WEED RESEARCH ORGANIZATION

TECHNICAL REPORT No. 45

METHODS OF ANALYSIS FOR DETERMINING THE EFFECTS OF HERBICIDES ON SOIL MICRO-ORGANISMS AND THEIR ACTIVITIES

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January 1978

Price - £4.00

Agricultural Research Council Weed Research Organization, Begbroke Hill, Yarnton, Oxford, OX5 1PF

ISBN 0 7084 0076 0

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JULY 1980 ADDITIONS TO TECHNICAL REPORT NO. 45

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INTRODUCTION

The aim of this report is to describe the methods which are in routine use by the Microbiology Group at the Weed Research Organization (WRO). The methods have been developed or introduced during the work of the Group on assessment of the effects of herbicides on soil micro-organisms. Particular attention has been paid to methods of examining aspects of soil microbiology which may be important to soil fertility.

The descriptions are intended to be guidelines rather than rigid rules. Inter-laboratory variations in availability of media or apparatus may necessitate modifications. In our experience, however, these methods are reproducible, sensitive and require a minimum input of labour.

Where specific pieces of equipment have been found to be preferable or essential the names and addresses of the manufacturers are given. In other instances any equipment of the description given will suffice. Where media or equipment are described as sterile they have been autoclaved at 121^{o}C for 15 min. Pipettes are sterilized by heating dry at 160^{o}C for 3h.

SOIL SAMPLING

At WRO the effects of herbicides on the soil microflora are measured on two soils (see Table 1 in the Appendix). Special sites are maintained for provision of bulk samples of these soils for laboratory experiments. These are in arable and permanent grassland fields of the WRO farm. Each site is 24m x 12m and is divided into 12 strips of 12m x 2m; each strip acts as a source of soil for 1 year. Before use as sampling areas strips are rotary cultivated monthly for 2 years to a depth of 15cm to ensure decomposition and even distribution of plant residues. During the sampling period rotary cultivation is maintained at regular intervals to control weeds. Those areas of the arable site which are not being prepared for, or in use as, sampling areas are cropped each year with spring barley. Weed control in the cropped area is achieved by push-hoeing between the rows and hand-weeding within the rows of crop. Similarly, the grassland site is kept under grass except for the areas being prepared for sampling. After samples have been taken from strips in the grassland site for one year they are not cultivated further and the natural grass flora is allowed to reinvade. These strips are then not grazed for at least one year. The remainder of the site under grass is cut for silage once or twice each year and is also grazed by beef cattle. Both sites, apart from the areas being rotary cultivated, receive appropriate fertilizer applications each year.

Bulk samples are obtained by combining 12 to 20 samples taken randomly over the sampling area. The surface trash is removed and the soil sampled to a depth of 12cm using a spade for very large samples or a 2.5cm diameter corer for smaller samples. The bulked soil is thoroughly mixed and sieved through

a 3mm mesh. Where the soil is too wet to allow easy sieving it is spread in a thin layer on polyethylene sheet and lightly air-dried at about 20°C. Fresh soil should be used whenever possible but if it cannot be used immediately after sampling it should be stored, unsieved, in large polyethylene bags at 4°C. Some soils can be stored for longer periods in large containers (eg 40cm plant pots) plunged in sand or soil conveniently near to the laboratory. This method should only be used after checks have been made to ensure that gross changes of soil and/or microbiological properties do not occur during the storage period. In general storage of soil should be reduced to a minimum and in normal circumstances should not exceed 1 week.

SOIL TREATMENT WITH HERBICIDES

In routine experiments to determine the effects of herbicides on the soil microflora, it is desirable for the soil to be uniformly treated with herbicide. To achieve this, soils are spread as a 2.5cm layer on trays covered with polyethylene sheet and sprayed by the laboratory pot sprayer described by Grossbard and Wingfield (1975). After spraying the soils are mixed thoroughly.

The herbicide is always sprayed onto moist soil. The total volume of water and herbicide used is calculated to produce the desired final soil moisture content, usually 60% moisture holding capacity.

Granular formulations of herbicides are ground in a pestle and mortar with a small amount of dry soil (10g/3kg of soil to be treated). The soil-herbicide mixture is then spread as evenly as possible over the soil to be treated and thoroughly mixed.

Reference

Grossbard, E. and Wingfield, G.I. 1975 , pp 235-256 In 'Some methods for microbiological assay'. Eds., R.G. Beard and F.W. Lovelock, Academic Press, London.

HERBICIDE CONCENTRATIONS

Clearly the choice of herbicide concentrations used will depend on the purposes of the experiment. For routine work, however, soils are sprayed at the manufacturer's highest recommended field rate and at ten times that rate.

HERBICIDE FORMULATION

In experiments to determine the effects of herbicides on the soil microflora the experimenter must decide whether to use relatively pure herbicide or a formulation of it. This decision can only be made after careful thought as to the objectives of the work.

At WRO soil incubation experiments normally use the formulated herbicide. Where potentially serious effects are found experiments are repeated using the pure chemical and, if possible, the separate formulation compounds.

In assays of general toxicity to pure cultures of micro-organisms (p 43) the simplicity of the method allows simultaneous testing of the formulated chemical and its components if required.

HERBICIDE ANALYSIS

In order to evaluate the effects of a herbicide on the soil microflora it is essential to know the concentration of residues throughout an experiment. At WRO soils in such microbiological experiments are analysed by the Chemistry Group. The methods used are described in Byast, Cotterill & Hance (1977).

Reference

Byast, T.H., Cotterill, E.G. and Hance, R.J. Methods of analysis for herbicide residues. Technical Report Agricultural Research Council Weed Research Organization, 1977, 15, 2nd Edition, pp. 58.

INCUBATION TECHNIQUES

Soil samples (200g) are incubated in square 7cm plastic plant pots lined with polyethylene bags at 19 $\pm$ 1 C in the dark. Square pots are used to facilitate packing and save space but any similar sized container will suffice.

Groups of pots, 4 replicates of each treatment, are kept on trays inside large polyethylene bags to reduce moisture loss during incubation. The bags are flushed with air each week using a compressed-air line. Moisture losses are checked by weighing and corrected by adding deionized water as required; in practice this is rarely more frequent than every two weeks. Weed seedlings are removed as they appear.

At intervals a group of pots is taken and the soil in each individual pot mixed and sub-divided into appropriate sized samples for the required analyses (eg nitrogen and phosphate levels, enzyme activity determination and enumeration of microbial populations).

