Chapter 5
Water analysis

Irrigation water always contains some soluble salts irrespective of its source. The suitability of waters for a specific purpose depends on the types and amounts of dissolved salts. Some of the dissolved salts or other constituents may be useful for crops. However, the quality or suitability of waters for irrigation purposes is assessed in terms of the presence of undesirable constituents, and only in limited situations is irrigation water assessed as a source of plant nutrients. Some of the dissolved ions, such as NO_3, are useful for crops.

IMPORTANT CHARACTERISTICS OF IRRIGATION WATER

The most important characteristics that determine the quality of irrigation water are:

- pH;
- total concentration of soluble salts assessed through EC;
- relative proportion of Na to other cations such as Ca and Mg, referred to as the sodium adsorption ratio (SAR);
- concentration of B and other elements that may be toxic to plants;
- concentration of carbonates and bicarbonates as related to the concentration of Ca plus Mg, referred to as residual sodium carbonate (RSC);
- content of anions such as chloride, sulphate and nitrate.

The analytical data on the above parameters are used to describe the quality of irrigation water taking standards fixed for each aspect as an index.

Water coming from industries as effluents and domestic wastewater as sewage may contain some specific plant nutrients. These waters may be useful for irrigation of field crops if assessed for toxic/pollutant metals and organic and microbial constituents with regard to their suitability or otherwise. Determination of organic constituents is generally carried out under two categories: (i) organic substances that quantify an aggregate amount of organic C; and (ii) individual or specific organic substances, such as benzene, DDT, methane, phenol and endosulphan. Important determinations are the chemical oxygen demand (COD), which gives the total organic substances, and the biochemical oxygen demand (BOD), which gives the amount of total biodegradable organic substances in the water sample.

The limits of purity established for drinking-water, water for industrial purposes and water for agriculture are different. Therefore, water that is not good for drinking and industrial uses may be suitable for irrigation. However, as the focus of this guide is on service laboratories, it examines only those parameters

of practical utility for assessing the quality of commonly used irrigation waters. Thus, it does not cover aspects pertaining to the use of effluents and sewage waters, nor does it assess water as a carrier of plant nutrients.

Electrical conductivity

The concentration of total salt content in irrigation waters, estimated in terms of EC, is the most important parameter for assessing the suitability of irrigation waters. Generally, all irrigation waters with an EC of less than 2.25 mS/cm are considered suitable except in some unusual situations, e.g. very sensitive crops and highly clayey soils of poor permeability. The ideal value is less than 0.75 mS/cm (Richards, 1954).

Sodium adsorption ratio

The SAR is calculated in order to determine the sodicity or alkalinity hazard of irrigation waters:

$$SAR = \frac{Na^{+}}{\left[\frac{Ca^{2+} + Mg^{2+}}{2}\right]^{1/2}}$$

where the concentration of cations is in me/litre.

Based on the SAR value, waters can be rated into different categories of sodicity (Richards, 1954) as follows:

- safe: < 10;
- moderately safe: 10–18;
- moderately unsafe: 19–26;
- unsafe: > 26.

Residual sodium carbonate

The RSC index is important for carbonate-rich and bicarbonate-rich irrigation waters. It indicates their tendency to precipitate Ca as $CaCO_3$. The RSC is calculated by:

$$RSC\ (me/litre) = (CO_3^{2-} + HCO_3^{-}) - (Ca^{2+} + Mg^{2+})$$

Concentrations of both cations and anions are in me/litre. The sodicity hazard in terms of RSC is categorized (Richards, 1954) as follows:

- safe: < 1.25;
- moderate: 1.25–2.5;
- unsafe: > 2.5.

The limits can vary depending on the type of soil, rainfall and climate conditions. Higher RSC values can be considered safe for sandy soils in a high rainfall area (> 600 mm/year).

Ratio of magnesium to calcium

It is widely reported that Ca and Mg do not behave identically in soil systems, and the Mg deteriorates soil structure particularly where waters are sodium-dominated and highly saline. A high level of Mg usually promotes a higher development of exchangeable Na in irrigated soils. Based on the ratio of Mg to Ca, waters are categorized as:

- safe: < 1.5;
- moderate: 1.5–3.0;
- unsafe: > 3.0.

Boron content

Crops require B in very small quantities. It becomes toxic where present beyond a certain level. In relation to B toxicity, water quality ratings are:

- low hazard: < 1 µg B/ml;
- medium hazard: 1–2 µg B/ml;
- high hazard: 2–4 µg B/ml;
- very high hazard: > 4 µg B/ml.

Each of the above parameters has a bearing on the quality of irrigation water. However, each water source will have its specific suitability or hazardous nature depending on the presence (and the degree) or absence of each of the constituents. Different chemical constituents interact with one another and cause a complex effect on soil properties and plant growth.

Waters with a low SAR and low EC are widely suitable. However, when a value of any one of these parameters (or both) increases in its content, the waters become less suitable for irrigation purposes. The selection of crops for such situations becomes critical. Salt-tolerant crops can be grown in such areas. Soil type is also an important consideration under such situations.

Table 18 indicates the upper permissible limits of EC, SAR, RSC and B for soils with different amounts of clay and for growing tolerant and semi-tolerant crops. These limits are based on extensive trials conducted by Paliwal and Yadav (1976).

TABLE 18
Suitability of irrigation water for semi-tolerant and tolerant crops in different soil types

Texture	Upper permissible limit							
	EC		SAR		RSC		B	
	(dS/m)		*(me/litre)*				*(µg/ml)*	
	ST	T	ST	T	ST	T	ST	T
> 30% clay	1.5	2.0	10	15	2	3	2	3
20–30% clay	4.0	6.0	15	20	3	4	2	3
10–20% clay	6.0	8.0	20	25	4	5	2	3
< 10% clay	8.0	10.0	25	30	5	6	3	4

Note: ST = semi-tolerant crop; T = tolerant crop.

Trace elements

The presence of trace elements or heavy metals reduces crop growth where their concentration increases beyond a certain level in irrigation waters and where such waters are used continuously. However, such elements are not normally a problem in common irrigation waters. They can be of concern where industrial effluent water is used for irrigation.

COLLECTION OF WATER SAMPLES

A representative water sample (500 ml) is collected in a glass or polyethylene bottle, which should be properly washed/rinsed with the same water that is being sampled. Floating debris or any other contaminant should be avoided while collecting the sample. After proper labelling (e.g. source of water, date of collection, and type of analysis required), the sample should be sent to the laboratory without undue delay.

Some of the anions such as SO_4 and NO_3 may be quite low in irrigation waters. Hence, the large volume of the sample has to be first concentrated by evaporating it to about 100 ml in order to obtain their detectable amounts.

ANALYTICAL METHODS

pH

The pH is determined by putting about 50 ml of the water sample in a clean 100-ml beaker and using a pH meter as described in Chapter 3.

Electrical conductivity

A conductivity meter cell is filled with the water sample, and the EC is determined as described in Chapter 3.

Calcium and magnesium

The usual method for determination of Ca and Mg is by versenate (EDTA) titration (Cheng and Bray, 1951). The estimation of Ca and Mg can also be done using an AAS, as described in Chapter 4. Estimation of Ca and Mg in water by the EDTA method is described below.

The apparatus required consists of:

- a porcelain dish;
- some volumetric flasks;
- a burette.

The reagents required are:

- Standard versenate solution (EDTA): Prepare an approximately 0.01N solution of EDTA disodium salt (versenate) by dissolving 2.0 g in distilled water, add 0.05 g of magnesium chloride ($MgCl_2.6H_2O$) and dilute to 1 litre. Standardize against 0.01N calcium chloride solution prepared by weighing 0.500 g AR-grade $CaCO_3$ (oven-dried) and dissolving in minimum excess of dilute HCl (AR), followed by making the volume up to 1 litre with distilled water.

- Ammonium chloride-ammonium hydroxide buffer (pH 10): 67.5 g of pure ammonium chloride dissolved in 570 ml of concentrated ammonium hydroxide and made up to 1 litre; pH adjusted to 10.
- EBT indicator: 0.5 g of EBT and 4.5 g of hydroxylamine hydrochloride (AR) dissolved in 100 ml of 95 percent ethyl alcohol.

