Chapter 2

EXPERIMENTAL PROCEDURES
2.1 Theoretical Consideration

Any system for evaluating pesticidal effect must reflect the situation that occurs in the natural environment. In soil many factors affect the interactions that occur between pesticides, microorganisms, and their environment (Anderson, 1978). For instance, clay content, cation exchange capacity, pH, and organic matter all influence the adsorption, leaching, and volatility of pesticides and hence may increase or decrease any potentially harmful effects. Competition between organisms may be of major importance. Pure culture systems have some technical advantages and often provide valuable biochemical information, but their results can not be extrapolated to the natural habitat; so the influences of pesticides on soil microorganisms must be determined in actual soil samples.

All tests apply pesticide to freshly sampled soil in the laboratory, in preference to treating soil in the field, then sampling at intervals and transporting the samples to the laboratory for testing. In the field, fluctuations in temperature and moisture, and local difference in physical and chemical soil properties, may modify microbial
responses to pesticides. Indirect consequences such as the removal of plant cover by a herbicide and the subsequent changes in soil moisture, temperature, etc., may have a far greater impact on the microorganisms than the pesticide application itself. In contrast, laboratory conditions allow greater control of variables. With suitable processing, replicate laboratory soil samples show less variation than replicate field samples. Also, larger organisms such as earthworms and arthropods, whose presence or absence might confuse the interpretation of field data, may be removed.

Thus laboratory tests are considered to be more rigorous than the field tests and are more likely to reveal pesticide effects, if any exists.

2.1.1 Soil preparation and pesticide treatment

Freshly sampled field soil, from the top 15 to 25 cm excluding any plant cover, is dried slowly if necessary to facilitate sieving through a 2 mm mesh to remove earthworms, arthropods, stones, plant roots, and other debris. The soil is mixed and deionized water is added to achieve a moisture content of about 50-60 percent of its maximum moisture holding capacity.
(mhc). This produces homogeneous and reproducible soil samples which are kept in pots with loosely fitting lids to permit gas exchange, but reduce drying. Disruptive actions like drying, sieving, mixing, and moistening are known to affect microbial activity (Craswell and Waring, 1972; Jenkinson and Powlson, 1976; Rovira and Creacen, 1957); so the microflora are allowed to equilibrate for 7-10 days at 28°C.

After equilibration, the pesticide is applied at normal and abnormally high rates and quickly and uniformly dispersed in the soil. Liquid formulations are applied in a sufficient volume of water to adjust the final moisture to 50 to 60 percent of mhc. Powdery formulations are first mixed with a small amount of dry soil which is then incorporated into the bulk soil before final adjustment of moisture. Control soil is subjected to the same procedures. If the tests require the addition of a substrate, it is mixed into the soil immediately after pesticide treatment or added to subsamples at suitable intervals as each method demands.

2.1.2 Soil incubation: conditions and duration

For the nitrogen turnover studies and enumeration
of microorganisms, the soil is incubated in bulk; at suitable intervals subsamples are taken to assess pesticidal effects. Soils are incubated in the dark at 28°C, and soil moisture is maintained at 50-60 percent of mhc.

The duration of incubation depends on the persistence and biological availability of the parent pesticide or the possible formation of harmful metabolites, and upon the progress of each test. Generally, a particular test will be terminated when an equilibrium state is reattained after the disruption of the treatment or the increased activity from additions of substrate. Should a pesticide have an effect, then incubation will usually continue until microbial activity returns to control soil level or until it is established that the effects are persistent (Johnen and Drew, 1977).

2.2 Materials and Methods

2.2.1 Pesticides

The thirteen commercial insecticide compounds examined were aldrin, BHC, endosulfan, demeton, fenthion, parathion-methyl, chlorpyrifos, monocortophoe,
phosphamidon, quinalphos, malathion, dimethoate, and carbaryl. Detailed information about them is presented in Table 1.

All pesticides except BHC, carbaryl and malathion, were in a commercially formulated liquid. These were diluted with glass distilled water and applied to soil as part of the moisture required to adjust the soil to 50 or 60 percent of its water holding capacity. The diluted pesticide emulsion was pipetted into the experimental container before addition of the semi-dry soil. The moisture uptake by soil was relied upon to distribute the pesticide evenly. To avoid compaction, stirring of the moist soil was avoided (Atlas et al., 1978). BHC, carbaryl and malathion were in formulated state as dusts. These were first mixed with a small amount of dry soil which was then incorporated into the bulk soil before final adjustment of moisture (Johnen and Drew, 1977).

