Click here for previous

heated at 80°C for 10 min in a water bath. The dilutions to be heated are prepared in screw-cap test tubes and the caps are sealed with PVC tape so that the tubes may be completely submerged. After heating the tubes are cooled rapidly by submersion in cold water and the dilutions are then inoculated onto agar plates as described above.

Calculation of results

$$\text{No. of propagules/g dry soil} = \frac{\text{Av. No. colonies per plate x dilution level}}{\text{dry weight soil}}$$

ENUMERATION OF MICROBIAL PROPAGULES IN SOIL: MINIATURIZED MOST PROBABLE NUMBER (MPN) TECHNIQUE

Amongst the many disadvantages of the 'plate-dilution' method for counting microbial propagules is the large input of manpower and materials. This disadvantage can be overcome by the use of a miniaturized MPN technique which also greatly speeds the processing of samples and allows more to be handled.

Medium

Nutrient broth (CM1) (Oxoid Limited, Basingstoke, Hants, RG24 OPW)

Apparatus

Colworth 400 'Stomacher' (A.J. Seward, Bury St. Edmunds, Suffolk, IP33 3PD)
Microdiluter rods - 25 µl capacity (Gibco: Bio-cult Ltd., Hounslow, Middlesex)
Manual diluting apparatus (Darbyshire, Wheatley, Greaves & Inkson, 1974)
AM 81 Mini Pipetter (Gibco: Bio-cult Ltd., Hounslow, Middlesex)
Illuminated Petri-dish viewer
Microtitre microtitration plates, 96 - well rigid polystyrene, U-wells.
(Gibco: Bio-cult Ltd., Hounslow, Middlesex)
200 ml medical flat bottles containing 90 ml deionized water (sterile)
1 ml graduated pipettes (sterile)
Pasteur pipettes calibrated to deliver about 100 µl/3 drops

Procedure

As manual filling of microtitration plates with liquid medium is a tedious procedure, prone to operator error, it is advisable to use one of the many semi-automatic, repetitive pipetting devices available. In our experience the best method, especially when large numbers of plates are to be filled, is using a multi-channel dispenser such as the Mini Pipetter.

Medium (100 µl) is dispensed into all the wells on a microtitration plate with the exception of those in the first row. The wells of the first row each receive about 100 µl of soil suspension from Pasteur pipettes. This suspension is prepared by treating 1g soil in 100 ml sterile deionized water for 1 min in the 'Stomacher' as described on p.29 and then diluting 10 ml of this suspension with 90 ml sterile deionized water.

Preparation of a dilution series from the soil suspension in the first row of wells is achieved using 25 µl microdiluters. The heads of these rods are calibrated to pick up exact volumes of solution by capillary action. There is some risk when using soil suspensions, especially those of highly organic soils, that particles may become lodged in the microdiluter head. To reduce this risk it is essential that the microdiluters used are those of the 'tulip claw' type; the newer designs with enclosed chambers are less satisfactory when using soil suspension.

The microdiluter rods are dipped into the suspension in the first row of wells, rotated 15 times in approximately 6 s to mix the suspension without causing frothing, and transferred to the medium in the second row of wells. The microdiluters are rotated again and transferred to the third row. This is repeated to effect transfers to all 12 rows of wells. Each transfer of 25 µl from one well to the next produces a five-fold dilution of the suspension carried over. When the dilution series down all 12 rows of wells in a microtitration plate has been achieved the lid of the plate is replaced and the plates

incubated in the dark at 19 ± 2°C for 5 days.

It is important that the microdiluters are immersed in the suspension to exactly the same extent at each transfer, in particular they should never be immersed below their shoulders. While it is quite simple to manipulate up to 8 microdiluters manually it is much preferable to use a mechanical system which will guarantee reproducibility of operation. A suitable system can be bought (eg The 'Titertek' Multidiluter, Flow Laboratories Ltd. Irvine, Ayrshire, KA12 8NB) or a cheap, effective alternative can be constructed quite easily. This latter is described by Darbyshire, Wheatley, Greaves and Inkson (1974).

Preparation of the microdiluters before use is of considerable importance (see note on p. 46). The heads (not the shafts) should be heated to an incandescent red and then allowed to cool in air. They are then wetted with distilled water, blotted lightly, dipped in absolute alcohol and flamed to sterilize them. This dries the heads and care must then be taken to ensure that the heads wet properly when immersed in the soil suspension and that air is not trapped between the claws. This is not normally a problem if the microdiluters are rotated properly. The microdiluters should be flamed with alcohol before every dilution plate and should be heated to red heat after about every 10 dilution plates and before putting away at the end of each work session.

After incubation any bacterial growth in the wells, shown by turbidity of the medium or aggregated growth at the bottom of the well, is recorded. This is facilitated by using an illuminated Petri-dish viewer. Where there is doubt about the growth in any well a sample is taken and examined using a microscope.

When it is necessary to incubate for longer periods than 5 days, or when higher incubation temperatures are required, it is advisable to place the plates inside plastic boxes with damp filter paper in the base. The plate lids are secured in place with rubber bands. These precautions reduce moisture loss from the medium in the wells and prevent drying out.

Calculation of results

An estimation of the numbers of bacteria in the soil suspension is made by recording the presence or absence of growth in all the replicates. At dilution levels where the concentration of cells is on average 1 per replicate, some of the replicates will show no growth. At present there are no statistical tables which aid the estimation of microbial densities for 5-fold dilution series and the estimation must be done by the method of Finney (1951) as described by Darbyshire, Wheatley, Greaves and Inkson (1974).

Other uses of the method

This method has been used successfully to estimate populations of algae and fungi, using the media described on pages 40-41. In addition it can be used to count different physiological groups of bacteria (eg non-symbiotic nitrogen-fixers) by using the appropriate medium.

References

Darbyshire, J.F., Wheatley, R.E., Greaves, M.P. and Inkson, R.H.E., 1974, Rev. Ecol. Biol. Sol., 11, 465.

Finney, D.J., 1951, J. Hyg. Cam., 49, 26.

ENUMERATION OF MICROBIAL PROPAGULES IN SOIL: MICRO-ORGANISMS IN THE ROOT REGION

Micro-organisms growing on or near plant roots can be counted by the 'dilution-plate' or miniaturized MPN techniques described previously.

The methods differ only in the preparation of the initial suspensions from which the dilution series are prepared. These methods are described below.

Apparatus

Pre-dried plates of agar medium or 'Microtitre' microtitration plates filled with suitable media
Wrist action flask shaker
Colworth 400 'Stomacher' (A.J. Seward, Bury St. Edmunds, Suffolk, IP33 3PD)
'Cryovac' bags 7x12 inch, 400G(" " " " " " ")
Aluminium weighing tubes (6.5x3.0cm), screw capped, lined with filter paper, sterile
250 ml conical flasks containing 100 ml deionized water, sterile
250 ml conical flasks containing 100 ml deionized water and 4g glass beads (2-3mm diameter), sterile
Medical flat bottles containing 100 ml deionized water, sterile
Rubber bungs for 250 ml conical flasks, sterile

Procedure

Rhizosphere soil. With plants grown in the field the roots are lifted gently from the soil with a garden fork. When plants are grown in pots the entire contents of the pot are tipped out. In both cases the roots are teased gently from the soil mass and any large lumps of soil gently crushed with the fingers and removed. Great care should be taken to minimise damage to the roots. The root systems are then shaken gently to remove loosely- adhering soil. At this stage any soil remaining on the root systems can be regarded as rhizosphere soil.

This procedure works satisfactorily in most soils at about 60% moisture holding capacity but may have to be varied slightly in very dry or especially in very wet soils. In wet soils simple shaking may not remove much soil and more vigorous treatment may be required to remove 'non-rhizosphere' soil. In general the greatest rhizosphere effect is considered to extend to only 1 or 2 mm from the root surface. In dry soils shaking may remove too much soil to allow a big enough sample of adhering soil to be obtained from the root.

Portions (up to 0.5g) of root, normally complete primary laterals or entire seedling root systems, are placed in 100 ml sterile water in a conical flask. The flask is closed with a bung and shaken vigorously on a wrist-action shaker for 5 min. The resulting soil suspension is serially diluted and the micro-organisms counted as described previously.

The weight of rhizosphere soil obtained is found after removing the root material from the suspension using a wire loop. The suspension is then dried in an evaporating basin on a boiling water bath. The dried soil is cooled in a desiccator and weighed. If required the dilutions of this suspension can be combined and dried down with it, but in practice it is usually satisfactory to mathematically correct the dry weight of soil in the initial suspension for the amount removed in preparation of the dilution series.

N.B. Recovery of roots from the rhizosphere soil suspensions using a wire loop will leave behind small fragments of fine roots whose weight will contribute to the weight of the soil. In practical terms this error can be ignored.