Incubation of soils for measurement of respiration (carbon dioxide evolution) and cellulose decomposition is done as described on pages 9 and 27 respectively.

Replacement for p 4

Issued July 1980

SOIL pH MEASUREMENT

Reagents

0.01 M $CaCl_2$ (AR)

Apparatus

pH meter
25 ml measuring cylinder
Vortex mixer ('Whirlimixer', Fisons Scientific Apparatus, Loughborough, Leics. LE11 ORG)
4 ml pipettes
50 ml beakers
Specimen tubes with polypropylene caps
Measuring scoop, 45 mm x 16 mm ID

Procedure

For large samples measure out approximately 10 g of soil with the measuring scoop by filling it with soil, tapping sharply on the bench and then striking off across the top with a knife spatula. Transfer to a 50 ml beaker, add 20 ml 0.01 M $CaCl_2$ and stand for 30 min with periodic stirring. Allow to settle and read the pH of the supernatant solution using a pH meter. Avoid stirring at this stage as the electrodes may be damaged by abrasion from the soil.

For small soil samples suspend 2.0 g in 4 ml 0.01 M $CaCl_2$ in a capped specimen tube and mix on a vortex mixer for 30 seconds. Read the pH using the meter and taking care that air is not trapped under the electrode.

Soil pH values may also be determined using distilled water instead of $CaCl_2$ by the above method. The $CaCl_2$ method is generally considered to give a truer measure of soil pH than that using water.

ORGANIC CARBON IN SOIL

Reagents

All reagents should be of analytical reagent (AR) grade.
$K_2Cr_2O_7$ solution. Dissolve 49.0g $K_2Cr_2O_7$ in distilled water and make to 1000ml; it is not necessary to dry the $K_2Cr_2O_7$.
Conc. H_2SO_4
20% v/v H_2SO_4
Sucrose standards. Dissolve 11.8755g sucrose in distilled water and make to 100ml (= 50mg C/ml). Dilute this solution with distilled water to produce standards in the range 2.5-12.5mg C/ml.
Standards should be freshly made for each batch of analyses.

Apparatus

Technicon AutoAnalyzer. (Technicon Instruments Co. Ltd., Basingstoke, Hants, RG21 2YE)
Log chart recorder paper
Glen Creston S80 Disc Mill. (Glen Creston Ltd., Stanmore, Middlesex, HA7 4DL)
Hot plate
2 ml pipettes
10 ml pipettes with a safety bulb
25 ml and 100 ml measuring cylinders
1000 and 100 ml volumetric flasks
250 ml conical flasks

Procedure

N.B. WEAR SAFETY GLASSES AND RUBBER GLOVES DURING THIS PROCEDURE

Dry soil samples at 105°C overnight and then grind finely in the disc mill. Between 0.2 and 2.0g soil, depending on C-content, is weighed into a 250ml conical flask and 10ml potassium dichromate solution added from a pipette fitted with a safety bulb, followed by 20ml conc. H_2SO_4 added quickly from a measuring cylinder. Mix thoroughly and stand on a hot plate for 5 min. Heating for longer than 5 minutes will result in decomposition of the dichromate. Cool and add 70ml distilled water. Stand overnight to allow particles to sediment then decant the clear solution for analysis - DO NOT FILTER.

A standard calibration curve is prepared by treating 2ml samples of standard solutions as described above. A blank of 10ml potassium dichromate solution and 20ml conc. H_2SO_4 is also prepared.

The samples are analysed on the AutoAnalyzer using the manifold shown in Fig. 1 in the Appendix.

N.B. Run 20% H_2SO_4 through the AutoAnalyzer for at least 1 h before running the samples (sample-cup volume 8-10 ml). Standard solutions should be run first followed by 2 washes of 20% H_2SO_4 and then the samples. A wash of 20% H_2SO_4 should be run after every tenth sample. The AutoAnalyzer is run at 40 samples/h with a sample: wash ratio of 2:1.

Calculation of results

Draw a calibration curve of mg C against optical density. The standards

obey Beer's law, but do not go through the zero as the dichromate gives a small reading. Calculate the slope of the line and use this to calculate mg C in each sample as follows:

$$\text{mg C} = (\text{peak height} - \text{Blank}) \times \text{slope}$$

Replacement for p7, 8 Issued July 1980

AVAILABLE PHOSPHATE IN SOILS

Reagents

All reagents should be of analytical reagent (AR) grade.
0.05% w/v aqueous polyacrylamide, M.W. $> 5 \times 10^6$ (B D H Chemicals Ltd., Poole, Dorset BH12 4NN).
0.5 M $NaHCO_3$ solution. Dissolve 210 g $NaHCO_3$ in deionized water, add 25 ml 0.05% w/v aqueous polyacrylamide solution and make up to 5 l. Adjust to pH 8.5 with 50% NaOH solution.
4% manoxol OT solution. Dissolve 4 g manoxol OT in 100 ml deionized water.
0.43% w/v $(NH_4)_6 Mo_7O_{24} 4H_2O$ solution. Dissolve 4.3 g powdered $(NH_4)_6 Mo_7O_{24} 4H_2O$ in 800 ml deionized water. Slowly add 52 ml conc. H_2SO_4 and make up to 1 l. Add approximately 1 ml 4% manoxol OT solution. Store in a dark bottle in a cool place.
1.5% w/v ascorbic acid solution. This reagent must be made up on the day of use.
Approx. 1.5 M H_2SO_4.
Wash solution. Add 5 drops 4% manoxol OT to 1 l. 0.5 M $NaHCO_3$ solution.
Standard solutions. Dissolve 0.879 g KH_2PO_4 (dried at 105°C for at least 2 h) in 150 ml deionized water. Add 1 ml conc. H_2SO_4, make up to 200 ml and add 1 drop toluene. 5 ml of this solution is diluted to 250 ml with 0.5 M $NaHCO_3$ to give a 20 µg P ml^{-1} solution for preparation of standards. Standards in the range 1-7 µg P ml^{-1} are made by diluting the appropriate volumes of the 20 µg P ml^{-1} solution to 100 ml with 0.5 M $NaHCO_3$.

Apparatus

Technicon AutoAnalyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants. RG21 2YE)
pH meter
2, 5, 10 and 25 ml pipettes
100, 250, 1000 and 5000 ml volumetric flasks
50 ml automatic dispenser
500 ml measuring cylinder
250 ml bottles
End-over-end shaker
Filter funnels, 7 cm diam.
Whatman No. 2 filter papers
100 ml conical flasks

Procedure

Weigh 5 g air dry soil, ground to pass a 2 mm mesh sieve, into 250 ml bottles and add 50 ml 0.5 M $NaHCO_3$ with the automatic dispenser. Shake for 45 min and then filter through Whatman No. 2 filter papers. Transfer to AutoAnalyzer cups and analyse at 40 samples h^{-1} with a 2:1 sample to wash ratio. (See manifold diagram, Fig. 2 in the Appendix).