Rates of application of pesticides to soil have been standardized as kilograms (kg) or litres per hectare (ha). In case of soil all conversions from other units to kg or litre ha$^{-1}$ have been based upon $10^8$ grams of soil ha$^{-1}$ at a depth of 1 cm,
<table>
<thead>
<tr>
<th>Active ingredient (1)</th>
<th>Chemical name</th>
<th>Commercial formulation and source</th>
<th>Proportion of (1) in (2)</th>
<th>Rate of application (kg or litre a.i., ha⁻¹)</th>
<th>Other than population study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin</td>
<td>1,2,3,4,10,10-hexachloro 1,4,4a,5,6,8a-hexahydro-exo 1,4-endoo-5,6-dimethano naphthalene</td>
<td>Aldrex National Organic Chemical Industries Ltd.</td>
<td>30%</td>
<td>1 and 10</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>BHC</td>
<td>1,2,3,4,5,6-hexachloro-cyclohexane</td>
<td>Benzene Sheret Pulverising dust</td>
<td>50%</td>
<td>10* and 100</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>6,7,8,9,10,10-hexachloro 1,5,5a,6,9,9a-hexahydro 6,9-methano 2,4,3-benzox(e) dioxathiepin-3 oxide</td>
<td>Thiodan Hoechst Pharmaceuticals</td>
<td>35%</td>
<td>1 and 10</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Demeton</td>
<td>Demeton-o-methyl sulphoxide-o,o -dimethyl o-2 (sulphonyl) ethyl phosphorothionate</td>
<td>Metaxystox Bayer (India) w/w</td>
<td>25%</td>
<td>1.8* and 18</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Penthion</td>
<td>Penthion-o,o-dimethyl o-3(3-methyl-4 methyl thlophenyl) phosphorothioate</td>
<td>Labaycid 1000 Bayer (India) w/w</td>
<td>80%</td>
<td>1 and 10</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Parathion-methyl</td>
<td>o,o-dimethyl o-4-nitrophenyl phosphorothioate</td>
<td>Parathionmethyl Pesticide India EC</td>
<td>50%</td>
<td>3* and 30</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>o,o-diethyl o-3,5,6 trichloro-2 pyridyl phosphorothioate</td>
<td>Curaban Motile Pesticide (India) Pvt. Ltd.</td>
<td>20%</td>
<td>1 and 10</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Monocrotophos</td>
<td>3-(Dimethoxyphosphinomy)-N-methyl-ciscrotonamide</td>
<td>Nuvacron Ciba-Geigy of India Ltd. w/w</td>
<td>36%</td>
<td>1 and 10</td>
<td>1,10 and 100</td>
</tr>
<tr>
<td>Phosphamidon</td>
<td>o-2-chloro-2-diethyl carbamoyl -1-methyl vinyl-o,o-diethyl phosphate</td>
<td>Dimcron Ciba-Geigy of India Ltd.</td>
<td>85%</td>
<td>1 and 10</td>
<td>1,10 am 100</td>
</tr>
<tr>
<td>Oxamidophos</td>
<td>o,o-diethyl-o-(quinoxalinyl) -2 thionophosphate</td>
<td>Ekelux Sandox (India) Ltd. w/w</td>
<td>25%</td>
<td>1 and 10</td>
<td>1,10 am 100</td>
</tr>
<tr>
<td>Malathion</td>
<td>S-(1,7-diethoxy carbonyl)ethyl dimethyl phosphorothiocholine</td>
<td>Malathion 10% Sheret Pulverising dust</td>
<td>10%</td>
<td>7.5* and 15*</td>
<td>1,10 am 100</td>
</tr>
<tr>
<td>Dimethoate</td>
<td>dimethyl S-(N-methyl carbamoyl methyl) phosphorothiocholthionate</td>
<td>Rogor MalHit India Ltd.</td>
<td>30%</td>
<td>1 and 10</td>
<td>1,10 am 100</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>Carbaryl-1-naphthyl methyl carbamate</td>
<td>Sevin Union Carbide</td>
<td>50%</td>
<td>2.5* and 28</td>
<td>1,10 and 100</td>
</tr>
</tbody>
</table>

Insecticides are generally applied at 1 litre (Kg) ha⁻¹ (Brown, 1978).