Root surface. Roots which have been washed free of rhizosphere soil are transferred from the flask to a sterile aluminium screw-cap weighing tube (6.5x3.0cm) lined with filter paper and weighed. Gentle shaking in this tube removes much of the surface moisture held by the roots. The roots are then transferred to 250 ml flasks containing 100 ml sterile water and 4 g glass beads (2-3mm diameter). The empty tube is weighed and the weight of the roots found by difference. The flask with glass beads and roots is closed with a sterile rubber bung and shaken vigorously on the wrist-action shaker for 20 min. The abrasive action of the beads serves to remove organisms which are adhering to the outer surfaces of the roots. The resulting suspension is diluted serially and the numbers of organisms present estimated.

Root interior. Micro-organisms colonizing the tissues of the root can be estimated in suspensions prepared by macerating the root. Roots are removed from the flask containing glass beads (above) with a wire loop and transferred to a sterile 'Cryovac' bag, having weighed them in sterile, filter-paper lined aluminium tubes as before. 100 ml sterile water is added to the bag and the roots are then macerated in the 'Stomacher' for 5 min. The resulting suspension is diluted serially and the numbers of organisms estimated.

We have found 5 min to be suitable for removing and macerating the epidermal and cortical tissue from cereal roots. It will be necessary to determine the optimum time for other types of root system by preliminary experimentation. The degree of removal and maceration is conveniently judged with low power (x20) magnification.

References

Leuw, H.A. and Webley, D.M., 1959., J. appl. Bact., 22, 216.
Webley, D.M., Duff, R.B., Bacon, J.S.D. and Farmer, V.C., 1965, J. Soil Sci., 16, 149.

ENUMERATION OF MICROBIAL PROPAGULES IN SOIL: DIRECT COUNTING BY FLUORESCENCE MICROSCOPY

Reagents

All reagents should be of analytical reagent (AR) grade.

0.5 M carbonate-bicarbonate buffer, pH 9.6. Dissolve 106 g anhydrous Na_2CO_3 in 1 l. deionized water (solution A). Dissolve 84.0 g $NaHCO_3$ in 1 l. deionized water (solution B). Mix 160 ml A with 340 ml B and make to 2 l.

0.01 M phosphate buffer, pH 7.2. Dissolve 3.12 g $NaH_2PO_4.2H_2O$ in 1 l. deionized water (solution X). Dissolve 2.839 g Na_2HPO_4 in 1 l. deionized water (solution Y). Mix 280 ml X with 720 ml Y and make to 2 l.

0.85% (w/v) NaCl solution

Fluorescein isothiocyanate, (FITC)

5% (w/v) $Na_4P_2O_7$ solution

Glycerol-saline buffer. Glycerol adjusted to pH 9.6 by mixing with carbonate-bicarbonate buffer and NaCl solution in the proportions 4:1:1 by volume.

Staining solution. Dissolve 1.0 mg FITC in a mixture of 0.25 ml carbonate-bicarbonate buffer, 1.1 ml phosphate buffer and 1.1 ml NaCl solution. The solution is mixed at room temperature and used immediately or stored for a MAXIMUM of 6 h at 4°C

Apparatus

Fluorescence microscope fitted for incident illumination with a 200 W mercury vapour lamp, excitation filter BG 12/4 mm and barrier filter Zeiss No. 50 or Schott OG4. A squared eyepiece-graticule and linear scale stage graticule are required. The most useful objective has been found to be a x40, oil-immersion, Planapochromat with iris diaphragm.

Colworth 400 'Stomacher' (A.J. Seward, Bury St. Edmunds, Suffolk, IP33 3PD)

'Cryovac' bags (" " " " " " ")

Microscope slides marked with a 1 cm^2 area using a diamond pencil

0.1 ml pipettes graduated to 0.01 ml divisions

Procedure

The method is that of Babiuk and Paul (1970) as modified by Greaves, Wheatley, Shepherd and Knight (1973).

Samples (1g) of fresh soil are suspended in 100 ml sterile water in 'Cryovac' bags by treating in the 'Stomacher' for 1 min (see p. 29). For microscopic examination, 0.01 ml aliquots of this suspension are spread over the 1 cm^2 areas marked on microscope slides. The smears are air-dried and then lightly fixed in a flame.

The fixed smears are stained for 3 min with the FITC staining solution, then washed in carbonate-bicarbonate buffer for 10 min followed by $Na_4P_2O_7$ solution for 2 min. The smears are drained, the back of the slide dried with tissue, and mounted in glycerol-saline buffer at pH 9.6. This mountant reduces fading of the fluorescence during observation on the microscope. (Nairn, 1969).

The micro-organisms in the stained soil smears fluoresce bright apple green and are readily visible. All such cells falling within the area outlined by the eyepiece graticule are counted. Normally 10 graticule areas, selected

within the 1 cm^2 smear at random, are counted for each smear. Only those organisms which have the morphology of bacteria are counted though obviously this may include some micro-organisms which are not actually bacteria.

The area of smear bounded by the eyepiece graticule is measured using a linear stage graticule.

Calculation of results

$$\text{Nos. of organisms/g dry soil} = \frac{\text{Av. No. cells/area counted} \times \frac{\text{(smear area)}}{\text{(area counted)}} \times \frac{(100)}{(0.01)}}{\text{dry wt. soil in orig. suspension}}$$

References

Babiuk, L.A. and Paul, E.A. 1970. Can.J.Microbiol., 16, 57.

Greaves, M.P., Wheatley, R.E., Shepherd, H. and Knight, A.H. 1973. Soil Biol. Biochem., 5, 685.

Nairn, R.C. 1969. Fluorescent Protein Tracing (R.C. Nairn, ed.) p.303 E. and S. Livingstone, Edinburgh.

MEDIA FOR DIFFERENT MICROBIAL PROPAGULES: GENERAL

Reagents

As shown for individual media

Apparatus

The following list is of apparatus required to prepare all the media described.

Ball mill with 3 mm glass beads
Steam sterilizer, Koch pattern
pH meter and glass electrode
Sieve, 2 mm mesh size
Stainless steel bucket, 13 l. capacity
3 l. conical flasks
500 ml medical flat bottles
9 cm Petri dishes
Membrane filters, 0.22 µm pore diam.
Buchner funnels (27 cm diam.) and filter flasks
Pulped filter paper - conveniently prepared by cutting filter paper off-cuts into small pieces and soaking in water. The resulting sludge is placed in the Buchner funnels and the moisture drained off under vacuum to provide a pulp layer about 1 cm thick.
pH test papers, range pH 1 to 5
Glass rods, sterile

MEDIA FOR DIFFERENT MICROBIAL PROPAGULES: CELLULOLYTIC FUNGI

(Modified from Skinner, F.A., 1960, J. gen. Microbiol., 22, 539.)

Reagents

a) Whatman cellulose powder CC41	40.0g	(B D H Chemicals Ltd., Poole, Dorset, BH12 4NN)
b) K_2HPO_4	65.0g	
KH_2PO_4	35.0g	
c) Carboxy methyl cellulose (7 HOP)	5.0g	(Hercules Powder Co. Wilmington, Delaware, USA)
$(NH_4)_2SO_4$	5.0g	
$CaCl_2$ (fused granular)	0.5g	
d) $MgSO_4.7H_2O$	0.5g	
Na Cl	10.0g	
Yeast extract (L21)	1.0g	(Oxoid Ltd., Basingstoke, Hants, RG24 0PW)
e) Agar (Oxoid No. 3)	150.0g	(Oxoid Ltd., Basingstoke, Hants, RG24 0PW)
f) Chlortetracycline - HCl	0.3g	
Water (deionized)	10 l.	

Procedure

a) 40g cellulose powder is ball-milled at 70 rev/min with 600 ml water for 72 h at 4°C using 170 g 3 mm diam. glass beads. The cellulose is separated from the glass beads by washing the slurry through a 2 mm mesh sieve with 1400 ml water.

b) The phosphate components are dissolved in 1 l. water and autoclaved.

c) The carboxy methyl cellulose is dissolved by heating in 400 ml water. The mixture is stirred continuously to ensure formation of a clear homogenous solution.

d) These components are dissolved individually in small volumes of water and the resulting solutions poured into a larger volume (about 6 l.) of water in a container of suitable volume. A 13 l. stainless steel bucket is convenient.

The carboxy methyl cellulose solution (c) is added and the total volume made to 7 l. This solution is dispensed equally into five 3 l. conical flasks.

e) The agar powder is added (30g/flask) and the flasks heated at 100°C for 30 min in a steam sterilizer to dissolve the agar.