Calculation

µg Pg^{-1} dry soil = µg ml^{-1} (from calibration curve) x 10

N.B. This technique has been found to be sensitive to temperature and to the method and time of mixing. It should therefore be used only for producing comparative results within complete experiments.

References

Olsen, S.R., Cole, C.V., Watanabe, F.S. and Dean, L.A., 1954, U.S. Dept. Agric. Circular No. 939

Murphy, J. and Riley, J.P., 1962, Analytica Chim. Acta, 27, 31.

MAFF Technical Bulletin, 1973, 27.

Banderis, A., Barter, D.H. and Henderson, K., 1976, J. Soil Sci., 27, 71.

RESPIRATION. CARBON DIOXIDE EVOLUTION

Reagents

All reagents should be of analytical reagent (AR) grade.
0.5 M H_2SO_4
0.05 M H_2SO_4 prepared from a concentrated volumetric solution.
M NaOH prepared by dilution from a 50% (w/v) solution. This reduces contamination by carbonates which are present on NaOH pellets and which precipitate from the concentrated solution.
Molecular sieve, type 5A $\frac{1}{8}$ inch pellets. ('Union Carbide', B D H Ltd., Poole, Dorset, BH12 4NN).

Silica gel.
Soda-asbestos, 6-12 mesh. ('Carbosorb' brand, B D H Ltd., Poole, Dorset, BH12 4NN)

CO_2-free water. (Distilled water boiled for 1 h and stored in a bottle fitted with a soda-asbestos trap).

Apparatus

Scrubbing system as illustrated. (Fig. 3 in the Appendix)
Respirometer flask units as illustrated. (Fig. 4 in the Appendix)
Automatic titrator and pH meter (Radiometer, V.A. Howe & Co. Ltd., London, SW6 3EP)

50 ml automatic dispenser
50 ml pipettes
100 ml volumetric flasks
125 ml Drechsel bottles

Procedure

Samples (100g) of control or treated soil are placed in respirometer flasks and connected to the gas flow lines from the scrubbing system. Gas flow rate is controlled largely by the capillary tube (A in Fig. 4) but a fine adjustment to 1-2 bubbles/sec in the Drechsel washbottle is obtained by moving the adjustable inlet pipe through the washbottle head.

In our experience 4 replicates of each treatment and 4 blanks (flasks with no soil) are sufficient.

NaOH (40 ml) is placed in the Drechsel bottles using an automatic dispenser. The bottles are replaced at 1 or 2 week intervals and the NaOH titrated to determine dissolved CO_2. The titration can be done manually but an automatic titrator is desirable as it allows larger numbers of samples to be handled.

The NaOH from the Drechsel bottle is made to 100 ml in volumetric flasks with CO_2 - free water and 50 ml then pipetted into the titration vessel of the automatic titrator. The solution is adjusted to approximately pH 9.5 with 0.5M H_2SO_4 and then to pH 8.3 with 0.05M H_2SO_4. The volume of 0.05M H_2SO_4 required to titrate this solution between pH 8.3 and 3.8 is noted.

Calculation of results

Carbon absorbed as CO_2 is calculated as :-

$$\text{ppm C as } CO_2 = \frac{[T-B] \times 12 \times 2 \times 100}{100 - M}$$

where T = titre of sample (ml)
B = titre of blank (ml)
M = moisture content of soil (%)

RESPIRATION: OXYGEN UPTAKE

Reagents

All reagents should be of analytical reagent (AR) grade.
M NaOH
0.2% (w/v) glucose solution

Apparatus

Gilson respirometer (Anachem Ltd.,Luton, Beds, LU2 7QE)
Respiration flasks are standard Quickfit 50 ml Erlenmeyer flasks with central wells (2.5 ml) fixed to the base.
2 ml graduated pipettes.

Procedure

Equilibrate the respirometer at the required temperature, normally 20°C. Place 20g treated or control soil in the respiration flasks taking care not to get any in the central well. A fluted filter paper wick is placed in the well of each flask so that it protrudes by 5mm and 0.5ml M NaOH put in each well. The flasks are then fitted to the respirometer with the appropriate reduction adaptors (Quickfit No. DA 13) and allowed to equilibrate for at least 30 min. After equilibration the taps on the respirometer are opened and readings taken every 30 min for 4-5 h.

With soils having low oxygen uptake rates 2.0 ml of 0.2% (w/v) glucose solution can be added to increase uptake. Care must be taken in interpreting results obtained by this method as the glucose treatment can seriously modify the soil microflora.

Calculation of results

Oxygen uptakes are calculated as:-

$$\mu l\ O_2/g\ soil/hour = \frac{100R \times \frac{273p}{760(t+273)}}{W(100-M)T}$$

where R = uncorrected O_2 uptake readings (μl)
p = barometric pressure (mmHg)
t = temperature of respirometer water bath (°C)
W = wet weight of sample (g)
M = moisture content of soil (%)
T = time (h)

Replacement for pp 12 and 13 Issued November 1984

NITROGEN TRANSFORMATIONS: AMMONIUM, NITRITE AND NITRATE ANALYSIS

Reagents

All reagents should be of analytical reagent (AR) grade unless stated.
2M KCl. 745.5 g dissolved in distilled water and made up to 5 l.
Add 1ml chloroform to inhibit nitrification in the soil extract prepared with the KCl.

NaOH. Dissolve 20 g NaOH in distilled water and make up to 1 l.
Add 0.1 ml Brij 35.

Sulphanilic acid. Dissolve 3 g sulphanilic acid in 300 ml distilled water. Cool, add 100 ml conc. HCl and make up to 500 ml.

Acetone/water. Dilute 125 ml AR or redistilled acetone to 1 l with distilled water.

Hydrazinium sulphate. Dissolve 1 g hydrazinium sulphate in distilled water and make up to 1 l.

$CuSO_4$. Dissolve 0.8 g. $CuSO_4\ 5H_2O$ in distilled water and make up to 1 l.

N.E.D. Dissolve 0.4 g. N(1-naphthylethylenediamine dihydrochloride) in distilled water and make up to 1 l.

NaOH/potassium sodium tartrate. Dissolve 1 g NaOH and 5 g potassium sodium tartrate in distilled water and make up to 1 l.