* Indicates recommended field rate.
assuming an average bulk density for soil of 1.0

2.2.2 Soil and soil preparation

The soil used throughout was sandy clay loam from the top 25 cm of a fallow plot in the Botany Garden of the School of Sciences, Gujarat University, Ahmedabad. Mechanical and chemical analyses (sand 74.183%, clay 22.079%, silt 3.739%, organic matter 0.608%, organic carbon 0.352%, total nitrogen 0.0304%, pH 7.8, water holding capacity 37.39%) were carried out at the Ground Water Division No.1, Ahmedabad, and at the Gujarat Agricultural University, Navsari Campus.

Freshly prepared soil was dried in the laboratory. The dried soil was sieved to separate crumbs of size range 2-4 mm. The soil was mixed and glass distilled water was added to achieve a moisture content of about 40 percent of its maximum moisture holding capacity. This produced homogenous and reproducible soil samples which were kept in plastic containers with loosely fitting lids to permit gas exchange, but reduce drying (Johnen and Drew, 1977). Disruptive action like drying, sieving, mixing, and moistening
are known to affect microbial activity (Craswell and Waring, 1972; Jenkinson and Fowlson, 1976; Rovira and Creacen, 1957); so the microflora were allowed to equilibrate for 7 days at 28°C.

After equilibration, the pesticides were applied at normal and abnormally high rates (Table 1). Control soil was subjected to the same procedures.

For all studies, except soil dehydrogenase activity, glass distilled water was added to the soil to obtain 60% mhc and evaporation losses were compensated weekly. Sixty percent mhc was used to represent optimal conditions for the activity of aerobic soil microorganisms (Atlas et al., 1978). In testing of soil dehydrogenase activity 2g soil samples were mixed with 0.2 ml of 0.3% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) and 1.0 ml of distilled water (Klein et al., 1971). Soil moisture was determined by oven drying at 105°C to a constant weight.

2.2.3 Enumeration of microorganisms

One hundred grams of soil samples treated with two doses of each insecticide (Table 1) were put in
each wide mouth glass container. These samples, prepared according to processes mentioned earlier, were incubated at 28°C for 6 weeks. The population of bacteria, streptomycetes and fungi were assessed at weekly intervals following the standard serial dilution plate method.

One g soil subsample from each treatment, was suspended in sterile distilled water. The primary suspensions were shaken manually for 5 minutes and subsequent serial dilutions for 1 minute each. Three dilutions were used for each plating to obtain about 100 colonies of bacteria and streptomycetes and about 40 colonies of fungi per plate (Mathur et al., 1976).

Bacteria were counted on Thornton's agar medium (Thornton, 1922). Composition of the medium is:

\[
\begin{align*}
K_2HPO_4 & \quad 1g \\
KNO_3 & \quad 0.5g \\
MgSO_4 \cdot 7H_2O & \quad 0.2g \\
CaCl_2 \cdot 6H_2O & \quad 0.1g \\
NaCl & \quad 0.1g \\
FeCl_3 \cdot 6H_2O & \quad \text{trace} \\
\text{asparagine} & \quad 0.5g \\
mannitol & \quad 1.0g \\
\text{agar} & \quad 25g \\
distilled water & \quad 1000 ml.
\end{align*}
\]

After the agar and salts have been dissolved, mannitol is added and pH adjusted to 7.4. The medium was sterilized under 15 psi at 121°C for 15 minutes.

Streptomycetes were assessed on a medium proposed by Küster and Williams (1964). Composition
of the medium is: glycerol (starch) 10g; casein (Difco vitamin free) 0.3g; NaCl 2g; K$_2$HPO$_4$ 2g; MgSO$_4$·7H$_2$O 0.05g; CaCO$_3$ 0.02g; FeSO$_4$·7H$_2$O 0.01g; agar 25g; distilled water 1000 ml; pH 7.0 to 7.2. The medium was sterilized under 15 psi at 121°C for 10 minutes.