Cellulose suspension ((a), 400 ml/flask) is then added to the agar solution which is dispensed as 360 ml volumes into 400 ml medical flat bottles and autoclaved. If the agar is to be used immediately, 40 ml sterile phosphate solution (b) is added aseptically to each bottle after autoclaving. If the agar is to be stored the phosphate solution is mixed with the melted agar immediately prior to use.

f) Before pouring plates of this medium 1 ml of a sterile solution of chlortetracycline - HCl containing 12 mg/ml (0.3 g dissolved in 25 ml of a mixture (2:1 v/v) deionized water and acetone) is added to the cooled (c. 55 °C), melted medium. The antibiotic solution is best sterilized by passage through a 0.22 µm pore diameter membrane filter.

The pH of the medium made in this way is naturally pH 7 and it is not necessary to adjust it by addition of acid or alkali.

Plates are surface-dried by incubating at 37 °C for 2 to 3 days immediately prior to use.

MEDIA FOR DIFFERENT MICROBIAL PROPAGULES: BACTERIA AND ACTINOMYCETES, FUNGI

(Bunt, J.S. and Roviŗa, A.D., 1955, J.Soil Sci., 6, 119)

Reagents

K_2HPO_4	0.4 g
$(NH_4)_2HPO_4$	0.5 g
$MgSO_4.7H_2O$	0.05 g
$MgCl_2.6H_2O$	0.1 g
$FeCl_3.6H_2O$	1 drop of a 1% w/v solution
$CaCl_2.2H_2O$	0.1 g
Peptone (Balanced Peptone No.1)	1.0 g (lab m Ltd., London EC3R 7QJ)
Yeast extract	1.0 g (Oxoid Ltd., Basingstoke, Hants, RG24 OPW)
Soil extract	250 ml
Water (deionized)	750 ml
Agar (Oxoid No.3)	12-15 g (Oxoid Ltd., Basingstoke, Hants, RG24 OPW)
Chlortetracycline HCl	2.5 ml containing 12 mg/ml (for fungi only)

Procedure

Soil extract is prepared by autoclaving 1 kg of a loam soil with 1 l tap water. The slurry is filtered under vacuum through a 1 cm deep layer of pulped filter paper in large Buchner funnels. The volume of the filtrate is made to 1 l. with tap water.

Bacteria and actinomycetes. The components of the medium are dissolved individually in small aliquots of water and each solution poured into the remaining water, followed by the soil extract. The agar powder is mixed into the solution with stirring and the medium is then heated at 100°C for 30 min to dissolve the agar. After cooling to about 60 to 70°C the pH is checked using a pH electrode and adjusted to 6.8 by the addition of 1 M NaOH or HCl as necessary. The medium is then autoclaved and 11 ml aliquots poured into Petri dishes. The dishes are stored in sealed polyethylene bags at 4°C and then surface-dried immediately prior to use.

Fungi. The components of the medium with the exception of the agar are dissolved individually in small aliquots of water which are each poured into a larger volume of water. This solution is made to a total volume of 250 ml with water and the soil extract added. The pH of this solution is adjusted to 5.5 with 1 M HCl using a pH electrode and then autoclaved. The agar is dissolved and sterilized in 500 ml water by autoclaving. After autoclaving the solutions are cooled to 60 to 70°C and combined aseptically. Chlortetracycline - HCl is added to each litre of medium to suppress bacterial growth and the medium immediately dispensed in 11 ml quantities into Petri dishes. The dishes are stored in sealed polyethylene bags at 4°C and then surface-dried immediately prior to use.

MEDIA FOR DIFFERENT MICROBIAL PROPAGULES: ALGAE

(Flent, E.A., 1958, N.Z. J.Agric.Res., 1, 991)

Reagents

NH_4NO_3	0.2 g
K_2HPO_4	0.1 g
$MgSO_4.7H_2O$	0.1 g
$CaCl_2.6H_2O$	0.1 g
$FeCl_3.6H_2O$	1 drop of a 1% w/v solution
Water (deionized)	1000 ml
Agar (Oxoid No.3)	12-15 g (Oxoid Ltd., Basingstoke, Hants RG24 0PW)

Procedure

The components of the medium are dissolved individually in small aliquots of the total volume of water required and poured into the remaining water. The agar is mixed into the solution with stirring and dissolved by heating at 100°C for 30 min in a steamer. The solution is cooled to 60 to 70°C and the pH adjusted to pH 7.0 with 0.1 M NaOH using a pH electrode. The medium is then autoclaved and immediately poured in 11 ml amounts into Petri dishes. The dishes can be stored in sealed polyethylene bags at 4°C and then surface-dried immediately prior to use.

MEDIA FOR DIFFERENT MICROBIAL PROPAGULES: YEASTS

(Di Menna, M.E., 1958, N.Z. J. Agric. Res., 1, 939)

Reagents

Glucose	40g
Peptone (Balanced Peptone No.1)	10g (1ab m Ltd., London, EC3R 7QJ)
Agar (Oxoid No. 3)	15g (Oxoid Ltd., Basingstoke, Hants, RG24 0PW)
Water (deionized)	1 l.

Procedure

The glucose and peptone are added directly to the 1 l. water. The agar is then added with stirring and dissolved by heating at 100°C for 30 min in a steamer. The agar medium is sterilized by autoclaving and then adjusted to pH4 by adding 10% HCl. The pH is measured using appropriate pH test paper by transferring aliquots of the medium to the paper using a sterile glass rod. Immediately after adjustment of the pH the medium is poured, in 11ml amounts, into Petri dishes. After cooling the dishes can be sealed in polyethylene bags and stored at 4°C. They are surface-dried immediately prior to use.

TOXICITY OF HERBICIDES TO MICRO-ORGANISMS: GENERAL

The method allows convenient, simultaneous assessment of the effects of herbicides on large numbers of micro-organisms in pure culture. It is suitable for bacteria, actinomycetes, fungi, algae and yeasts. Since the method is miniaturized and involves multipoint inoculation it is economical of time and materials.

Reagents

Growth media. Any liquid medium that supports growth of the micro-organisms being used is suitable. We find a liquid version of Bunt and Rovira (1955) bacterial medium at pH 6.8 (see page 40) is suitable for actinomycetes, bacteria, fungi, algae and yeasts from soil.

Herbicides. The amount of each herbicide formulation (see p. 2) to give the required final concentration is added to sterile growth medium. Liquid preparations of herbicide, which are normally obtained at high concentration, are generally sterile and thus can be added directly to the sterile medium. Dry herbicide preparations are sometimes contaminated and are sterilized by suspending in 70% (v/v) methanol (3.3 ml methanol/100mg herbicide) for 24 h at 4°C before diluting to the required concentration with sterile medium. An equivalent amount of methanol is added to the control medium.
The concentration of herbicide in the media is a matter of choice. At WRO, concentrations in the range of 1 to 50 ppm are normally used.

Micro-organisms. The organisms used at WRO were isolated from dilution plates prepared for enumeration of micro-organisms in soil.
For bacteria and actinomycetes, which are difficult to identify, all the colonies appearing on dilution plates carrying about 50 colonies were taken and purified by streaking onto fresh plates. This procedure is considered to give a suitable spread of the predominant organisms in the soil used. Fungi, yeasts and algae were isolated more selectively, individuals being transferred from dilution plates to fresh plates for purification and where possible identification. A selection was then made deliberately so that as wide a range of species as possible was available for subsequent testing. When it is deemed necessary to use specific micro-organisms, especially those which are not easily isolated from dilution plates, cultures are obtained from appropriate official culture collections or by specific isolation methods reported in the literature.
Cultures of isolated bacteria and yeasts are maintained in semi-solid forms (0.3% w/v agar) of the appropriate media given on pages 40 and 42. All other organisms are kept on agar slopes of appropriate media. Cultures are kept at 4°C for a maximum of 6 months, subcultured onto agar plates to check purity and viability and then inoculated back onto maintenance medium for further storage.
Cultures of bacteria and yeasts to be used as inocula are grown in liquid forms of the maintenance media for 7 days at 20°C. Inocula of fungi and actinomycetes are prepared from the maintenance slopes. Sporulated cultures are shaken with 5 ml sterile 0.9% (w/v) NaCl solution and resulting spore suspensions used as inocula. Non-sporulating cultures are broken up with a wire loop and shaken vigorously in 5 ml sterile 0.9% (w/v) NaCl solution to produce an inoculation of mycelial fragments.

Apparatus

Microtitration plates - sterile (Gibco:Bio-Cult Ltd., Hounslow, Middlesex)
96-pin multipoint inoculator (" " " " " ")
AM 81 Mini Pipetter (" " " " " "
Pasteur pipettes

Procedure

The 96 wells in the microtitration plates are dosed with 100 µl of herbicide medium or control medium using the automatic pipetter. Master inoculation plates are prepared by putting approximately 100 µl of each microbial inoculum in separate wells of a sterile microtitration plate using sterile Pasteur pipettes. The 96-pin multipoint inoculator is then used to pick up inocula from the master plate and transfer them to the test plates. Inoculated test plates are incubated at 20°C for 7 days and the growth of each organism in the herbicide medium then compared with that in the control. Any inhibition or stimulation of growth is noted. Toxicity of the herbicide is judged on the basis of the percentage of the cultures which are inhibited.