Akaline phenate solution. Dissolve 62.4 g phenol in 100 ml 27% (w/v) NaOH solution. Cool, add 20 ml acetone and make up to 1 l. This reagent should be made up as freshly as possible (i.e. it should not be kept longer than 1 day).

Sodium hypochlorite solution. Dilute 50 ml concentrated sodium hypochlorite (containing 10-14% w/v available chlorine) to 1 l with distilled water.

Standard solutions.
a) KNO_3 (1000 µg NO_3^--N ml^{-1}). 3.611 g dried at 105°C for at least 2 h and dissolved in 500 ml deionized water. Add 1 ml chloroform and store at 4°C.
b) $(NH_4)_2SO_4$ (1000 µg NH_4^+-N ml^{-1}). 2.3475 g dried at 105°C for at least 2 h and dissolved in 500 ml deionized water. Add 1 ml chloroform and store at 4°C.
c) Mixed standard. 10 ml solution a) is mixed with 20 ml solution b) and made to 200 ml with 2M KCl solution.
d) Working standards are prepared in the range 1 µg NH_4^+-N + 0.5 µg NO_3^--N ml^{-1} to 30 µg NH_4^+-N + 15 µg NO_3^--N ml^{-1} by diluting appropriate volumes of mixed standard c) with 2 M KCl. These working standards may be stored for not more than 7 days at 4°C.
e) $NaNO_2$ (500 µg NO_2^--N ml^{-1}). 1.2321 g dried at 105°C for at least 2 h and dissolved in 500 ml deionized water. Add 1 ml chloroform and store at 4°C. 2 ml of this solution is diluted to 100 ml with 2 M KCl to give a 10 µg NO_2^--N ml^{-1} standard solution. Working standards in the range 0.05 to 0.6 µg NO_2^--N ml^{-1} are prepared by diluting the 10 µg NO_2^--N ml^{-1} solution with 2 M KCl.

Apparatus

Technicon AutoAnalyzer with 2 channels. (Technicon Instruments Co. Ltd., Basingstoke, Hants. BG21 2YE).
Log chart recorder paper
End-over-end shaker
100 ml conical flasks
50 ml pipettes
7 cm filter funnels

Procedure

Samples (25 g) of treated and control soils are extracted by shaking with 50 ml of 2M KCl for 1 hour on the shaker. The extract is filtered through a Whatman No. 1 paper into 100 ml conical flasks and the filtrate analysed on the AutoAnalyzer using the manifolds shown in Fig. 5. Nitrate and ammonium are analysed simultaneously at 20 samples h^{-1} on Channels 1 and 2 of the AutoAnalyzer (see Fig. 5 in the Appendix for manifold diagram). Sample: wash ratio is 1:2. In this method nitrate is reduced to nitrite. The colour measured is then equivalent to $NO_2^- + NO_3^-$ in the sample and NO_3^- is found by subtraction. Nitrite samples are analysed at 70 samples h^{-1} on Channel 1 with a sample : wash ration of 2:1 and with the hydrazinium sulphate and $CuSO_4$ tubes pumping water. Since nitrite is not normally detected this sample rate is satisfactory. If nitrite is present in measurable quantities it will be necessary to analyse at 20 samples h^{-1} with a sample : wash ratio of 1:2. It is necessary to use a X4 range expander on the recorder when measuring soil nitrite values.

N.B. All reagents should be run through the AutoAnalyzer for at least 30 minutes before the samples are run. Standard solutions should always be run before and after a batch of samples and, if batches are large, at intervals throughout the samples.

Calculation of results

Draw a graph of $\mu g\ N\ ml^{-1}$ against optical density using the values for the standard solutions. This should obey Beer's law at low concentration. The slope of this calibration graph can be used as a factor for calculating N concentrations in the unknowns as follows:-

$$\mu g\ N\ g^{-1}\ \text{dry soil} = S \times F \times D \times \frac{(200+M)}{(100-M)}$$

Where S = peak height
F = factor (slope of calibration graph)
D = dilution factor of sample
M = moisture content of soil (%)

References

Selmer-Olsen, A.R., 1971, The Analyst, 96, 565

Chapman, B., Cooke, G.H., & Whitehead, R., 1967, Journal of the Institute of Water Pollution Control, 2, 3-7.

NITROGEN TRANSFORMATIONS: TOTAL NITROGEN IN SOIL

Reagents

All reagents should be of analytical reagent (AR) grade.
Kjeldahl copper catalyst tablets
Kjeldahl selenium catalyst tablets
Conc. H_2SO_4, nitrogen free
40% w/v NaOH solution
0.005M standard H_2SO_4
4% w/v H_3BO_3 solution

Apparatus

Kjeldahl digestion stand
30 ml Kjeldahl flasks
Semi-micro Kjeldahl distillation apparatus
Automatic titrator and pH meter (Radiometer, V.A. Howe & Co. Ltd., London, SW6 3EP)

80 mesh sieve
3 ml safety pipettes
20 ml pipettes
50 ml volumetric flasks

Procedure

Air dry the soil and grind to pass an 80 mesh sieve. Weigh 1g ground soil and place in a 30 ml Kjeldahl flask. Add approximately 1 ml water and when this has wetted the soil add 3 ml conc. H_2SO_4 with a safety pipette. Gently boil off the water, then add half a copper catalyst tablet and half a selenium catalyst tablet. Transfer to the Kjeldahl digestion stand and heat strongly until the liquid clears. If solid adheres to the walls of the flasks wash down with approx. 1 ml conc. H_2SO_4. Continue heating for a further 4 h. Cool, dilute with water, pour into a 50 ml volumetric flask together with the washings and make up to the mark. Do not filter.

Transfer 20 ml solution into the distillation apparatus, add approximately 10 ml of 40% NaOH and steam distil into 5 ml of 4% H_3BO_3. Titrate the distillate to pH 5.2 with 0.005M H_2SO_4 using the automatic titrator.

Calculation

$$\% \text{ N in dry soil} = \frac{T \times 0.014}{W}$$

T = titre (ml)
W = weight of dry soil

NITROGEN TRANSFORMATIONS: LEGUME NODULATION

Those herbicides intended for use in legume crops can be tested for their effects on the legume - Rhizobium symbiosis. These tests can be done in pot or field experiments.