The procedure is:

1. Put 5 ml of the water sample in a porcelain dish (8 cm in diameter).
2. Dilute to about 25 ml with distilled water.
3. Add 1 ml of ammonium chloride-hydroxide buffer and 3–4 drops of EBT indicator.
4. Titrate with the standard versenate solution. The colour change is from wine-red to bright blue or bluish green. At the end point, no tinge of the red colour should remain.

From the volume of 0.01N EDTA (standardized against 0.01N $CaCl_2$) solution required for titration, the concentration of Ca and Mg is obtained directly as follows:

$$\text{Ca} + \text{Mg (me/litre)} = \frac{\text{ml versenate (EDTA) used x normality of EDTA} \times 1000}{\text{ml aliquot taken}}$$

$$\text{Ca} + \text{Mg (g/litre)} = \frac{\text{Ca} + \text{Mg in me/litre} \times \text{equivalent wt.}}{1000}$$

$$= \frac{\text{Ca} + \text{Mg in me/litre} \times 32.196}{1000}$$

or:

$$\text{Ca} + \text{Mg (g/litre)} = \frac{\text{ml versenate (EDTA) used} \times \text{normality of EDTA} \times 1\,000 \text{ x equivalent wt.}}{\text{ml aliquot taken} \times 1000}$$

$$= \frac{\text{ml versenate (EDTA) used} \times \text{normality of EDTA} \times 32\,196}{\text{ml aliquot taken}}$$

Sodium

A small amount of Na is generally present even in the best-quality irrigation water. The concentration of Na may be quite high in saline water with an EC higher than 1 mS/cm and containing relatively less Ca and Mg. Its estimation is of interest when the water sample tests saline (i.e. an EC higher than 1.0 mS/cm at 25 °C). The determination of Na is carried out directly with the help of a flame photometer using appropriate filters and standard curves prepared by taking a known concentration of Na.

The apparatus required consists of:

- a flamephotometer;
- some volumetric flasks;
- some beakers.

The reagent required is NaCl (AR-grade).

The procedure is:

1. Preparation of the standard curve:

- Take 2.5413 g of NaCl (AR), dissolve in water to make to the volume up to 1 litre; this gives a solution of 1 000 µg Na/ml. From this solution, take 100 ml and dilute to 1 litre to obtain 100 µg Na/ml as stock solution.
- To prepare working standards, take 5, 10, 15 and 20 ml of stock solution in 100-ml volumetric flasks and make up the volume. This gives 5, 10, 15 and 20 µg Na/ml.
- Feed the standards on the flamephotometer one by one in order to obtain a standard curve, with absorbance on the y-axis and the respective concentrations of Na on the x-axis.

2. Feed the water samples on the flamephotometer, and record the absorbance for each sample.
3. Observe the concentration of Na against each absorbance (in µg Na/ml).

The relevant calculation is:

$$\text{Content of Na in mg/litre of water} = \frac{A \times 1\,000}{1\,000} = A$$

where:

- A = absorbance reading (µg/ml) from the standard curve.

It is important to note that:

- If a water sample is diluted for estimation, the quantity of Na as observed on the standard curve is multiplied by the dilution factor.
- If the water sample is concentrated before estimation, the quantity noted from the standard curve is divided by the concentration factor.
- Normally, no dilution or concentrated is required.

Carbonates and bicarbonates

The estimation is based on simple acidimetric titration (Richards, 1954) using different indicators that work in the alkaline (higher than 8.2) or acidic pH range (lower than 6.0).

The apparatus required consists of:

- a porcelain dish;
- a burette.

The reagents required are:

- Phenolphthalein indicator: 0.25 percent solution in 60 percent ethyl alcohol.
- Methyl orange indicator: 0.5 percent solution in 95 percent alcohol.
- Standard sulphuric acid (0.01M).

The procedure is:

1. Put 5 ml of the water sample (containing not more than one milli-equivalent of carbonate plus bicarbonate) in a porcelain dish.
2. Dilute with distilled water to about 25 ml.
3. A pink colour produced with 2–3 drops of phenolphthalein indicates the presence of carbonate, and it is titrated with 0.01M sulphuric acid until the colour just disappears (phenolphthalein end point) because of alkali carbonate having been converted to bicarbonate. This is called the half-neutralization stage. This burette reading (volume used) is designated Y.

4. To the colourless solution from this titration (or to the original sample of water if there was no colour with phenolphthalein), add 1–2 drops of methyl orange indicator, and continue titration with brisk stirring to the methyl orange end point (yellow); the final reading (volume used) is designated Z.

The relevant calculation is:

$$\text{Carbonates (me/litre)} = 2\,(\text{Volume of } H_2SO_4) \times \text{Molarity of } H_2SO_4 \times \frac{1000}{\text{ml of aliquot}}$$

$$= 2Y \times 0.01 \times \frac{1000}{5}$$

$$= 2Y \times 2 = 4Y$$

$$\text{Carbonates (g/litre)} = \frac{2\,(\text{Vol. of } H_2SO_4) \times \text{Molarity} \times 1000 \times \text{Eq. wt. of } CO_3\,(30)}{\text{ml of sample} \times 1000}$$

$$= \frac{2Y \times 0.01 \times 30}{5} = 0\ 12Y$$

The volume of acid used for half-neutralization of carbonate is *Y*. Hence, for full neutralization it has been assumed as *2Y*:

$$\text{Bicarbonates (me/litre)} = (Z - 2Y) \times \text{molarity of } H_2SO_4 \times \frac{1000}{\text{ml of aliquot}}$$

$$= \frac{(Z - 2Y) \times 0.01 \times 1000}{5}$$

$$= (Z - 2Y) \times 2$$

Where carbonate is absent: $Z \times 2$.

Residual sodium carbonate

This is an important characteristic for assessing the suitability of irrigation water in consideration of a probable sodium hazard. It is calculated from the analysis data for carbonates, bicarbonates and Ca plus Mg as follows (all expressed in me/litre):

$$\text{RSC (me/litre)} = (CO_3^{2-} + HCO_3^{-}) - (Ca^{2+} + Mg^{2+})$$

Boron

The method for B estimation is same as described in Chapter 3. The determination is carried out using the azomethine-H colorimetric method. It can also be estimated on an AAS. Suitable quantities of the sample should be taken depending on the B content in the waters.

Chloride

Mohr's titration method is the one most commonly used for chloride estimation. It depends on the formation of a sparingly soluble brick-red silver chromate ($AgCrO_4$) precipitate at the end point when the sample is titrated against standard silver nitrate ($AgNO_3$) solution in the presence of potassium chromate (K_2CrO_4)

as indicator. Initially, the Cl ions are precipitated as AgCl and the dark brick-red precipitate of Ag_2CrO_4 starts just after the precipitation of AgCl has finished.

The apparatus required consists of:

- some beakers;
- a porcelain dish;
- a burette.

The reagents required are:

- Potassium chromate (K_2CrO_4) indicator (5 percent) solution: Dissolve 5 g of K_2CrO_4 in about 75 ml of distilled water, and add saturated solution of $AgNO_3$ drop by drop until a slight permanent red precipitate is formed. Filter and dilute to 100 ml. With high-purity AR, the indicator solution can be prepared directly.
- Standard silver nitrate solution (0.05M): Dissolve 8.494 g of silver nitrate ($AgNO_3$) in distilled water, and make the volume up to one litre. Standardize it against standard NaCl solution, and keep it in an amber-coloured bottle away from the light.

The procedure is:

1. Put 5 ml of the sample in a 100-ml beaker or a porcelain dish, and dilute to about 25 ml with distilled water.
2. Add 5–6 drops of K_2CrO_4 indicator (making it dark yellow), and titrate against the standard $AgNO_3$ solution with continuous stirring until the first brick-red tinge appears.
3. Run a blank to avoid error caused by any impurity in the chemicals.

