Obrigawitch *et al.*, 1982) and isofenphos (Racke & Coats, 1987) and the literature relating to the biological problem is likewise extensive (for a short review see Walker & Suett, 1986). The potential problems of pest control arising as a result of enhanced degradation are very significant but it is only in the past decade that this has been noted (Rahman *et al.*, 1979; Gunsolus & Fawcett, 1980).

A number of workers have reported enhanced degradation of the insecticide carbofuran in soil (Felsot *et al.*, 1981; Harris *et al.*, 1984; Read, 1983; Suett, 1986). This has also been demonstrated with the dicarboximide fungicides iprodione and vinclozolin. The fungicides were found to be unable to control *Allium* White Rot (*Sclerotium cepivorum*).

(Entwistle, 1983) and subsequent soil degradation studies revealed that the compounds were degrading at an extremely rapid rate in soils treated previously with the same compounds (Walker et al 1984; Walker et al., 1986; Walker, 1987).

It is well-known that microorganisms have the ability to catabolise a plethora of synthetic organic compounds (Gibson, 1984), including pesticides, and in instances of enhanced degradation it has frequently been shown that the elevated rates of pesticide dissipation are due to stimulation of the appropriate soil microflora (Felsot et al., 1981; Read; 1983; Kaufman et al., 1984). It has been suggested that transfer of degradative ability may be plasmid mediated (Waid, 1972; Lee, 1984; Racke & Coats, 1987; Kaufman, 1987). This study demonstrates this is potentially the case with carbofuran, iprodione and vinclozolin degradation.

MATERIALS AND METHODS

Microbial cultures were obtained using a shake flask enrichment technique. Mineral salts medium (Barnett & Ingrams, 1955) (20ml) adjusted to pH 6.5 dispensed in 100ml Erlenmeyer flasks provided with the appropriate formulated pesticide ($10mg.l^{-1}$ a.i.) were inoculated with samples of soil (0.1g) which degraded the compound of interest rapidly. In addition to cultures obtained using this methodology, carbofuran-degrading cultures were also obtained from a soil column, through which a carbofuran containing solution ($10mg.l^{-1}$) was continuously percolated. Pesticide-degrading cultures were maintained by weekly subculture in liquid mineral salts medium containing pesticide ($10mg.l^{-1}$) as the sole source of carbon and energy. Pure isolates of pesticide degrading bacteria were obtained by streaking or plating out dilutions (10^{-2}-10^{-8}) of enrichment cultures on solid nutrient media. Pesticide residues in culture supernatant were analysed by h.p.l.c. using a modification of the method of Cabras et al., (1982).

Screening of cultures for the presence of plasmid DNA was accomplished using the method of Wheatcroft and Williams (1981) which has been shown to be successful in revealing plasmids from environmental isolates of bacteria (Robson, 1986). Lysates of Pseudomonas arvilla PaW1 cells known to harbour the TOL plasmid pWW0 of molecular mass 78 Md (117 kilo base pairs [kbp]) were run as a positive control on agarose gels.

RESULTS AND DISCUSSION

Mixed enrichment cultures of microorganisms capable of metabolising all three pesticides examined were readily obtained from soils showing enhanced degradation of the compounds (Figure 1.). Pure cultures of carbofuran-degrading bacteria were also obtained which degraded the insecticide to completion within 70h. Fungicide-degrading cultures were found to produce 3,5-DCA (3,5-dichloroaniline) in near stoichiometric amounts from the parent compound, an observation previously noted in soil degradation studies (Walker et al., 1984; Walker, 1987). Unidentified metabolites were also produced during the course of fungicide degradation. Metabolites with a u.v. absorbance were not detected during metabolism of carbofuran provided at $10mg.l^{-1}$ but at higher concentrations ($500mg.l^{-1}$) the culture fluid became deep red brown in colour, an

observation previously made by other workers (Venkateswarlu & Sethunathan, 1984). The culture fluid was analysed by t.l.c. and shown to contain eight distinct components (unpublished data) which have yet eluded identification.

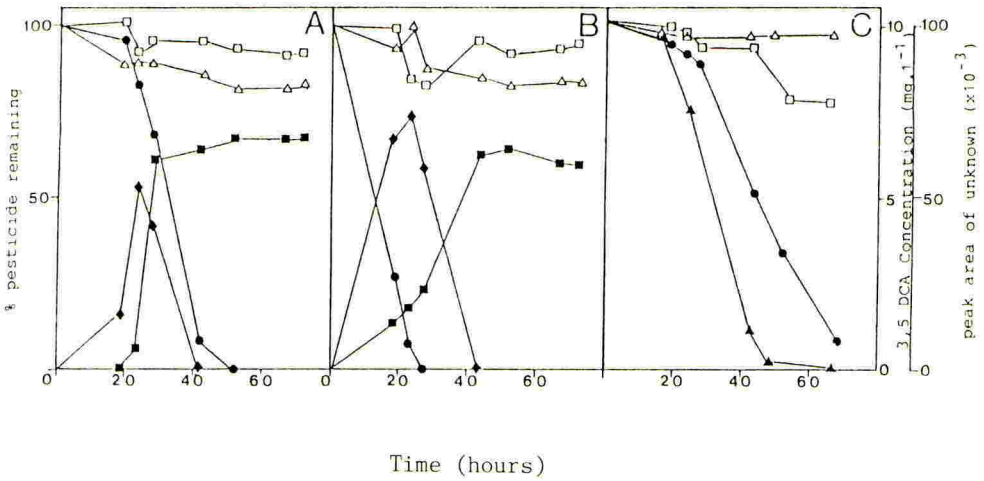

Figure 1. Degradation of (A) iprodione ($10mg.l^{-1}$), (B) vinclozolin ($10mg.l^{-1}$) and (C) carbofuran in mineral salts medium by microorganisms enriched from field treated soils. Pesticide (●); 3,5 DCA (■); unidentified metabolite (◆); pesticide in uninoculated (△) and sodium azide (0.1% wt/vol.) inhibted (□) controls. Carbofuran degradation by pure strain of bacterium (▲).

Carbofuran-degrading pure isolates showed similar degradative capacity to mixed enrichments. Pure isolates failed to grow on methylamine as sole carbon source although this compound has been found to be produced by microbial hydrolysis of carbofuran (Rajagopal et al., 1984; Karns et al., 1986) and carbofuran-degrading bacteria which utilise methylamine as a carbon source have been isolated (Chaudhry & Ali, 1988). We have isolated a bacterial strain from mixed cultures which grows on methylamine as sole source of carbon and energy but is unable to metabolise carbofuran. The carbofuran-degrading bacteria are Gram-negative rods which produce an orange pigment and have been tentatively identified as Flavobacterium sp.

All attempts to isolate fungicide degrading pure strains have failed and mixed enrichment cultures have proved unstable, their degradative ability being lost suddenly after several months of consistently degrading the compounds. This has caused problems in maintaining viable fungicide degrading cultures and on more than one occassion re-enrichment of cultures from soil has been necessary.

Screening for extrachromosomal DNA revealed the presence of plasmids in pure isolates of carbofuran-degrading bacteria and less consistently in fungicide-degrading mixed cultures (Figure 2.). The fact that fungicide degrading ability is spontaneously lost in mixed cultures suggests that the plasmids visualised from mixed cultures may encode genes specifying fungicide degradation. This has been difficult to confirm as

cultures which actively degrade the fungicides often fail to contain readily visualised plasmids, this is in part due to difficulties in obtaining sufficient biomass from these cultures. The carbofuran-degrading bacteria fall into two groups based on their plasmid profile. These correlate with the enrichment technique employed. Those enriched using a soil column contain three large plasmids and several smaller ones which are not always clear on agarose gels. Isolates from shake flask enrichments carry only two large plasmids of similar size which often appear as a single band on photographs, but close examination of agarose gels reveals two bands.

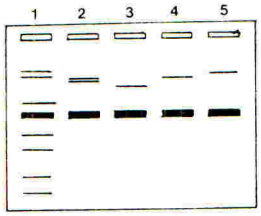

Figure 2. Diagramatic summary of plasmid content of pesticide degrading bacteria and Pseudomonas arvilla PaW1. DNA extracted from (Lane 1) carbofuran-degrading bacterium isolated by soil column enrichment; (2) carbofuran-degrading bacterium isolated by shake flask enrichment; (3) Pseudomonas arvilla PaW1; (4) iprodione degrading enrichment culture; (5) vinclozolin-degrading enrichment culture.

The degradation of several pesticides has been found to be plasmid encoded; these now encompass 2,4D (Pemberton & Fisher, 1977); EPTC (Tam et al., 1987); organophosphorus insecticides (Serdar et al., 1982); chloridazon (Kreis et al., 1981) and Phenyl carbamates (Vega et al., 1988). Evidence presented by Karns et al., (1986) suggests this is also the case with carbofuran-degradation and have recently isolated a large plasmid (100 kilobase pairs) from an Achromobacter sp. capable of hydrolysing carbofuran using the methylamine generated as a nitrogen source. Their attempts to associate the plasmid with carbofurnan degradation have so far failed. Our work has demonstrated the presence of a number of plasmids in carbofuran-degrading bacteria which utilise the compound as a carbon source. Association of these plasmids with pesticide degrading ability is now being examined and if successful the evolutionary relatedness of plasmids from the different groups of carbofuran-degrading organisms will be assessed. The proliferation of these organisms or a specific gene sequence associated with carbofuran metabolism will be used to chart the evolution and spread of pesticide metabolising ability. The effect of environmental parameters on the spread of pesticide degrading ability through the soil microflora should provide an understanding of the factors governing enhanced degradation of pesticides and allow preventive measures to be formulated.

ACKNOWLEDGEMENTS

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THE METABOLISM OF AZINPHOS-METHYL IN APPLES AND APPLE CELL SUSPENSION CULTURES

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ABSTRACT

The metabolism of [phenyl-UL-^{14}C]azinphos-methyl in apple and apple cell suspension cultures was studied. In the apple, azinphos-methyl was metabolized to a small degree; 71 % of the applied radioactivity consisted of unchanged parent compound at the time of harvest on day 35. In the organic phases of the apple cell culture extracts and the nutrient media azinphos-methyl was also the main radioactive compound. Azinphos-methyloxon was also identified as a metabolite in these fractions by cochromatography with the authentic reference compound. In the aqueous phase of the peel extract two major metabolites (M 1 and M 2) were detected. These were identical by cochromatography with the polar metabolites of the cell extracts of the apple cell cultures. The major one of the two metabolites (M 1) consisted of a novel conjugate of mercapto-methyl-benzazimide with 2-(1-glucopyranosyl)-propionic acid, and the minor one (M 2) of the monodesmethyl compound of azinphos-methyl.

INTRODUCTION

Azinphos-methyl is a broad spectrum insecticide and acaricide registered for use in a wide variety of fruits (for example apple), vegetable and field crops as well as ornamentals, forest and shade trees. It acts as a skin and contact poison. It is not systemic.

This investigation was designed to study the metabolism of azinphos-methyl in apples. Since earlier studies had shown that only a small metabolic conversion rate was to be expected, a heterotrophic apple cell culture study was conducted at the same time. Cell cultures often have the advantage of a better metabolite/matrix ratio compared to whole plant parts thus facilitating the purification and isolation of metabolites. After demonstrating the identical nature of the metabolites in both systems by cochromatography, the structural elucidation of the metabolites from the cell cultures would serve to identify the corresponding degradation products in the whole apple.

MATERIAL AND METHODS

Radiochemical

The investigations were conducted with [phenyl-UL-^{14}C] azinphos-methyl. The radioactive ingredient had a specific radioactivity of 87.6 µCi/mg, and a radiochemical purity of > 99 % according to tlc and hplc. For the cell culture experiments it was diluted with the non-labelled compound to a final specific activity of 2.76 µCi/mg. For the experiments with the apples a 25 WP formulation was made containing 20.1 % active ingredient.

Plant material, application and sampling

29 apples of the variety "James Grieve" were used. 20.6 µCi were applied evenly onto each apple with a brush. Samples were taken after 7, 21 and 35 days.

The cell culture experiments were made with heterotrophic apple cell suspension-cultures of the variety "Boskop". The cell cultures were grown in a modified Murashige/Skoog-medium [Murashige & Skoog 1962] in 200 Erlenmeyer flasks containing 40 ml nutrient solution in a rotatory incubator at 25 °C and 130 rpm. After 7 days the cells were transferred into fresh nutrient solution. A sterile acetonitrile solution of the active ingredient was applied directly to the cell cultures. The amount of solvent added did not exceed 100 µl. The concentration of the active ingredient was 632 µg / 40 ml (= 50 µM) corresponding to 1.75 µCi / 40 ml. The cell cultures were incubated for 48 h. The active ingredient was stable in the nutrient solution alone.

Extraction

After surface washing with chloroform, the apples were separated into peel and pulp. Both were extracted with acetone/water and acetone. The acetone/water extract was partioned with chloroform to yield an organic and a water phase.

In the cell culture experiments, nutrient solution and cells were separated by filtration. The nutrient solution was extracted with ethyl acetate, the cells with acetonitrile/water (8:2). The cell extract was further partioned with ethyl acetate to yield an organic and a water phase.

Thin layer chromatography (tlc)

Merck 0.25 mm silica gel F_{254} plates were used with the following solvent systems:

LS 1: toluene / ethyl acetate / water = 50 : 50 : 1 (v/v),
LS 2: chloroform / isopropanol = 95 : 5 (v/v) and
LS 3: n-hexane / toluene / acetone = 40 : 30 : 30 (v/v).

TLC-spots were detected 1. by quenching of the u.v.-induced fluorescence (254 nm), 2. by autoradiography and 3. by linear analyzer scan (IM 3000, Raytest). The quantitation of the radioactivity on the tlc-plates was done using the linear analyzer.

High performance liquid chromatography (hplc)

HPLC chromatography was performed with an HP 1090 instrument (Hewlett Packard) with u.v.- and radioactivity detectors (Ramona 4, Raytest) in line. Column: RP-8, Lichrospher 100 CH-8/II, particle size: 5 um; flow: 1.0 ml/min.; solvent system: mobile phase A: 0.1 M ammonium acetate in water, mobile phase B: acetonitrile; typical gradient: 0-5 min. 5 % B, 5-25 min. 5 % to 35 % B in A, 25-30 min. 35 % to 70 % B in A. The ammonium acetate was removed from the samples by hplc with water as mobile phase A.

Spectroscopic Methods

Mass spectrometry (ms)

mass spectrometer: Finnigan MAT 8230
EI-spectra: electron energy: 70 eV, emission: 1 mA, source temperature: 200 °C, direct inlet mode;
CI- and DCI-spectra: source temperature: 200°C, emission: 0.2 mA, electron energy: 70 eV, reagent gas: isobutane or ammonia;
FAB-spectra: source temperature: non-heated, matrix: glycerol, FAB-gas: xenon (supplier Matheson).

^{1}H-NMR-spectroscopy (nmr)

NMR spectrometer: Bruker, AC 300
internal standard: D_2O: 3-(Trimethylsilyl)propionic acid-d_4-sodium-salt (TMP).

RESULTS

Distribution of radioactivity

The treated apples were harvested after 7, 21 and 35 days. The major part of the radioactivity (in % of the recovered radioactivity) was found in the chloroform washings (day 7: 91.8 %, day 21: 79.8 %, day 35: 68.0 %). In the organic fractions of the peel and the pulp a small amount of radioactivity was detected (less than 3 % at all days of harvest). In the water phases the radioactivity increased during the time of the experiment (peel: day 7: 2.7 %, day 21: 6.7 %, day 35: 13.8 %; pulp: day 7: 1.0 %, day 21: 4.8 %, day 35: 8.6 %).

In the cell culture experiment the radioactivity balance yielded the following results (in % of recovered radioactivity): cell extract organic phase 36.5 %, cell extract water phase 34.9 %, unextractable 2.6 %, nutrient solution organic phase 14.5 % and nutrient solution water phase 5.7 %.

Identification of metabolites

In the investigation of the apples the metabolites were analyzed by tlc. The only radioactive component in the surface washings was the parent compound. The metabolic pattern of peel and pulp were identical. In the organic extracts of the peel, unchanged parent compound constituted the major part of the radioactivity. Azinphos-methyl-oxon was detected as a metabolite along with bisbenzazimide-sulfide and bisbenzazimide-disulfide as possible artifacts. These metabolites represented less than 1 % of the radioactivity recovered from the whole apple.

The organic phases of the cell extract and the nutrient solution of the cell culture experiments were analyzed by tlc (solvent systems 1, 2 and 3) and by hplc. Azinphos-methyl was found to be the main radioactive compound in these extracts. The main metabolite was azinphos-methyl-oxon representing 2 respectively 7 % of the recovered radioactivity.

The metabolites of the water phase of the peel extracts (M 1 and M 2) were isolated by XAD-4-adsorption chromatography, medium pressure chromatography and hplc both in the reversed phase mode. Because of the unfavorable metabolite/plant matrix ratio the metabolites M 1 and M 2 could not be isolated pure enough and in such quantities that their structural elucidation by spectroscopy was possible.

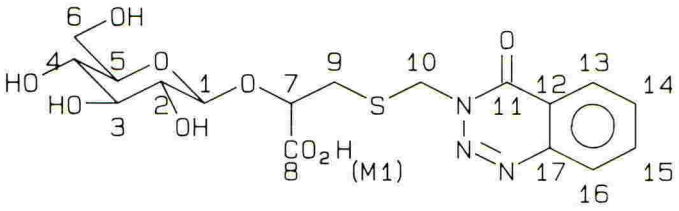
The identity of these two metabolites M 1 and M 2 with the polar metabolites from the cell extract (water phase) of the cell culture experiments was established by tlc- and hplc-co-chromatography. They were isolated from these cell extracts by XAD-4-adsorption chromatography (stepwise gradient of methanol in water) followed by semipreparative hplc.

M 2 being the less polar of the two was identified as monodesmethyl-azinphos-methyl by comparison of its ^{1}H-nmr- and ms-spectra with those of the authentic reference compound.