Changes in growth characters, cell morphology, spore production and pigmentation can also be observed if necessary. If microtitration plates with wells with flat bottoms are used an inverted microscope allows observation of these characteristics directly in the wells. Alternatively it is a simple matter to transfer small samples from each well to microscope slides for observation in a standard microscope.

Reference

Cooper, S.L., Wingfield, G.I., Lawley, R. and Greaves, M.P., 1978, Weed Research, 18.

TOXICITY OF HERBICIDES TO MICRO-ORGANISMS: DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

This method is used routinely with the green unicellular alga Chlorella sp. but there is no reason why it cannot be used with any micro-organisms that will produce, or can be treated to produce, a suspension of viable units.

Reagents

Growth medium. Flent's medium - see p.41.
Herbicides. Stock solutions of herbicides in Flent's medium (sterilized if necessary) as described on p.43.
Chlorella sp. The strain used at WRO was isolated from soil. However, cultures of Chlorella from many sources are suitable. A stock culture of the organism is maintained in Flent's medium at 20°C in the light from a north-facing window or in an illuminated incubator with light intensity of c. 11,500 lux. It is subcultured at about 6 week intervals into fresh medium.

Apparatus

Microtitration plates (sterile) (Gibco:Bio-Cult Ltd.,Hounslow, Middlesex)
Sealing tape for microtitration plates (" " " " ")
AM 81 Mini Pipetter (" " " " ")
Microdel bottles delivering 25 µl drops (" " " " ")
96-pin multipoint inoculator (" " " " ")
Illuminated incubator
Manual diluting apparatus (Darbyshire, Wheatley, Greaves & Inkson, 1974)
25 µl pipettes

Procedure

All the wells in each microtitration plate are dosed with 25 µl of sterile Flent's medium from Microdel bottles. Herbicide solutions at the required initial concentration are then pipetted (25 µl/well) into the wells in the first row. Up to 8 replicates can be done with one plate but we find it convenient to use 3 different herbicides and two controls on one plate putting duplicate samples in adjacent wells on the first row. The herbicide in the wells of the first row is then diluted by 2-fold stages using the microdiluter rods of the manual diluting apparatus as described on pp.31-32 . Obviously by changing the volume of medium in the wells the dilution rate can be changed as required, for example to the 5-fold rate used for enumerating bacteria. After completion of the dilution operation all the wells are inoculated with Chlorella sp. The inoculum is prepared by shaking a stock culture to disperse the cells, and adjusting to a cell density of about 10^6 cells/ml. Normally this is achieved by concentrating the cells by centrifuging at about 5000g for 10 min, removing the supernatant and resuspending the cells in a small volume of fresh growth medium. The cell density is measured by counting the cells using a microscope and haemocytometer and the suspension diluted to give the required cell density. This suspension is placed in the 96 wells of a sterile microtitration plate and a 96-pin multipoint inoculator used to transfer inocula from this plate to those containing the dilution series of herbicides. This method gives sufficient inoculum to allow good growth of the alga without giving an initial green colour to the medium in the wells.

Inoculated plates are incubated for 3-7 days at 25°C in an incubator with a light intensity of about 11,500 lux. The relative humidity in the incubator is raised by placing open trays of water on its lowest shelf. In addition the

microtitration plates can be closed with transparent sealing tape. These precautions prevent the loss of water from the small volumes of medium in the wells, and thus ensure that the herbicide concentration does not change during the incubation period.

N.B. The microdiluter rods used in this method are precision instruments and, if used properly, will produce accurate dilutions. However their accuracy will be impaired if handled roughly or if not cleaned efficiently. The manufacturer's recommendations on cleaning, sterilizing and handling must be followed closely. Failure to do this may result in cumulative dilution errors.

Results

Results are assessed visually, the minimum inhibitory concentrations of the herbicides under test, in the formulation used, being detected easily as the first dilution level at which growth of the alga does not occur.

References

Darbyshire, J.F., Wheatley, R.E., Greaves, M.P. and Inkson, R.H.E., 1974, Rev. Ecol. Biol. Sol., 11, 465.

Cooper, S.L., Wingfield, G.I., Lawley, R. and Greaves, M.P., 1978, Weed Research, 18, (in press).

APPENDIX 1

Table 1. Soils used at the Weed Research Organization for testing effects of herbicides on the soil microflora.

Characteristic	Soil 1	Soil 2
Soil type	Sandy loam	Sandy loam
Cropping history	Permanent grass	Continuous spring barley
Mechanical analysis		
% Coarse sand	27	35
% Fine sand	37	35
% Silt	18	16
% Clay	18	14
pH (in 0.01 M $CaCl_2$)	4.8	5.9
Organic C %	3.7	1.1
Total N %	0.34	0.10
NH_4^+-N, µg/g dry soil	5	1
NO_3^--N, µg/g dry soil	56	24

N.B. The figures given are the means of several analyses.

APPENDIX 2.

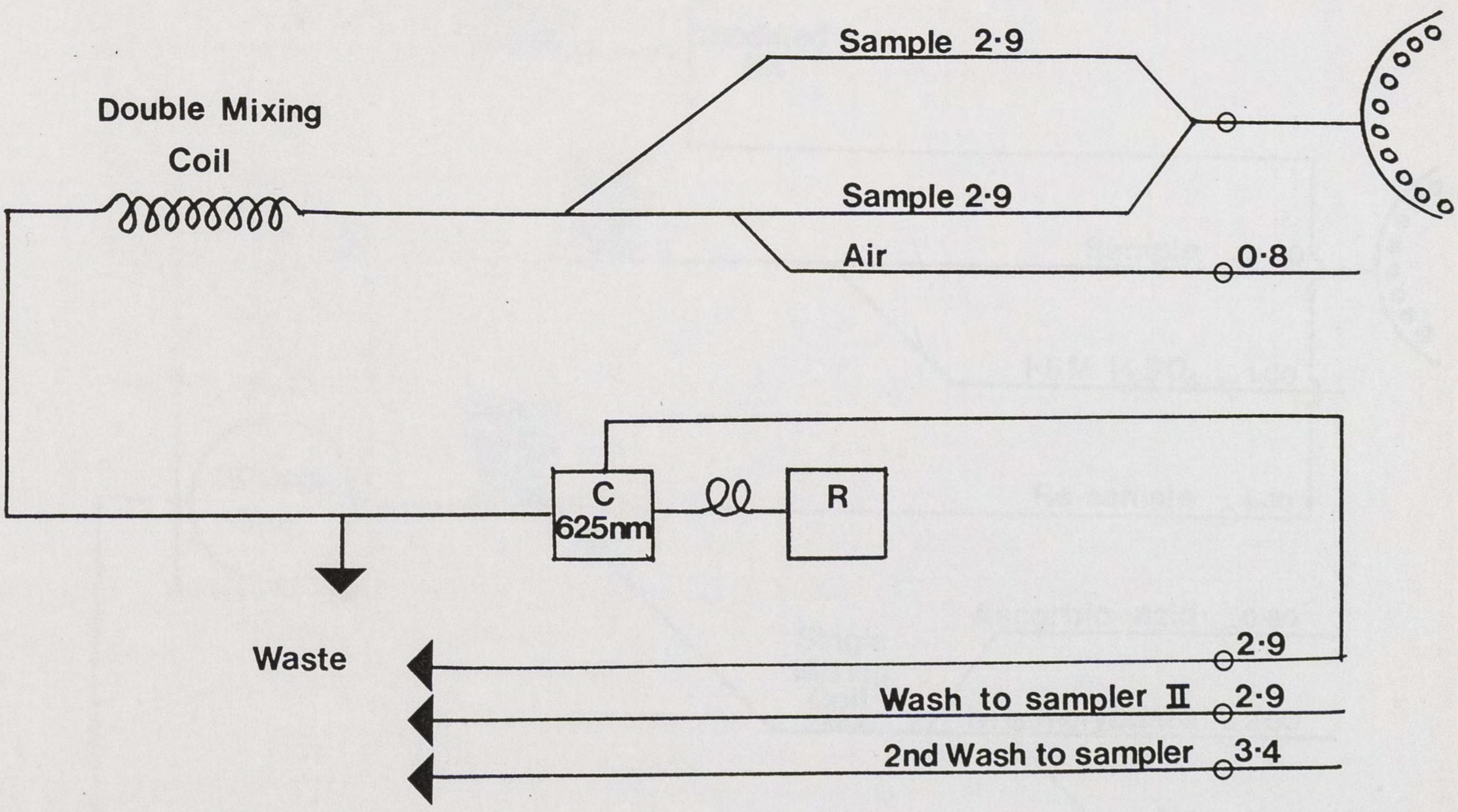
Key to labels on AutoAnalyzer manifold diagrams.