The relevant calculation is:

$$\text{Cl mg/litre of water} = X \times 1.775 \times \frac{1000}{\text{ml of sample}}$$

where:

- ml of water sample taken = 5;
- X = ml of 0.05M $AgNO_3$ consumed in titration;
- 1.775 = factor representing mg of Cl in aliquot/sample as calculated below:
  - 1 ml of 1M $AgNO_3$ = 1 me of Cl;
  - 1 ml of 0.05M $AgNO_3$ = 0.05 me of Cl = 35.5 × 0.05 = 1.775 mg of Cl (in aliquot).

Sulphate

While traces of sulphate occur universally in all types of waters, its content may be appreciably high in several saline waters showing an EC higher than 1 dS/m at 25 °C. Sulphate can be determined gravimetrically, colorimetrically, turbidimetrically or titrimetrically. Here, the turbidimetric method is described.

Sulphate content is determined by the extent of turbidity created by precipitated colloidal barium sulphate suspension. Barium chloride solid crystals are added to ensure fine and stable suspension of $BaSO_4$ at a pH of about 4.8. This also eliminates interference from phosphate and silicate. This fine suspension of

$BaSO_4$ is stabilized by gum acacia, and the degree of turbidity is measured with a turbidity meter or estimated spectrophotometrically at 440 nm.

The apparatus required consists of:

- a spectrophotometer;
- some beakers;
- some volumetric flasks.

The reagents required are:

- Sodium acetate – acetic acid buffer: Dissolve 100 g of pure sodium acetate in 200 ml of distilled water. Add 31 ml of glacial acetic acid, and make the volume up to 1 litre. Adjust the pH to 4.8.
- Gum acacia: Dissolve 2.5 g of gum acacia in 1 litre of distilled water. Keep overnight and filter.
- Barium chloride: Grind pure $BaCl_2$ crystals to pass through a 0.5-mm sieve but be retained on a 0.25-mm sieve.
- Potassium sulphate solution: To make a stock solution of 10 me S/litre, weigh 1.74 g of pure K_2SO_4 salt and dissolve in 1 litre water.

The procedure is:

1. Put 5 ml of the water sample (of < 1 me S/litre) in a 25-ml volumetric flask. If the EC of water is > 1 dS/m, dilute it with distilled water to bring the EC to lower than 1 dS/m.
2. Add 10 ml of sodium acetate – acetic acid buffer to maintain the pH at about 4.8.
3. Add 1 ml of gum acacia and 1 g of $BaCl_2$ crystals, and shake well.
4. Make the volume up to 25 ml with distilled water.
5. Invert the flask several times, and measure the turbidity with a spectrophotometer at 440 nm using a blue filter.
6. Preparation of the standard curve: For 0, 1, 2, 3, 4 and 5 me S/litre, pipette 2.5, 5, 7.5, 10 and 12.5 ml from stock solution containing 10 me S/litre into 25-ml volumetric flasks. Then develop the turbidity and measure its intensity as in the case of samples. Draw a curve showing S concentration on the x-axis and absorbance on the y-axis.

The S content of the samples is calculated using the standard curve, taking into account the dilution factor of five (5 ml made to 25 ml) and expressed as me S/litre of water.

Nitrate nitrogen

This method depends on the reduction of nitrate to ammonia by adding Devarda's alloy and alkali. The nitrites (NO_2^-) (if present) in the sample are also reduced and determined along with NO_3^--N.

The apparatus required consists of:

- a Kjeldahl distillation assembly;
- an electric muffle furnace;
- a desiccator.

The reagents required are:

- Magnesium oxide (MgO): Heat the MgO at 65 °C for 2 hours in an electric muffle furnace to remove any traces of $MgCO_3$ that may be present. Cool in a desiccator over solid KCl, and store in a tightly stoppered bottle.
- Boric acid with mixed indicator: Weigh 20 g of boric acid, and add about 900 ml of hot distilled water. Cool and add 20 ml of mixed indicator, and make the volume up to 1 litre.
- Mixed indicator: Dissolve 0.066 g of methyl red and 0.099 g of bromocresol green in 100 ml of alcohol.
- Standard sulphuric acid (0.02M).
- Devarda's alloy: Mix Cu, Al and Zn in the ratio of 50:45:5, and grind to pass through a 0.15-mm sieve.

The procedure is:

1. Put 50 ml of the water sample in the distillation flask.
2. Add 0.5 g of MgO and 0.2 g of Devarda's alloy.
3. Put the heaters on and collect the NH_4 (NO_3 converted into NH_4 by reducing agent – Devarda's alloy) in boric acid (20 ml) having mixed indicator into a conical flask, which is connected with the distillation apparatus.
4. Continue distillation to collect about 35–40 ml.
5. Remove the distillate first and then switch off the heating system.
6. Titrate the distillate against 0.02M H_2SO_4 until the pink colour appears.
7. Carry out a blank simultaneously.

The relevant calculation is:

$$NO_3^- - N \text{ (mg/litre)} = \frac{(X - Y) \times 0.56}{50 \text{ (ml of sample)}} \times 1000$$

where:

- X = volume (ml) of 0.02M H_2SO_4 consumed in sample titration;
- Y = volume (ml) of 0.02M H_2SO_4 consumed in blank titration;
- 0.56 = factor. 1 litre 1M H_2SO_4 = 28 g N; therefore:

$$1 \text{ ml } 0.02M\ H_2SO_4 = \frac{28 \times 0.02 \times 1000}{1000} \text{ mg N} = 0.56 \text{ mg N}$$

Chapter 6

Mineral and organic fertilizer analysis

The main objective in analysing fertilizers is to assess their quality. The analysis examines both their physical and chemical composition. The quality of fertilizers is stated by the manufacturers and, in most countries, it is statutorily notified. Hence, analysis is carried out to determine whether the stated quality meets the statutorily notified standards or not. Together with the statutory notified composition of the fertilizers, the testing methods are specified. In situations where the testing methods are not notified, the prevalent standard methods are followed. Generally, the term fertilizer refers to mineral fertilizers, which are manufactured chemical products of standard composition, while the term organic fertilizers refers to organic manures, compost, agro-industrial wastes, etc. The compositions of organic fertilizers, unlike mineral fertilizers, are quite variable and, thus, difficult to regulate precisely.

Fertilizer quality is notified in terms of physical and chemical characteristics. The physical parameters include moisture content and particle size. The chemical parameters refer to the amount and form of nutrients, and to various impurities that may be toxic to plants above a critical limit, e.g. biuret in urea. The efficiency of a fertilizer depends on its form of nutrient content. A phosphatic fertilizer may have water-soluble, citrate-soluble, water-insoluble or citrate-insoluble forms of phosphate. A nitrogenous fertilizer may contain ammoniacal, nitrate and amide forms of N in various proportions.

The accompanying ions also reflect the quality of a fertilizer, such as SO_4 ions in ammonium sulphate, potassium sulphate, superphosphates and many micronutrient fertilizers. They supply S in addition to the main nutrient. Many fertilizers are fortified with micronutrients such as boronated single superphosphate (SSP) and zincated urea.

Therefore, in fertilizer analysis, in addition to estimating total nutrient content, it is necessary to estimate the forms of nutrients and other associated compounds in order to assess their quality properly. For organic fertilizers, the C content and the total content of nutrients are considered relevant and not their forms as they are low-analysis materials.

The analytical methods for fertilizers as described are applicable to most common fertilizers and the forms of nutrient content in them. The procedures as applicable to a particular nutrient could be applicable to any fertilizer with the

nutrient in that particular form. To conform to the scope of this guide, this chapter considers only commonly used solid fertilizers and their major constituents, including impurities.

SAMPLE COLLECTION AND PREPARATION

The collection of a representative sample of a fertilizer is an important step in fertilizer analysis and quality control. The method of sample collection depends on the type and source of fertilizer. Fertilizers are manufactured commodities. They are stored in silos at the manufacturing site for different periods. They are transported using various means (e.g. ship, rail and road) and stored again at various locations including port godowns, warehouses and dealers' shops. Fertilizers are stored/moved both in bulk and as bagged. Therefore, the sample collection methods can be classified broadly as concerning:

- collection from bulk stock in godowns;
- collection from ship hatches or while the ship is being loaded/unloaded;
- collection from bagged stock in godowns of various types;
- sample collection from damaged stock.