Pot experiments

Legume seeds are sown in 12.5 cm plant pots filled with a soil which is known to give full nodulation of the legume. The number of seeds planted will vary according to seed size (clovers, 5 seeds; peas and beans 2 seeds). The pots are kept in a glasshouse with a daylength of 12 hours (obtained using suitable fluorescent, mercury vapour or sodium lamps in the winter months) and a temperature minimum of c. 10°C. After germination the seedlings are thinned to 1 per pot leaving a stand of uniformly sized seedlings. Sufficient pots are prepared to allow 10 replicate plants to be taken at each sampling date. Pots to be treated with herbicide are sprayed when the legume reaches the stage of growth at which the manufacturer recommends treatment. Alternatively the herbicide can be uniformly incorporated into the soil by spraying a 2-3 cm layer as described on p. 2. Spray rates used are 1x and 4x the manufacturer's recommended rate.

Field experiments

Legume seeds are sown thinly in rows 60 cm apart. After germination seedlings are thinned out to 60 cm spacing within the rows. This enables single plants to be sampled when required. Herbicide is applied at the time and rate recommended by the manufacturer and at four times recommended rate.

Sampling

In both pot and field experiments plants are sampled at intervals after spraying. 10 plants are taken from each treatment and from controls. Plants grown in the field should be lifted from the ground using a spade and taking care to damage the root system as little as possible.

Soil is washed from the root systems with a jet of water taking care to avoid damaging the roots.

Plants are normally grown to the fruiting stage and sampled at 2 to 4 week intervals.

Assessment of results

Numbers of nodules per root system are counted and then excised from the roots and weighed. The nodules are then dried at 80°C for 16-18 h and reweighed. Roots and aerial parts of the plants are weighed similarly. When fruits are formed they are weighed separately from the aerial parts to give a figure for final yield.

All parts of the plant are analysed separately for total nitrogen content. (see method on p. 16).

NITROGEN TRANSFORMATIONS: TOTAL NITROGEN IN PLANTS

Reagents

All reagents should be of analytical reagent (AR) grade.
Digestion mixture: 500 ml conc. H_2SO_4 + 2g selenium metal powder. Heat the mixture until the selenium dissolves, cool and transfer to the automatic dispenser.
H_2SO_4 conc.
H_2O_2, 100 vol.
Sodium phenate. Dissolve 250g phenol in 200 ml deionized water with gentle warming. Dissolve 135g NaOH in 500 ml deionized water and cool. Add the NaOH slowly to the phenol solution, make the total volume to 1 l. and shake well. This solution may be stored in a polyethylene bottle at 4°C for a few days but preferably should be used fresh.
Sodium hypochlorite solution. Dilute a conc. solution (containing 10-14% w/v available chlorine) to give a 5-7 (w/v) solution.
Standard solutions. Dissolve 4.7162 g $(NH_4)_2SO_4$ (dried at 105°C for at least 2h) in 100 ml deionized water (store at 4°C). 10 ml of this solution is diluted to 1 l. with deionized water to prepare the basic 100 µg N/ml, solution for preparation of standards. The standards, in the range 5-25 µg N/ml are made by mixing the appropriate volume of 100 µg N/ml solution with 2 ml conc. H_2SO_4 (use a pipette with a safety bulb) and diluting to 100 ml.

Apparatus

Technicon AutoAnalyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants, RG21 2YE).
Log chart recorder paper
Kjeldahl digestion block. An aluminium block, 10 x 10 x 33.5 cm lagged on sides and ends with asbestos sheet and drilled with 56 holes 9 cm deep, 16.5 mm in diameter, and a central thermometer well.
Hotplate capable of heating the block to 330°C.
Drying oven
Glen Creston Micro-hammer mill. (Glen Creston Ltd., Stanmore, Middlesex, HA7 4DL).
50 ml automatic dispenser
Pyrex test tubes, rimless, thick walled 16mm x 150 mm.
2 ml and 1 ml pipettes with safety bulb
10 ml pipettes
1000, 100 & 50 ml volumetric flasks
Thermometer, 0-360°C

Procedure

Dry all samples at 80°C overnight and grind in the hammer-mill. Dry the ground material at 105°C for 2 h prior to analysis. Large samples from mature plants should be thoroughly mixed before sub-sampling for analysis. Weigh the ground material from whole seedlings, or 0.06-0.1 g sub-samples from large plants, into 16mm x 150mm rimless pyrex test tubes (the actual weight taken depends on the amount of nitrogen expected). Add 2 ml of the digestion mixture from the 'Zipette' dispenser. Place the tubes in the digestion block and heat to 150°C on a hot-plate. Carefully add 2 drops 100 vol H_2O_2 by letting the solution run down the side of the tube. Follow this with a further 0.5 ml H_2O_2

again letting the solution run down the side of the tube. (N.B. IT IS RECOMMENDED THAT A FACE MASK AND GLOVES ARE WORN WHILST ADDING THE H_2O_2). Heat the tubes to 330°C and maintain at this temperature until the digestion is complete (3-3½h). Cool, add 10 ml deionized water and transfer to a 50 ml volumetric flask add the washings from the tube and make up to 50 ml. DO NOT FILTER. Digestions from sub-samples of large plants should be made up to 100 ml.

The digested samples are transferred to the cups on the sample plate of the AutoAnalyzer and analysed using the manifold shown in Fig. 6 (see Appendix). It is recommended that the following sequence is used on the sample programme; Standards, 2 water washes, samples. A water wash should be used after every ten samples and standards should be run after every 40 samples. The instrument is run at a rate of 40 samples/h with a sample to wash ratio of 2:1. The reagents should be run through the AutoAnalyzer for at least 30 min before the samples.

Calculation of results

Draw a calibration curve of optical density against µg N standards and read samples from this.

$$\% N = \frac{R \times 100}{10W} \times \frac{V}{100} = \frac{R \times V}{10W}$$

where R = ppm N in solution (from graph)
W = weight of sample (mg)
V = volume to which the sample is made up (ml)

Reference

Varley, J.A., 1966, The Analyst, 91, 119.

NITROGEN TRANSFORMATIONS: NITROGENASE ASSAY

Reagents

All reagents should be of analytical reagent (AR) grade.
Ethylene, C_2H_4, 99.8% pure
CaC_2
10% (w/v) $CuSO_4$ solution
Acid dichromate solution
Calcium carbide

Apparatus

Gas chromatograph with a 2 x 0.003m diam. stainless steel column packed with 80-100 mesh Poropak N.
Chart recorder
Universal bottles (30ml)
Wide-mouth reagent bottles (60ml)
'Suba-seal' rubber closures (William Freeman & Co. Ltd., Barnsley, Yorks)
1, 5, 10 and 20 ml disposable plastic hypodermic syringes
Hypodermic needles (25mm long, 25G)
Rubber bungs
Acetylene generator

Procedure

Assays should be done immediately after sample collection.