The ^{1}H-nmr-spectrum of metabolite M 1 recorded in D_2O as solvent showed signals corresponding to 4 aromatic protons at δ = 8.36 - 8.01 ppm (multiplets) characteristic for the unsubstituted benzene ring of the benzazimide moiety. A singlet at δ = 5.66 ppm was assigned by comparison with the ^{1}H-nmr-spectrum of the mercaptomethyl-benzazimide to the CH_2-group attached to the amide nitrogen atom of the benzazimide moiety. Protons which indicated the presence of a glucopyranosyl-conjugate had the following chemical shifts: H1 4.38 ppm (d); H2, H3, H4 3.35 ppm (m); H5 3.25 ppm (m); H6 3.80 and 3.68 ppm (AB-system). An additional CH_2-group was detected as an AB-system at 3.20 and 3.12 ppm. A double-doublet at 4.25 ppm belonged to a CH-group. Decoupling experiments showed that these two groups were attached to each other.

The FAB-mass-spectrum yielded a molecular weight for M 1 of 465 ($[M+H]^+$ at m/z 466, $[M+Na]^+$ at m/z 488) probably being a sodium salt because the matrix indicated a high amount of sodium. After silylation with MSTFA the CI-mass-spectrum with ammonia as reagent gas yielded a $[M+NH_4]^+$ ion at m/z 821 which indicated the addition of five trimethylsilyl groups. 4 of these silyl groups were assigned to the glucopyranose moiety which the ^{1}H-nmr-spectrum had shown to be present in metabolite M 1. According to the remaining difference in the molecular weights the 5th silyl group was attached to a carboxyl function.

After measuring a ^{13}C-nmr-spectrum of the metabolite M 1 it was possible to connect the identified molecular parts. The following structure was proposed:



According to the ^{13}C-nmr-spectrum the CH_2-group at C10 at 54.67 ppm was located next to a sulfur and not an oxgen atom shown by comparison of the corresponding signal of the mercaptomethyl-benzazimide (44.73 ppm) with that of the hydroxymethyl-benzazimide (74.17 ppm). The resonance signal at 82.12 ppm for C7 gave additional proof of the neighboring carboxyl group. The signal at 104.85 ppm for C1 of the glucopyranose showed that it was attached via oxgen to C7. The corresponding signal in the related sulfur analog glucopyranosyl-thiomethyl-benzazimide was located at 88.32 ppm. Conjugation of the glucopyranosyl-part via an ester instead of an ether bond was excluded because the proton signal of H1 in the ^{1}H-nmr-spectrum would have been at a lower field. Because of the small amount of material available (approx. 0.5 mg) the ^{13}C-signals of C8 and C11 were not detected. ^{13}C-nmr-data in ppm : 146.87 (C17), 139.11 (C15), 136.70 (C14), 130.86 (C16), 127.57 (C13), 122.20 (C12), 104.85 (C1), 82.12 (C7), 78.74 (C2), 78.43 (C3), 75.76 (C4), 72.17 (C5), 63.31 (C6), 54.67 (C10), 36.80 (C9).

DISCUSSION

On the whole azinphos-methyl showed a comparable metabolic pattern in the apple cell culture and in the whole apple (Figure 1). Following the application of [phenyl-UL-^{14}C]azinphos-methyl to apples the major part of the radioactivity remained on the peel as unchanged active ingredient. In the apple extracts azinphos-methyl also was the main radioactive component. During the course of the study azinphos-methyl was degraded to the corresponding azinphos-methyl-oxon and 2 major polar metabolites. These were shown to be identical by tlc- and hplc-cochromatography with the polar metabolites in the water extracts of the cell cultures. Besides formation of the oxon-compound the metabolism of azinphos-methyl starts with a dealkylation step to form monodesmethyl-azinphos-methyl. Degradation then proceeds (possibly via the undetected mercaptomethyl-benzazimide) to yield the doubly conjugated metabolite M 1.

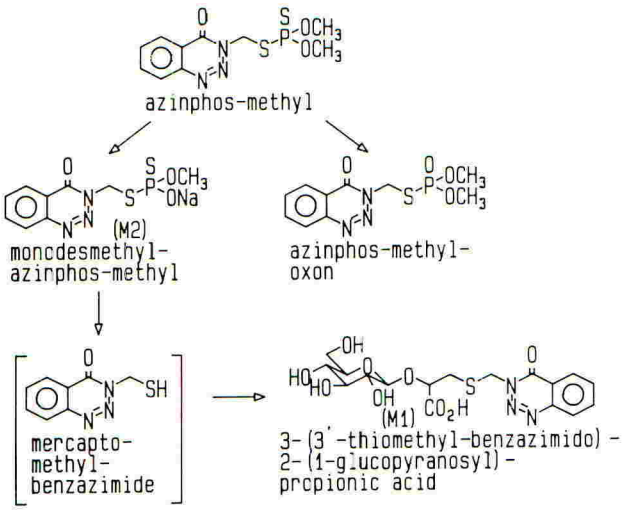


FIGURE 1

Metabolism of azinphos-methyl in apples and apple cell suspension cultures.

The formation of M 1 can be explained by the stepwise conjugation of mercaptomethyl-benzazimide with 3-phosphoglycerate in a nucleophilic displacement reaction followed by the conjugation of the hydroxy group of the propionic acid with glucopyranose. Without the easy access to the metabolite M 1 in an amount of approx. 0.5 mg through the cell culture experiments the structure of this metabolite would not have been established so unambiguously. Thus in the case of azinphos-methyl the apple cell culture and the study on the whole apple complemented one another in an ideal way. This is yet another example in the long row of pesticides showing a similar degradation in whole plant parts as in cell cultures [Sandermann et al. 1984, Mumma 1987, Swisher 1987].

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IMPROVED TECHNOLOGY FOR SAMPLING IN PESTICIDE FIELD VOLATILIZATION STUDIES

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ABSTRACT

A field study was conducted at four locations in the US involving a novel approach to the direct measurement of volatilization of a test chemical into the air. Emphasis was on measuring pesticide concentration at different heights above the plot surface and crop. A special air monitoring system was constructed and centrally located within large plots. Air samples were taken by drawing air through TENAX packed micro-pipette cartridges over 12 hour periods of time. Sampling was continuous over 24 hours commencing at pesticide application. Additional method validation included the use of field spiked cartridges for sampling and shipping.

1. INTRODUCTION

A wide range of methods for collecting volatilized chemicals has been described in the literature for both laboratory and field use. The technique presented herein represents a cheap and effective approach to the "trapping" of chemical vapors.

While a number of radiolabelled and "cold" studies are frequently performed in the laboratory and field to measure the degradation of chemicals in soil, only limited information exists on actual field vapor losses. There is considerable information in the literature on the factors affecting volatilization in the field (ie. soil moisture, temperature, bulk density, air flow etc..), however, data interpretation is often complicated by field sampling problems. The method described herein was designed to attempt to minimize sampling variables as much as possible and attempt to determine actual test chemical flux from a large scale field trial using a compound applied at planting.

2. METHODS

2.1 Field preparation

Plots were prepared using normal agricultural practices and were planted to the typical corn variety grown in the area. Planting was begun as early in the day as practical to provide for maximum sampling of first day vapor. A chemical herbicide such as SUTAN was applied early on to minimize weed cover over the field. The test plots were flat areas not subject to run-off, square and approximately 1 ha (2.5 acres) in size. Soil types evaluated were silt loam, sandy loam and a silty clay loam.

2.2 Application

Treated granules were applied with field corn at planting using a banded application treatment and again three weeks later using a broadcast technique. The first application was made at planting by placing granules in a 7-inch band directly behind the planter shoe (in front of the press wheel). The granules were incorporated by using a set of tines mounted behind the press wheel.

2.3 Air Sampler and Weather Station

Air samples were taken continuously by drawing air through micro pipette cartridges during 12-hour intervals over 24 hours. Immediately after planting the air monitoring system was installed centrally in the field and sampling begun within 30 to 35 minutes. Prior to planting/application, a minimum of four hours of air sampling (pre-treatment) was conducted at each site.

The air sampling system components consist of a vacuum pump, a vertical PVC tower/vacuum reservoir, a sampling tube holder equipped with flow meters (see Figure 1) and cartridge sampling tubes (see Figure 2). In addition, a Campbell Scientific weather station was located adjacent to the sampler (see Figure 3). Wind speed, wind direction, relative humidity and air temperature were taken at three heights above the field surface. Other weather parameters measured included 2 and 8 inch soil temperatures, solar radiation and rainfall.

The sampling tower is a 15 cm wide PVC pipe, 2 meters in height with eight usable ports. Sampling height adjustments could therefore be made for changes in crop canopy height. A minimum of 2 sampling positions over the canopy was maintained during the sampling period. A vacuum was created in the tower by the vacuum pump and maintained at about 30 centibars by a pressure valve. The vacuum (air flow) through the cartridges was regulated by flow meters for each cartridge.

2.4 Cartridge Installation

Cartridges were installed into the sampling arm cartridge holder and secured by the use of a thick "O" ring to provide the vacuum seal. Air sampler arms were 1/2 inch (1.27 cm) CPVC tubing and sloped to minimize rain water/moisture intake. In the field the sampling tube opening was located between the corn rows. Sampling tubes were positioned at about 25 cm heights starting at 10 cm above the plot surface.

Cartridges were replaced every 12 hours (+/- 2 hours) at 0600 and at 1800 hours. Accordingly, a "daytime" vs "nighttime" sampling set was produced. Each cartridge unit consisted of 2 pipette cartridges joined together with heat shrink tubing. The second tube being a back-up sample cartridge designed to

confirm no chemical "break through" from the first pipette. Following cartridge installation into the arm, cartridge air flow was measured before the test period and just prior to cartridge removal. Air flow through each cartridge was set at about 500 mls per minute.

2.5 Analytical Validation

The use of TENAX cartridges as an air monitoring device was researched and validated. Additional work using "spiked" cartridges inserted in the sampling tower during 12 hour intervals showed excellent recovery and storage stability of test chemical.

3. RESULTS

Excellent consistency in air flow volumes through the sample cartridges was noted throughout the test period under varying temperature and weather conditions. Also, no failures of the 4 different sampling units occurred while operating under typical field conditions. This testing has shown the units to be reliable through about 6 weeks of continuous operation in the field.

4. CONCLUSIONS

The system and procedures worked well in the field with no major difficulties noted despite the fact that a large number of samples were collected. The system affords an economical and effective method for determining field chemical vapors. Total cost of the system excluding the weather station was about $1100.

A tremendous advantage of the system is the time savings from the use of a micro-pipette cartridge. Cartridge insertion and removal from the system was both quick and easily done. Also, the analyst was able to elute the sample directly in the lab with no other sample prep operations.

A material and equipment list is available for interested persons.

5. ACKNOWLEDGEMENT

Research on the validation and use of the TENAX micro-pipette cartridge was done with the help of Dr. J. P. Leahey ICI PPD, Jealotts Hill Research Station, Berkshire, England