Scale of sampling

The number of samples to be collected from a given stock depends on the quantity available in the stock. In a ship, each hold/hatch is treated separately from the others, and samples are collected accordingly. In practice, one representative sample is taken from 100 tonnes of material. A similar scale of sampling is followed for factory silos where bulk fertilizers are stored.

In the case of bagged material, stored in smaller quantities (e.g. at dealers' godowns), generally, 1 sample (minimum) is drawn from 10 bags; 2 samples from 100 bags, 6–7 samples from 1 000 bags; and 10 samples from 2 000 bags. There is no fixed number of samples that can be defined to represent a given quantity. However, it is necessary to ensure that the sample is truly representative of the lot it designates.

In the case of bulk material being loaded into or unloaded from ships, samples are taken from the conveyor belt at certain intervals of time. The material so collected is stored in a container, and from such quantity collected, three representative samples each weighing about 400–500 g are taken.

Samples from the bulk material are collected with the help of sampling cups made of corrosion-free metal. Bagged fertilizers are sampled with the help of a sampling probe/tube. This is often a slotted double tube with a solid cone tip made of stainless steel or brass, about 60–65 cm long, about 1.5 cm in diameter, and with a slot width of about 1.2–1.3 cm. The sampling probe should be inserted diagonally from one corner to another, keeping the slit down and rotated while withdrawing.

The samples collected from the bags or bulk representing one lot is composited and, through the quartering method of leaving aside the portions of two opposite

quarters successively, three identical samples of about 450–500 g each are retained for analysis. These samples are labelled with details showing:

- the name of the fertilizer;
- the source;
- the date when collected;
- the signature, name and details of authority of the person who collected the sample.

Unlike soil, plant and water samples, fertilizer samples are generally collected in triplicate. Of the three samples, one goes to the designated laboratory for analysis; another goes to the owner of the fertilizer (e.g. ship's captain, godown manager, or dealer), and the third sample is kept for further reference by the sample collector. Such a procedure is necessary because in the event of deficiencies in the quality parameters, there has to be provision to analyse the reference sample before holding the party concerned responsible for the poor quality of the fertilizer manufactured/imported/sold to the farmers.

Sample preparation for analysis

The sample received for analysis is recorded in the laboratory with adequate details, and a laboratory code number is assigned in order to identify the sample and to keep its identity confidential.

About half of the sample is ground, sieved through a 1 mm sieve, and stored in a sample bottle for analysis. The remaining half is kept unground for particle size estimation. The samples are stored in an airtight glass bottle or taken for analysis in a moisture-free room (fitted with a dehumidifier) as most fertilizers are hygroscopic in nature.

ANALYTICAL METHODS

There are a number of estimation methods available for each of the constituents. Fertilizer analysis is carried out primarily for quality control and statutory purposes. Each country has adopted certain methods in its fertilizer statute, and only these methods are relevant for that country.

Fertilizer is a widely traded commodity, and it is necessary that the methodology adopted in checking quality be acceptable to all concerned. While there are no internationally accepted or common methods, the methods adopted, verified and notified by the AOAC are widely used (Motsara, 1984). The AOAC adopts a method after its verification and validation by a large number of analytical laboratories in various countries. In view of this, this guide describes the AOAC-based methods of fertilizer analysis in most cases, with simplification of the procedures for ease of comprehension.

Moisture

Two important forms of water present in fertilizers are: (i) absorbed/adsorbed water; and (ii) free water. They are interchangeable depending on the degree of moisture saturation and temperature. Some fertilizers also contain water as an

integral part of their composition, which is referred to as water of crystallization, as in the case of $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$. As fertilizers are generally hygroscopic in nature, they tend to absorb moisture from the atmosphere (depending on the relative humidity and their packing and storage conditions).

Excessive moisture may damage the granular structure of fertilizers, affect their quality and influence their nutrient content by increasing the weight of fertilizers in a given container.

Therefore, moisture estimation is critical to determining the quality of a fertilizer. The method used depends on the type of fertilizer and the nature of moisture held by it. Some common methods are:

- gravimetric method;
- vacuum desiccator method;
- Karl Fischer titration method.

With the gravimetric or oven-drying method, the loss of water on heating fertilizer samples at a certain temperature is estimated. This method is suitable for fertilizers such as ammonium sulphate, sodium nitrate, superphosphates, muriate of potash (MOP) and sulphate of potash (SOP). It is not suitable for fertilizers that yield volatile substances (such as NH_4) other than moisture on drying at a specified temperature, e.g. calcium ammonium nitrate and di-ammonium phosphate (DAP).

With the vacuum desiccator method, the free moisture present in the fertilizer is absorbed by the desiccant (sulphuric acid), and the loss in weight is reported as moisture. This method is suitable for fertilizers such as calcium ammonium nitrate, DAP, and NPK complexes.

The Karl Fischer titration method is suitable for fertilizers such as nitrophosphates, urea, and urea-based fertilizers, which do not withstand high temperatures.

Gravimetric method

Moisture is estimated by the gravimetric method where the loss in weight at a constant temperature of 100 °C ± 1 °C for 5 hours is measured, e.g. zinc sulphate, and copper sulphate. In the case of sodium nitrate, superphosphates, ammonium sulphate, SOP and MOP, the heating is at 130 °C ± 1 °C. For urea and urea-based fertilizers, the heating is at 70 °C. However, heating at 70 °C does not reflect full moisture content. Therefore, another method such as the Karl Fischer method is preferred.

The apparatus required consists of:

- a glass weighing bottle;
- an electronic balance;
- a temperature-controlled oven.

The procedure is:

1. Weigh 2.0 g of fertilizer sample in a pre-weighed glass weighing bottle.
2. Heat in a temperature-controlled oven for about 5 hours at the specified temperature, as given above for different types of fertilizers.

3. Cool in a desiccator, and weigh.

The relevant calculation is:

$$\text{Moisture \% by weight} = \frac{(B - C) \times 100}{B - A}$$

where:

- A = weight in grams of the empty sample bottle;
- B = weight in grams of the bottle plus material before drying;
- C = weight in grams of the bottle plus material after drying.

Vacuum desiccator method

In this method, the sample is kept in a vacuum desiccator over sulphuric acid. Free moisture present in fertilizers is absorbed by the acid, and the loss in weight of the sample is recorded as the moisture content in the sample.

The apparatus required consists of:

- a vacuum desiccator;
- a porcelain dish;
- a balance.

The procedure is:

1. Weigh (accurately) 5 g of sample in a porcelain dish, and keep it in a desiccator for 24 hours.
2. Take the weight again after 24 hours. The loss in weight is equal to moisture content in the sample.

The relevant calculation is:

$$\text{\% moisture} = \frac{B - C}{B - A} \times 100$$

where:

- A = weight in grams of the porcelain dish;
- B = weight in grams of the porcelain dish plus the fertilizer sample;
- C = weight in grams of the porcelain dish plus the fertilizer sample after desiccation for 24 hours.

Karl Fischer method

The apparatus required consists of:

- a Karl Fischer titrator;
- a balance;
- a beaker or flask;
- a graduated cylinder.

The reagents required are:

- Karl Fischer reagent (pyridine-free).
- Disodium tartrate dihydrate ($Na_2C_4O_62H_2O$) – AR-grade.
- Methanol – Karl Fischer grade / spectroscopy grade containing less than 0.05 percent water.

The procedure is:

1. Standardization of Karl Fischer reagent:
   - Set up the instrument.
   - Add about 25 ml of methanol to the titration vessel until the electrodes are dipped, and titrate with Karl Fischer reagent to a pre-set end point that persists for 30 seconds.
   - Add 100 mg of the disodium tartrate dihydrate to the titration vessel carefully, and titrate with Karl Fischer reagent to a pre-set end point (the end point should persist for 30 seconds). Note the volume (ml) of Karl Fischer reagent used as V_1.
2. Weigh accurately about 1 g of the prepared sample, transfer it carefully to the titration vessel, and stir until dispersed.
3. Titrate with Karl Fischer reagent to the same pre-set end point as above, and note the volume (ml) of Karl Fischer reagent used as V_2.