Adhering soil is removed from root systems by gentle shaking or, if necessary, by washing the root system gently in water and lightly blotting the roots dry with filter paper. (N.B. a surface film of water or air-drying reduces nitrogenase assay of root nodules). If the root samples differ widely in size similar weights of each should be taken for assay. Root samples are placed in suitable containers (30 ml Universal bottles or 60 ml wide-mouth reagent bottles depending on sample size) for incubation. Universal bottles are sealed with a metal screw-cap with a rubber liner. A small hole (c. 0.5cm diam.) is cut in the metal cap to expose the rubber liner and allow sampling with hypodermic syringes. The reagent bottles are closed with 'Suba-seals'.

Undisturbed samples, such as soil cores or plants growing in pots should be placed in airtight containers of appropriate size. Dart, Day and Harris (1972) have described a suitable system.

Air is withdrawn from the sample container, using a hypodermic syringe, and replaced with an equal volume of acetylene to give a final acetylene concentration of 10% (v/v). A known amount of propane, to give about 100 vpm, may also be injected. This acts as an internal calibration standard to measure the gas volume of the assay chamber accurately and to monitor leaks and other losses of C_2H_4.

N.B. Acetylene from commercial sources frequently contains impurities and it is preferable to generate the gas as required. Calcium carbide is treated with water and the acetylene produced bubbled through 10% (w/v) $CuSO_4$ solution followed by acid dichromate solution. The purified gas is then collected over water.

Chambers containing samples and acetylene are incubated at 20 $\pm$ 1°C (30 min for nodulated roots; up to 24 h for soils) and gas samples (1ml) then withdrawn using hypodermic syringes. These gas samples are analysed immediately or stored for not more than 3h after sealing the needles by stabbing them into rubber bungs.

N.B. Before re-use these rubber bungs must be steamed at 100°C for 15 mins and then left at room temperature for 24 h to ensure removal of all traces of ethylene. Similarly syringes and needles must be placed in a chamber which is evacuated several times before they are re-used.

The ethylene and acetylene contents of the gas samples are analysed on the gas chromatograph. The instrument is set with the column oven at 100°C, a nitrogen carrier gas-flow rate of 45ml/min and a hydrogen/air flame ionization detector. After expelling excess gas sample from the syringes exactly 1 ml is injected through the rubber septum onto the column.

N.B. In order to ensure penetration well into the column it is recommended that 25 mm hypodermic needles are used. The rubber septum should be replaced after 6 or 7 injections to minimize the risk of leakage from the column.

Gas production from samples with no added acetylene should be assayed to account for endogenous production of ethylene. In addition blank incubations of acetylene alone should be measured.

If an internal standard of propane is not used the gas volume of the chambers plus samples should be measured by water displacement, using a burette to add the water.

Calculation

The heights of peaks on the chromatograph's recorder which correspond to ethylene are measured and the gas produced is calculated from:

$$\mu M\ C_2H_4/h = \left[H_s \times \frac{Vs}{V} \times T \times K \right] - \left[H_b \times \frac{Vb}{V} \times K \right]$$

Where H_s = peak height for sample
Hb = peak height for acetylene blank
Vs = volume of gas in sample container
Vb = volume of gas in blank container
V = volume injected into gas chromatograph
T = assay time (h)
K = conversion factor obtained using standard C_2H_4 gas mixture to calibrate the chromatograph.

e.g. for 100 vpm C_2H_4 standard, K is derived as follows :

1ml 100 vpm C_2H_4 = 100×10^{-6} ml C_2H_4 and gives peak ht. (H)
22.41 C_2H_4 at STP = 1 mole C_2H_4

1 ml of 100 vpm C_2H_4 = $\frac{100 \times 10^{-6}}{22.4 \times 10^{3}}$ moles C_2H_4

$$= 0.00446 \ \mu \text{ moles } C_2H_4 = H$$

$$\text{Then } K = \frac{0.00446}{H} \ \mu \text{ moles } C_2H_4$$

Reference

Dart, P.J., Day, J.M. and Harris, D., 1972. In FAO/International Atomic Energy Agency. Technical Booklet on Grain Legume Production.

SOIL ENZYMES: PHOSPHATASE

Reagents

All reagents should be of analytical reagent (AR) grade.
0.5% w/v disodium phenyl phosphate solution. Store at 4°C.
0.3% w/v Al $K(SO_4)_2.12H_2O$
Sodium borate buffer, pH 9.4. Dissolve 3.81g di-sodium tetraborate decahydrate in 1 l. deionized water. Adjust to pH 9.4 with M NaOH. This reagent can be stored for 1 month at 4°C.
Gibbs reagent. Dissolve 0.125g 2,6-dibromoquinone-4-chloroimide in 100 ml absolute alcohol. Do not keep this reagent for more than 8 h.
Phenol standard solutions. A 1000 µg phenol/ml stock solution is prepared by dissolving 0.25g phenol in 250 ml deionized water. The solution may be stored at 4°C. Working standards in the range 2.5 to 25 µg/ml are prepared from the stock solution by dilution with deionzed water.

Apparatus

Technicon AutoAnalyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants, RG21, 2YE)

Log chart recorder paper
20 ml pipettes
50 ml automatic dispenser
100 ml conical flasks
7 cm filter funnels
Whatman No. 1 filter papers

Procedure

Samples (5g) of treated or control soils are mixed with 20 ml disodium phenyl phosphate solution, prewarmed to 30°C, in 100 ml conical flasks. The suspensions are incubated at 30°C for 2 h then mixed with 100 ml $AlK(SO_4)_2$ solution and filtered through Whatman No. 1 paper. The phenol content of the filtrate is measured using the AutoAnalyzer with the manifold shown in Fig. 7 in the Appendix. All reagents should be run through the AutoAnalyzer for at least 30 min before starting the analyses.

The sample tray (8-10 ml cups) should be loaded with the standards followed by two water washes and then the soil extracts. A water wash is used after every tenth soil extract. The instrument is run at 40 samples/h with a sample wash ratio of 2:1.

Calculation of results

Draw a calibration graph from the optical densities of the standards and calculate the slope of the graph.

Phosphatase activity is expressed as mg phenol released / g dry soil

$$\text{mg phenol/g dry soil} = \frac{(S-B) \times F \times 120}{1000 \times W}$$

Where S = peak height
B = baseline height*
F = slope of calibration graph
W = weight of dry soil (g)

* Baseline drift occurs with this technique and must therefore be allowed

for in the calculation.