SAMPLING TOWER

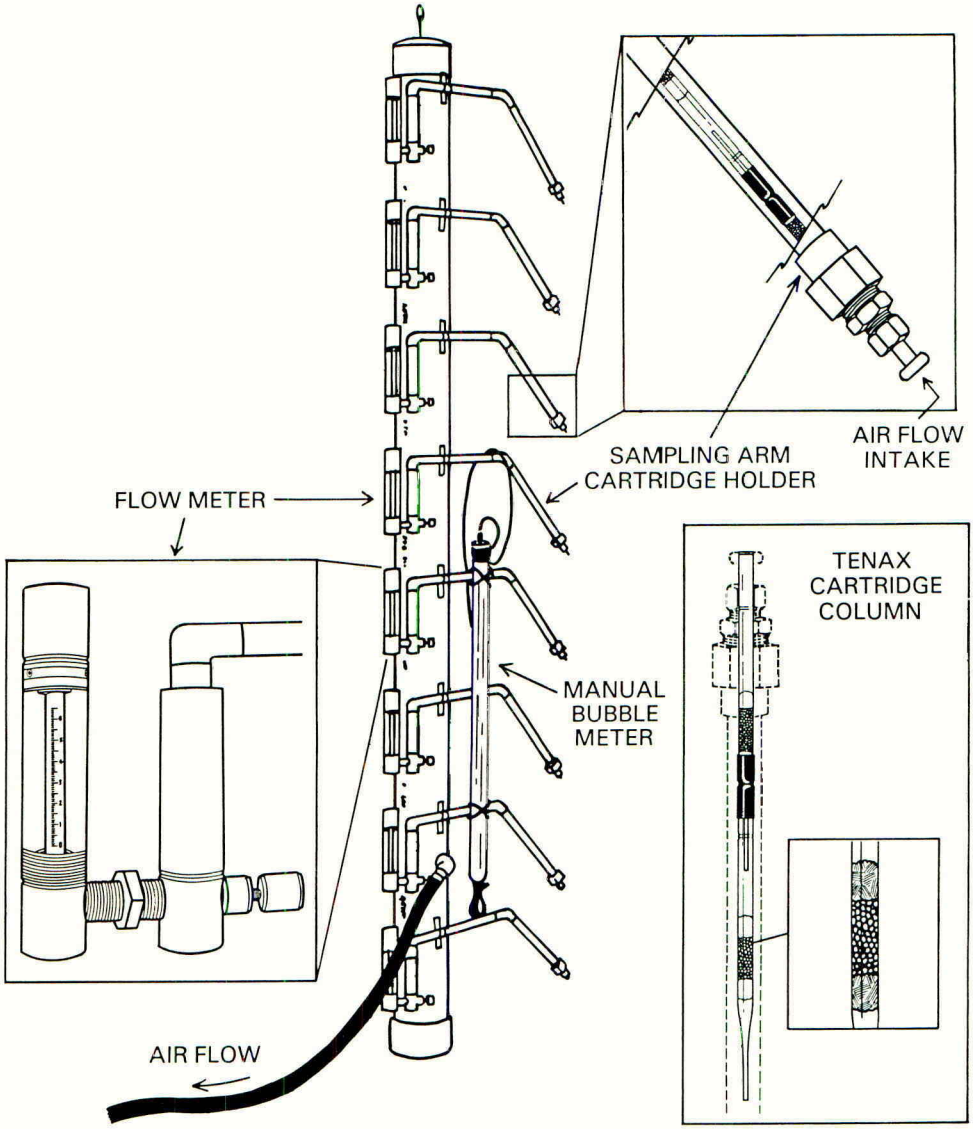

FIGURE 1

DUAL TENAX SAMPLING CARTRIDGE

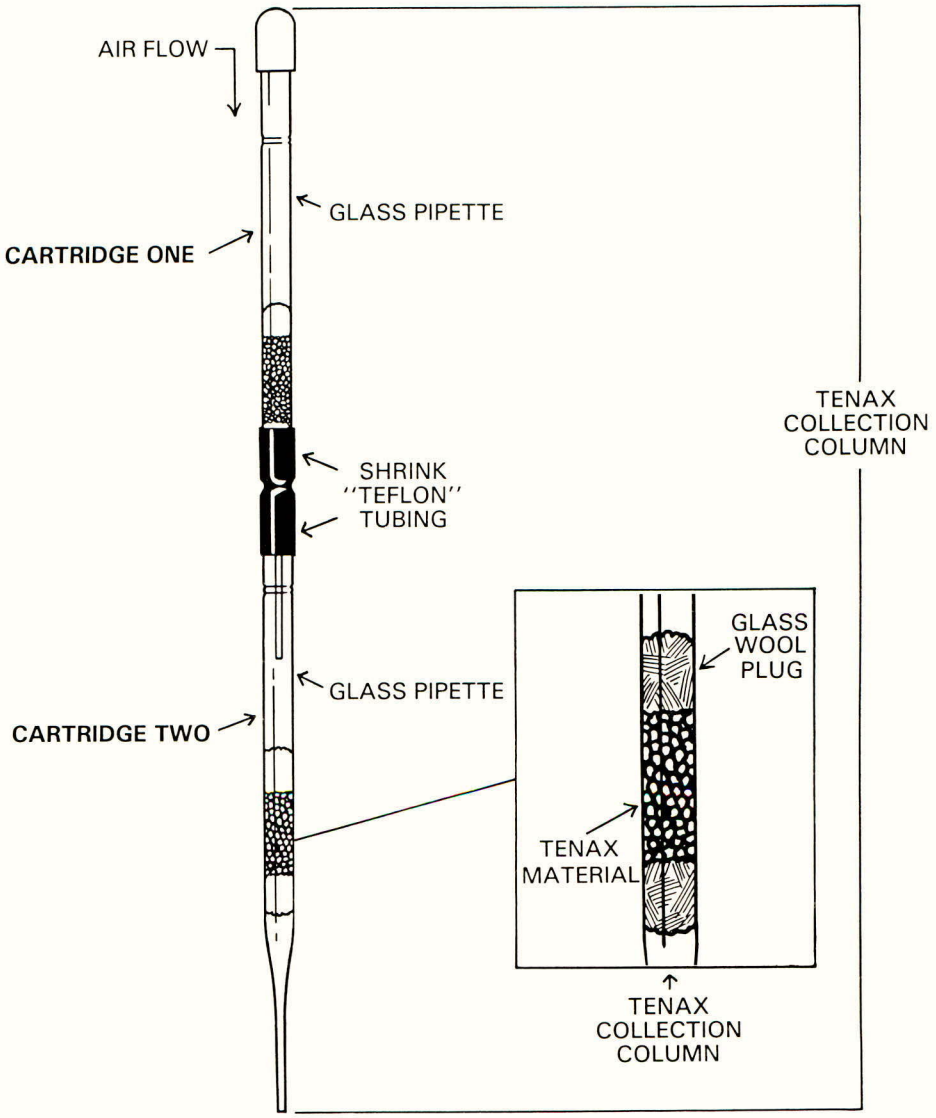

FIGURE 2

WEATHER STATION / SAMPLING TOWER

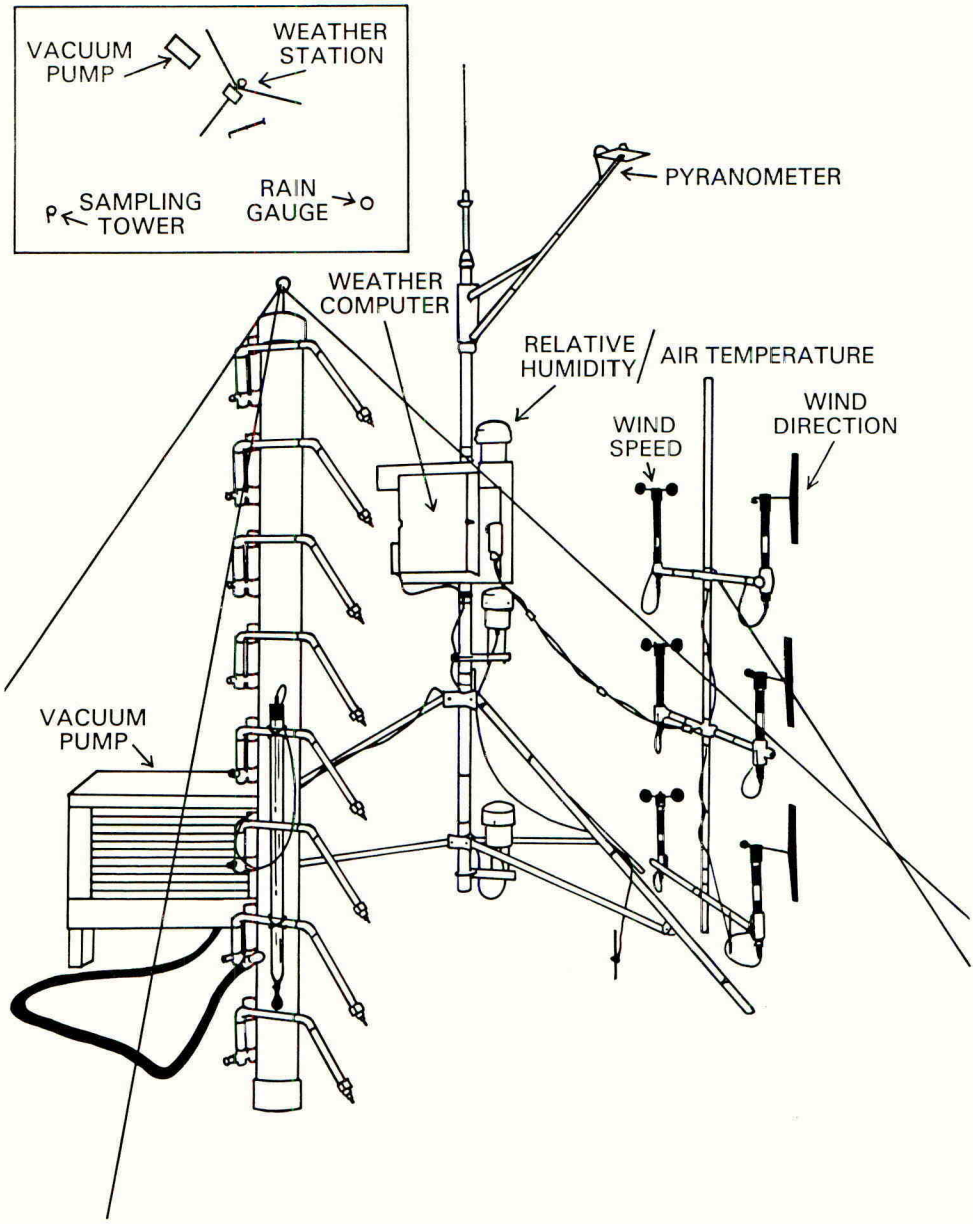

FIGURE 3

ANALYSIS OF PESTICIDE RESIDUES IN DEVELOPING COUNTRIES

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ABSTRACT

The complexities of pesticide residue analysis and the cost of undertaking such analyses are well known. There is a requirement for a well equipped laboratory staffed by trained chemists and support personnel with a budget sufficient to cover the full running costs taking into account the replacement of consumable materials, the replacement of old and unserviceable items and future expansion. These difficulties are often increased manyfold for Developing Countries faced with the need to establish such facilities.

ODNRI is, through its Chemical Control and Pesticide Analysis Section, making an active contribution towards reducing the problems by:-

* Developing appropriate guidelines and specifications for the establishment and maintenance of laboratories for the analysis of pesticide residues.

* Developing suitable procedures for residue analysis that minimise the quantities of consumable materials required.

* Developing a greater awareness of the commitment and resources required through the provision of expert advice based on research findings and accumulated expertise.

INTRODUCTION

There is a need for countries to be able to determine residues of pesticides for the purpose of consumer safety, the collection of data for registration and the monitoring of environmental contamination. Samples can originate from agricultural trials, market basket surveys, the routine examination of drinking water or be in support of legislation on the control of pesticide use. The necessity to conduct residue analysis of exported and imported foodstuffs has also taken on a particular significance in International trade. Any doubts as to the accuracy of such analyses may cause the results of expensive experimental programmes to become valueless.

Pesticide residue analysis is one of the most demanding forms of analysis with high capital equipment and maintenance costs and with a requirement for large quantities of reagents and other consumable materials. Additionally there is a need for them to be staffed by well-trained, well-qualified and highly motivated personnel. The establishment of such laboratories poses difficulties in developed countries but for the developing countries the problems are often greatly intensified. Not only are the establishment and recurrent costs prohibitively high, with the

great majority of equipment and materials having to be imported, but the climatic conditions can also affect working patterns and procedures.

ODNRI has encountered many cases of laboratories, often established with Aid funds or through grants, that have floundered or are largely inoperative through inadequate funding, poor maintenance of equipment and laboratory facilities or the lack of trained staff. In most cases such failures could have been prevented if there had been a greater understanding of the overall requirements for establishment and operation at the planning stage. Funding is generally available for the initial establishment of facilities but as little attention is paid to the funding of future running costs, laboratories become inoperative through a lack of materials. Similarly too little attention may be paid to the nature of the equipment itself and its operational environment, to the accommodation and to the staffing. This paper reviews the difficulties that are faced in the successful establishment of a pesticide analytical laboratory in a developing country and ways of overcoming these.

ESTABLISHMENT OF A LABORATORY

Awareness of the problems to be faced in establishing and maintaining a pesticide laboratory at the planning stage will increase the chances of its success. Such an appreciation must cover all aspects of policy development, laboratory management, the provision of finance on a long-term basis and the recruitment and retention of staff. The initial project appraisal must address the nature of the work programme, the nature of the analyses that are required and the anticipated sample throughput, the accommodation available together with laboratory services, the appropriate equipment for the laboratory, finance for replacement of equipment and general running costs and the training of laboratory staff. All of these items are vital but the need for adequate recurrent funds cannot be overstressed; the costs of the operation, both initial and the annual costs for materials and the replacement of equipment through age/breakdown must not be underestimated although in practice they invariably are. More laboratories in developing countries fail because of lack of recurrent funds than for any other reason. A laboratory that cannot function because of a lack of, say, reagents will decline rapidly with accelerated instrument failure and the demotivation and loss of staff. Any proposals to establish a laboratory must guarantee adequate funding for its anticipated lifespan.

These considerations apply to most laboratories but they are exacerbated in developing countries by the limitations on finance, public services and remoteness from major suppliers. It must be recognised that it is not possible just to translate a laboratory specification from a temperate developed country to a tropical developing country without substantial reappraisal and modification because of these special factors.

A comprehensive guide has been prepared by ODNRI to assist organisations intending to establish pesticide analytical laboratories. The guide details the stages for the laboratory appraisal so that no factor can be overlooked and contains detailed equipment and material specifications for laboratories of different sizes and with different projected sample throughputs. It also details minimum space requirements for such laboratories and discusses how the individual operations should be separated.

Particular attention has been given to the effect of climatic conditions on laboratory instruments and procedures and the recommendations for equipment and reagents reflect these considerations. Temperature and humidity are major factors. Laboratory temperatures of 30-40 degrees centigrade can be encountered and together with high relative humidities these can greatly affect the performance of "standard" procedures necessitating suitable modifications to them if adequate air conditioning cannot be installed in the laboratory area. The deactivation of prepared adsorbents or thin layer chromatography plates are ready examples of the effects of humidity. Laboratory solvent vapour levels can also become a problem necessitating the extensive use of fume extraction hoods. Piped water supplies can also have similar temperatures which pose difficulties for the condensation of solvent vapours with consequential implications for laboratory safety. Water chilling and circulating units are required for most operations together with eg water jacketed columns for chromatography. Water supply pressures are also often inadequate and pumps have to be installed to bring them to operational levels. Such factors are often overlooked but with adequate planning can be catered for. However, this obviously increases the cost of establishing and operating the laboratory with consequent implications for initial capital and recurrent expenditure.

DEVELOPMENT OF SUITABLE ANALYTICAL PROCEDURES

It has been suggested in some quarters that laboratories in developing countries should use methods of pesticide residue analysis which do not require the sophisticated gas chromatographic equipment which is seen in equivalent laboratories in the developed world. This in fact occurs in, for example, India where extensive use is made of thin layer chromatography for the final analysis of sample extracts. There are also possibilities of using "ELISA" techniques. However, it is inevitable that for most purposes methodology will continue to be based on that used by laboratories in developed countries, so that no doubts on reliability can be cast on results.

Collaboratively tested "referee" methods are often expensive in terms of both labour and materials. Whereas in developed countries materials are generally cheap with staff time being the major cost factor, the position is usually reversed in most developing countries. There is a requirement, therefore, to develop reliable analytical procedures for routine use which minimise the use of consumable materials and where possible utilise solvents of higher boiling point to reduce solvent vapour effects at high ambient temperatures. There will still, of course, be a need to use the referee methods for the analysis of certain samples but considerable savings in resources can be made through the careful selection of analytical procedures.

The miniaturisation of standard procedures at the sample clean up stage (not at the extraction stage where the requirements of representative sampling must be preserved) offers an attractive way of reducing requirements for solvent and adsorbents, but this is not without its problems. Disposable pre-packed cartridges are commonly available for this purpose but this can be more costly than conventional column chromatography. However, small glass columns (or empty pasteur pipettes) can be repacked and there is only the need to change the adsorbent. These small scale operations however do require care and are particularly prone to temperature changes and the effects of high humidity.

The use of adsorbents which can be used more than once has an obvious attraction and the use of Gel Permeation Chromatography is becoming popular. Manual systems for GPC operation, of relevance for developing countries, have been established with little loss of efficiency from that of the automated systems.

For analytical procedures to be widely applicable, the originators must bear in mind the fact that other laboratories may not be as well-equipped as their own. Procedures should be developed with economy in mind and using commonly available equipment where possible. There will inevitably be the need to use more sophisticated techniques for some separations or determinations but there is undoubtedly a tendency for over-elaboration with some modern procedures. The needs for sophisticated analytical equipment must be kept to a minimum where servicing is difficult and the choice of equipment becomes of particular importance. There is a need to balance sophistication with robustness and this may necessitate purchasing equipment that is tried and tested rather than at the forefront of developments - not always a popular decision.

The Gas Liquid Chromatograph ranks high in the analysts armoury but requires supplies of good quality gas for its operation. These are commonly supplied from cylinders but this is an expensive and inconvenient method for many developing countries who do not produce gases of the correct quality and need to import their supplies. The use of gas generators has been evaluated at ODNRI and found to be a more than adequate substitute even when used with the electron capture detector. Although hydrogen generators have been around for some time they have not always been reliable. Modern models are, however, far more satisfactory. The use of air compressors to produce air supplies is becoming more popular and the development of a nitrogen generator has been a major breakthrough. Most gas needs can now be supplied by generator and this is particularly important for overseas laboratories.

STAFFING OF LABORATORY

It is essential that the analytical staff are good graduates with appropriate training in pesticide analysis for a minimum of six months. It must also be recognised that this is a minimum and that further on-the-job training must be allowed for. The initial training is best undertaken in an established laboratory with follow up visits from a suitable consultant once the analyst has returned to the parent laboratory. Close contact must be maintained with other reputable laboratories, particularly for participation in the collaborative analysis of treated samples.

Training of staff is a long and expensive process and every effort must be made to motivate and retain them so that a good return is obtained on the investment and the laboratory continues to be effective. Lack of career opportunities, which results in key staff moving into other areas of work is often a problem, to which management should give particular attention. This may well be due to a lack of recognition of the professional skills required of the analyst who is often perceived as offering a purely support service, rather than being a participating scientist. Loss of staff in this way may be avoided by according adequate status and remuneration, perhaps on a personal basis.

RODENT RESIDUE ASSESSMENT IN FLOCOUMAFEN BAITED RICEFIELDS IN THE PHILIPPINES

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ABSTRACT

During 1987 a large scale (154 ha) rodenticide trial was carried out in a rice growing area of Laguna in the Philippines (Hoque and Olvida, 1988). As part of this trial a study was conducted to assess the residues of the rodenticide (flocoumafen) in the baited population of rodents. Rodents were trapped at intervals after baiting, sacrificed, preserved by freezing, and tissue samples subsequently analysed for flocoumafen residues.

Total residues of flocoumafen in the rodents were highest shortly (2 days) after baiting (range <0.02-1.2 mg/kg). Residues in rodents collected 10-25 days after baiting were much lower (<0.02-0.03 mg/kg) indicating that secondary hazard was likely to be limited to the period of a few days after baiting. While some domestic animals may potentially have been at risk from exposed bait this primary hazard was minimised by adequate concealment of bait blocks.

INTRODUCTION

A major consideration on the use of rodenticides in agricultural areas is their safety to humans, domestic animals and wildlife, and the potential hazards associated with their use. Registration requirements reflect the active ingredients used in the "second generation" anticoagulant baits. The ingredients (difenacoum, bromadiolone, brodifacoum and flocoumafen) are classified as highly toxic owing to their low LD50's in certain vertebrate species. Baiting methods for these rodenticides have been developed to minimize possible hazards when controlling rodents in urban areas and croplands. (Dubock, 1982).

Flocoumafen has been reported to be both highly palatable and potent against most rodent pests, including those tolerant to warfarin (Bowler et al. 1984; Buckle, 1986; Garforth and Johnson, 1987; Rowe et al., 1985). A recent field trial in the Philippines demonstrated that the application of 3.5g flocoumafen wax block baits (STORM) provided very effective rodent control with only 1.175 kg bait/ha/season when applied over a large area (Hoque and Olvida, 1988). None of the wild animals (included birds) observed regularly during the 95-day observation period showed a significant decrease in number after baiting. A few domestic animals were attracted to the baits but no casualties were reported.

Although the flocoumafen baiting did not cause any undesirable impact on domestic animals and wildlife, it was considered important to quantify the levels of rodenticide ingested by rats in the field following baiting. Although none of the observed rat carcasses showed evidence of scavenging, rats dying following treatment could be hunted by farmers' dogs in the ricefields. Thus this study was conducted to assess the residues of flocoumafen in the field population of rodents baited with wax block bait in the trial previously reported (Hoque and Olvida, 1988).

METHOD AND MATERIALS

Description of Site

This study was conducted together with the efficacy study of flocoumafen (STORM) wax block baits which has been reported previously (Hoque and Olvida, 1988). The site was located in Tubuan, Pila, Laguna, Philippines. The irrigated ricefields consisted of 154 ha, in the centre of which was 6 ha of village housing interspersed with coconut groves, fruit trees and vegetable plots. For operational purposes the treated site was divided into 4 sub-plots, one of which was the village area and the other three were rice fields.

Application of flocoumafen baits

A commercial STORM wax block bait formulation was used. Each block, 3.5 g, contained 0.005% flocoumafen on a rice cereal base with the addition of a bright blue warning dye and a human taste deterrent. All baiting was carried out by local villagers who had received prior instructions on baiting techniques. Four groups of villagers, each supervised by a member of the research team, was assigned to one of the four sub-plots. The group allocated to the village also baited surrounding crop plots. In addition, all householders were given 10 blocks and were instructed to place half of them inside and half outside the house in such a way that they would be well concealed from children and domestic animals. The three groups baiting the ricefields laid one block every 10 to 15 m along each bund. This resulted in bait usage of only 80 to 100 blocks/ha (about 280 to 350 g of bait/ha).

Bait was applied on five occasions starting July 30 1987. All of the treated areas were baited on the first two occasions 14 days apart but subsequent applications were only made to sub-plots where there was still heavy rat infestation as judged by bait monitoring. A total of 181 kg of bait was used in the rice fields and 5.7 kg in the village area.

Rat collection

The first two collections ware made over the entire area, while succeeding collections were made only in areas that required additional baiting (see above). Live animals were collected with snap traps set in the late afternoon and retrieved the following morning. Species were *Rattus rattus mindanensis*, *Rattus norvegicus* and *Rattus exulans*. The

carcasses were weighed, sexed and marked. Half the total number were packed in dry ice and shipped to England for residue analysis while the other half was kept deeply frozen at the NCPC.

Residue analysis

Tissue samples were prepared and analysis for flocoumafen carried out in accordance with Shell Analytical Methods Series (SAMS) 419-3.

RESULTS

The residues of flocoumafen in trapped rat carcasses collected from the village and ricefields are summarised in Figure 1. The highest residues were observed in rodents caught two days after the first baiting (range <0.02-1.2 mg/kg, mean 0.36 mg/kg). It is likely from LD50 values (e.g. LD50 Rattus norvegicus - 0.46 mg/kg) that a number of these rats had ingested a sufficient amount of bait to cause subsequent death. Residues of flocoumafen in rats trapped later (i.e. 10-25 days) after baiting were much lower (0.02 - 0.03 mg/kg). The limit of determination was 0.02 mg/kg.