Fungi were counted on peptone rose bengal agar medium (Martin, 1950). Composition of the medium is: glucose 10g; peptone 5g; KH$_2$PO$_4$ 1g; MgSO$_4$·7H$_2$O 0.5g; streptomycin 30mg; agar 25g; rose bengal 0.035g; distilled water 1000 ml. The medium without streptomycin was sterilized under 15 psi at 121°C for 10 minutes. Streptomycin (3ml of 1% sterile solution) was added after sterilization.

The plates were incubated at 28°C. Microbial counts on these media were recorded after 7 days. All microbial counts are reported as number/g soil on dry wt. basis. Results are the averages of three plates from each of the triplicate samples.

2.2.4 Ammonification study

Three hundred grams soil samples treated with three doses of each insecticide (Table 1) were put in
each wide mouth plastic container. Reagent grade peptone was added at the rate of 300 ppm-N in each container. Soil sample without pesticide treatment was taken as control. Each treatment was replicated thrice. Ammonification was studied by rate of formation of ammoniacal-N from added peptone upto 4 weeks. Ammoniacal-N was determined by the following procedures:

1) Extraction of ammonia from pesticide treated and control soils.

The procedure adopted was the same as reported by Sahrawat and Prasad (1975) and consist of mechanically shaking 5g soil from each sample, with 25 ml of Morgan's reagent (100g of sodium acetate + 30 ml of glacial acetic acid, diluted to one litre with distilled water and adjusted to pH 4.8) and 0.25g of activated charcoal, for 30 minutes. The solutions were filtered through whatman No.1 filter paper.

2) Determination of NH$_4^{+}$-N was done using 5 ml aliquot of the above extracts and following the procedure of Nesslerization (Jackson, 1962).
Aliquots of the extracts of each pesticide treated and control soils, were placed in a series of 100 ml volumetric flasks together with 2 ml of 10 percent sodium tartarate solution and the same amount of acidified sodium chloride solution. Water was added to make about 93 ml total volume. Then 5 ml of the Nessler reagent was added with rapid mixing. The solution was brought to volume (100 ml), mixed and read at the end of 25 minutes in a colorimeter at 410 nm wavelength.

3) Preparation of standard colorimetric curve

Exactly 1.337g ammonium chloride was dissolved in 1000 ml of water, giving 0.025 meq of NH$_4^+$ per ml. A dilute NH$_4^+$ standard containing 0.001 meq of NH$_4^+$ per ml, was prepared from 20 ml of the first standard solution diluted to 500 ml. The latter standard solution contained, 18 ppm NH$_4^+$ per ml. Aliquots (3 to 30 ml) of the 18 ppm NH$_4^+$ standard solution, were placed in a series of volumetric flasks for the estimation of NH$_4^+$ by Nesslerization. The standard series was diluted to 100 ml during the process and contained 0.54 to 5.4 ppm of NH$_4^+$-nitrogen.
The transmission percentage of the standard solutions was read at 410 nm in a colorimeter. The standard graph was prepared by plotting transmission percentage on a log scale against concentration on a linear scale.

4) Calculation of results

The results are reported in parts of NH$_4^+$-N per million parts of oven-dry (O.D.) soil. The concentrations of the NH$_4^+$ in each pesticide treated and control soil extract as ppm were obtained from the standard curve. Then the calculation was done as follows.

$$\text{ppm NH}_4^+ \text{ in soil} = \text{ppm NH}_4^+ \text{ in test solution} \times \frac{\text{aliquot dilution} \times \text{soil dilution}}{\frac{\text{ml final colour volume}}{\text{ml aliquot extracted}} \times \frac{\text{ml extraction solution}}{\text{g O.D. soil extracted}}}$$

$$= \text{ppm NH}_4^+ \text{ in test solution} \times \frac{100}{5} \times \frac{25 + \text{ml } H_2O}{5 - \text{ml } H_2O}$$
The dilution of the extracting solution by the soil moisture percent in the original moist sample was taken into account in equation as \((+\) and \((-\) ml H\(_2\)O. In this way the results were based in the oven-dry weight of the soil.

5) Reagent used

Needed reagents (all analytical grade) include 10 percent sodium tartrate (100g of Na\(_2\)C\(_4\)H\(_6\)O\(_6\)\cdot2H\(_2\)O per litre of solution), the standard ammonium chloride solution (1.337g of ammonium chloride in 1000 ml of water containing 1 ml of chloroform), 10 percent acidified sodium chloride (acidified to pH 2.5 with hydrochloric acid), and the following special reagents.