Reference

Halstead, R.L. 1964 , Can. J. Soil Sci., 44, 137.

SOIL ENZYMES: DEHYDROGENASE

Reagents

All reagents should be of analytical reagent (AR) grade.
3% w/v 2,3,5-triphenyl tetrazolium chloride (TTC) solution. Dissolve 0.3g TTC and 0.6057g tris-(hydroxymethyl)-methylamine in 10 ml deionized water. This solution should be made up immediately before use and kept in the dark.
Methanol
Standard solutions. Dissolve 10 mg 2,3,5-triphenyl tetrazolium formazan (TPF) in 1 l. methanol. This solution (10 µg TPF/ml) is stable if stored in the dark at 4°C. Working standards are prepared by diluting this stock solution with methanol to give TPF concentrations in the range 2 to 8 µg TPF/ml.

Apparatus

Unicam SP500 Spectrophotometer with automatic sample changer (Pye-Unicam Ltd., Cambridge, CB1 2PX)
Vortex mixer ('Whirlimixer', Fisons Scientific Apparatus, Loughborough, Leics, LE11 0RG)
End-over-end shaker
Centrifuge
25 ml volumetric flasks
15 ml screw-cap specimen tubes
0.5 ml graduated pipettes

Procedure

Samples (1g) of treated or control soils are weighed into the screw-cap specimen tubes and 0.2ml TTC solution and 0.5 ml deionized water added. The soil suspensions are then mixed for 15 seconds on the vortex mixer and incubated in the dark at 30°C for 18 h.

N.B. It is important that subsequent stages in the procedure are done in subdued light to avoid fading of the colour of the TPF solutions.

Add 10 ml of methanol to each tube and shake on the end-over-end shaker for 30 min. Centrifuge the extracts at 2000g for 20 min and transfer the supernatant to 25 ml volumetric flasks. Make the volumes to 25 ml with methanol. Measure the optical densities at 485 nm using the spectrophotometer.

Use of a flow cell in the spectrophotometer and an automatic sample changer allows a much larger throughput of samples (80 samples/hour). The sample plate is loaded in the following order: blank (methanol), 10 µg/ml TPF standard, blank, whole range of TPF standards, blank, soil extracts with blanks after every 10 extracts. The initial 10 µg/ml TPF standard is used to adjust the pen deflection on the chart recorder of the spectrophotometer.

Calculation of results

TPF concentrations are obtained from comparison with a standard curve of TPF in methanol. Results are recorded in volumes of hydrogen transferred during the reduction of TTC to TPF in 1g dry soil according to the equation:-

2,3,5-triphenyltetrazolium chloride + 2H $\longrightarrow$ triphenyl formazan + HCl

The formation of 1mg of TPF requires 150.35 µl H.

$$\text{Thus dehydrogenase activity} = \frac{(S-B) \times F \times 25 \times 0.15035}{W}$$

Where S = peak height
B = baseline height
F = slope of calibration graph
W = weight of dry soil (g)

References

Stevenson, I.L., 1959, Can. J. Microbiol., 5, 229.
Klein, D.A., Loh, T.C. and Goulding, R.L., 1971, Soil Biol. Biochem., 3, 385.
Ross, D.J., 1971, Soil Biol. Biochem., 3, 97.

SOIL ENZYMES: UREASE

Reagents

All reagents should be of analytical reagent (AR) grade.
All solutions should be made in glass-distilled water.
0.4% w/v sodium azide. Store at 4°C.
0.5 M tris/0.5 M maleic buffer. Dissolve 6.057g tris (hydroxy-methyl) methylamine and 5.8035 g maleic acid in 100 ml water. Adjust the pH to 7.0 with M NaOH solution. This solution can be stored at 4°C for not more than 1 week.
Boric acid / indicator solution. All glassware used in the preparation of this solution should be rinsed with ethanol. Dissolve 0.0084 g bromocresol green and 0.016 g methyl red in 10 ml ethanol. Dissolve 4 g boric acid in water with heating, cool, transfer to 200 ml volumetric flask, add 4 ml indicator solution and make up to volume with water. Store at 4°C in the dark.
0.01 M Ag_2SO_4. Dissolve 0.3118 g in 100 ml water with heating (avoid boiling). Store in dark at room temperature.
6.0 M urea. Make up immediately before use.
45% w/v K_2CO_3. $1\frac{1}{2}$ H_2O
0.2 M & 0.02 M HCl prepared from concentrated volumetric solutions.

Apparatus

Conway microdiffusion dishes
0.5, 1, 2 and 3 ml pipettes
1.0 ml self-filling burettes (0.01 ml graduations)

The Conway dishes and pipettes should be washed overnight in 2M HCl.

Procedure

Weigh samples (1g) of control or treated soil and place in the outer well of the Conway dishes. Add 0.5 ml sodium azide solution and leave for 1 h. Add 2 ml tris/maleic buffer to the soil and mix using a glass rod. Add 3 ml boric acid/indicator solution to the centre well. Care must now be taken not to move the dishes to avoid mixing the contents of outer and inner wells. Add 1 ml urea to the soil-buffer mixture in each dish. Prepare blanks by adding 1 ml distilled water in place of the urea. Incubate the dishes at room temperature (about 20°C) for 1 to 2 h, depending on the soil urease activity. Add 0.5 ml Ag_2SO_4 to the soil mixture and stir with a glass rod. It is important that the incubation time is exact for each dish and thus additions of the urea and Ag_2SO_4 should be done in a timed sequence. Add 1 ml K_2CO_3 to the soil mixture, cover immediately and leave for 18 hours. Some of the soil mixture in the outer well can be removed before the dishes are moved as it must not be spilt into the indicator solution. Titrate the indicator solution from green back to red using first 0.2 M HCl dropwise until the indicator starts to turn purple and finally with 0.02 M HCl till the red end point is reached. Calculate the total volume of HCl taken in terms of 0.02 M HCl.

Calculation of results

$$\text{Urease activity, as } \mu \text{ moles } NH_3 \text{ evolved /g dry soil/hour} = \frac{(t-b) \times 20}{W \times T}$$

t = titre, ml 0.02M HCl
b = blank titre, ml 0.02M HCl
W = weight dry soil (g)
T = incubation time (h)

Reference

Pettit, N.M., Smith, A.R.J., Freedman, R.B. and Burns, R.G., 1976, Soil Biol. Biochem., 8, 479.