The relevant calculation is:

$$\text{Moisture \% in sample} = \frac{F \times V_2}{\text{Wt. of sample (g)}} \times \frac{100 \text{ (for \%)}}{000 \text{ (for mg to g)}}$$

where:

- F (factor) in mg H_2O/ml of Karl Fischer reagent =

$$\frac{0.1566 \times \text{mg of sodium tartarate dihydrate added}}{V_1}$$

Disodium tartrate dihydrate contains 0.1566 percent moisture.

Particle size

Fertilizers are manufactured with varying degrees of particle size. This property of fertilizer has a bearing on its efficiency when used in various types of soil for crop production. The size and strength of the particle determine its dissolution time when applied in soil. Most fertilizers are highly water soluble; hence, they dissolve quickly when they come into contact with soil moisture. Fertilizers can be crystalline or granular. With a view to reducing losses caused by rapid dissolution, fertilizers with large granules are also being manufactured, e.g. granular urea and super granular urea.

Granular fertilizers are considered superior for machine application, for preparing bulk blends with greater homogeneity and uniformity, and they are also less vulnerable to adulteration.

Therefore, particle size estimation is an important aspect in determining the fertilizer quality. Most granular fertilizers range between 1 and 4 mm, with a specific particle size for a specific fertilizer.

The apparatus required for particle size estimation consists of sieves of various size.

The procedure consists of sieving through a given sieve size. The material is passed through a sieve with a mesh equal to the maximum particle size prescribed

for a given fertilizer. The material so sieved is retained on a sieve with a mesh equal to the minimum particle size prescribed for that fertilizer. For example, a fertilizer is sieved through a 4 mm sieve and is retained on a 1 mm sieve, kept below the 4 mm sieve. The material retained on the 4 mm sieve is larger than 4 mm in size and that passed through the 1 mm sieve is less than 1.0 mm in size. The material retained on the 1 mm sieve is that with a particle size of between 1 and 4 mm.

Generally, 250 g of the fertilizer is taken and sieved as per the requirement. Sieving can be done mechanically or manually.

Nitrogen

Nitrogen in fertilizers may be present in various forms such as NH_4-N, NO_3-N, urea-N (amide) and organic N. The estimations are carried out for total N and its forms. For urea fertilizer, the total N estimation method is followed. The principle of N estimation is based on the Kjeldahl method. For including or excluding a particular form of N in total N estimation, specific chemicals/catalysts are used. For example, in nitrate-containing fertilizers, 2 g of salicylic acid and 5 g of sodium thiosulphate are added in the digestion mixture. This helps to bind the NO_3-N in the form of nitrosalicylic acid, and it is converted eventually into NH_4-N in the presence of H_2SO_4 and is estimated along with other forms of N present in the sample. Devarda's alloy (2/3 g per sample) can also be used instead of salicylic acid and thiosulphate.

Total nitrogen by the Kjeldahl method

The method and procedure are the same as described for total N estimation in soil (Chapter 3). Specific care is required for sample size. It may vary between 0.2 and 0.5 g depending on the N content of the sample. A smaller amount of sample may be taken for high-analysis fertilizers (e.g. urea) and a larger amount for low-analysis fertilizer (e.g. ammonium sulphate).

Ammoniacal nitrogen by the distillation method

The apparatus required consists of:

- a Kjeldahl distillation unit;
- some flasks, beakers and pipettes;
- a burette.

The reagents required are:

- Freshly ignited carbonate-free MgO.
- Standard acid (0.1M HCl).
- Standard alkali (0.1M NaOH).
- NaOH (40 percent) for distillation.
- Methyl red indicator.

The procedure is:

1. Put 0.5 g of the sample in a 600 ml distillation flask with about 250 ml of water.

2. Add 2 g of freshly ignited carbonate-free MgO or 5 ml of NaOH solution (40 percent) by tilting the flask and through the side of the flask so that the contents do not mix at once.
3. Connect the flask to a condenser by a Kjeldahl connecting bulb and connecting tube.
4. Start heating, and distil about 100 ml of liquid into a measured quantity of standard acid (0.1M HCl).
5. Titrate the distillate with standard NaOH (0.1M) to determine the remaining amount of unused acid, using methyl red indicator. The acid used to neutralize ammonia is equivalent to the N content in the sample.
6. Carry out a blank.

The relevant calculation is:

$$\text{Percent } NH_4 - N = \frac{(A - C) - B}{W} \times 0.0014 \times 100$$

where:

- A = ml of standard acid (0.1M HCl) taken to receive ammonia;
- B = ml of standard alkali (0.1M NaOH) used in titration;
- W = weight of the sample taken;
- C = ml of standard alkali used in the blank.
- 1 ml 0.1M HCl = 0.0014 g N

Ammoniacal plus nitrate-nitrogen by the distillation method

Devarda's alloy (50 percent Cu, 45 percent Al, and 5 percent Zn) reduces NO_3 to NH_4 in an alkaline condition. The method is same as for NH_4-N estimation (above), except that 2–3 g of Devarda's alloy is added before distillation in order to take into account the NO_3 by reducing it to ammonia form.

Nitrate-nitrogen

In fertilizers containing both NH_4 and NO_3-N, first ammoniacal nitrogen is estimated followed by NH_4 plus NO_3 estimation. From the combined value of NH_4 and NO_3, the value of ammoniacal N is subtracted to obtain the nitrate-N content.

Urea nitrogen

The urea form of N can be estimated together with total N by digestion with sulphuric acid. For example, total N is estimated for urea fertilizer. However, for some NPK complexes, urea N has to be estimated separately. In such cases, it is done by the urease method.

The apparatus required for the urease method consists of:

- some beakers;
- some flasks;
- a Gooch crucible;
- some filter paper.

The reagents required are:

- Neutral urease solution: Shake 1 g of jack bean meal with 100 ml of water for 5 minutes. Transfer 10 ml of the solution to a 250 ml Erlenmeyer flask, dilute with 50 ml water, and add 4 drops of methyl purple indicator. Titrate with 0.1M HCl to reddish purple, then backtitrate to green colour with 0.1M NaOH. From the difference in volume used, calculate the amount of 0.1M HCl required to neutralize 10 ml of solution. Based on the calculated acid required, add 0.1M HCl to the remaining 90 ml of solution (about 2.5 ml of acid is required per 100 ml of solution), and shake well.
- HCl (0.1M): Dilute 100 ml of concentrated HCl to 1 litre, and titrate with the standard alkali to establish the exact strength of the acid.
- NaOH (0.1M): Dissolve 4 g of NaOH in 900 ml of water in a 1-litre volumetric flask, make the volume up, and standardize with the standard acid.
- Sodium carbonate (10 percent).
- Barium hydroxide (saturated).

The procedure is:

1. Weigh 10 ± 0.01 g of the sample and transfer it to 15 cm No. 12 fluted filter paper.
2. Leach with about 300 ml of water into a 500 ml volumetric flask.
3. Add 75–100 ml of saturated barium hydroxide solution to precipitate phosphates.
4. Let it settle, and test for complete precipitation with a few drops of saturated barium hydroxide solution.
5. Add 20 ml of 10 percent sodium carbonate solution to precipitate excess barium and any soluble Ca salts.
6. Let it settle, and test for complete precipitation (when the addition of a few more drops of sodium carbonate does not show further precipitation).
7. Dilute to volume, mix, and filter through 15 cm No. 12 fluted paper.
8. Transfer 50 ml of aliquot (equivalent to 1 g of sample) to a 200 or 250 ml Erlenmeyer flask, and add 1–2 drops of methyl purple indicator.
9. Acidify solution with 0.1M HCl, and add 2–3 drops in excess (after colour change is noticed).
10. Neutralize (titrate) solution with 0.1M NaOH to the first change in colour of the indicator.
11. Add 20 ml of neutral urease solution, close flask with rubber stopper, and let it stand for 1 hour at 20–25 °C.
12. Cool the flask in ice water slurry, and titrate at once with 0.1M HCl to full purple colour, then add about 5 ml in excess.
13. Record total volume added, backtitrate excess HCl with 0.1M NaOH to neutral end point.