All figures show flow rates of the tubing used in ml/min. Unless indicated otherwise the tubing used is clear standard Technicon tubing.

B	Heating bath
C	Colorimeter
D	Dialyzer
DC	Delay coil
R	Recorder

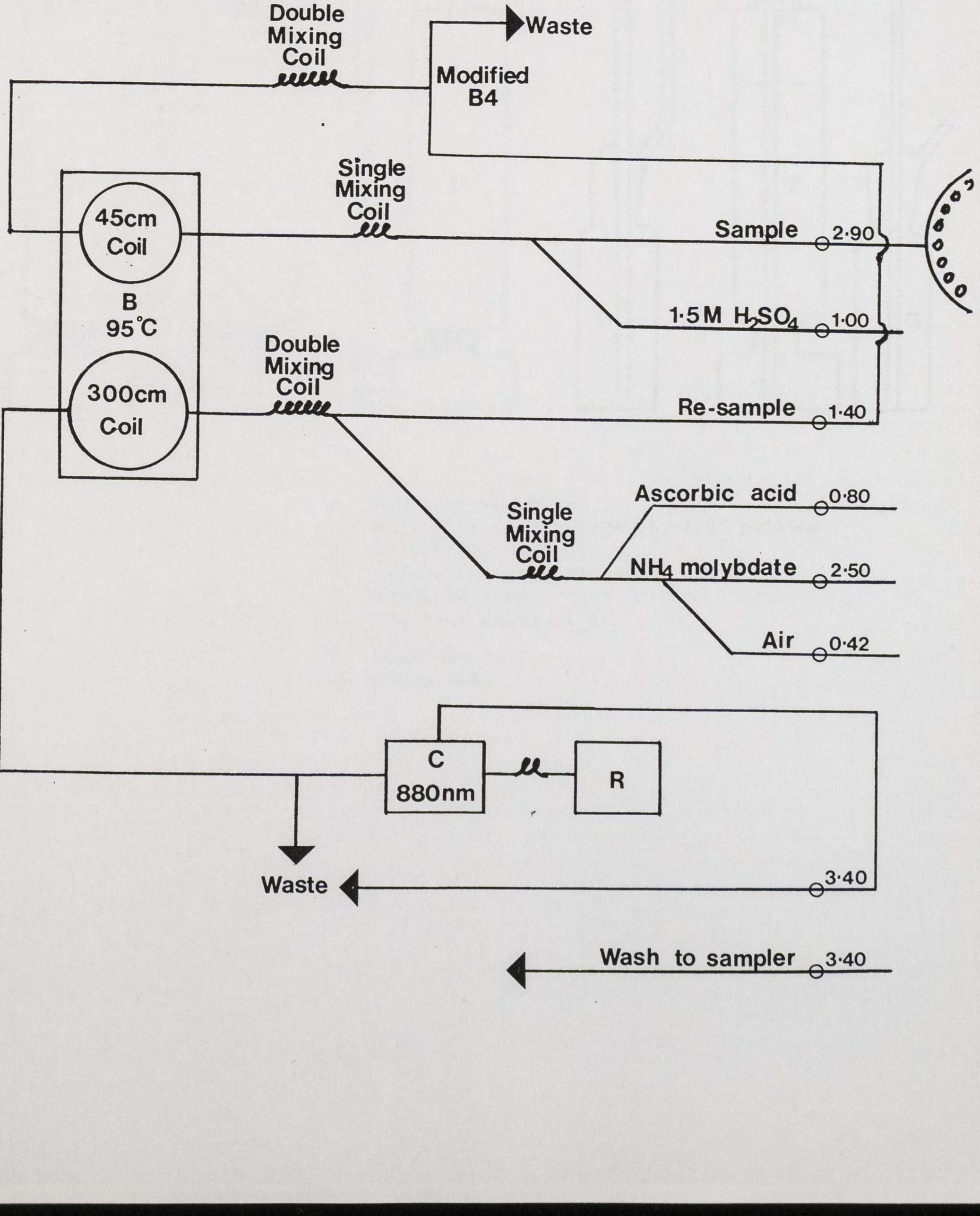
APPENDIX 3.

Fig.1. AutoAnalyzer manifold - organic carbon



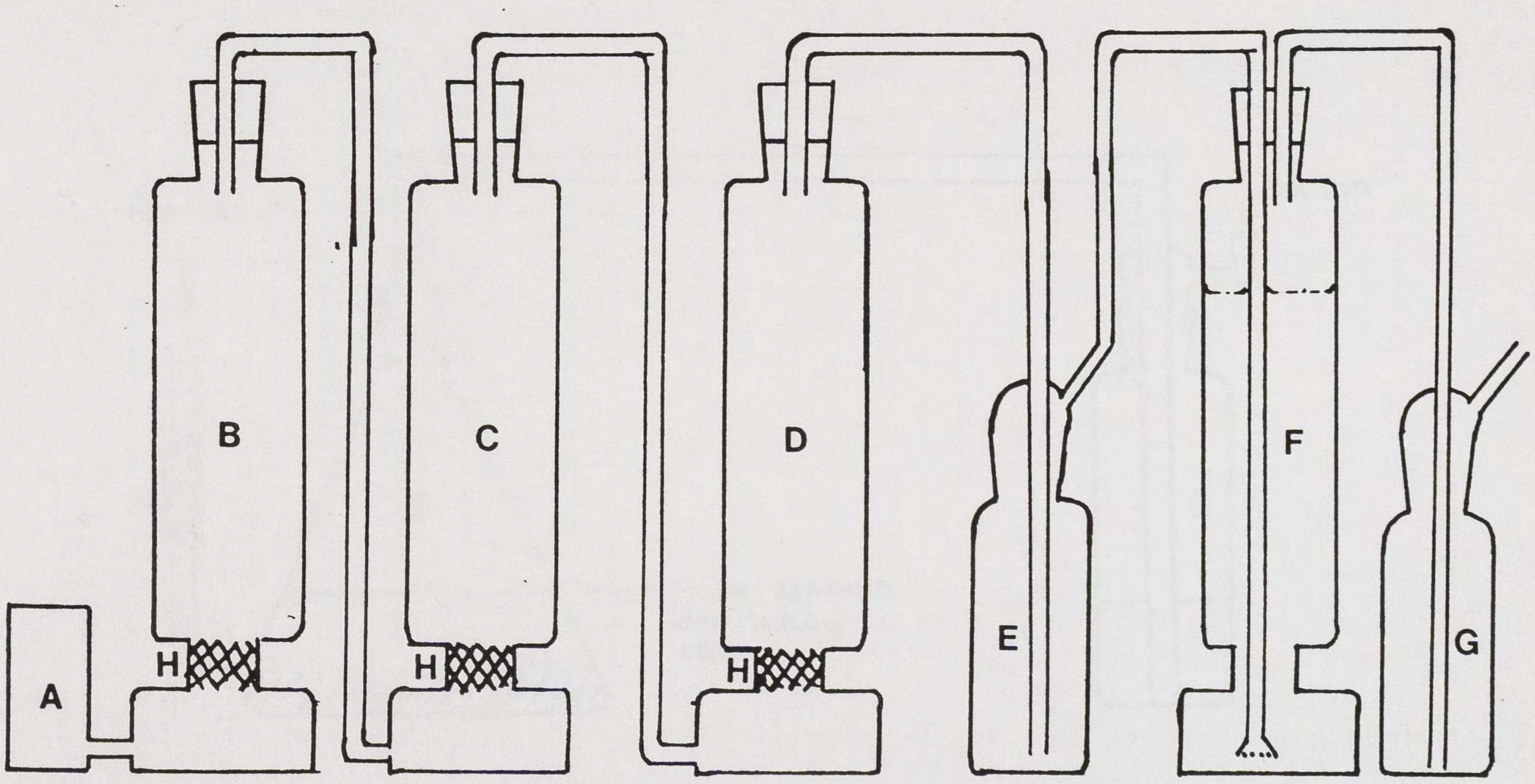
Replacement for Appendix 4. Issued July 1980

Fig.2. AutoAnalyzer manifold - phosphate



APPENDIX 5.