(i) Nessler reagent

This reagent was prepared by dissolving 45.5g of mercuric iodide and 35.0g of potassium iodide in a few ml of water:

\[
\text{HgI}_2 + 2\text{KI} \rightarrow \text{K}_2\text{HgI}_4
\]

The solution was washed into a 1 litre volumetric flask.
Then, 112g of potassium hydroxide was added and the volume was brought to about 800 ml. The solution was mixed, cooled, and diluted to 1 litre with water. The solution was allowed to stand for a few days, and the clear supernatant liquid (Nessler's reagent) was decanted off into an amber coloured bottle for use.

(ii) Extraction reagent

This reagent was prepared by mixing 100g of sodium acetate and 30 ml of glacial acetic acid. It was then diluted to 1 litre with glass distilled water and adjusted to pH 4.8.

Instead of distilled water, glass distilled water was used in the preparation of reagents and in the final washing of glasswares.

2.2.5 Nitrification study

Three hundred grams soil samples treated with three doses of each insecticide (Table 1) were put in each wide mouth plastic container. Reagent grade ammonium sulphate was added at the rate of 100 ppm-N in each sample. Each treatment was replicated thrice.
Soil sample without pesticide treatment was taken as control.

Nitrification was studied by rate of formation of nitrate-N from added ammonium sulphate upto 4 weeks. Nitrate-N was determined by the following procedures:

1) Extraction of nitrate from pesticide treated and control soils.

The procedure adopted was the same as reported by Sahrawat and Prasad (1975) and consists of mechanically shaking 5g soil from each sample with 25 ml of Morgan's reagent (100g of sodium acetate + 30 ml of glacial acetic acid, diluted to one litre with distilled water and adjusted to pH 4.8), and 0.25g of activated charcoal for 30 minutes. The solutions were filtered through whatman No. 1 filter paper.

2) Determination of NO$_3$-N

This was done using 10 ml of the above extract and following the procedures of Harper (1924) and Prince (1945), described by Jackson (1962).
Aliquots of the extracts of each pesticide treated and control soils, were placed in separate 8 cm evaporating dishes and evaporated to dryness on the steam bath in an atmosphere free from nitric acid fumes. The dishes were cooled, and 1 ml of phenol disulphonic acid was added rapidly, directly in the centre of each. The dish was rotated to effect contact with all of the residual salt, and the reagent was allowed to act for 10 minutes. Then 15 ml of cold water was added, and the solution was stirred with a glass rod until all the residue was in solution. After the dishes were cooled, 10 ml of 6N ammonium hydroxide was added slowly for the development of a yellow colour. This solution was then diluted to 100 ml with water. Addition of 1 ml of phenol disulphonic acid and 10 ml of 6N ammonium hydroxide, follows the procedure given by Nicholas and Nason (1957).

The transmission percentage of the nitrate solution was read in a colorimeter at 420 nm wavelength.

3) Preparation of standard colorimetric curve

Exactly 0.7221g pure dry potassium nitrate was dissolved in water and the solution was diluted to
exactly one litre, giving 0.1 mg N per ml, or 100 ppm stock solution. This stock solution was then diluted 20 ml to 200 ml in a volumetric flask. This latter solution contained 0.01 mg N per ml, or 10 ppm. Aliquots (2, 4, 6, 8, 10, 12 and 15 ml) of the 100 ppm N standard nitrate solution were placed in separate 8 cm porcelain evaporating dishes and evaporated to dryness on the steam bath in an atmosphere free from nitric acid fumes. Colour development follows the procedure given for nitrate-N determination in soil extract. The standard solutions were diluted to 100 ml in the final adjustment of volume, and contained 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 ppm of nitrate-nitrogen.

A calibration curve was plotted from the standard nitrates on semilogarithmic paper, the log scale being employed for the transmission percentage readings.

4) Calculation and results.