The relevant calculation is:

$$\% \text{ Urea} - \text{N} = \frac{(\text{ml } 0.1\text{M HCl} - \text{ml } 0.1\text{M NaOH}) \times 0.0014 \times 100}{\text{Wt. of sample (1 g contained in 50 ml aliquot)}}$$

Biuret

Biuret ($C_2O_2N_3H_5$) is a chemical compound formed by the combination of two molecules of urea with a release of a molecule of ammonia when the temperature during the urea manufacturing process exceeds the controlled level. Fertilizer-grade urea contains biuret, which usually varies between 0.3 and 1.5 percent. Biuret is toxic to plants particularly when applied through foliar spray.

The apparatus required for estimating biuret consists of:

- a water-bath shaker;
- a spectrophotometer;
- some beakers and flasks;
- a burette.

The reagents required are:

- Alkaline tartrate solution: Dissolve 40 g NaOH in 50 ml of cold water and 50 g of $NaKC_4H_4O_6.4H_2O$, and dilute to 1 litre. Let it stand for 1 day before use.
- Copper sulphate solution: Dissolve 15 g of $CuSO_4.5H_2O$ in CO_2-free water, and dilute to 1 litre.
- Biuret standard solution (1 mg/ml): Dissolve 100 mg of reagent-grade biuret in CO_2-free water, and dilute to 100 ml.
- Standard H_2SO_4.

The procedure is:

1. Preparation of the standard curve:
   - Transfer a series of aliquots, 2–50 ml of standard biuret solution, to a 100 ml volumetric flask.
   - Adjust the volume to about 50 ml with CO_2-free water. Add one drop of methyl red, and neutralize with 0.1M H_2SO_4 to a pink colour.
   - Add, with swirling, 20 ml of alkaline tartrate solution and then 20 ml of $CuSO_4$ solution.
   - Dilute to volume. Shake for 10 seconds, and place in a water-bath for 15 minutes at 30 °C ± 5 °C.
   - Also prepare a reagent blank.
   - Determine absorbance of each solution against the blank at 555 nm on the spectrophotometer with a 2.4 cm cell, and plot the standard curve.
2. Stir continuously 5 g of the sample in 100 ml of water for 30 minutes.
3. Filter and wash in 250 ml volumetric flask and dilute to volume.
4. Transfer 25 ml of aliquot to 100 ml volumetric flask and proceed as given under preparation of standard curve.

The relevant calculation is:

$$\text{Biuret \%} = \frac{C \times 100}{W} \times df$$

where:

- C = concentration in mg/ml of biuret as read from the standard curve;
- W = weight of sample;

- *df* = 200 (5 g of fertilizer extracted to 250 ml, and 25 ml taken for further dilution to 100 ml).

Determination of free acid in ammonium sulphate (as H_2SO_4)

The presence of free acid needs to be regulated in fertilizers such as ammonium sulphate. It is generally kept below 0.025 percent. Its estimation is necessary in order to check the quality of fertilizers.

The apparatus required consists of:

- some beakers and flasks;
- a burette.

The reagents required are:

- Standard sodium hydroxide solution (0.02M).
- Methyl red indicator: Dissolve 0.15 g of water-soluble methyl red in 500 ml of water.
- Methyl red – methylene blue mixed indicator solution: Mix equal volumes of 0.2 percent solution of methyl red and 0.1 percent solution of methylene blue in rectified spirit.

The procedure is:

1. Dissolve about 20 g of the prepared fertilizer sample, accurately weighed, in about 50 ml of cold water.
2. Filter and make the volume up to about 200 ml.
3. Titrate with standard sodium hydroxide solution, using 1–2 drops of methyl red as indicator.
4. If a satisfactory end point (change from red to yellow-orange) with methyl red is not obtained, methyl red – methylene blue mixed indicator may be used.

It is important to use a microburette for this titration. The filtering medium should be neutral and should not contain any alkaline material (which would neutralize free acid).

The relevant calculation is:

$$\text{Free acid as } H_2SO_4 \text{ \% by weight} = \frac{4.9 \times A \times M}{W}$$

where:

- A = volume in millilitres of standard NaOH solution;
- M = Molarity of standard NaOH solution;
- W = weight in grams of prepared sample taken for the test;
- 4.9 = factor (1 M 1 000 ml NaOH = 49 g H_2SO_4; thus, 1 M 100 ml NaOH = 4.9 g H_2SO_4).

Phosphorus

Phosphate (P_2O_5) in fertilizers may be present in different forms: (i) water soluble; (ii) neutral ammonium citrate soluble or insoluble; (iii) citric acid soluble or insoluble; and (iv) acid soluble. Phosphate is generally present as bound with Ca as monocalcium phosphate, dicalcium phosphate and tricalcium phosphate. It may be

present with other metal elements, e.g. aluminium phosphate and iron phosphate. In phosphate rock, it is present as apatite of chlorine and fluorine. Being in water-soluble form, monocalcium phosphate is considered available, while dicalcium phosphate becomes available in slightly acidic situations. Tricalcium phosphate is in an unavailable form and can be available only in acidic situations. Similarly, the aluminium and iron phosphates are also in plant-unavailable forms.

Neutral ammonium citrate soluble form is also considered as available, which includes both monocalcium phosphate and dicalcium phosphate.

In view of the variability in availability to plants, the estimation of different forms of phosphate is critical.

For the so-called "available" forms of P, appropriate extractants have been designed to extract P from fertilizers under a set of well-defined sampling conditions: extractant ratio, temperature, time of extraction, shaking period, etc. The form of P as a fraction of the total P is extracted by a particular method. Estimation of the extracted P utilizes various testing methods: (i) gravimetric; (ii) volumetric; and (iii) colorimetric.

The following methods are used for P estimation in fertilizers:

- gravimetric ammonium phosphomolybdate;
- gravimetric quinolinium phosphomolybdate;
- volumetric ammonium phosphomolybdate;
- volumetric quinolinium phosphomolybdate;
- spectrophotometric vanadium phosphomolybdate.

All the methods are used in various laboratories. For total phosphate estimation, the gravimetric quinolinium phosphomolybdate method is generally preferred because of the minimal interference of other ions and its accuracy and simplicity. Another common method providing acceptable accuracy and simplicity is volumetric ammonium phosphomolybdate.

Gravimetric quinolinium phosphomolybdate method

Various forms of P present in fertilizers are first converted into orthophosphate through chemical treatments. On reaction with quimociac reagent, the orthophosphate precipitates as quinolinium phosphomolybdate [$(C_9H_7N)_3$ $H_3PO_4.12\ MoO_3$] in a boiling medium. The precipitate is weighed gravimetrically, which gives the P content of the sample.

In the gravimetric method, Ca, Fe, Mg, alkali metals and citrates do not affect the analysis. The citrate in the reagent complexes the ammonium ions, thus preventing interference from precipitation of ammonium phosphomolybdate by the ammonium salts usually present in mixed fertilizers. The citrates also reduce interference from soluble silica.

The apparatus required consists of:

- a volumetric flask;
- some beakers;
- a Gooch crucible;
- some filter paper;

- an analytical balance.

The reagents required are:

- Concentrated nitric acid.
- Concentrated hydrochloric acid.
- Magnesium nitrate solution (9 percent): Dissolve 90 g of P-free $Mg(NO_3)_2$ in water, and dilute to 1 litre.
- Acetone.
- Citric acid.
- Sodium molybdate dihydrate.
- Quinoline.
- Quimociac reagent: Dissolve 60 g of citric acid in a mixture of 85 ml of HNO_3 and 150 ml of water, and cool. Dissolve 70 g of sodium molybdate dihydrate in 150 ml of water. Gradually add the sodium molybdate solution to the citric acid – nitric acid mixture, with stirring. Dissolve 5 ml of synthetic quinoline in a mixture of 35 ml of HNO_3 and 100 ml of water. Gradually add this solution to the molybdate citric-nitric acid solution, mix, let it stand for 24 hours, and filter. Add 280 ml of acetone, dilute to 1 litre with water, and mix well. Store in a polyethylene bottle.