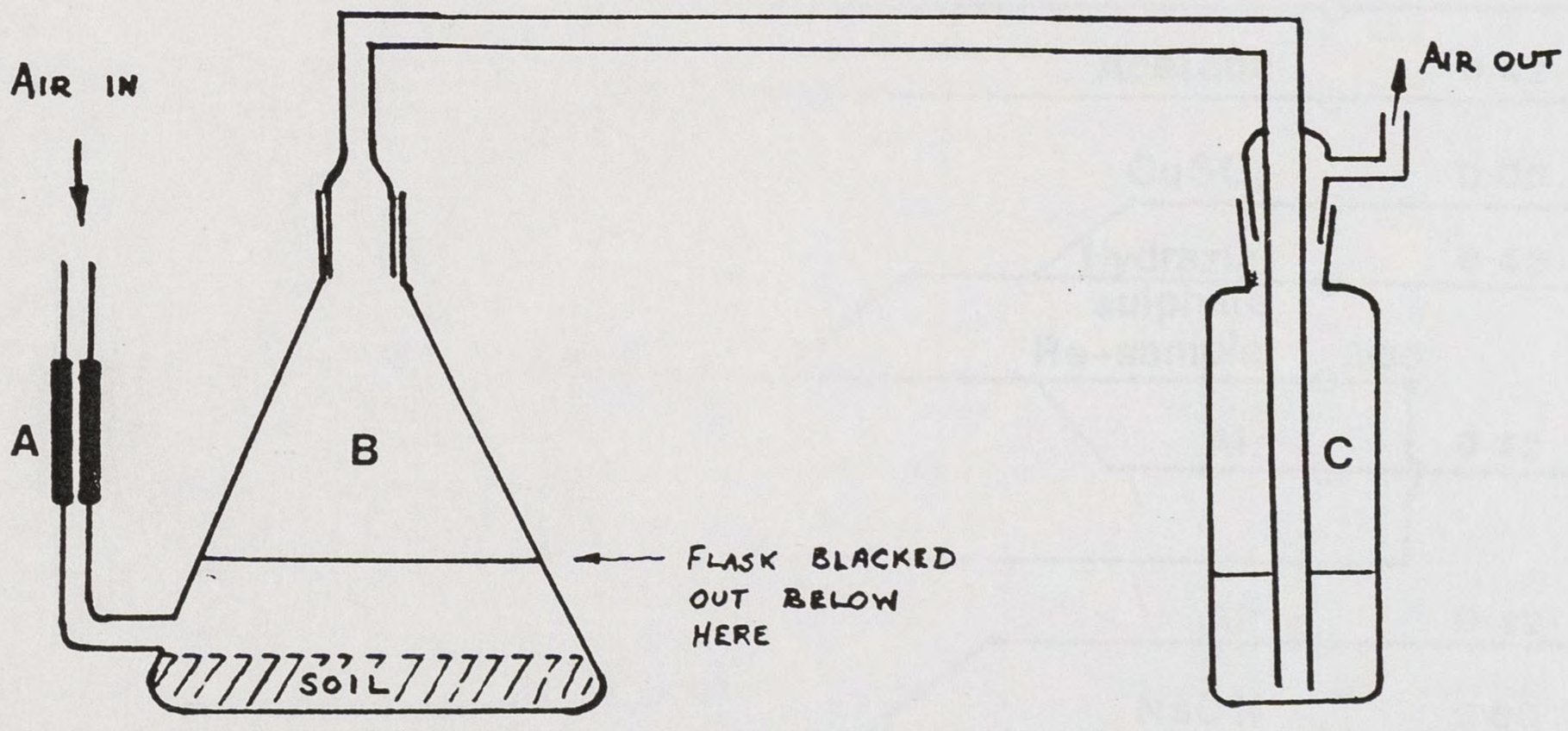
Fig. 3. Gas scrubbing system



A. Aquarium air pump
B. Molecular sieve, type 5A, 1/8" pellets
C. Silica get (250 g)
D. Soda asbestos, 6-12 mesh, 250 g
E. Moisture trap
F. CO_2-free water, 1 l
G. Moisture trap
H. Glass wool

APPENDIX 6.

Fig.4. Respirometer flask unit

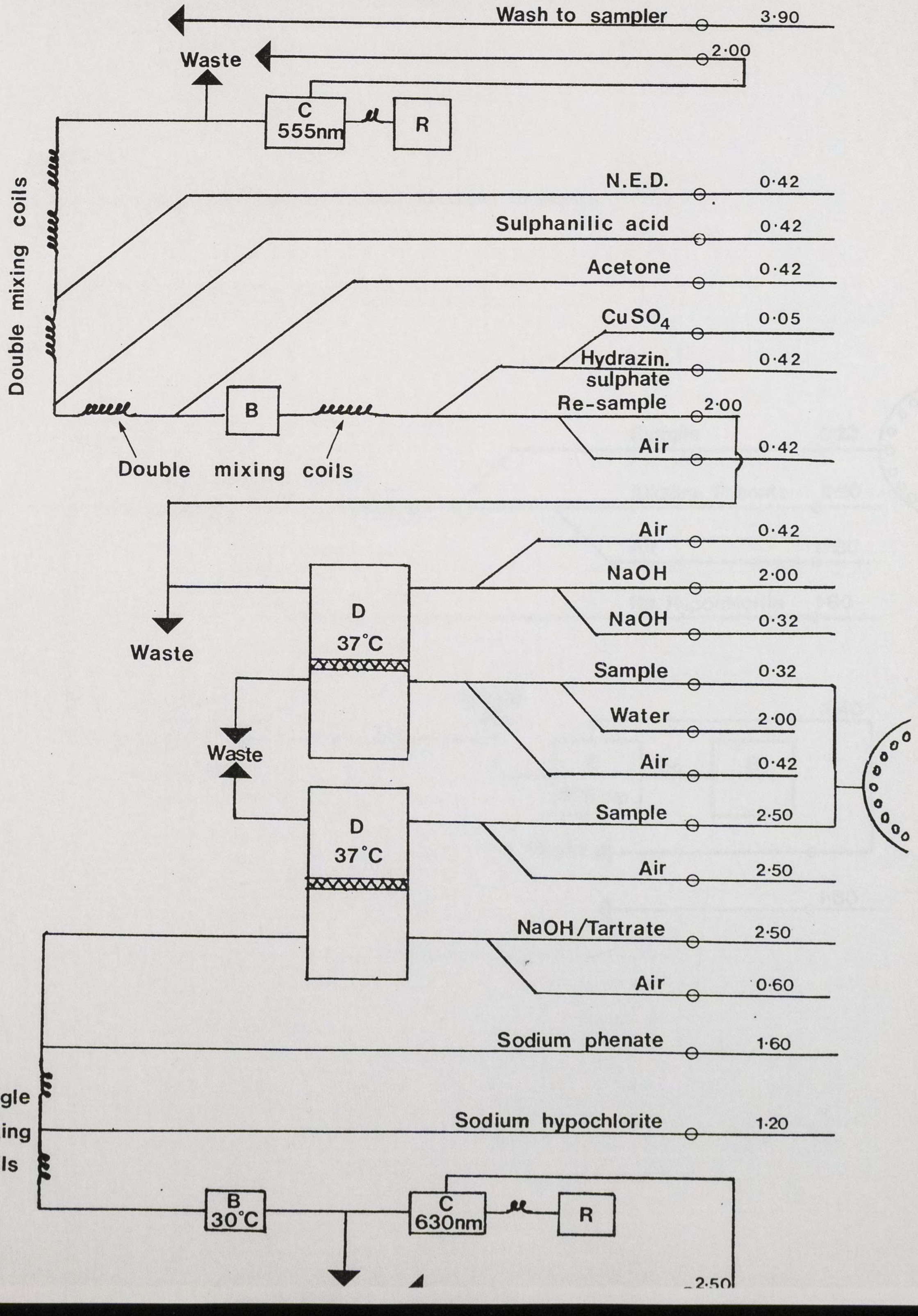


A. Prescision bore capillary (flow-rate 0-10 ml/min) as supplied with capillary flowmeters
B. 500 ml conical flask with side-arm
C. 125 ml Drechsel bottle containing 40 ml M NaOH