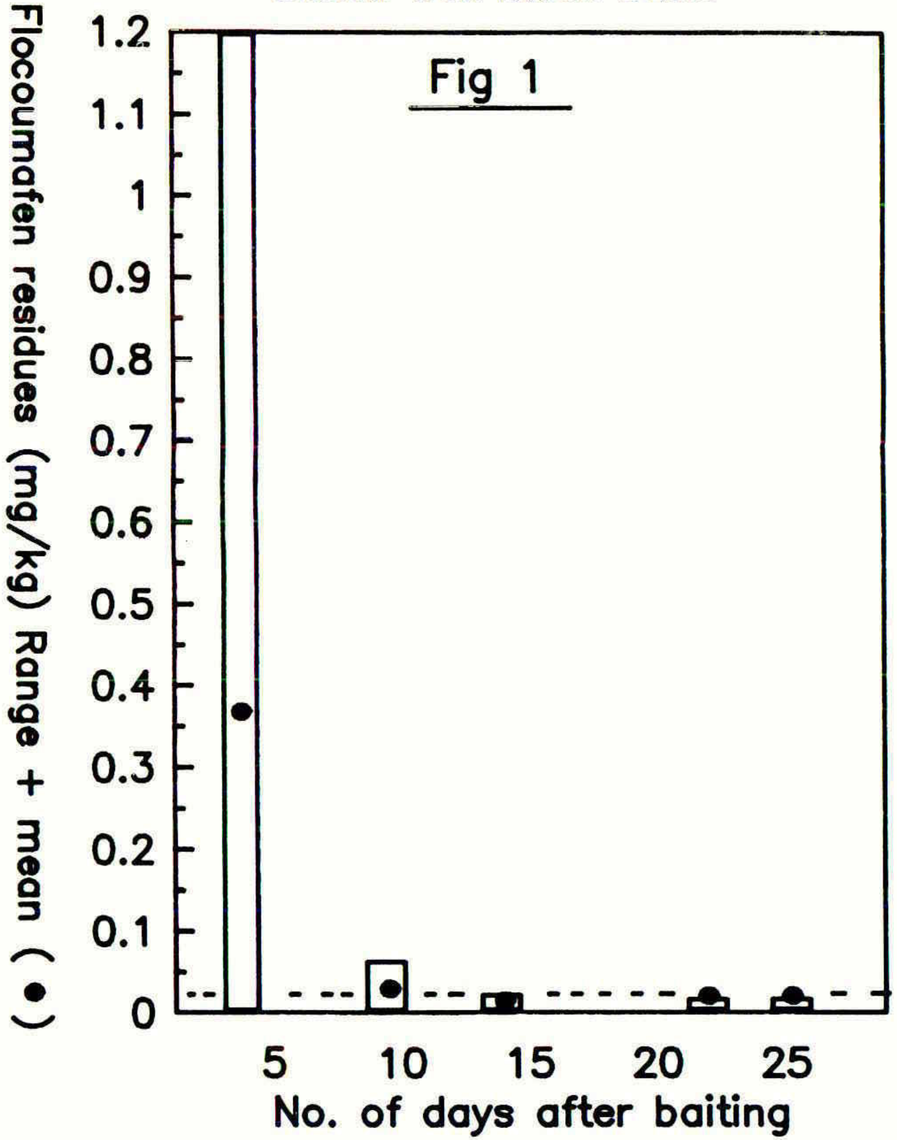
The low proportion of the trapped rats having residues of flocoumafen (11 of 28) coupled with the low residue levels in rats collected between 10-25 days after baiting would seem to indicate that secondary hazard is likely to be limited to the period of a few days after baiting. None of the observed carcasses during the trial showed evidence of scavenging. If domestic animals, e.g. dogs and cats, fed on dead rats two days after baiting, the possible risk of death would be low. For example, supposing a dog with a body weight of 6 kg ate a rat of 150 g with the maximum residue level of 1.2 mg/kg as detected in the sample. This particular dog would have ingested a total of 0.03 mg/kg computed as follows:

$$\text{amount ingested} = \frac{\text{residue level (1.2 mg/kg)} \times \text{weight of rat (.15 kg)}}{\text{body weight of dog (6 kg)}} = 0.03$$

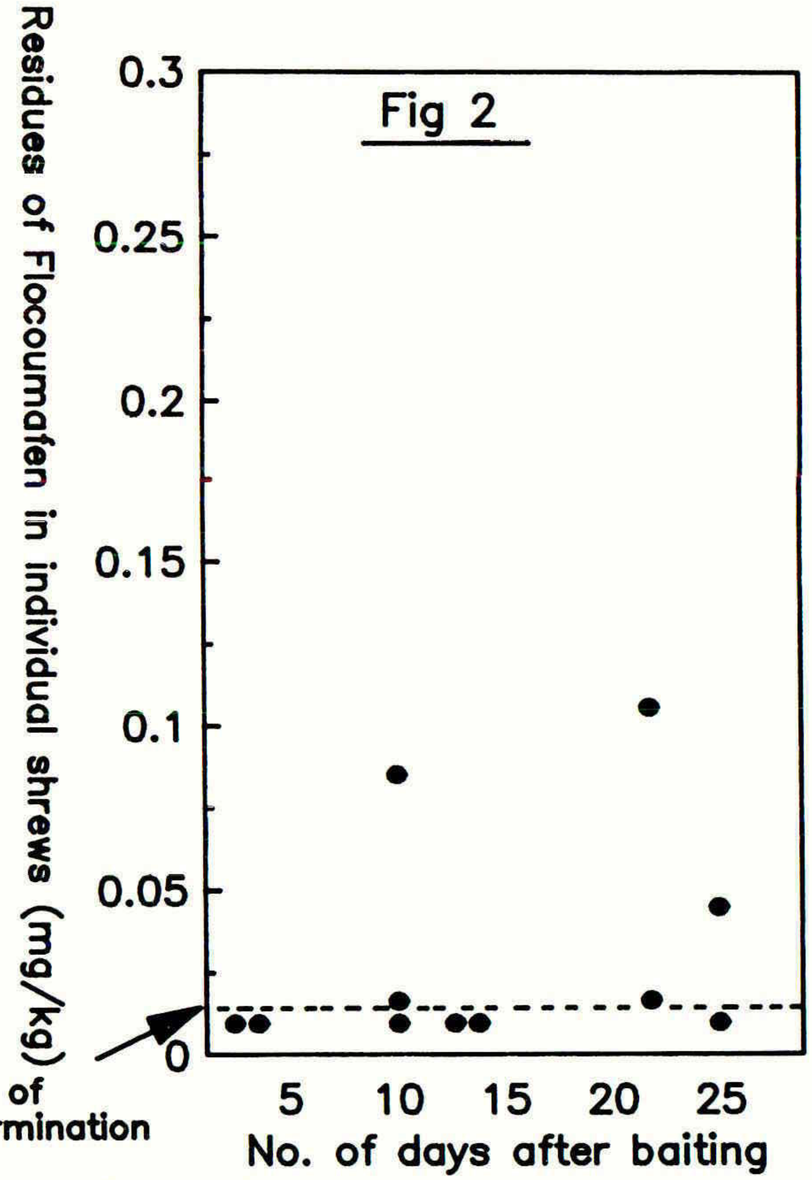
For a most susceptible dog to be affected it would need to eat 2.5 rats to obtain the equivalent of the LD50 of flocoumafen to this species which is in the range of 0.075 to 0.25 mg/kg (Johnson and Scott, unpubl.). If the mean residue level for this study of 0.36 mg/kg is taken a minimum of 8 rats would be necessary to affect the most susceptible strain of dog. Likewise, if a cat weighing 3 kg ate a rat of 200 g and with the highest residue level of 1.2 mg/kg, this particular cat would ingest .08 mg/kg of the toxicant. The LD50 of the compound to the cat is >10 mg/kg. (Johnson & Scott, unpubl.), and so this species is less likely to be affected than the dog.

The only non-target species collected in the village and rice fields during the trapping were shrews (Suncus luzonensis) (Figure 2). A total of 12 shrews were collected, 5 of them did not show residues and in the others the residues were low (.02-.11 mg/kg). This species is known to be

Flocoumafen residues in carcasses of three species of rodent pests (1) collected after two area wide and three spot appls. of 3.5 g Storm wax block baits



Flocoumafen residues in carcasses of shrews *(Suncus luzonensis)*



1 *Rattus rattus mindanensis , R. norvegicus + R. exulans*

an omnivore. They may have accumulated residues from eating blocks or from eating insects which had fed on blocks or rat carcasses. This species could therefore be at risk during flocoumafen baiting.

DISCUSSION AND CONCLUSION

The total residues of flocoumafen in a field population of rodents (n = 28) was highest shortly (2 days) after baiting (range <0.02-1.20 mg/kg, mean 0.36 mg/kg). Mean residues in rodents collected 10-25 days after baiting were much lower (<0.02-0.03 mg/kg). The total residues of flocoumafen in a non-target species, shrew (n = 11) ranged from 0.01-0.11 mg/kg (mean 0.03 mg/kg).

The results of the residue analyses confirmed the previous observations that secondary hazard arising from flocoumafen baiting was low (Hoque and Olvida, 1988). At the maximum residue level of 1.2 mg/kg, a 6-kg dog would have to eat between 2 and 6 rats to be able to ingest doses equivalent to the LD50 of flocoumafen to dogs (0.075 to 0.25 mg/kg). However, this observation must be tempered by consideration of the No Effect Level (NOEL) which is generally taken as 20% of the LD50 and accordingly reduces the permissable intake of affected rats. In previous observations on farmers' dogs at the trial site none of the dogs observed preyed on rodents. Dogs that are trained to hunt rats would generally just kill the rats and often are well fed animals. They may be more at risk if they feed directly on the bait. Previously only one out of 10 dogs attempted to eat the blocks (Hoque and Olvida, 1988), thereby suggesting that the majority of the dogs did not find the baits attractive. None of the domestic animals observed during the trial died due to the rodenticide. Only one case was recorded which involved a dog that had eaten an unknown number of blocks while the farmer was baiting his ricefields. As a precautionary measure, the dog was given 10 vitamin K tablets (10 mg) and checked daily for the next two weeks. No symptoms of poisoning were observed at any time and the farmer was happy that the dogs health had not been affected.

While a few domestic animals, dogs, chickens and ducks, may be potentially at risk from exposed baits, adequate concealment of the blocks should reduce even this small risk to a negligible level. Interestingly where there was a high activity of domestic animals, householders tended to completely conceal the baits. It was concluded that adequate concealment of blocks, the relative unattractiveness of STORM baits to domestic animals, and the low residues in carcasses, results in an acceptably low risk to non-target domestic animals around houses.

The baiting trial did not cause observable reduction on the wildlife population, birds, skinks, toads and frogs (Hoque and Olvida 1988). No dead birds were reported during the carcass search conducted at the trial site. The only non-target carcasses found during the study were two dead shrews (Suncus spp.). This observation coupled with the residue data indicate that this species may be at risk during flocoumafen baiting.

This residue assessment has completed the study of flocoumafen baiting in village and ricefields. The efficacy trial (Hoque and Olvida 1988) showed that flocoumafen performed well in protecting rice yields

from rodent damage. The results of carcass analysis now confirm the low hazard observed during the field trial to wildlife and domestic animals.

ACKNOWLEDGEMENTS

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RESIDUE- AND GROUNDWATER ANALYSIS: NEW TECHNIQUES

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ABSTRACT

An enzyme-immunoassay (EIA) was developed for the chlorinated hydrocarbon insecticide endosulfan. The EIA is based on antibodies raised against the diol of endosulfan coupled to a carrier protein. The limit of detection for endosulfan using this assay is 3 ng/ml. With this simple assay residues of endosulfan in different biological samples could be rapidly screened.

New regulations require groundwater analysis with limits of detection of 0.1 µg/l. There is therefore a need for an inexpensive and rapid multi-residue method suited to detect a broad range of pesticides. We are currently working with the AMD-technique (basically an automated hptlc), and our recent results show that this method is highly suited for groundwater analysis.

INTRODUCTION:

In order to fully assess the environmental fate of a pesticide large numbers of samples are analysed for both the pesticide and its metabolites. Ever increasing requirements by regulatory authorities necessitate analyses to lower levels, resulting in more time consuming and costly analyses.

A new regulation of the EC has been enacted by the FRG (Oct.1988, low level contamination of groundwater: 0.1 µg/l per single pesticide). Other European countries will follow. This means that pesticides producers have to adapt their methods to this low level. Often an extremely difficult task!

Full automation of these methods can be established but only with an initial large outlay of capital. Two new analytical techniques recently used in our laboratories could be used to analyse large numbers of samples while reducing resource and results in cost savings, Enzyme Immunoassay (EIA) and Automated Multiple Development (AMD).

The use of the simple inexpensive and highly automated EIA technique allows a rapid screen of the levels of the residues in samples like fruits, soil, water. Positive samples will be subsequently selectively analysed by other physico-chemical method as confirmatory analyses.

The AMD-technique is based on High Performance Thin Layer Chromatography (hplc). Up to 12 samples can be handled simultaneously. Potentially, this method seems highly suited to become a standard method in groundwater analysis.

EXPERIENCES

1 Enzyme-Immunoassay

Environmental analytical studies for residues of endosulfan and its degradation products have so far relied on conventional analytical techniques; eg. silica gel column chromatography and subsequent quantitative determination by infrared spectroscopy (Weinmann, 1970), or gas chromatography; the latter techniques containing a time consuming clean up stage (Zweig, 1960). The use of a simple and sensitive method would therefore be highly desirable. The application of immunological methods especially in the analysis of pesticides has been described in great detail by Hammok and Mumma (1980), and reviewed by Schwalbe-Fehl (1986).

Recently, immunological methods such as enzyme linked immunosorbent assay has been shown to be applicable to the field of environmental analytical chemistry. An enzyme immunoassay (EIA) has been developed at Battelle Frankfurt Laboratories for this insecticide and its degradation products. The EIA is based on antibodies raised against the diol of endosulfan by immunizing rabbits with a keyhole limpet haemocyanin (KLH) endosulfandiol conjugate (Fig. 1).

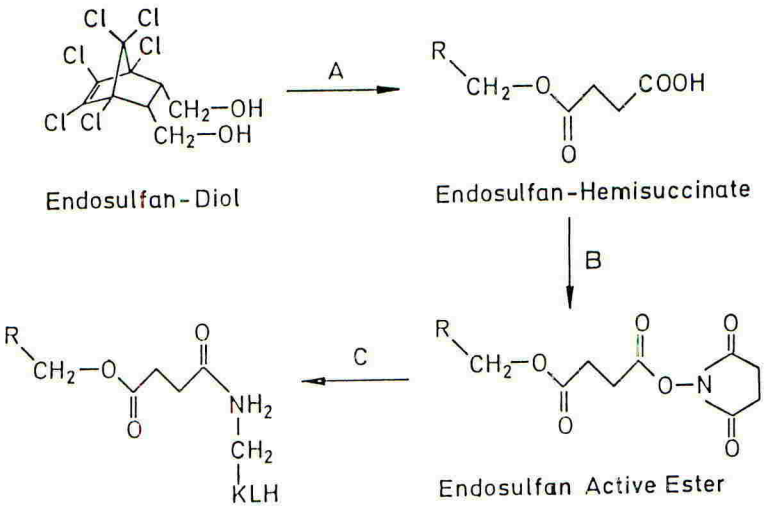

Fig. 1: Synthetic route to endosulfan protein conjugagte (A succinic anhydride in pryridine, (B) dicyclohexylcarbodiimide and N-hydroxysuccinimide, (C) Keyhole limpet hemocyanin (KLH)

With this method, endosulfan was detected in aqueous solutions at a level of 3 ng/ml without any sample extraction procedure (Fig. 2). The measuring range was found to be between 3 and 400 ng/ml. The cross reactivity of other similar

chlorinated hydrocarbon pesticides was tested. Only low cross reactivities were shown by Lindan, Alodan and Aldrin, whereas Endrin demonstrated a cross reactivity of 180 %.

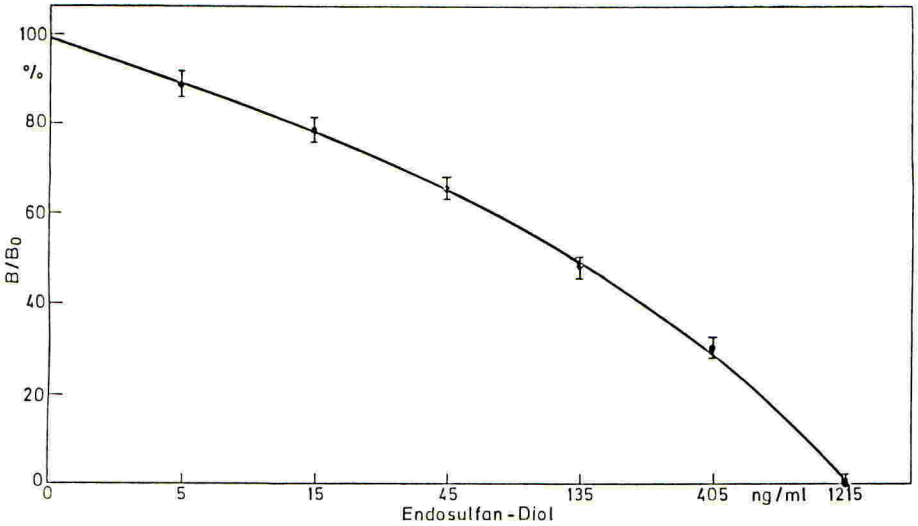

Fig. 2: Typical standard curve for endosulfan. The curve represents the mean and standard deviation of an 11 fold determination
B/B_0 = binding of the antibody to the coated microliter plate at concentration x of the the antigen divided by binding with no antigen in the assay

So far, the present study demonstrates the first development of an EIA-method to detect chlorinated hydrocarbon pesticide (Dreher et al, 1988). There is no doubt that this assay system could now be adapted to an even higher sensitivity and to residue samples from different biological matrixes, e.g. soil.

Our recent experiences with other pesticides of a very different chemical nature clearly demonstrate that the potential of this simple and cost-effective technique in the field of residue analysis of pesticides is just beginning to be realized.

2 AMD-Technique

To comply with the new regulatory requirements for groundwater analysis two different approaches are principally possible: development of highly specific methods for each single compound (or a small group of compounds) or development of universal multi residue methods suited to detect a broad range of different compounds.