The results are reported in parts of nitrate-N per million parts of oven-dry soil. The concentrations of the nitrate in each pesticide treated and control extract as ppm are obtained from the standard curve.
Then the calculation was done as follows:

\[
\text{ppm N in soil (NO}_3\text{ form)} = \frac{\text{ppm N in test solution X aliquot dilution (from curve)}}{X \text{ soil dilution}} \times \frac{X \text{ soil dilution}}{\text{ppm N in test solution X}} \times \frac{\text{ml final colour volume}}{\text{ml aliquot evaporated}} \times \frac{\text{ml extraction solution}}{g \text{ oven dry soil extracted}}
\]

\[
= \frac{\text{ppm N in test solution X}}{100} \times \frac{25 + \text{ml } H_2O}{10} = \frac{\text{ml } H_2O}{5} = \text{ml } H_2O
\]

The dilution of the extracting solution by the soil moisture present in the original moist sample was taken into account in equation as (+) and (-) ml $H_2O$. In this way the results were based on the oven dry weight of the soil.

5) Reagents used

Needed reagents (all reagents were analytical grade) include 6N ammonium hydroxide, activated charcoal (Darco G 60), sodium acetate, glacial acetic acid, and the following special reagent:
Phenol 2,4-disulphonic acid

Twentyfive g pure phenol (crystal white in colour) was dissolved in 150 ml of concentrated sulphuric acid. Then 75 ml of fuming sulphuric acid was added. This solution was mixed and heated by placing the flask in boiling water for 2 hours. The resulting phenoldisulphonic acid \([ \text{C}_6\text{H}_3\text{OH} \,(\text{HSO}_3)_2 ]\) solution was stored in a brown bottle.

2.2.6 Sulphur oxidation study

Two hundred grams soil samples treated with three doses of each pesticide (Table 1) were put in each wide mouth plastic container. Reagent grade sulphur powder was added at the rate of 0.5 percent in each sample. Each treatment was replicated thrice. Soil sample without pesticide treatment was taken as control.

Sulphur oxidation was studied by rate of formation of sulphate-\(\delta\) from elemental sulphur upto 49 days. This follows the procedure reported by Winiarska et al. (1975). The sulphate content was measured according to Massoumi and Cornfield (1963).
1) Procedure

Sulphate was extracted by placing 2g soil from each treatment and 20 ml water in 6 inch X 1 inch boiling tubes, stoppered with a rubber bung and shaken in a mechanical reciprocating shaker for 15 minutes and then 0.04g purified animal charcoal was added in each tube and the tubes were placed in shaker for another 15 minutes. The soil suspensions were filtered through a whatman No. 42 filter paper. A suitable portion of the filtrate (not more than 15 ml and containing not more than 120 \( \mu \) g of sulphate-sulphur) from each tube was placed in a 6 inch X 3/4 inch test tube calibrated at 25°C and 2.5 ml of 25% nitric acid and 2 ml of acetic-phosphoric acid were added. The content in each tube was diluted to about 22 ml. After proper shaking, 0.5 ml of the barium sulphate seed suspension (which must be shaken just before use) and 1g barium chloride crystals were successively added. Then the bungs were inverted and the tubes were inverted three times. After 10 minutes the tubes were inverted 10 times and after another 5 minutes, inverted 5 times. After another 5 minutes 1 ml of the gum-acacia-acetic solution was added and diluted to volume (25 ml). The tubes were inverted three times,
and set aside for 1½ hours. Then the tubes were inverted 10 times. The optical density was measured at 440 nm of a colorimeter.

2) Reagents

I) Nitric acid, 25% v/v prepared from analytical reagent grade nitric acid.

II) Acetic acid-phosphoric acid: 100 ml of analytical grade acetic acid was mixed with 300 ml of analytical grade orthophosphoric acid.

III) Gum acacia-acetic acid solution

This was prepared by dissolving 5g of gum acacia in 500 ml of hot water. The hot solution was filtered through whatman No.42 filter paper. The filtrate was allowed to cool and then diluted to 1 litre with analytical grade acetic acid.

IV) Barium sulphate seed suspension

This was prepared by dissolving 18g of analytical grade barium chloride in 44 ml of hot water and 0.5 ml of the concentrated standard solution. This was brought to the boil and then cooled quickly. Then 4 ml of the gum acacia-acetic acid solution was added. The seed suspension was prepared freshly each day before use.
V) Concentrated standard sulphate solution, 2 mg/ml: 1.088 g of oven-dried analytical grade potassium sulphate was dissolved in water and diluted to 100 ml.

VI) Working standard sulphate solution, 10 μg/ml. Each day 5 ml of concentrated standard solution was diluted to 1 litre with water.