Replacement for Appendix 7

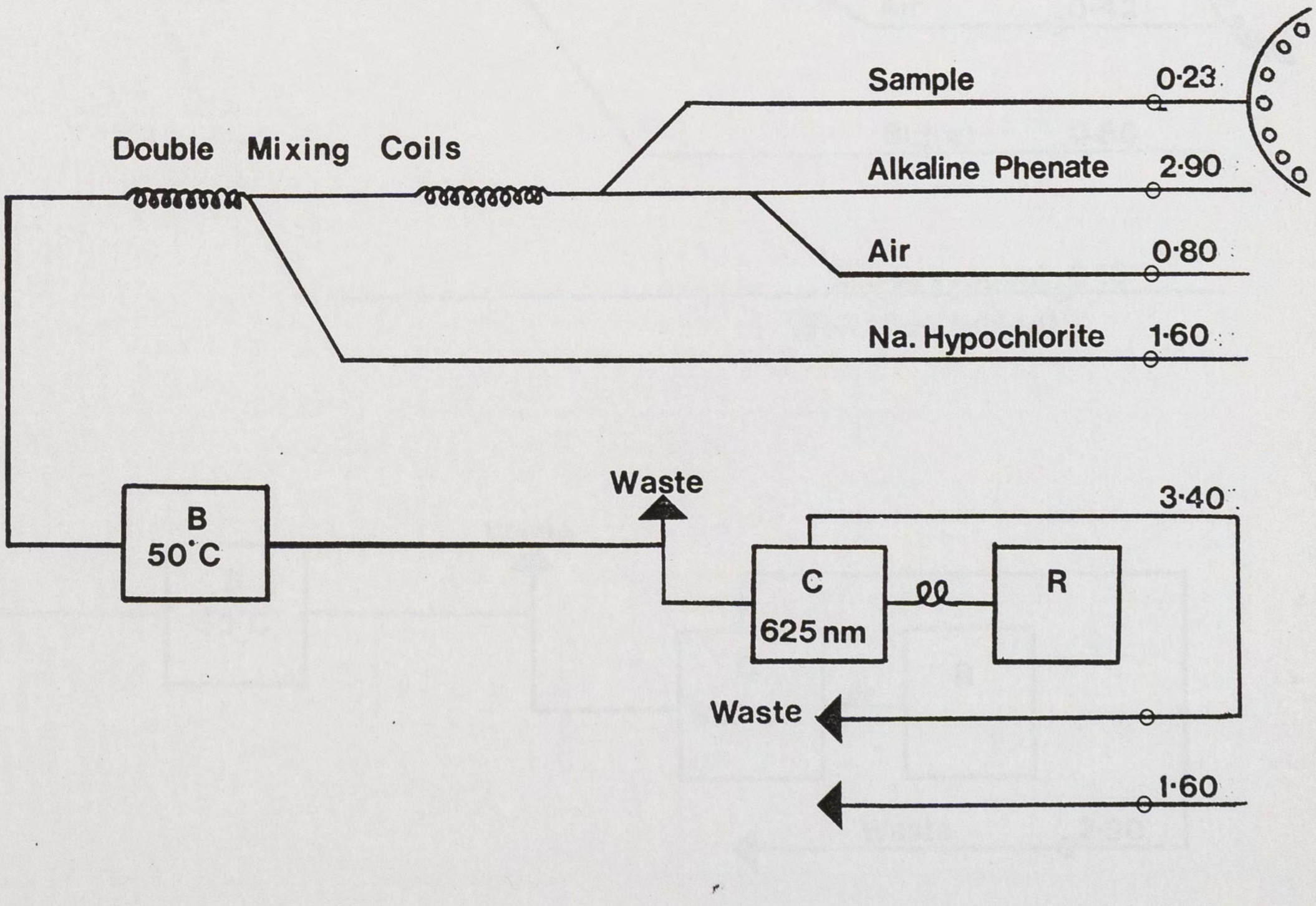
Issued November 1984

Fig.5. Auto-Analyzer manifold - ammonium, nitrite and nitrate



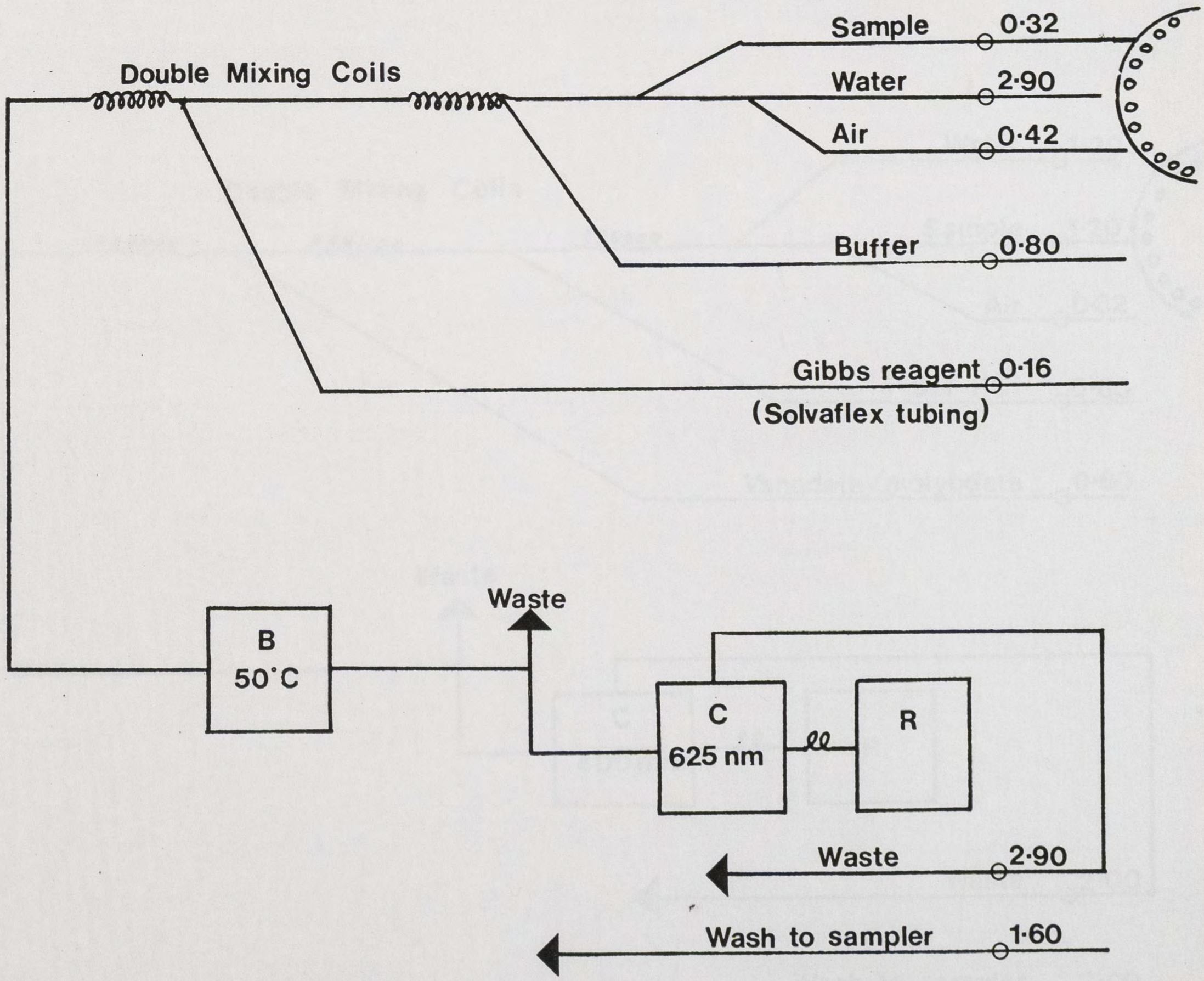
APPENDIX 8.

Fig.6. AutoAnalyzer manifold - total nitrogen in plants



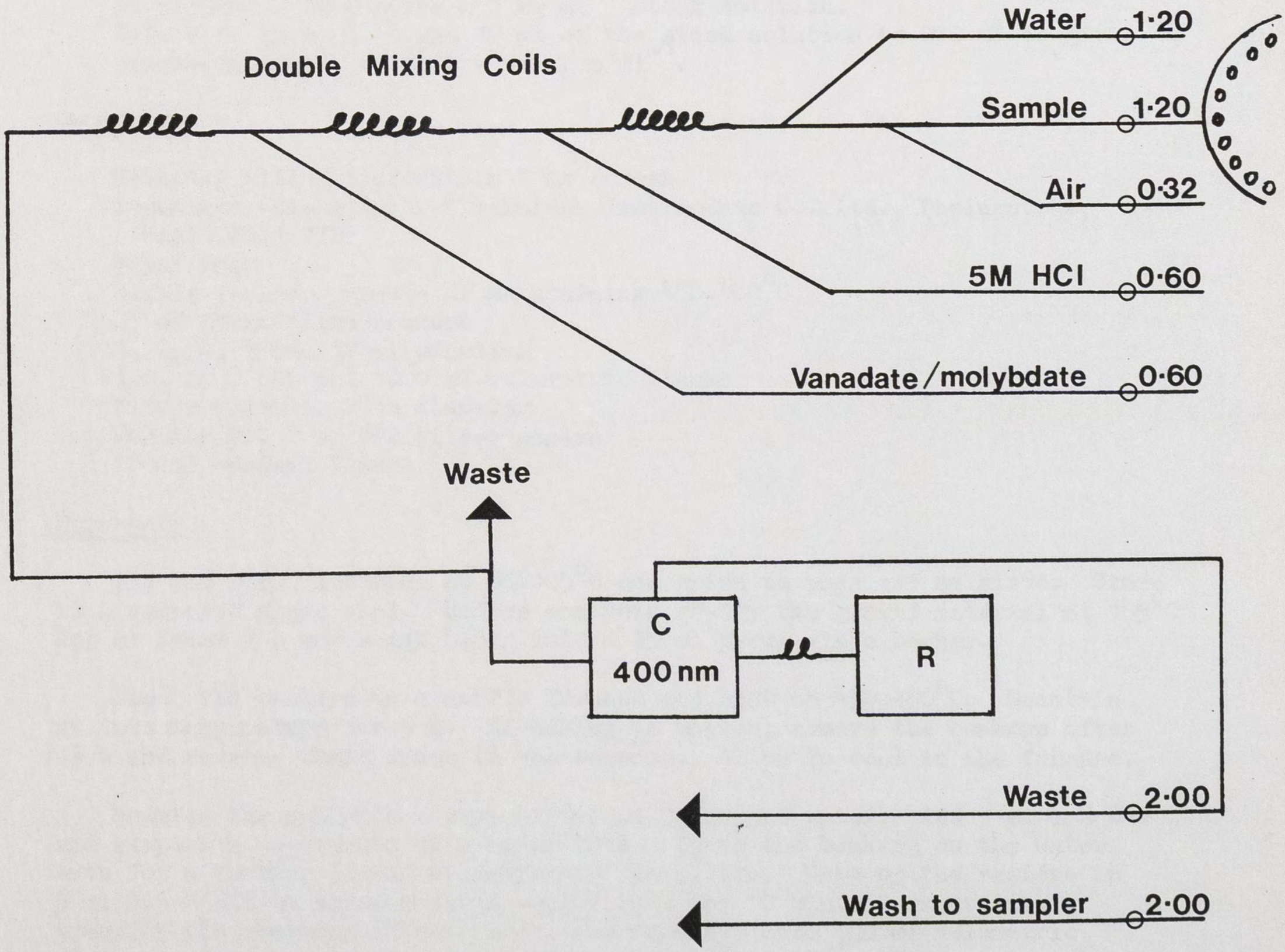
APPENDIX 9.