According to the nature of the fertilizer, the sample solution should be prepared using one of the following methods:

- For materials and fertilizer mixtures with a high OM content: Put 1 g of the sample in an evaporation dish. Add 5 ml of $Mg(NO_3^-)_2$ solution, and evaporate to dryness. Ignite to destroy the OM, and dissolve in 10 ml of HCl.
- For materials with a low OM content: Put 1 g of the sample in a 50 ml beaker. Add 30 ml of HNO_3 and 5 ml of HCl, and boil gently until the OM is destroyed and red-brown fumes cease to appear.
- For basic slag and fertilizers containing iron or aluminium phosphate: Treat 1 g of the sample with 30 ml of HCl and 10 ml of HNO_3, and boil gently until red-brown fumes disappear.

Cool the solution, prepared by any of the above three methods, dilute to 250 ml, mix, and filter through a dry filter, if required (may contain some insoluble material).

The procedure is:

1. Pipette 5–25 ml of aliquot (sample solution) depending on the P content (containing not more than 25 mg P_2O_5 in the aliquot) into a 250 ml beaker, and dilute to 100 ml with distilled water.
2. Add 50 ml of quimociac reagent, cover with a watch glass, place on a hotplate, and boil for 1 minute.
3. Cool the material to room temperature, swirl carefully 3–4 times during cooling.
4. Filter the precipitate with fibreglass filter paper (or Gooch crucible G4) previously dried at 250 °C and weighed. Wash 4–5 times with 25 ml portions of water. Dry the crucible/filter paper and contents for 30 minutes at 250 °C. Cool in a desiccator to a constant weight.

5. Run a reagent blank with each batch. Subtract the weight of the blank from the weight of the sample precipitate.

The relevant calculation is:

$$\text{Total phosphate (as } P_2O_5\text{) \%} = \frac{3.207(S - B)}{100} \times df \times \frac{100}{W} = \frac{3.207(S - B)}{W} \times df$$

where:

- S = weight of sample precipitate in grams;
- B = weight of blank precipitate in grams;
- df = dilution factor for aliquot taken:
  - suppose volume of the aliquot (solution) taken for estimation = 5 ml, and total volume of fertilizer solution prepared = 250 ml;
  - $df = \frac{\text{Total volume of fertilizer solution}}{\text{Aliquot taken}} = \frac{250}{5} = 50$
- For percentage $= \frac{100}{\text{Wt.}}$
- W = weight of sample taken in grams;
- Factor 3.207 = the quinolinium phosphomolybdate precipitate contains 3.207 percent P_2O_5 on weight basis.

In cases where $MoO_3.Na_2MoO_4.2H_2O$ (quinoline) is not of standard quality, the exact volume of quimociac reagent to be added for precipitation should be calculated by running a series of known standards and observing the phosphate recovery in them.

Volumetric ammonium phosphomolybdate method

Phosphorus is precipitated from the acidic solution as ammonium phosphomolybdate $[(NH_4)_3PO_4.12MoO_3]$ by adding ammonium molybdate solution. The precipitate is dissolved in a measured excess of the standard alkali after filtration and washing until free of the acid.

The apparatus required consists of:

- some volumetric flasks / beakers;
- a burette;
- a shaker;
- a water-bath;
- some No. 44 filter paper.

The reagents required are:

- Magnesium nitrate solution (9 percent): Dissolve 90 g of P-free $Mg(NO_3)_2$ in water, and dilute to 1 litre.
- Concentrated nitric acid.
- Concentrated hydrochloric acid.
- Ammonium molybdate solution (3 percent): Dissolve 30 g of ammonium molybdate in hot distilled water, and make the volume up to 1 litre.
- Standard NaOH solution (0.1M): Dissolve 4 g of NaOH in 1 litre of water, and standardize against standard acid.

- Standard H_2SO_4 solution (0.1M): Take 5.6 ml of concentrated H_2SO_4 and make the volume up to 1 litre. Standardize against a primary standard alkali such as Na_2CO_3 (procedure as detailed in Chapter 3).
- Sodium nitrate (2 percent): Dissolve 20 g of AR-grade sodium nitrate in 1 litre of distilled water.
- Phenolphthalein indicator (1 percent): Dissolve 1 g of phenolphthalein in 100 ml of 95.5 percent ethanol.
- Ammonium nitrate (AR-grade).
- Sodium carbonate (AR-grade).

The sample solution should be prepared using one of the methods indicated for the gravimetric quinolinium phosphomolybdate method (above).

The procedure is:

1. Pipette 5–25 ml of aliquot (sample solution) depending on the P content (containing not more than 25 mg P_2O_5 in the aliquot) in a 250 ml beaker, and dilute to 100 ml with distilled water.
2. Add about 5–10 ml of concentrated HNO_3 and about 10 g of ammonium nitrate.
3. Heat this mixture on a water-bath at 55–60 °C for 10 minutes.
4. Add 3 percent ammonium molybdate solution in the beaker drop by drop with the help of a burette. Continue stirring with a glass rod until about 50 ml of molybdate solution is added. Stir for another few minutes until the yellow precipitate appears to become granular.
5. Cover the beaker with glass and allow it to settle for some time. Decant the clear solution through No. 44 filter paper, and wash the precipitate with 2 percent sodium nitrate solution, agitate thoroughly, and allow the precipitate to settle. Transfer the precipitate to the filter paper, and wash with $NaNO_3$ solution until free from acid (by test with a litmus paper).
6. Transfer the precipitate and filter paper to a beaker, and add 10 ml of 0.1M NaOH at a time by pipette until the precipitate becomes soluble.
7. Add 1–2 drops of 1 percent phenolphthalein, and titrate the excess of alkali against 0.1M sulphuric acid.
8. Run a reagent blank with each batch.

The relevant calculation is:

Total phosphate (as P_2O_5) percent = $F \times (V_1M_1 - V_2M_2) \times df$

$$= 0.0031 \times (40 \times 0.1 - 10 \times 0\ 1) \times \frac{250}{5} \times \frac{100}{1} = 46.5$$

where:

- F = factor for P_2O_5 corresponding to 1 ml of 1M alkali (NaOH). The calculation is as follows: 23 g equivalent of NaOH = 31 g P = 71 g P_2O_5 (P × 2.29 = P_2O_5)

$$1\,\text{equivalent of NaOH or } 1000\,\text{ml of } 1\,\text{M NaOH} = \frac{71}{23}\,\text{g}\ P_2O_5$$

$$1\,\text{ml of } 1\text{M NaOH} = \frac{71}{23} \times \frac{1}{1000}\,\text{g}\ P_2O_5 = 0.0031\,\text{g}\ P_2O_5$$

- V_1 = volume of 0.1M NaOH required to dissolve the precipitate (e.g. 40 ml);
- V_2 = volume of 0.1M H_2SO_4 used for titration to neutralize excess alkali (e.g. 10 ml);
- M_1 = molarity of the standard alkali (NaOH);
- M_2 = molarity of the standard acid (H_2SO_4);
- *df* = dilution factor for aliquot taken:
  - suppose, volume of the aliquot (solution) taken for estimation = 5 ml; total volume of fertilizer solution prepared = 250 ml.

$$df = \frac{\text{Total volume of fertilizer solution}}{\text{Aliquot taken}} = \frac{250}{5} = 50$$

- For percentage $= \frac{100}{\text{Wt.}}$

Water-soluble phosphate (P_2O_5)

The water-soluble phosphate is obtained from the sample by dissolving it in distilled water or by washing the sample successively with distilled water. As a procedure, put 1 g of the sample on a filter paper fitted on a 12 cm funnel. Wash with small portions of water at a time to collect about 250 ml of filtrate and make up the exact volume.

Pour water into the funnel only when the earlier portion has drained fully. Otherwise, filtration and complete washing may be prolonged (which should be completed in 1 hour). The filtrate so obtained is used for estimation of phosphate by the gravimetric quinolinium phosphomolybdate method or volumetric ammonium phosphomolybdate method as described above.

The residue remaining on the filter paper contains the water-insoluble portion of P in the sample.

Available phosphate (neutral ammonium citrate-soluble P_2O_5)

For estimating available phosphate, an indirect method is followed whereby total, water-soluble and ammonium citrate-insoluble fractions are estimated. By subtracting citrate-insoluble P from total P, estimates are made for the available P.