VII) Purified animal charcoal

Twenty gram of Darco G 60 charcoal with about 200 ml of concentrated hydrochloric acid was boiled for about 10 minutes. It was filtered under suction and washed until free of chloride. The washed charcoal was dried in an oven at about 100°C.

3) Preparation of standards

0.1, 3, 5, 8, 10 and 12 ml of the working standard sulphate solution, were placed in separate 25 ml calibrated test tube and the above procedure was followed. Each time a batch of solution was analysed, standards were prepared and a reagent blank test was also run.

Standard graph was prepared by plotting transmission percentage on a log scale against concentration on a linear scale.
4) Calculation of results

The results are reported in mg of sulphate-sulphur in 100g of oven dry soil. The concentrations of sulphate in each pesticide treated and control soil extract were obtained from the standard curve. The values thus obtained, were then multiplied by the aliquot dilution and soil dilution factors.

2.2.7 Soil dehydrogenase activity

One hundred grams soil samples treated with three doses of each insecticide (Table 1) were put in each wide mouth glass container. Each treatment was replicated thrice. Soil sample without pesticide treatment was taken as control.

Experimental procedure for the study of soil dehydrogenase activity was designed according to Atlas et al. (1978) and Johnen and Drew (1977). The method adopted for determination of soil dehydrogenase, combines features of those of Klein et al. (1971), Ross (1971) and Glathe and Thalman (1970). Two grams pesticide treated and control soils were placed in screw cap tubes and mixed with 0.2 ml of 3% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.5 M tris buffer and 1.0 ml distilled water. The
tubes were shaken for 15 seconds and incubated in the dark at 30°C for 24 hours. Dehydrogenase enzymes convert 2,3,5-triphenyl tetrazolium chloride to 2,3,5-triphenyl formazan (TPF). The incubated samples were extracted with acetone (Glathe and Thalman, 1970). This was done in dimlight as sunlight caused loss of the red colour of extracted formazan (Wingfield et al., 1977). Acetone (3 ml) was added to each tube shaken for about 15 seconds, and the suspension filtered through glass fiber paper (whatman GF/A) into 25 ml volumetric tubes. The tubes were washed out with more acetone and the washings filtered. Residual formazan was carefully washed out of the filter paper with acetone and the final volume of the filtrate made to 25 ml. Colour density of the filtrate was measured colorimetrically at 485 nm.

Standard curves were prepared, each time an assay was done, using TPF (2,3,5-triphenyl tetrazolium formazan, Budapest, Hungary) at concentrations from 2-10 μg per 1 ml in acetone.

Units of dehydrogenase activity were calculated as μl H₂ g⁻¹ soil day⁻¹, 150.35 μl H₂ being required for formation of 1 mg TPF (Pramer and Schmidt, 1965).
2.2.8 Statistical methods

The results obtained for the pesticide effects on non-target soil microflora and on their activities were analysed for the statistical significance. Interaction analysis was carried out for all the data and the method was adopted from Salmon and Hanson (1964).

The discussion in this section is limited to the statistical analysis of the response of soil bacteria to the different levels of aldrin. The same methods were applied to analyse other data, where only mean values of three replications have been given in the tables.
TABLE 2
Effect of aldrin on soil bacteria

<table>
<thead>
<tr>
<th>Levels of aldrin (litre a.l ha⁻¹)</th>
<th>Days of incubation</th>
<th>Replications (number x 10⁷)</th>
<th>Total</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>I</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>15.5</td>
<td>14</td>
<td>12.5</td>
</tr>
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<td>0</td>
<td>7</td>
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<td>30.3</td>
<td>33.7</td>
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<tr>
<td>0</td>
<td>14</td>
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<td>1.76</td>
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<td>1.5</td>
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<td>21</td>
<td>0.63</td>
<td>0.7</td>
<td>0.62</td>
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<tr>
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<td>28</td>
<td>0.23</td>
<td>0.32</td>
<td>0.35</td>
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<td>35</td>
<td>1.2</td>
<td>1.3</td>
<td>0.5</td>
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<tr>
<td>10</td>
<td>42</td>
<td>1.05</td>
<td>1.25</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Total 236.7 232.47 229.08 698.25
The correction (c) is \[ \frac{698.25^2}{63} = 7738.93 \]

and (1) the total sum of squares is;
\[ 15.5^2 + 14^2 + \ldots + 1.3^2 - C \]
\[ \text{total ss} = 18509.64 - 7738.93 = 10770.71 \]