CELLULOSE DECOMPOSITON: SOIL BURIAL TEST

Apparatus

Shirley Test Cloth. (The Shirley Institute, Didsbury, Manchester, M20 8RX).
Disposable glass microscope slides (75x26mm)
Plastic 'sandwich' boxes (145x115x70mm)
Polyethylene bags (260x310mm)
Desiccator
Graticule - a rigid transparent plastic strip (70x26mm) marked in rectangles each of which is about 1% of the total area.
'Araldite' epoxy-resin adhesive (Ciba-Geigy (UK) Ltd.,Cambridge, CB2 4QA)

Procedure

Mount strips of test cloth (100 x 26mm) on glass slides fixing the ends to the backs of the slides with epoxy-resin adhesive. 'Araldite' has been found to be suitable.

Line the plastic boxes with polyethylene and place a 10 mm layer of moist soil in the bottom. Place five cloth-covered slides on edge in this soil and fill the box with more moist soil using a glass rod to make the soil firm between the slides. This ensures good contact between soil and cloth. The boxes are each covered with polyethylene bags which are secured in place with elastic bands and then inflated using a compressed air supply. The resultant polyethylene 'tent' over each box reduces moisture losses during incubation. The boxes are weighed and incubated at $19 \pm 2^{o}C$ for up to 8 weeks. Moisture losses are checked every two weeks by weighing and corrected by adding water from a hypodermic needle pushed through the polyethylene 'tent'. At the same time the air in the 'tents' is changed by inserting a rubber tube and flushing with compressed air. The time of incubation will vary according to the activity of the soil. If the cloth strips are incubated for too long they will disintegrate and recovery from the soil will be impossible.

Following incubation the slides are carefully removed from the soil and soil particles gently removed from the cloth using a small artist's brush. A 70mm long piece of cloth is removed from each slide and air-dried for 18 h at room temperature in a desiccator. The strip is weighed and the weight loss calculated as a percentage of the weight of a piece of cloth of equal size which has not been buried.

The microbial colonization of the cloth strip can be assessed crudely on the basis of the areas of cloth showing different colours (eg areas invaded by Fusarium spp. or Gliocladium roseum are frequently pink while black areas usually denote colonization by Stachybotrys chartarum or Acremonium spp.). These coloured areas are measured by covering the cloth strip with the transparent plastic graticule and counting the number of rectangles covering each coloured area.

The graticule can also be used to give an approximate estimate of the area of cloth missing due to complete decomposition. Similarly partial decomposition can be estimated with the cloth strip held in front of a light source when small holes and areas of 'thinning' can be easily seen.

The scoring scale used is :-

0 - no effect
1-3 - some thinning of the material

4-6 - small holes appearing
7-9 - large areas of cloth disintegrated
10 - complete disintegration

When required the micro-organisms colonizing the cloth can be isolated for further study. Fungal hyphae and/or sporing structures can be isolated using fine glass needles while observing the cloth with a stereoscopic microscope and transferred to suitable agar medium (see p. 37). Other micro-organisms can be isolated by removing small fibres and placing these on agar plates. Alternatively pieces of cloth can be treated in water using a 'Stomacher' and the suspensions plated out (see section on counting micro-organisms p. 29).

Reference

Grossbard, E. and Wingfield, G.I., 1975, Soc. appl. Bact. Tech. Ser. 8, p. 236.

ENUMERATION OF MICROBIAL PROPAGULES IN SOIL: 'DILUTION-PLATE' TECHNIQUE

Apparatus

Pre-dried plates of agar media (see pp.37-42 for suitable growth media)
Colworth 400 'Stomacher' (A.J. Seward, Bury St. Edmunds, Suffolk,IP33 3PD)
'Cryovac' bags, 7x12 inch, 200 G(" " " " " " ")
Temperature-controlled water bath, controllable from 20 - 90°C.
Vortex mixer ('Whirlimixer', Fisons Scientific Apparatus, Loughborough, Leics., LE11 ORG)
Illuminated incubator at 25°C
Incubator at 19°C
1 ml and 10 ml graduated pipettes (sterile)
Illuminated Petri-dish viewer
Glass spreading rods (sterile)
200 ml medical flat bottles (with polypropylene caps) containing 100 ml and 90 ml deionized water (sterile)
Rimless test tubes with polypropylene caps containing 9 ml deionized water (sterile)
Test tubes with screw caps containing 9 ml deionized water (sterile)

Procedure

Samples (1g) of control or treated soil are placed inside 'Cryovac' bags with 100 ml sterile water. (The inner surfaces of 'Cryovac' bags are sterile when the bags are new). The soil suspension is then treated in the 'Stomacher' for 1 min to remove and suspend micro-organisms adsorbed onto particle surfaces. We have found 1 min to be long enough to give maximal bacterial counts in a range of soils including sandy loam, clay loam and fenland peaty soil. It is advisable, however, to test the 'Stomacher' on each soil used and determine the time required to give maximal counts. With soils it is necessary to use a double 'Cryovac' bag to avoid puncture during treatment in the 'Stomacher'.

This initial suspension of soil is diluted ten-fold by pipetting 10 ml into 90 ml sterile water and shaking the suspension by hand for 1 minute. A 1 ml aliquot is transferred to 9 ml sterile water in a test tube with a polypropylene cap and the suspension mixed on the vortex mixer for 15 s. This suspension is further diluted in the same way and the procedure repeated as often as necessary to produce the required range of dilutions. We normally use dilutions of 10^{-2}, 10^{-3} and 10^{-4} for fungi and algae and 10^{-4}, 10^{-5} and 10^{-6} for bacteria and actinomycetes. In practice, however, a preliminary experiment will be necessary to find the suitable dilution levels for each soil used.

To count the micro-organisms present 0.1 ml volumes of the appropriate dilutions are pipetted onto the surface of plates of suitable agar medium (see pp. 37-42 for media used). Before use the plates are kept at 37°C for 2 to 3 days to dry any surface moisture on the agar and promote rapid absorption of the soil dilution into the agar. Three replicate plates are prepared for each dilution. Plates are incubated at 19 ± 2°C for 7 days (total fungi and cellulolytic fungi) or 14 days (bacteria and actinomycetes). Plates for counting algae are incubated for 14 days at 25 ± 1°C in an illuminated incubator. After incubation the numbers of colonies per plate (or, in the case of cellulolytic fungi, the numbers of colonies producing clear zones in the agar) are counted on an illuminated colony counter.

Spore forming bacteria are counted in soil dilutions which have been

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