Both scenarios have been investigated on several groups of pesticides (i.e. phenyl ureas, carbamate derivatives, methylcarbamates, N-heterocyclic compounds). The specific methods are based on glc and hplc-methods, whilst a multi-residue method for the same set of compounds has been developed on hptlc using the AMD experiment. (Burger, K., 1984).

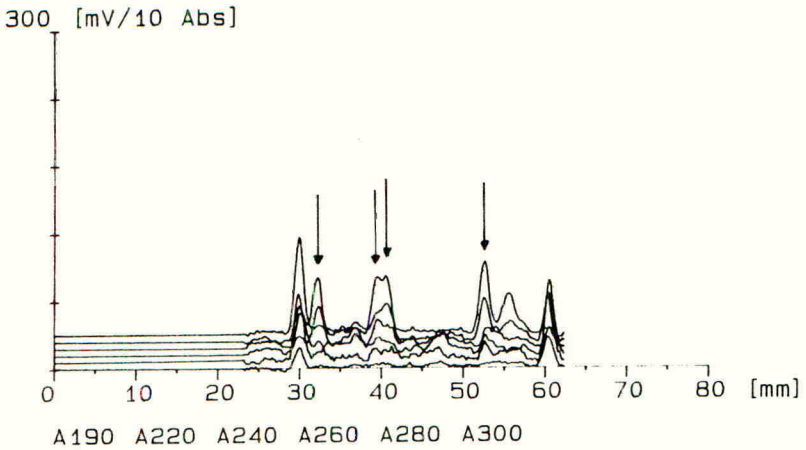

Fig. 3: Typical sample run of five (N-heterocycle type) pesticides Samples are detected an evaluated at 190, 220, 240, 260, 280, and 300 nm. 0.1 µg/l each, extracted from 1 l spiked water.

The general outline of the hptlc multi-method used in our laboratories is as follows:

sample
(1 l groundwater)
↓
solid phase extraction (C_{18})
↓
hptlc: Development with AMD equipment
↓
Detection and evaluation
(UV scanning at 6 wavelengths)

A typical chromatogram is shown in Figure 3.

Characteristics:
- One method for all compounds usually determined by either glc or hplc
- No transformation (i.e. hydrolysis) of the pesticide
- Sensitivity is usually about 0.05 µg/l
- UV detection at multiple wave length, even at 190 nm
- Many steps can be performed automatically (development, scanning, evaluation)
- Simple clean-up (solid phase extraction, evaporation)

Limitations:
- Lack of specificity if complex mixtures (15 compounds) must be analyzed
- Extremely polar compounds (i.e. carbamates) can only be determined at the 0.1 µg/l level because of interferring natural compounds (i.e. humic acids)

CONCLUSIONS

Both EIA and AMD have shown considerable potential in the field of residue analysis. EIA offers a cost effective technique which allows a large number of samples to the rapidly processed while AMD has been shown to have many advantages as a multi-residue method in groundwater analyses.

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IMPACT OF PESTICIDES ON NON-TARGET FUNGI OF PEACH TWIGS

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ABSTRACT

The mycoflora of peach twigs was qualitatively altered when a pesticide program (including treatments with copper oxychloride , thiram, captan, dinocap, benomyl and methomyl) was applied to cv "Baby Gold" peach trees during two consecutive years. Changes in the populations of non-target fungi were detected by the spot-frequency method. The dominant fungi on non-treated twigs were Cladosporium spp., Alternaria spp. and Penicillium spp. Fungal populations on treated-twigs were less diverse than on control twigs. Populations of Cladosporium spp. and Alternaria spp. were reduced by the applied pesticides, while species of Penicillium, particularly P. frequentans appeared most frequently on the sprayed twigs. The population decrease of the other species and the differences in the LC50 among species to each chemical could explain part of these results.

INTRODUCTION

Chemicals are used in pest-control programs for high-value crops such as peaches. In earlier studies attemps were made to determine the influence of spray treatments on the phyllosphere mycoflora of peach trees and indications were obtained that some fungicides have a wide spectrum of activity against non-pathogenic epiphytic fungi (Jailloux and Froidefond 1979). This impact of pesticides on non-target microorganisms constitutes one type of side-effect that is of ecological significance since the changes produced in the ecosystem could have favorable or/and unfavorable implications in the development of diseases (Hislop 1976) and on the food of non-target arthropods.

Previous studies on the epiphytic mycoflora of peach twigs showed that it includes beneficial fungi that, by antagonistic interactions with peach pathogens, could inhibit infection and pathogenesis (Melgarejo et al 1986). It is the practice in the major peach producing areas of Spain to make several fungicide applications to the trees each year, to control the different fungal pathogens. The study of the effects of these chemicals on microorganisms of the peach tree phyllosphere is basic to achieving the most suitable pesticide schedule for an integrated control strategy.

This study tries to determine the effects of a commonly used pesticide schedule on the different species of fungi present on peach twigs.

MATERIALS AND METHODS

Experimental field design

Our research was conducted for two years, 1986 and 1987, on a 5 year old peach orchard cv "Baby Gold" located in Montañana, Zaragoza, Spain. Two experimental plots, separated 10 m, were established in the orchard. Each plot contained a row of five trees. During the time of experiment, one of the plots received several phytotherapeutic treatments and the other, the control plot, was unsprayed. Pesticides used were: 3.7 g/l a.i. copper oxychloride, 1.6 g/l a.i. thiram, 1.25 g/l a.i. captan, 0.2 g/l a.i. dinocap, 0.25 g/l a.i. benomyl and 0.4 g/l a.i. methomyl (Fig. 1). Sprays were applied by a hand-held 17 l, hydraulic sprayer at about 10 Kg/cm^2. Trees were sprayed uniformily to the "run-off" stage. Three twigs of about 10 cm long per tree, were sampled at random 3-5 days after each treatment.

Evaluation of the fungal population

Total fungal populations were assesed by the washing twig method (Andrews and Kenerley 1978) with modifications. Twigs were shaken individually in 150 ml sterile, 0.01 M phosphate buffer, pH 7.1 for 30 min. Dilutions of the washings were then plated, in 0.1 ml aliquots, on the surface of potato-dextrose agar (PDA) and malt-extract agar (MEA), both with 0.5 g/l of streptomycin sulphate. After four days of incubation at 20-25 ºC in the dark, qualitative data on the frequencies of each species of fungi were obtained by the spot-frequency analysis (Andrews and Kenerley 1978). Ten fungal colonies were chosen at random from the undiluted replicates on 10 plates each of PDA and MEA under 6-12 x magnification. After microscopic examination one colony of each different type was isolated, purified, identified and stored at 4 ºC on PDA. Relative abundance of a particular microorganism was then determined by recording the percentage of colonies from which it was isolated.

Sensitivity of fungal isolates to pesticides

The sensitivity of several isolates of species of fungi which appear more frequently on twigs, which were more disturbed by treatments and which had potential for biocontrol Monilinia laxa (Aderh et Ruhl) Honey (Melgarejo et al 1986) to captan, dinocap, benomyl and methomyl was determined (Fraile et al 1986). The different chemicals (about 100 % a.i. technical material, except 88 % a.i. for dinocap) were dissolved in 1 % acetone and added to autoclaved, warm (40-45 ºC) PDA before plates were poured to give at least three different concentrations per chemical. Mycelial plugs were cut from the margin of actively growing, 7-day-old colonies, of each isolate on PDA and transferred to Petri dishes with PDA or PDA amended with chemicals. Four replicates were made for each concentration of chemical and fungal isolate. Plates were incubated for 10 days at 20-25 ºC in the dark and each day the percent-inhibition of growth was calculated by using the formula $PIG = D_1 - D_2 / D_1 \times 100$, where D_1 and D_2 were the diameter of control and the diameter of each isolate.

Mean data of percentages of the relative toxicity of different concentration levels of each a.i. towards each isolate were processed by the probit-analysis method (Finney 1971). The effective concentrations (LC) were calculated in mg/l.

RESULTS

Isolations

The dominant flora in pesticide-treated and control twigs are shown in Table 1. Fungal populations on treated twigs were less diverse than on control twigs (Table 1).

TABLE 1

Fungi isolated from pesticide-treated and control twigs(*)

From control twigs only	From treated twigs only
Lepthosphaeria coniothyrium Sacc.	Alternaria chlamydospora Mouchacca
Phoma sp.	Alternaria tenuissima Wiltshire
Penicillium ardesiacum Novobranova	Alternaria sp.
Nitchkea sp.	Penicillium decumbens Raper et Thom
Gliomastix murorum (Corda) Hughes	
Aspergillus candidus Link	

From both treated and control twigs
Cladosporium cladosporioides (Fres.) de Vries
Cladosporium herbarum (Pers.) Link per Fr.
Alternaria alternata (Fr.) Keissler
Alternaria ramulosa (Sacc.) Joly
Penicillium frequentans Westling
Penicillium chrysogenum Thom
Penicillium citrinum Thom
Penicillium purpurescens (Sopp) Biourge

(*) Only species isolated with a frequency of at least 15 % at any sampling date have been recorded.

Numbers of populations of the different species isolated from twigs varied along the year. Species on the genera Penicillium and Epicoccum were mostly isolated in late winter and spring while Fusarium and Aspergillus in summer. Genera Alternaria and Cladosporium were isolated all over the year although Cladosporium was more abundant on old twigs than on young twigs (Fig. 1 and data not shown).

Treatments had different influence on each species of fungi present on twigs. Treatments with thiram and copper oxychloride did not affect the mycoflora (Fig. 1). Treatments with captan, dinocap, benomyl and methomyl reduced populations of Cladosporium spp. and Alternaria spp. However, in July populations of Alternaria spp. were higher in treated than in control twigs. Penicillium spp. were isolated most frequently from treated twigs (Fig. 1). Penicillium frequentans was the species of the genus Penicillium predominantly isolated from control twigs throughout the year, except in July, when P. chrysogenum was mostly isolated. Populations of P. frequentans on twigs treated with captan, dinocap, benomyl and methomyl increased largely (Data not shown).

These effects of treatments were maintained 60 days after the last spray, except for Alternaria spp. (Fig. 1).

Differences of isolations in PDA and MEA were not observed.

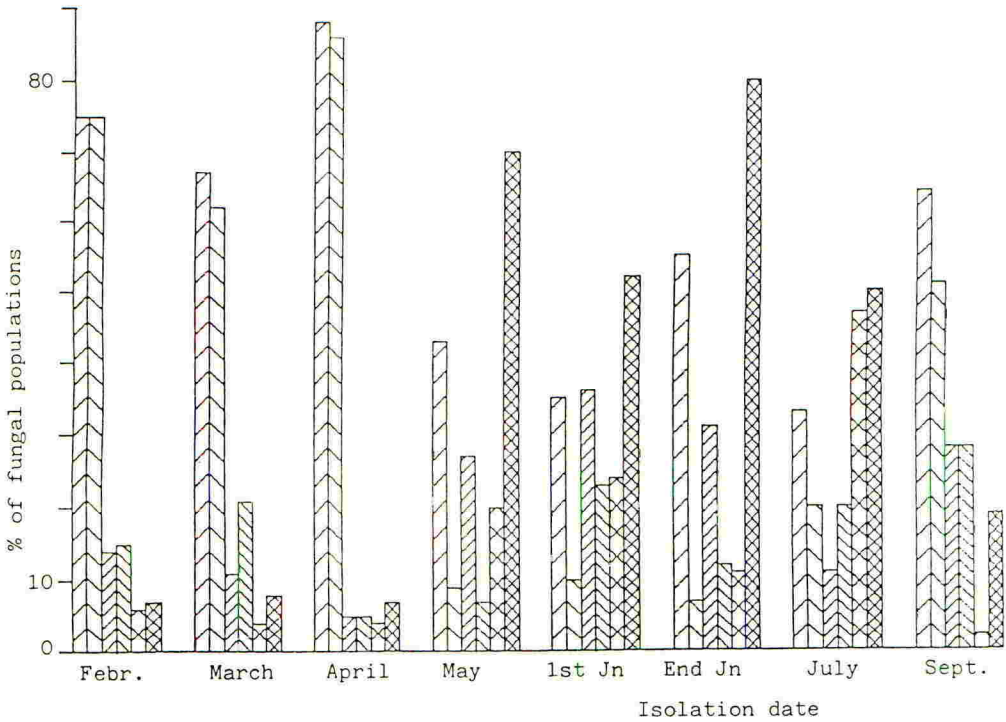

FIGURE 1:

Effects of treatments on populations of Cladosporium spp., Alternaria spp. and Penicillium spp. Data are the mean of the percentage of these genera of total fungi isolated from control and treated twigs during 1986 and 1987 in PDA and MEA: Cladosporium spp. on control twigs, Cladosporium spp. on treated twigs, Alternaria spp. on control twigs, Alternaria spp. on treated twigs, Penicillium spp. on control twigs, Penicillium spp. on treated twigs. Treatments were made 3-5 days before each sampling date and were: copper oxychloride (March), thiram (April), captan + dinocap (May), captan + dinocap (1st June), captan + benomyl + methomyl (End June) and dinocap + benomyl + methomyl (July).

Sensitivity of fungal isolates to pesticides

Data of estimation of concentration required to inhibit 50 % of test growing (LC50) are shown in Table 2. Sensitivity to chemicals of different isolates varied within each species. LC50 values of methomyl were higher than 10,000 mg/l for every isolate tested. LC50 values of captan were similar for all species, the smallest being for Cladosporium spp. However, LC50 values of dinocap were very different for each species, being the smallest for Alternaria spp. and the highest for Penicillium spp., Fusarium spp. and Aspergillus spp. Benomyl, was highly effective to every species except to Alternaria spp. (Table 2). Benomyl affected largely the genera Cladosporium, Aspergillus, Fusarium, and Penicillium, while dinocap did Epicoccum and Alternaria (Table 2).

TABLE 2:

Sensitivity of fungal species isolated from peach twigs during 1986 and 1987 to fungicides (*).

Species	Fungicides		
	Captan	Dinocap	Benomyl
Cladosporium cladosporioides	35-135	6-13	0.1-0.3
Cladosporium herbarum	35-40	10^{-6} 1-2	0.2-0.25
Alternaria alternata	115-425	10^{-6} -1	140-300
Alternaria tenuissima	150-400	0.25-0.75	125-130
Alternaria chlamydospora	425-1450	10^{-6} -1.7	100-120
Alternaria ramulosa	375-380	0.035-0.25	435-440
Epicoccum nigrum	125-265	10^{-6} -0.35	1.95-2.70
Penicillium frequentans	100-325	150-2900	0.015-0.025
Penicillium purpurescens	365-780	45-220	0.1-0.15
Penicillium citrinum	90-100	25-190	0.025-0.055
Penicillium chrysogenum	370-450	7-10	0.06-0.07
Aspergillus flavus	105-110	2100-2125	0.3-0.35
Aspergillus niger	150-160	1275-1300	0.3-0.35
Fusarium oxysporum	185-215	135-1500	0.6-0.8

(*) Data are the intervals of LC50 values (mg/l) for several isolates of each fungal species. LC50 values are estimated by the probit-analysis method (Finney 1971).

Data of LC90 values are not presented because the differences among values for each fungicide and each isolate are similar to LC50 values.

DISCUSSION

The epiphytic fungi on peach twigs were altered by the pesticide program. These results are in accordance with similar studies on different hosts (Andrews and Kenerley 1978, Jailloux and Froidefond 1979, Fokkema and Nooij 1981).

The pesticide schedule applied here is commonly used in peach orchards in Spain. Our studies, both evaluation of the effects of this pesticide-schedule in the epiphytic mycoflora of peach twigs as a whole in the field and sensitivity of different fungal isolates to the individual pesticides, permit a good approximation of what is occurring in orchards.

Although other authors observed differences in magnitude of effects of other pesticide programs on microflora sampled in different years (Andrews and Kenerley 1978), we have not observed significant differences ($p \leq 0.05$) between results obtained in 1986 and 1987, perhaps because the weather in both years was similar. Thus, we have presented the pooled of data of both years.

As occurred in other studies the fungicides captan, dinocap and benomyl were largely responsible for deppression of microbial populations (Jailloux and Froidefond 1979).

Species of Cladosporium were the most frequently isolated along the year, and also the most affected by treatments. The three fungicides captan, dinocap and benomyl inhibit the growth with LC50 values lesser than the concentrations applied in field treatments. Numbers of populations of Alternaria were also reduced with treatments in May and June due to its high sensitivity to dinocap. However, in July, after a treatment with benomyl and dinocap, populations of Alternaria spp. were increased. Benomyl affects Alternaria spp. in lesser degree than other genera (Table 2), being also better adapted to the summer weather conditions than hyaline fungi such as Penicillium (Melgarejo et al 1985). The fact of that populations of Penicillium spp., and in particular P. frequentans, were present more frequently on treated than on control twigs is perhaps due to its lack of sensitivity to dinocap and to its ability to exploit the twig niche better than the other species.

These results have both ecological significance and potential implications for control of the brown rot fungi. In addition to lowering fungal populations, the treatments altered the mycoflora composition. They inhibited antagonistic species to M. laxa such as E. nigrum (Melgarejo et al 1985). However, P. frequentans, another powerful antagonist to M. laxa (De Cal et al 1988) is able to increase its numbers in twigs as a consequence of the pesticide-program applied. Hence, the importance of looking such studies in implementing to integrated control strategies.

ACKNOWLEDGEMENTS

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THE USE OF KEY INDICATOR PROCESSES FOR ASSESSMENT OF THE EFFECTS OF PESTICIDES ON SOIL ECOSYSTEMS

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ABSTRACT

There is a need by registration authorities for standardized methodologies for the assessment of the effects of pesticides on soil ecosystems. Three complementary methods of assessing the effects of pesticides on key indicator processes in soil are: (i) a method of assessing the effects of pesticides on the respiration of undisturbed soil cores in large Barcroft respirometers; (ii) methods of assessing the effects of pesticides on invertebrate biomass by extracting invertebrates from pesticide-treated soil cores in high gradient Tullgren funnels, then recording their oven-dry biomass; as well as of assessing the effects of pesticides on microbial biomass, by counting cells or hyphae and converting to biomass or by measuring evolution of CO_2 from microorganisms killed by chloroform and calculating biomass from carbon evolution. (iii) a method of assessing the effects of pesticides on the breakdown of organic matter in soil using batches of fifty leaf disks, enclosed in nylon mesh bags, buried in soil, and assessed for breakdown at regular intervals using photometric or gravimetric techniques.