(2) treatment sum of squares is;
\[ s.s.(Tr) = \frac{1}{3} (42^2 + 96^2 + \ldots + 3.6^2) - C \]
\[ = \frac{1}{3} (18449.63) - 7738.93 \]
\[ = 10710.7 \]

(3) replication sum of squares is;
\[ s.s.(R) = \frac{1}{21} (236.72^2 + 232.47^2 + 229.08^2) - C \]
\[ = \frac{1}{21} (162546.83) - 7738.93 \]
\[ = 1.39 \]

Finally sum of squares due to error
\[ = \text{total s.s.} - s.s.(Tr) - s.s.(R) \]
\[ = 10770.71 - 10710.7 - 1.39 \]
\[ s.s. (error) = 58.62 \]

Subdivisions of treatment sum of squares into components for factors A and B and for interaction can be facilitated by contributing the following two way table, where the entries are the totals in the right hand columns of the table giving the original (mean) data.
**TABLE 3**

<table>
<thead>
<tr>
<th>Factor A (rates of aldrin in litre s.i. ha⁻¹)</th>
<th>Factor B (days of incubation)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
<td>126</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>315</td>
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<tr>
<td>10</td>
<td>21</td>
<td>101.25</td>
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<td>28</td>
<td>79.2</td>
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<td>35</td>
<td>17.1</td>
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<tr>
<td></td>
<td>42</td>
<td>28.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.2</td>
</tr>
</tbody>
</table>

Using formula analogous to the ones with which we have computed sum of squares for various effects, we have,

(i) \[ s.s. A = s.s. \text{ due to factor } A = \frac{1}{b \times r} \sum T_i^2 - C \]

where, \( b \) = no. of replications = 3
and \( r \) = no. of columns for days = 7

\[
s.s. A = \frac{1}{3 \times 7} \left[ (364.5)^2 + (193.8)^2 + (139.95)^2 \right] - C \\
= \frac{1}{21} \left[ (190004.69) - 7738.93 \right] \\
s.s. A = 1308.91
\]
(ii) \( s.s. B = s.s. \) due to factor \( B \)

\[
\frac{1}{ab} \sum t_i^2 - C
\]

where, \( a \) = various rates of aldrin = 3

\( b \) = number of replication = 3

\[
s.s. B = \frac{1}{3 \times 3} (126)^2 + (315)^2 + \cdots + (312)^2
\]

\[
= 1/9 (133703.3) - 7738.93
\]

\[
= 7116.95
\]

now ss due to interaction

\[
s.s. (AB) = ss(Tr) - s.s.A - s.s.B
\]

\[
= 16710.7 - 1308.91 - 7116.99
\]

\[
= 2284.8
\]
The results are presented in the following analysis of variance table.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Variance</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>replication</td>
<td>2</td>
<td>1.39</td>
<td>0.695</td>
<td>0.474</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>1308.91</td>
<td>654.45</td>
<td>446.57</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7116.99</td>
<td>1186.16</td>
<td>809.39</td>
</tr>
<tr>
<td>interaction</td>
<td>12</td>
<td>2284.8</td>
<td>190.4</td>
<td>129.92</td>
</tr>
<tr>
<td>error</td>
<td>40</td>
<td>58.62</td>
<td>1.4655</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>62</td>
<td>10770.71</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F test for replication is not significant at 5% and 1% levels of significance. The other three F tests are significant at 1% level of significance.

Observation of Table 1 will show that the interaction is due to rates of aldrin and days of incubation. The fact that the rates (A) x days (B) interaction is highly significant shows that the differential response was greater than can reasonably be explained by random variation. Whether the
differential response is statistically significant or not, can be calculated by considering the standard error of difference,

\[
\text{the standard error for comparing treatments} = \sqrt{\frac{2 \times \text{error variance}}{3}}
\]

\[
= \sqrt{\frac{2 \times 1.4655}{3}}
= \sqrt{0.977}
= 0.988
\]

C.D. at 5% level = standard error \times t_{0.05.40}

\[
= 0.988 \times 2.02
= 1.996
\]
"RESULTS AND DISCUSSION"