Fig.7. AutoAnalyzer manifold - phosphatase



APPENDIX 10. Issued July 1980

Fig.8. AutoAnalyzer manifold, total phosphorus in plant material.



TOTAL PHOSPHORUS IN PLANT MATERIAL

Reagents

All reagents should be of analytical reagent (AR) grade.
Approximately 6 M HCl. Approximately 500 ml concentrated HCl made up to 1 litre with deionised water.
Approximately 5 M HCl. Approximately 430 ml concentrated HCl made up to 1 litre with deionised water.
Approximately 0.1 M HCl. Dilute 20 ml 5M HCl to 1 litre with deionised water.
Ammonium molybdate-ammonium vanadate reagent. Warm 25 g ammonium molybdate and 1.25 g ammonium metavanadate in approximately 300 ml deionised water until dissolved. Cool and make up to 500 ml. Filter if necessary. Add 0.5 ml manoxol OT. Standard phosphate solutions. Dissolve 0.879 g KH_2PO_4 (dried at 105°C for at least 2 h) in deionised water, add 1 ml conc. HCl and make up to 200 ml with deionised water. Add 1 drop of toluene. This gives a 1 mg ml^{-1} stock solution.
Dilute 1, 2, 4, 6, 8 and 10 ml of the stock solution to 200 ml to give standards in the range 5 - 50 µg p ml^{-1}.

Apparatus

Grinding mill fitted with a 1 mm screen
Technicon AutoAnalyzer (Technicon Instruments Co. Ltd., Basingstoke, Hants.RG21 2YE)
Water bath
Muffle furnace capable of maintaining 450-480°C
25 ml pyrex glass beakers
1, 2, 4, 5 and 10 ml pipettes
100, 200, 500 and 1000 ml volumetric flasks
Filter funnels, 7 cm diameter
Whatman No. 2 or 542 filter papers
100 ml conical flasks

Procedure

Dry the plant material at 85-105°C and grind to pass a 1 mm sieve. Store in a numbered glass vial. Before analysis re-dry the ground material at 105°C for at least 4 h and weigh 0.5 g into a 25 ml pyrex glass beaker.

Place the beakers in a muffle furnace and heat to 450-480°C. Maintain at this temperature for 3 h. If ashing is uneven, remove the beakers after $1\frac{1}{2}$ h and reverse their order in the furnace. Allow to cool in the furnace.

Moisten the ash with a drop or two of deionized water. Add 5 ml 6 M HCl and evaporate to dryness on a water bath. Leave the beakers on the water bath for a further 30 min to dehydrate the silica. Take up the residue in 5 ml 0.5 M HCl by warming on the water bath for 10 min. Quantitatively transfer the contents of the beaker and washings into 100 ml volumetric flasks and make up to the mark. After thoroughly mixing, filter the solution through a Whatman No. 2 or 542 filter paper and store in glass or polyethylene bottles. Transfer the samples to AutoAnalyzer cups and analyse at 40 samples h^{-1} with a 2 : 1 sample to wash ratio (see manifold diagram, Fig. 8 in the Appendix).

Calculation of results

Draw a calibration curve of optical density against $\mu gP\ ml^{-1}$ standards and read samples from this.

$$\% P = \frac{R \times 10}{W}$$

where

R = $\mu gP\ ml^{-1}$ (from graph)
W = weight of sample (mg)

ABBREVIATIONS

ångström	Å	freezing point	f.p.
Abstract	Abs.	from summary	F.s.
acid equivalent*	a.e.	gallon	gal
acre	ac	gallons per hour	gal/h
active ingredient*	a.i.	gallons per acre	gal/ac
approximately equal to*	≃	gas liquid chromatography	GLC
aqueous concentrate	a.c.	gramme	g
bibliography	bibl.	hectare	ha
boiling point	b.p.	hectokilogram	hkg
bushel	bu	high volume	HV
centigrade	C	horse power	hp
centimetre*	cm	hour	h
concentrated	concd	hundredweight*	cwt
concentration	concn	hydrogen ion concentration*	pH
concentration x time product	ct	inch	in.
concentration required to kill 50% test animals	LC50	infra red	i.r.
cubic centimetre*	cm^3	kilogramme	kg
cubic foot*	ft^3	kilo ($x10^3$)	k
cubic inch*	in^3	less than	<
cubic metre*	m^3	litre	l.
cubic yard*	yd^3	low volume	LV
cultivar(s)	cv.	maximum	max.
curie*	Ci	median lethal dose	LD50
degree Celsius*	^oC	medium volume	MV
degree centigrade	^oC	melting point	m.p.
degree Fahrenheit*	^oF	metre	m
diameter	diam.	micro ($x10^{-6}$)	μ
diameter at breast height	d.b.h.	microgramme*	μg
divided by*	÷ or /	micromicro (pico: $x10^{-12}$)*	μμ
dry matter	d.m.	micrometre (micron)*	μm (or μ)
emulsifiable concentrate	e.c.	micron (micrometre)*†	μm (or μ)
equal to*	=	miles per hour*	mile/h
fluid	fl.	milli ($x10^{-3}$)	m
foot	ft	milliequivalent*	m.equiv.
		milligramme	mg
		millilitre	ml

† The name micrometre is preferred to micron and μm is preferred to μ.

millimetre*	mm
millimicro* (nano: $x10^{-9}$)	n or mμ
minimum	min.
minus	-
minute	min
molar concentration*	M (small cap)
molecule, molecular	mol.
more than	>
multiplied by*	x
normal concentration*	N (small cap)
not dated	n.d.
oil miscible concentrate	o.m.c. (tables only)
organic matter	o.m.
ounce	oz
ounces per gallon	oz/gal
page	p.
pages	pp.
parts per million	ppm
parts per million by volume	ppmv
parts per million by weight	ppmw
percent(age)	%
pico (micromicro: $x10^{-12}$)	p or μμ
pint	pint
pints per acre	pints/ac
plus or minus*	±
post-emergence	post-em
pound	lb
pound per acre*	lb/ac
pounds per minute	lb/min
pound per square inch*	lb/in^2
powder for dry application	p. (tables only)
power take off	p.t.o.
precipitate (noun)	ppt.
pre-emergence	pre-em.
quart	quart
relative humidity	r.h.
revolution per minute*	rev/min
second	s
soluble concentrate	s.c.
soluble powder	s.p.
solution	soln
species (singular)	sp.
species (plural)	spp.
specific gravity	sp. gr.
square foot*	ft^2
square inch	in^2
square metre*	m^2
square root of*	$\sqrt{}$
sub-species*	ssp.
summary	s.
temperature	temp.
ton	ton
tonne	t
ultra-low volume	ULV
ultra violet	u.v.
vapour density	v.d.
vapour pressure	v.p.
varietas	var.
volt	V
volume	vol.
volume per volume	v/v
water soluble powder	w.s.p. (tables only)
watt	W
weight	wt
weight per volume*	w/v
weight per weight*	w/w
wettable powder	w.p.
yard	yd
yards per minute	yd/min

* Those marked * should normally be used in the text as well as in tables etc.

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