INTRODUCTION

Pesticides are biocides with a potential for killing or affecting the function of the diverse range of soil organisms that contribute to the biological processes that maintain soil structure and fertility (Edwards, 1973 a&b). The soil acts as a sink that pesticides eventually reach, whatever the method of application, and this contamination is reinforced by fall-out of residues from spraying operations or from the atmosphere in precipitation. It is important to avoid contamination of soils by chemicals that have serious effects on soil processes and thereby affect soil health, in order to maintain overall soil fertility.

The literature on the effects of chemicals on soil organisms and soil processes is voluminous. Moreover, it is extremely diverse, ranging from reports of the effects of chemicals on individual species of organisms, to those on overall populations of organisms and on individual biological systems using a great diversity of methods in both field and laboratory. Many experiments reported in the literature have been done *in vitro*, involve unrealistic doses of chemicals, methods of application, and modes of exposure, so it is virtually impossible to compare the results of experiments by different workers using the same chemical and reach a valid conclusion.

There have been a number of reviews on the effects of chemicals on soil organisms but most of these have addressed either effects on the soil fauna Edwards and Thompson, 1973; Thompson and Edwards, 1974; Eijsackers and van der Bund; 1980) or effects on the soil microflora (Greaves *et al*, 1978; Grossbard, 1976; Anderson 1978; Simon-Sylvestre and Fournier, 1979)

independently. Such approaches reflect a strict disciplinary approach to ecotoxicological research, which seldom considers the effects of chemicals on interactive processes that involve both the soil fauna and microflora and are fundamental to soil fertility and productivity. Edwards (1979 a, 1982) and Pimentel and Edwards (1982) addressed the issue of effects of pesticides on ecosystems, and Edwards (1983a) suggested that some soil processes could be used as indicators of soil health and fertility. Only one overall review published to date has considered the overall effects of chemicals on whole soil ecosystems, or even on both the soil fauna and microflora, to provide a common basis for evaluating environmental hazards caused by pesticides in soil (Edwards, 1988).

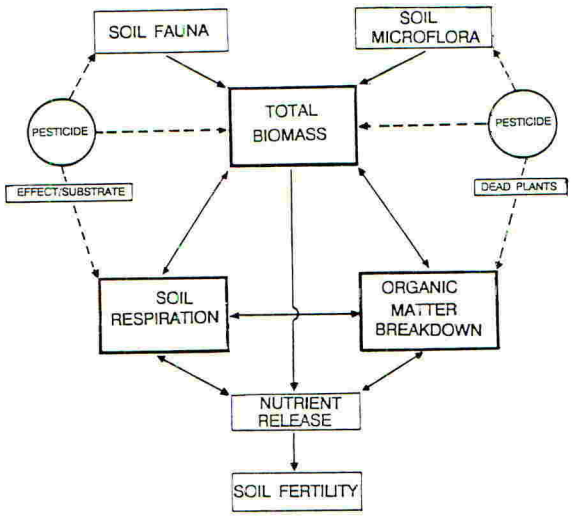

Figure 1. Impact of pesticides on key inputs and processes in soil ecosystems.

Registration authorities and international organizations have an urgent need for standardized or harmonized methodologies that will assess adequately the potential for hazard to soil ecosystems of any pesticide. Hence, there is a need for innovative methodologies involving key processes in soils. This has been recognized by international organizations such as the Organization for Economic Cooperation and Development (OECD), The European Economic Community (EEC), and the Food and Agriculture Organization of the United Nations (FAO) (Edwards, 1979b). These organizations have attempted to promote the use of more standardized testing methodologies, both in terms of "key indicator organisms" and "key soil processes" (Edwards, 1983b, 1984). The aim of the present paper is to describe key indicator soil processes that could be used in this way and give typical results from such methodologies. The processes involved are total soil biomass, organic matter breakdown and soil respiration (Figure 1).

METHODS

(i) Total Soil Biomass

The total soil biomass comprises both that due to microorganisms and that from soil invertebrates (Figure 2). It includes organisms which breakdown organic matter and incorporate it into soil and release nutrients, as well as pests and disease organisms and their antagonists. However, there is good evidence that a large total biomass is correlated so strongly with soil fertility that it could be a good overall indicator of adverse effects of pesticides (Anderson and Macfadyen, 1976; Dickinson and Pugh, 1974; Swift et al, 1979). The method involves taking undisturbed soil cores for biomass estimation, from field experiments, which compare pesticide-treated plots with untreated controls, within four weeks of application.

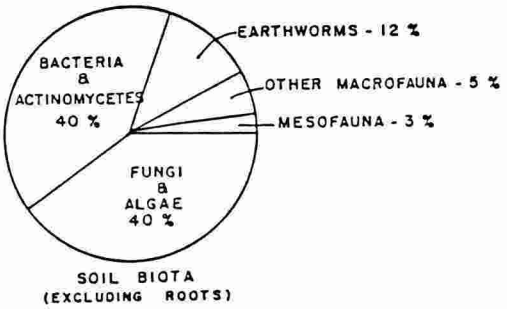

Figure 2. Biomass of soil organisms (From Odum, 1971)

a) Invertebrate Biomass

To assess invertebrate biomass, these animals must be separated from soil. The most efficient method of doing this is by use of high gradient Tullgren funnels (Edwards and Fletcher, 1970, 1972). Sixteen soil cores, 5 cm diameter by 15 cm deep, taken at random from field plots, provide representative samples for a pesticide-treated or control area in the field. The invertebrates are extracted from the soil samples in Rothamsted high gradient Tullgren funnels for four days, the alcohol evaporated, then oven-dried for 24 hours and weighed. The data are converted to mg dry weight of invertebrates per 100 g soil. The biomass of different functional groups of soil invertebrates can be calculated from regressions provided by Edwards (1967) if this information is required.

b) Microbial Biomass

The biomass of micro-organisms in soil can be determined by counting the number of microbial cells or length of mycelium, calculating the volume of such cells and correcting for specific gravity. For bacteria, the method involves grinding a soil sample, making a suspension in water, staining with fluoroscein isothiocyanate (Babiuk and Paul, 1970) and counting the cells. A similar method can be used for assaying the lengths of fungal mycelium in soil (Thomas et al. 1965). However, such methods are laborious and time-consuming.

A more direct method of estimating microbial biomass is that of Jenkinson (1966). He used a series of large-scale direct-reading Barcroft

respirometers that could accommodate 16 large soil samples (see later). After measuring the respiration of each 50 g soil sample, it is sterilized with chloroform and then re-inoculated with non-sterile soil. The carbon in the microorganisms that are killed by the chloroform is converted rapidly to carbon dioxide and the amount of oxygen consumed to produce this can be measured directly on the respirometer, if the CO_2 is absorbed by NaOH. If it is assumed that the flush of decomposition indicated by CO_2 production following fumigation, is due to the decomposition of killed organisms, and not to that of non-biomass organic matter the size of the flush should be related directly to the size of the soil biomass. Jenkinson derived the approximate relationship B = F/k, where B = soil biomass carbon (mg C per 100 g soil); F=flush of decomposition, i.e. CO_2-carbon evolved from fumigated soil minus that evolved by unfumigated soil incubated under the same conditions (mg CO_2-carbon per 100 g soil); and k = the fraction of the biomass carbon mineralized to CO_2 during the incubation following fumigation and inoculation with unfumigated soil. The value of k depends on microbial properties such as species of organism, phase of growth and nutritional status, and on soil factors such as aeration and pH. However, a value for k of 0.5 was proposed for all sections of the soil biomass; i.e. for a 10 day incubation at 25°C, B = F/0.3 (Powlson, 1975).

Typical microbial only biomass estimates based on the fumigation method range from 20 mg carbon per 100 g. oven dry soil in a long term arable soil to 330 mg carbon per 100 g in a highly organic soil under permanent grass. This is much higher when invertebrate biomass is included. The percentage of total soil carbon in the soil biomass ranges from 1.2 - 3.4%.

(ii) Organic Matter Breakdown

The breakdown of organic matter, its reincorporation into soil and release of mineral nutrients is a key process in the maintenance of soil fertility. The method used is based on cutting 5 cm diameter disks of filter paper or deciduous tree leaves e.g. oak, beech, or elm. Batches of fifty such disks are enclosed in nylon mesh bags of 5 mm aperture mesh (Edwards and Heath, 1963). Eight bags per pesticide treatment are buried in soil to a depth of 5 cm and dug up at regular intervals for assessment of the extent of breakdown. Losses due to invertebrates only, are assessed by washing disks and measuring their surface area by light transmission, after laying them out on a glass plate or moving translucent band. The amount of light passing through them is measured on a photoelectric cell and the data recorded. Each measurement was compared with that from an intact set of 50 disks. A more recent method of assessing disappearance is by the use of digital image analysis using a computer scanning system. After each measurement, disks are replaced in the mesh bag and reburied. For assessment of combined microbial and invertebrate degradation sets of disks from treated and control plots are washed, oven-dried and weighed. Sets of disks are buried in control plots and all pesticide- treated plots (Edwards and Heath, 1963 a&b, Heath, et al. 1964, 1966., Edwards, 1983,1988).

(iii) Soil Respiration

The method used is modified from one developed by Jenkinson and Powlson (1976). The apparatus uses a series of 16 large-scale Barcroft respirome- ters. Each respirometer flask takes a 50 g soil core. The

carbon dioxide produced is absorbed in 20 ml NaOH and the oxygen uptake measured directly manometrically. Small soil samples of moist soil weighing 25 g are treated with a pesticide using a fine, calibrated chromatographic spray, in suffi-cient water, to bring them to 55% of their water-holding capacity. Control samples are sprayed with only water to the same water-holding capacity. Two replicate samples are used for each treatment. The soil samples are incu- bated for 10 days at 25°C in the dark. Oxygen uptake in each respirometer is recorded daily. An alternative is to take small undisturbed soil samples from pesticide-treated and untreated field plots and intervals after treatment and assessing their respiration at 55% of water-holding capacity.

RESULTS

Soil Biomass

The overall results of the effects of diazinon and carbaryl on soil biomass are given in Table 1.

Control	Diazinon (2 mg/kg)	Carbaryl (2 mg/kg)
125.3 (±13.9)	109.9 (±10.8)	74.5 (±6.9)

Table 1. Effects of two pesticides on soil biomass (mg/100 gm soil)

Organic Matter Breakdown

The loss of leaf area for disks in each bag can be calculated as a percentage of the original leaf tissue (50 intact disks) and the results from 8 bags per treatment combined for an overall percentage disappearance, and a standard error calculated. The rate of breakdown in soil treated with a particular pesticide can be compared with that in an untreated control soil of the same type. Assessments are continued every 2 to 4 weeks to follow patterns of breakdown for an overall experimental period of 2-6 months (length depending on persistence of the pesticide) and results combined to give overall data. Typical results for the effects of two insecticides, diazinon and carbaryl, are given in Figure 3.

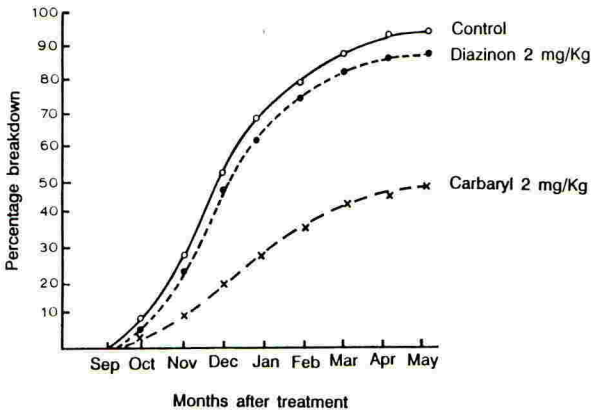

Figure 3. Effects of two pesticides on organic matter breakdown.

Soil Respiration

The experiment was run for a minimum of 21 days with manometric readings taken every four days at approximately the same time. This gave a direct measure of oxygen consumption. Data can be calculated as mg O_2/50 g soil or mg CO_2/50 g soil. Bulking of data can provide an overall assessment of depression (or stimulation if the pesticide provides a microbial substrate). Typical results for the effects of diazinon and carbaryl are given in Figure 4.

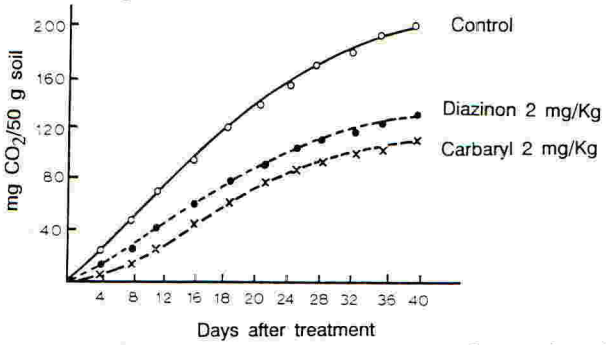

Figure 4. Effects of two pesticides on soil respiration.

DISCUSSION

The three methodologies give separate assessments of the effects of a pesticide on three closely-linked soil processes. Each methodology is open to some criticism. For instance, although the organic matter breakdown assessment gives consistent results and provides the opportunity for assessing the separate components of microbial and faunal contributions to decomposition, it has been suggested that the close juxtaposition of leaf disks in a mesh bag may alter the conditions for breakdown. Nevertheless, since the exposure of the disks is the same in both treated and control soil, the comparison of data should be valid. The respiration data can also be criticized justifiably. Disturbance of soil can change its respiration. Moreover, since the CO_2 is absorbed, the measurements of oxygen uptake are made in an atmosphere lacking freely available CO_2 which may modify the activity of the microflora (e.g. autotrophs). Finally, the microorganisms can use pesticides as substrates, so that if large doses are applied, the overall effect is a balance between the decrease in oxygen uptake due to the pesticide and the increase based on its use as a substrate. Nevertheless, if large undisturbed soil samples are used as was suggested by Parkinson and Coups (1963) and modified in the apparatus devised by Jenkinson and Powlson (1966) and low recommended doses of pesticides are used, the technique makes a valid comparison of the effects of a pesticide on soil respiration. Finally, there are no absolute methods of assessing faunal and microbial biomass. For invertebrates, although the method described is the most efficient currently available, no method of separating invertebrates from soil is 100% efficient. Similarly, neither the counting technique for microorganisms described nor the fumigation method of assessing microbial biomass are very efficient and may involve considerable errors. Nevertheless, the use of a standardized technique for comparative studies makes such data much more valuable. Where all three methodologies indicate a major impact of a pesticide, it could be grounds for serious consideration on its continued use.

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REDUCING PESTICIDE DRIFT INTO THE HEDGEROW BY THE INCLUSION OF AN UNSPRAYED FIELD MARGIN

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ABSTRACT

Two approaches are described for the forecasting of annual deposition rates of pesticide drift into field margins. Firstly, the cumulative annual level of deposition is calculated from experimental results which measured the proportion of the applied spray impinging on the hedgerow. Secondly, the maximum and minimum boundaries of annual drift deposition are estimated from data collected in an assessment of spatial variation in drift. Both techniques reveal the potential for reducing drift by the use of unsprayed headland strips.

INTRODUCTION

In order to evaluate the potential biological impact of spray drift on the hedgerow flora and fauna the level of pesticide deposition to which they are exposed must be assessed. It would be impractical to measure spray drift during each spray application in an annual cropping season; it is therefore necessary to forecast drift from a limited number of experimental samples. This paper outlines two approaches that might contribute towards these forecasts. These techniques can be used to evaluate the potential for reduced pesticide impact of conservation headlands (Sotherton et al., 1988), they may also help to focus attention on groups of the non-target flora and fauna that are at risk and determine the levels and combinations of pesticide to which they might be exposed experimentally.

MATERIALS AND METHODS

Cumulative annual drift deposition in hedgerows

The drift sampling took place during an autumn insecticide application to a winter barley crop. Application was made via an Everard tractor mounted sprayer fitted with a 24 m spray boom and albuz 110° flat fan nozzles calibrated to apply at a volume rate of 200 l/ha^{-1}. In order to detect drifting spray, fluorescein tracer was added to the spray tank to give a solution concentration of approximately 0.005% w/v. (Cook et al., 1986). A variety of targets of known collection efficiency were used. In this example, data collected from plastic drinking-straw targets (6.0 x 0.37 cm) was used. These were placed, in groups of four, at heights of 0.5, 1.0 and 1.5 m on 10 canes, set at 1 m intervals along the hedgerow. Following spray application, the straws were washed individually in 5 ml phosphate buffer and the level of spray deposition estimated using a calibrated spectrofluorimeter. Grasses at the hedgerow bottom were extracted in a similar way and the deposition rate per unit area estimated from measurements of the surface area of each sample following washing. Detailed meteorological records were taken during the spray application; the results of the complete study, including droplet size analyses are to be presented elsewhere. The experiment was carried out during a conventional spray pass, and with the boom at a distance of 6 m from the hedgerow.

The mean deposition rate of the pesticide formulation (in $\mu l/cm^{-2}$) at each of the four sampling heights was calculated and expressed as a percentage

of the theoretical maximum spray deposition per unit area. This value could then be used to estimate the mass of pesticide (in ng/cm^{-2}) that might be expected to land in a hedgerow following any application. The application regime for the experimental field was used in this example to provide an estimate of the cumulative deposition over a whole cropping season.

Variation in hedgerow drift deposition during individual applications

A simplified methodology was devised whereby the variation in drift deposition during individual sprays could be calculated. The targets and other components of the sampling system were supplied in kit form for use on a series of occasions throughout a summer spray campaign to cereal crops. 30 canes were set at 1 m intervals along "fully sprayed" or "conservation headland" field margins and glazed photographic paper collectors were placed on supports at the hedge bottom, 0.4 m, 0.8 m and 1.2 m above ground level. Spray application was carried out starting at least 50 m before and ending at least 50 m after the group of collectors. The spray formulation was labelled with fluorescein tracer at approximately 0.01% w/v. a sample being taken to calibrate the spectro-fluorimeter system prior to and following the assessment. Immediately after application the samples were washed off in phosphate buffer and placed within labelled vials. In the example given, the maximum and minimum values of drift detected in a single field experiment are given; full details of this work will be provided elsewhere. The "fully sprayed" regime in this example consisted of application to a 6 m margin strip followed by a full 24 m spray swath, the "conservation headland" regime left the 6 m strip unsprayed and was therefore for a single 24 m swath.

RESULTS

Table 1 gives the predicted levels of drift deposition over a complete annual spray regime in a winter cereals crop. The conversion factors for deposition at each stratum (ground, 0.5 m, 1.0 m and 1.5 m) were 0.67%, 0.46%, 0.31% and 0.22% in the fully sprayed headland and 0.03%, 0.12%, 0.12% and 0.16% in the conservation headland. This percentage conversion was applied to each pesticide spray in the regime and the mass of pesticide deposited calculated from the concentration of the formulation and the deposition rate in each position. In this example, there would appear to be a considerable reduction in deposition rate when the unsprayed headland was included.

Table 2 gives the maximum and minimum levels of drift deposition that occurred at each height in the fully sprayed and conservation headland treatments of the spatial variation study, again expressed as a percentage of the maximum field deposition rate. These values were used in conjunction with the spray regime in Table 1 to calculate the extremes of drift deposition that might occur. Table 3

DISCUSSION

These data provide, for the first time, a forecast of the annual level of exposure of a hedgerow to spray drift within one treatment regime. In particular they highlight the 70-75% reduction in drift that occurs at most heights in the hedgerow by the inclusion of an unsprayed 6 m headland strip. The data in Table 1 refer to a single experimental study from which the annual predictions were made. An independent check that these fall within the range of possible drift levels is provided by Table 3, where the variation in drift deposits over a 30 m transect was used to establish boundaries of potential deposition rates. The first example was within or

TABLE 1: Predicted drift deposition rates of pesticides at different levels in a hedgerow throughout an annual spray regime in winter cereals. Deposition estimated from a single experimental study at the time of autumn insecticide application. 1a Fully sprayed field margin. 1b Conservation headland

Chemical class	Active ingredient	Application date	Application rate ha^{-1}	Pesticide depositon in ng/cm^{-2} at different positions in the hedgerow				Mean hedgerow deposition rate per annum of each chemical class (ng/cm^{-2})
				0	0.5	1.0	1.5	
a) Herbicides	isoproturon	12.11.86	1500 g a.i. in 100 l.	99.9	68.9	47.0	32.4	85.6
	diclofop-methyl	12.11.86	570 g a.i. in 100 l.	37.9	26.2	17.8	12.3	
Fungicides	prochloraz	6.3.87	300 g a.i. in 30 l.	20.0	13.8	9.4	6.5	17.6
	propicona-zole	10.6.87	125 g a.i. in 200 l.	8.3	5.7	3.9	2.7	
Insecticides	fenvalerate	12.11.86	17.5 g a.i. in 100 l.	1.2	0.8	0.6	0.4	0.8
			TOTALS	167.2	115.4	78.7	54.3	104.0
b) Herbicides	isoproturon	12.11.86	1500 g a.i. in 100 l.	4.0	18.6	18.5	24.3	22.2
	diclofop-methyl	12.11.86	570 g. a.i. in 100 l.	1.5	6.7	7.0	9.2	
Fungicides	prochloraz	6.3.87	300 g a.i. in 30 l.	0.8	3.5	3.7	4.9	4.6
	propicona-zole	10.6.87	125 g a.i. in 200 l.	0.3	1.5	1.5	2.0	
Insecticides	fenvalerate	12.11.86	17.5 g a.i. in 100 l.	0.1	0.2	0.2	0 3	0.2
			TOTALS	6.7	29.5	30.9	40.7	27.0

TABLE 2

Predicted maximum and minimum drift deposition rates of pesticides at different heights in a hedgerow expressed as a percentage of the maximum field deposition rate. (Each value selected from 30 independent measurements).

		Target height above ground (m)			
		0	0.4	0.8	1.2
Fully sprayed headland	min.%	0	0.22	0.27	0.30
	max.%	0.74	11.49	7.07	4.68
Conservation headland	min.%	0.03	0.13	0.25	0.30
	max.%	0.53	1.36	2.70	3.72

TABLE 3

Predicted maximum and minimum deposition rate (ng/cm^{-2}) per annum for each class of pesticide from example in Table 1, using data from study of spatial variation in drift. (Data presented as mean deposition rate over all target heights).

	Herbicides		Fungicides		Insecticides		Total	
	min	max	min	max	min	max	min	max
Fully sprayed headland	38.28	1292.48	7.88	265.38	0.33	10.93	46.49	1568.79
Conservation headland	71.22	429.78	7.48	88.28	0.29	3.63	78.99	521.69

just below the lower extremes of the range provided in the second study. There could be several explanations for this however, it was noted that the mean wind speeds at 1 m in the two studies were 6.4 m/s and 4.0 m/s respectively; the higher windspeed in the former study may have lifted the spray drift cloud above the target zone.

These methods serve to provide a first, testable approximation of the exposure of field boundary flora and fauna to drift in one spray regime. The data may be used to identify organisms at risk of suffering side-effects and the zones of the hedgerow that receive the highest doses. They may also isolate the times of greatest potential impact when linked to the records of this or other spray regimes. Thus the potential levels of exposure to particular combinations of products can be estimated and used experimentally to examine biological effects.

The limitations of these techniques as a whole derive from the inherent variability in drift deposition. In these examples, the first experiment gave a mean drift deposit at or below the lowest level detected in the second study, indicating that a substantial amount of replication will be required to establish reliable boundaries. The data may also be specific to the type of hedgerow and the application equipment used in the investigation and alternatives of both of these should also be studied. Other important constraints on annual forecasting from a limited number of measurements include the degree of filtration of the spray cloud that is given by a growing crop; more spray being intercepted in a mature crop than a seedling one. These studies should therefore be repeated at different crop growth stages.

Given these limitations, these forecasts at least provide a basis for the experimental examination of the biological effect of spray drift on non-target flora and fauna of hedgerows. The experimental results reveal the potential for using unsprayed field boundaries as a method of reducing drift deposits in non-crop, field margin areas.

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A PHOTOGRAPHIC METHOD FOR ASSESSING THE RECOVERY OF AQUATIC MACROPHYTES IN DRAINAGE CHANNELS

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ABSTRACT

A simple photographic method is described for assessing the recovery of the aquatic plant species of a drainage channel after herbicide treatment with dalapon, relative to control sections. The recovery of the species was photographed monthly over the period May to October for three years following treatment. Photography of two contrasting species, Sparganium erectum and Alisma plantago-aquatica, is used to illustrate the value of the technique. The advantages and disadvantages of the method are considered.

INTRODUCTION

The effectiveness of aquatic herbicides is usually assessed on changes in the biomass or percentage cover of the target species. The success of treatment is inversely related to the amount of a target species developing in the recovery phase, however, there is little information on the nature of the recovery of either target or non-target species. For example, does the regrowth of the plant depend upon rhizomes, seeds or other propagules? A more comprehensive understanding of this process is important in improving the effectiveness of herbicide usage whilst minimising damage to other species, for example by paying more attention to flowering and fruiting stages in those species where regeneration by seed is significant.

A simple photographic technique using colour transparencies taken from the top of a stepladder is described for use in drains to record accurately the spatial changes in the stands or individuals of selected aquatic species before and after treatment. Although photography has been used in assessing terrestrial vegetation (e.g. Wimbush, Barrow and Costain, 1967) and in crops (e.g. Garcia et al., 1986; May, Harvey and Wright, 1985), the technique has not been widely used with aquatic plants. A regular tetrahedral balloon for assessing relative weed cover and weights of plants in a chalk stream was employed by Edwards and Brown (1960), and Thurling (1987) used a model plane carrying a remotely controlled camera for low level surveys of the aquatic vegetation of ponds. Both these techniques relied on specialised equipment and were also difficult, if not impossible to use in windy conditions. The use of a stepladder overcomes these limitations and is to be particularly suitable for the drain habitat.

The technique described was established as part of the methodology for assessing the impact of dalapon treatments on the aquatic macroflora of a drain on Romney Marsh, Kent. The width of the channel at water surface was 3.5 to 4.5m and the depth of water in the centre of the channel was 0.7 to 1.0m. Emergent stands of Sparganium erectum and Glyceria fluitans dominated one or both margins of the drain with a dense floating mat of Lemna and filamentous algae developing during the spring. Extensive beds of Zannichellia palustris and Potamogeton spp. were displaced by Ceratophyllum demersum and Lemna trisulca as the floating mat took over the water surface. The emergent vegetation of the channel was sprayed with dalapon (2,2 dichloropropionic acid) using a knapsack sprayer on 17 July 1985 and 15 July 1986. A Bradshaw weed cutting bucket was used to remove dead and decaying vegetation from the channel on 16 October 1985 and 15 September 1986, an operation known as brushing.

METHOD

A lightweight aluminium stepladder was placed on the top of the bank ensuring that all four feet of the ladder were in contact with the ground. When the soil was soft the ladder was pushed firmly into the soil in order to prevent it toppling over. A photograph was taken of a sample section from a standing position at the top of the ladder. In very windy weather the ladder was set up and ascended immediately to prevent its being blown over, and lowered to the ground after photography. The height of the camera above the water surface was 3.5 to 4.5m and the angle of the photograph relative to the mid point of the water surface was about 50^{o}. The photographs were taken using an Olympus OM2 camera with a lens of 35mm focal length using 36 x 24mm negative size colour transparency film. The 35mm lens comfortably included a 5m sample unit. Each sample unit was marked on one side of the drain using reference marker posts and a tape measure. A polarising filter was used to reduce the reflection from the water and to give a view of submerged plants. Photographs were taken and the percentage cover of each species recorded once a month during the growing season (May to November) over a period of three years (1985-87). The distribution of individual species was subsequently mapped out from the colour transparencies projected on to a screen (Figures 1, 2 and 3).

The level of water in the drain varied during the year and a procedure was devised to estimate the width of the channel at the water surface to enable accurate comparisons between a sample section at different sampling times. The procedure was based on the relationship between the actual width of the channel and the width as measured from the photographic image at a known magnification.

RESULTS

The photography was successful in providing a permanent record of the aquatic vegetation in the drain which clearly showed the gross changes in the emergent and floating vegetation; the detailed changes in the distribution of certain species which in some instances could be related to

individual plants; and the condition of the vegetation, e.g. flowering and senescence.

The application of the method can be illustrated by considering the results for two species with different growth strategies from certain 5m sample units: Sparganium erectum L. which develops dense emergent stands, and Alisma plantago-aquatica L., a species typically growing as individual plants. Neither plant is able to root in water depths greater than 50 cm. Sparganium erectum, a large plant, was easy to recognise in the colour slides throughout the whole year including its senescense. Figures 1 and 2 represent the changes in the distribution of Sparganium in an untreated section of the drain and a section treated with dalapon and brushed in both 1985 and 1986. In the control section Sparganium (Figure 1) developed into a substantial and, in the third year, continuous belt along one side of the drain. As the stand developed out into the body of the drain, Sparganium used the floating mat of filamentous algae and Lemna as a raft to support new growth.

In the treated section, Sparganium had already developed a continuous belt along the drain side (Figure 2). The dalapon effectively controlled this stand and there was little evidence of regrowth in the second year. The small amount of regrowth observed regenerated from rhizomes not killed by the herbicide rather than from seeds, a pattern observed in other sections of the drain. The Sparganium rhizomes can account for anything between 30 and 65% of the fresh weight biomass (Aario, 1933; Hejny, 1960).

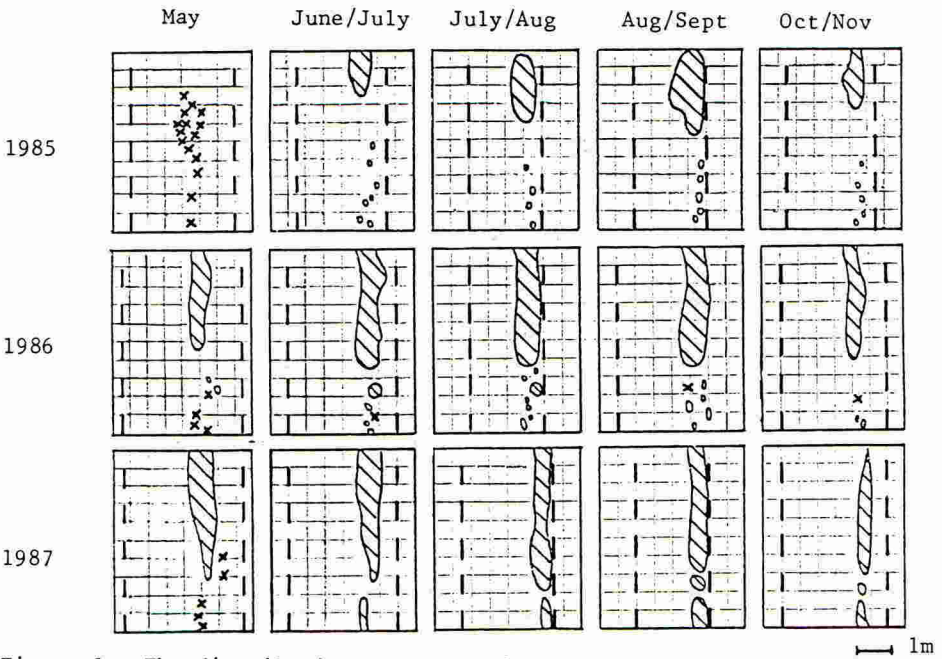

Figure 1. The distribution of Sparganium erectum in a 5m sample unit of drain (sample 3.3b) (X = an individual plant; --- = margin of drain).

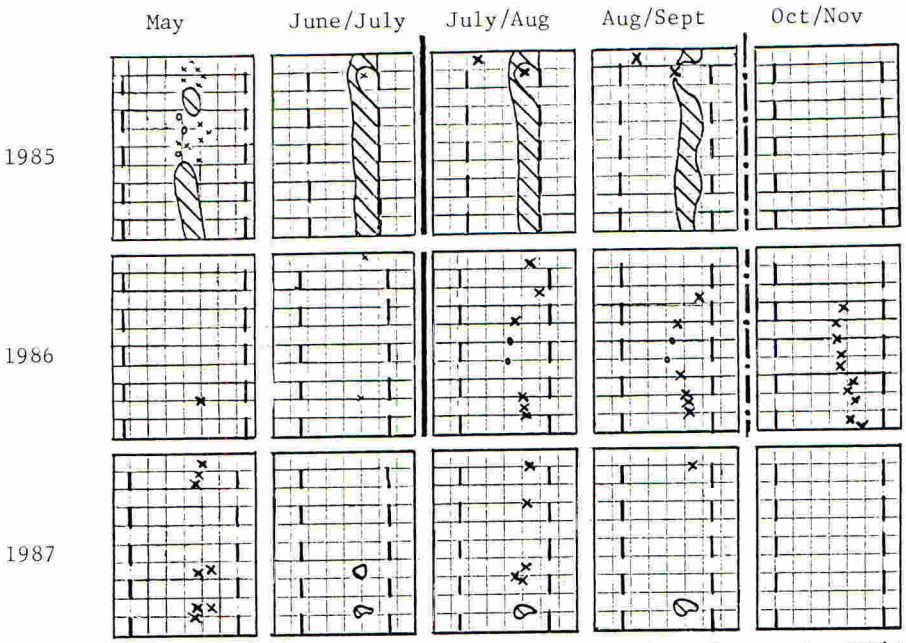

Figure 2. The distribution of Sparganium erectum in a 5m sample unit of drain (x = individual plant; --- = margin of drain; —— = dalapon treatment; ·-·-· = Bradshaw brushing).

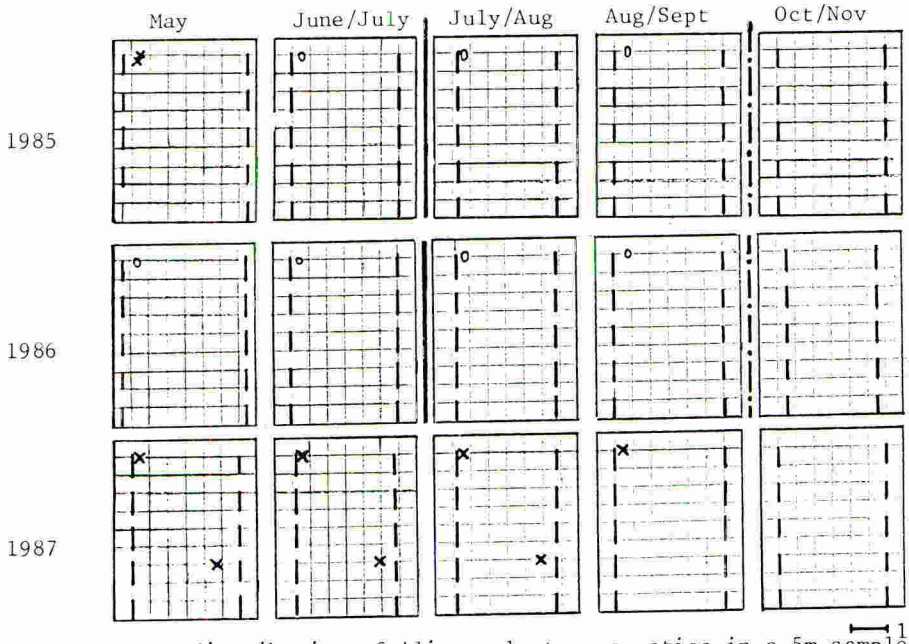

Figure 3. The distribution of Alisma plantago-aquatica in a 5m sample unit of drain. (For legend see Figure 2)

The distribution pattern in year three (1987) was similar to that of year two and it is difficult to determine the impact of the 1986 dalapon treatment. Regeneration was from the same rhizomes as in year two.

Alisma plantago-aquatica, occurred in the drain as individual plants (Figure 3). These plants were occasionally very small in the early stages of development and it was necessary to combine the use of recording sheets of species cover data and careful inspection of the slide to ensure no plants were missed. Alisma was unaffected in the year following herbicide treatment, regenerating from the underground parts of the plant which can account for about 40% of the fresh weight biomass (Aario, 1933; Hejny, 1960). Alisma growing in control sections exhibited a similar pattern with individual plants dying back over the winter and reappearing the following spring in exactly the same spot.

DISCUSSION

The photographic record of the aquatic vegetation in the drain provided an important aid in describing the recovery of the plant species after herbicide treatment and in comparing patterns of recovery with the growth in control sections. This has been illustrated with Sparganium and Alisma, and could be extended to other species along the whole 500 m length of drain used in the investigation.

The method was easy to use for the emergent vegetation, e.g. Sparganium and Typha, and floating vegetation, e.g. Callitriche but was of little value in describing the submerged species much of which was obscured by the floating plants. The ladder was light to carry around and the technique was used in very windy weather. The angle of 50^{o} gave a more detailed view of the near side bank than the far side bank and some plants were obscured due to emergent growth. These problems could have been reduced by photographing the drain from both sides.

The use of reference markers was essential and could have been extended to include markers on the other side of the drain and even permanent markers for the 5m sample lengths. Even with careful use of a tape measure there was some variation between sampling occasions for some sample sections. The polarising filter was important in removing most of the reflection from the water surface though dull weather necessitated using a faster film. This was unfortunate as it was preferable to use the same type of film throughout to aid interpretation of the slides. The 35mm lens was ideal for this study though for larger areas a lens with a wider angle would be needed. This could give distortion at the edges of the slides.

The recording of the percentage cover of the species present in each 5m sample section was necessary to provide a check for those species which were difficult to see on the slides. These cover data can be supplemented with notes to aid interpretation.

Four limitations were identified. The method was unsuitable for use in very wet weather, a problem which could be overcome by using a waterproof camera or camera housing. Shadows cast early or late in the day obscured plants and hindered interpretation. The method is best used in the middle part of the day. The profile of the bank created difficulties in maintaining the same distance between the camera and the water surface for each sample section. For any one sample section this distance was the same for different sampling occasions. Finally, some species were difficult to identify, or distinguish from other species, a particular problem for the inexperienced interpreter. On the other hand, photographs of excellent quality can be taken by a technician which can then be interpreted by an expert.

Photographic material such as that produced in this investigation could be valuable in the longer term should a site, or sites, be used again as part of another investigation.

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THE SHORT TERM EXPOSURE OF NON-TARGET INVERTEBRATES TO PESTICIDES IN THE CEREAL CROP CANOPY.

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ABSTRACT

Techniques for estimation of the direct and residual exposure of non-target invertebrates to pesticides in the cereal canopy are described. The direct exposure techniques were based upon the use of soluble and particulate fluorescent tracers. Residual exposure was estimated by the use of a field bioassay technique carried out at different levels of the crop and on the soil. The techniques demonstrated the variation in exposure that different species may be exposed to and provide some indication of the species most at risk following pesticide application.

INTRODUCTION

The effects of pesticides on non-target invertebrates, at the time of spray application, will be mediated by a variety of biological and operational factors (Jepson, 1988). This paper describes a research project which aims to quantify the direct contact and residual components of exposure to pesticides on a range of beneficial species in cereals. The data are primarily intended to provide a quantitative expression of the direct mortality of different species, to distinguish this from effects such as repellency or dispersal following food depletion. This may serve to identify species at risk of suffering long-term side-effects or harmful chemicals or usage tactics. The data may also however, provide an insight into the mechanisms of selective pesticide usage tactics within rational pest management programmes for arable crops.

MATERIALS AND METHODS

The methods below attempt to provide answers to the following questions.

(1) Are insects inhabiting different levels and positions in the crop canopy, potentially exposed to different levels of direct spray uptake?

(2) Are insects walking on sprayed plant or soil surfaces potentially exposed to different levels of uptake and to what extent is this affected by the choice of active ingredient?

Spray deposition in the crop canopy

The pattern of spray deposition in a mature cereal crop was measured using a technique adapted from Jepson *et al.*, (1987). Fluorescein fluorescent tracer (0.05% w/v + 0.1% wetting agent) was applied by tractor mounted and Azo gas-powered hydraulic sprayers via flat fan nozzles at rates equivalent to 200 l/ha^{-1}. The crop density was 336 tillers m^{-2} and application via tractor and plot sprayer took place on 18:07 and 31:07, 1988 respectively. Following spray application, samples were taken from the foliage; 25 whole ears, 25 whole flag leaves, 25 whole first leaves and 25 flag leaf sections placed across 10 cm glazed tiles at ground level were placed in individually labelled vials containing 10 ml of phosphate buffer solution for 8 hours. The samples were removed and the volume of tracer released into the buffer was determined by a comparison of the emission level of the solution at 490 nm in a fluorescent spectrophotometer with a

standard calibration curve obtained from measured amounts of original spray solution placed in a known volume of buffer. Data were converted to volume of tracer (in µl) per cm^2 from measurements of the exposed surface area of each plant sample. Data for the different strata and the two sprayers were analysed by analysis of variance followed by Tukeys test on significantly heterogeneous groups to separate individual means.

Direct measurement of deposition on invertebrates

Four species of beneficial insect were selected and placed at different positions in the crop canopy to assess the level to which they were exposed to direct contact spraying. Dead individuals were used to obtain control over position and orientation. At the time of fluorescein application, described above, 10 Coccinella septempunctata (Coleoptera; Coccinellidae) were placed respectively on the developing ear, the highest point of the flag leaf dorsal surface, directly beneath on the flag leaf ventral surface and on the dorsal surface of the first leaf. Ten each of Pterostichus melanarius, Nebria brevicollis and Harpalus rufipes (Coleoptera; Carabidae) were placed at ground level. Each insect was pinned to a 1 cm x 7.5 cm piece of glazed photographic paper. The tracer deposition on each insect was estimated using the method described above, with the amount of pesticide landing per unit insect area being calculated from estimates of the mean ground area coverage of each species.

A second method was used to calculate the mean number of droplets impinging on these species in the different crop strata. Stardust particulate fluorescent tracer was applied at a rate of 0.5% w/v in water with 0 1% wetting agent. Application was made via an Azo plot sprayer calibrated to apply 200 l/ha^{-1}. The same insect species and positions as above were employed with the addition of a standard species Bembidion lampros, (Coleoptera; Carabidae) in groups of four next to each individual of the other species. These insects were placed on water sensitive paper strips to obtain a comparative estimate of droplet number on a standard, two dimensional surface. Following application, the insects and paper strips were photographed using a fluorescent photography apparatus (to be described elsewhere) to visualise the individual droplets. The number of droplets per insect were again converted to a standardised area value from the mean ground area coverage of each species.

The persistence of different active ingredients at different strata within the crop canopy

The residual toxicity of different foliar pesticides on leaf and soil surfaces was evaluated by exposing insects to treated substrates for 24 h within leaf cages in the field and assessing mortality at 24 h intervals for 3 days. Adult B. lampros were used throughout. A randomised plot design with three insecticide treatments and an unsprayed control with four replicates was employed. The pesticides were applied by Azo plot sprayer at field rate in 200 l. of water; the winter wheat crop cv. Rendezvous with a tiller density of 520 m^{-2} was sprayed at GS 47 and the insects exposed to treated foliage and soil at 1,2,3,6 and 9 days after treatment. Seven adult B. lampros per treatment replicate were introduced to petri dishes lined with expanded polystyrene through which a channel had been cut to accommodate treated leaf surfaces. One set of dishes was placed on flag leaves, another set on the first leaf, of randomly selected plants in the sprayed area. A further set of dishes were sunk to soil surface level and remained open during spraying to expose the soil within them to direct spraying. The pesticides applied were deltamethrin, dimethoate and pirimicarb.

RESULTS

Table 1 gives the spray depositional data through the crop from a single example of this technique. Significant overall heterogeneity was detected ($F = 6.26$, $DF = 7,149$, $p < 0.001$) with trends for both sprayers to deposit more spray at ear and flag leaf level than first leaf or the soil surface. The major significant difference was between the tractor sprayed flag leaf and other sprayed surfaces. Tractor spray deposition was consistently although not significantly greater than Azo deposition at any level.

Table 2 gives the depositional data for insects, ranked in ascending order of deposition per unit area. Significant overall heterogeneity was found ($F = 18.86$, $DF = 13,13$, $p < 0.001$) with separations between the strata apparent especially insects on leaf undersides and at ground level having lower deposition than those on ears and leaves. No important differences were detectable between spraying methods or the different species at ground level, although deposition rates with the tractor sprayer were again higher than those with the Azo sprayer. The data for mean droplet number per cm^2 on beetles at different positions in the crop canopy is given in Table 3. Significant heterogeneity was again detected in this data ($F = 7.35$, $DF = 11,82$, $p < 0.001$) with significant differences between ear and flag leaf and the other strata. No major differences were detected between the species when comparisons were made in terms of numbers of droplets per unit area. *B. lampros* however tended to have higher rates of deposition than the other species on the plant.

Figures 1 a-c give the mean 24 h mortality data +/- 95% CL for each residual pesticide treatment and crop stratum. Deltamethrin and dimethoate gave similar levels of mortality throughout at all levels with some effects still detectable after 9 days. The highest levels of mortality were obtained on the flag leaf, with a lower level on the first leaf, this difference increasing as time progressed. The lowest levels of mortality were detected on the soil. Significant levels of mortality were only found with pirimicarb on the first and second days after treatment on the crop foliage, no effect was detectable at soil level.

DISCUSSION

The data indicate that there is likely to be some variation in the level of risk to different species inhabiting particular zones of the crop. The level of risk to a particular species of direct contact exposure will be mediated by its diel activity pattern and the zone of the crop that it inhabits. The guild of aphid specific predators and parasitoids inhabiting the upper levels of the crop would seem to be the most at risk, with ground dwelling polyphagous species, that are mainly nocturnal, being less exposed. Those species inhabiting the upper strata of the crop are again the most at risk of significant side-effects from residual exposure. The effects of the compounds on the soil level were lower in comparison with the effects on the foliage and this may offer indirect protection to ground dwelling species.

The data may be used to provide direct estimates of the levels of particular routes of exposure to pesticides and may thus be used to aid interpretation of laboratory derived dose-response data for non-target, beneficial species. They also provide testable predictions of potential mortality, when linked with toxicological data and may therefore be used to aid interpretation of field experiments where the contribution of lethal and sub-lethal effects to observed trapping levels of beneficial species is

TABLE 1

Deposition levels ($\mu l/cm^{-2}$) of fluorescent tracer applied at a rate of 200 l/ha^{-1} to winter wheat by Azo plot sprayer or tractor sprayer. Values sharing the same letter are not significantly different ($p > 0.05$)

Crop stratum	Mean deposition rate	S.E.		Mean deposition rate	S.E.	
	TRACTOR			AZO		
Ear	0.360	0.029	a	0.317	0.024	a
Flag leaf	0.495	0.053	b	0.297	0.037	a
First leaf	0.298	0.021	a	0.222	0.037	a
Ground level	0.336	0.017	a	0.288	0 024	a

TABLE 2

Deposition levels ($\mu l/cm^{2}$) of fluorescent tracer on insect species in different strata in winter wheat canopy. Azo (plot sprayer); Tra. (tractor sprayer); Ear, (insect on ear); Top. (insect on dorsal surface of flag leaf); Under (insect on ventral surface of flag leaf; Mid, (insect on dorsal surface of first leaf); Grd. (insect placed on ground) C.s. (C. septempunctata); P.m. (P. melanarius); H.r. (H. rufipes); N.b. (N. brevicollis). Values sharing the same letter are not significantly different ($p > 0.05$).

Sprayer/crop level/insect	Mean deposition rate	S.E.	
Tra/Ear C.s.	1.827	0.208	a
Azo/Ear C.s.	1.160	0.221	b
Tra/Top C.s.	0.901	0.169	bc
Azo/Top C.s	0.853	0.139	bcd
Azo/Mid C.s.	0.502	0.099	cde
Tra/Grd P.m.	0.412	0.066	cde
Tra/Mid C.s.	0.309	0.116	de
Tra/Grd H.r.	0.288	0.070	e
Azo/Grd H.r.	0.271	0.052	e
Tra/Grd N.b.	0.267	0.082	e
Azo/Grd P.m.	0.239	0.050	e
Azo/Grd N.b.	0.185	0.035	e
Tra/Under C.s.	0.044	0.044	e
Azo/Under C.s.	0.009	0.006	e

Figure 1a-c Mean percentage mortality +/- 95% CL for B. lampros placed for 24 h on flag leaf, first leaf or ground level after application of deltamethrin, dimethoate or pirimicarb at field rate to a winter wheat crop. Data given for assays at 1,2,3,6 and 9 days following treatment.

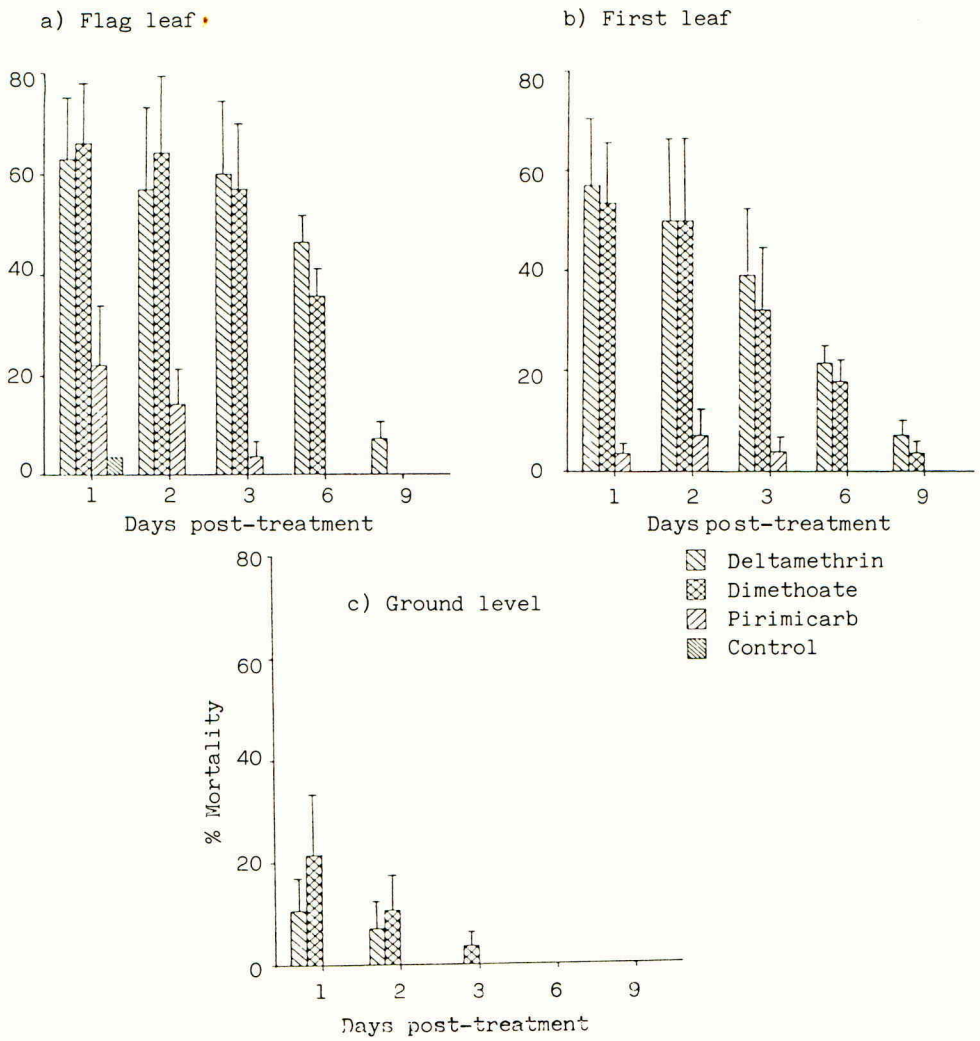

TABLE 3

Mean droplet number/cm^{-2} impinging on a range of representative non-target invertebrates placed in different strata of a winter wheat crop canopy (tracer applied by Azo plot sprayer @ 200 l/ha [1]) B.l. (B. lampros); C.s. (C. septempunctata); P.m. (P. melanarius); H.r. (H. rufipes); N.b. (N. brevicollis). See Table 2 for key to plant positions. Values sharing the same letter are not significantly different ($p > 0.05$).

Stratum	Mean droplet number +/- S.E.				
	B.l.	C.s.	P.m.	H.r.	N.b.
Ear	81.0 +/- 16.2 a	53.4 +/- 15.6 b			
Top	49.8 +/- 7.9 ab	32.0 +/- 4.06 b			
Under	9.8 +/- 4.8 b	9.6 +/- 4.1 b			
Middle	33.5 +/- 6.9 b	29.5 +/- 6.1 b			
Ground	17.2 +/- 2.86 b		11.8 +/- 1.9 b	22.8 +/- 5.1 b	18.2 +/- 2.9 b

rarely recorded. Finally, they may also be used to test theoretically, the value of reducing dosage levels to improve the survival of beneficial species (Taye and Jepson, this volume).

The techniques reported above have important limitations. The insects were for example placed in fixed positions whereas, in the real world, these species exhibit a vertical zonation in behaviour, travelling through different strata of the crop during their activity cycles. The behaviour of a species may also limit the degree to which it is exposed to a particular compound via, for example, repellency or food depletion which lead to dispersal away from the treated area. These data therefore provide a useful index of the short-term effects of a compound for use in the development of selective application practices or in quantifying side effects. They may also contribute to models predicting long-term effects (Jepson, 1988).

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