The apparatus required for estimation of citrate-insoluble P consists of:

- a volumetric flask / beaker;
- a burette;
- a water-bath-cum-shaker;
- a Büchner funnel.

The reagents required are:

- Concentrated HNO_3.
- Concentrated HCl.
- Concentrated H_2SO_4.
- Ammonium hydroxide.

- Ammonium nitrate (5 percent).
- Quimociac reagent (same as described in total P_2O_5 estimation).
- Filter paper.
- Neutral ammonium citrate solution: Dissolve 370 g of pure citric acid in 1 500 ml of distilled water. Add about 345 ml of 28–29 percent ammonium hydroxide so that the acid is neutralized. After neutralization, the solution must attain a pH of 7.0; if it does not, adjust the pH by adding NH_4OH or citric acid solution.

The procedure is:

1. Follow the procedure as described above for the preparation of a sample solution for estimation of water-soluble phosphate. Within 1 hour, transfer the filter paper and residue to a 250 ml conical flask containing 100 ml of ammonium citrate solution previously heated to 65 °C.
2. Close the flask tightly with a smooth rubber stopper, shake vigorously until the filter paper is transformed to pulp, and release pressure by removing stopper occasionally.
3. Agitate continuously the contents of the stoppered flask in a controlled temperature (65 °C ± 0.5 °C) water-bath-cum-shaker for 1 hour.
4. Exactly 1 hour after adding the filter paper and residue, remove the flask from the shaker, and filter immediately by suction as rapidly as possible through No. 5 filter paper or equivalent, using a Büchner or ordinary funnel.
5. Wash with distilled water at 65 °C until the volume of filtrate is about 350 ml, allowing time for thorough draining before adding more water. If the material is such that it will yield a cloudy filtrate, wash with 5 percent NH_4NO_3 solution.
6. Determine the P_2O_5 in the citrate-insoluble residue (remainder on filter paper) after digestion by one of the following methods:
   - Transfer the dry filter paper and contents to a crucible, ignite until all OM is destroyed. Digest with 10–15 ml of HCl until phosphates are dissolved.
   - Transfer the filter paper and residue to a 250 ml Kjeldahl flask, boil for 30–45 minutes with 30 ml of HNO_3 and 10 ml of HCl. Boil very gently until it is colourless and white dense fumes appear in the flask.
7. Dilute the solution to 250 ml, mix well, and filter through dry filter paper if required. Pipette out 25 ml of aliquot containing not more than 25 mg of P_2O_5 into a 500 ml Erlenmeyer flask, and proceed as described for estimation of total P_2O_5 using quimociac reagent (above).

The relevant calculation is:

$$\text{Citrate insoluble } P_2O_5\% = \frac{3.207\ (S - B)}{100} \times df \times \frac{100}{W}$$

where:

- S = weight of sample precipitate in grams;
- B = weight of blank precipitate in grams;
- W = weight of sample in grams;

- df = Dilution factor = $\frac{\text{Volume of fertilizer solution}}{\text{Aliquot taken}}$
- For percentage = $\frac{100}{W}$

Percent available (citrate-soluble) P_2O_5 = % total P_2O_5 - % citrate-insoluble P_2O_5

The procedure for total P_2O_5 estimation is described above.

Method for estimation of free phosphoric acid (as P_2O_5)

In the specifications of superphosphates, a certain content of free acid (a maximum of 4 percent as phosphoric acid) is allowed in order to check the conversion of water-soluble P into non-water-soluble P during long storage. Therefore, its estimation is essential in determining the quality of superphosphates.

The apparatus required consists of:

- a shaker;
- some flasks and beakers;
- a burette.

The reagents required are:

- Acetone.
- Standard sodium hydroxide solution (0.1M).
- Bromocresol green indicator solution: Dissolve 0.1 g of bromocresol green in 100 ml of rectified spirit.

The procedure is:

1. Weigh accurately about 2.5 g of the prepared sample in a 250 ml Erlenmeyer flask.
2. Add 100 ml of neutral acetone. Fix to a wrist-action shaker. Shake for 1 hour.
3. Filter rapidly through No. 1 filter paper into a 250 ml Erlenmeyer flask, wash with acetone 2–3 times, adding 10 ml of acetone each time.
4. Evaporate the acetone as much as possible.
5. Add about 50 ml of water and drops of bromocresol green indicator.
6. Titrate with standard NaOH solution until the colour changes from yellow to blue.

The relevant calculation is:

$$\text{Free phosphoric acid (as P O) percent by weight} = \frac{7.1 \times M \times V}{W}$$

where:

- M = molarity of standard NaOH solution;
- V = volume of standard NaOH solution;
- W = weight in grams of sample taken for the test;
- 7.1 = factor (1 ml 1M NaOH = 0.098 g H_3PO_4; thus, 100 ml 1M NaOH = 9.8 g H_3PO_4. The relationship between H_3PO_4 and P_2O_5 is 196:142 = 0.72. Hence, the factor is 9.8 × 0.72 = 7.1).

Potassium

In all potassic fertilizers, K is generally present in water-soluble form. Therefore, it is estimated directly in fertilizer solution either gravimetrically, volumetrically or flame photometrically. In manures and organic fertilizers, wet digestion with acid is required prior to determination of K in order to bring the element into solution form, as described for plant digestion (Chapter 4).

The methods used for K determination in fertilizers and manures are:

- gravimetric perchloric acid method;
- gravimetric chloroplatinate method;
- gravimetric and volumetric cobaltinitrite method;
- gravimetric and volumetric sodium tetraphenyl boron (STPB) method.

The AOAC-based STPB volumetric method is commonly used in laboratories because of its accuracy and simplicity.

STPB method

Potassium from the fertilizer sample is first extracted with water or ammonium oxalate. The K in extracted solution is precipitated with an excess of STPB as potassium tetraphenyl boron. The excess of STPB is backtitrated with benzalkonium chloride (BAC) or quaternary ammonium chloride using Clayton yellow as indicator: $Na[B(C_6H_5)_4] + K \rightarrow K[B(C_6H_5)_4] + Na^+$.

Interference of NH_4^+ takes place during K precipitation. It is avoided by complexing NH_4^+ with formaldehyde under slightly alkaline conditions before precipitation of K. The chlorides and sulphates do not interfere in the titration.

The apparatus required consists of:

- some volumetric flasks and beakers;
- a burette / semi-microburette;
- some filter paper.

The reagents required are:

- Sodium hydroxide solution (20 percent): Dissolve 20 g of NaOH in 100 ml of distilled water.
- Formaldehyde (HCHO) solution (37 percent).
- STPB solution (about 1.2 percent): Dissolve 12 g of STPB in about 800 ml of water. Add 20–25 g of $Al(OH)_3$, stir for 5 minutes, and filter through No. 42 filter paper (or equivalent) into a 1 litre volumetric flask. Rinse the beaker sparingly with water and add to the filtrate. Collect the entire filtrate, add 2 ml of 20 percent NaOH solution, dilute to volume (1 litre) with water, and mix. Let it stand for 48 hours, and then standardize (as described below). Adjust (by using K salt of known composition for prior standardization by trial and error) so that 1 ml of STPB = 1 percent K_2O. Store at room temperature.
- BAC or quaternary ammonium chloride solution (about 0.625 percent): Dilute 50 ml of 12.8 percent BAC to 1 litre with water, mix and standardize (as described below). If a different concentration is used, adjust the volume accordingly (BAC of 0.625 percent strength is required so the dilution can be done according to the concentration available).

- Clayton yellow (0.04 percent) indicator: Dissolve 40 mg of Clayton yellow powder in 100 ml of water.
- Ammonium oxalate solution [$(NH_4)_2\ C_2O_4$] (4 percent): Dissolve 40 g of ammonium oxalate in 1 litre of distilled water.

The procedures for standardizing the solutions are:

- BAC solution: Put 1 ml of STPB solution in a 250 ml Erlenmeyer flask; add 20–25 ml of water, 1 ml of 20 percent NaOH, 2.2 ml of HCHO, 1.5 ml of 4 percent ammonium oxalate, and 6–8 drops of Clayton yellow indicator. Titrate to pink end point with BAC solution, using a 10 ml semi-microburette. Adjust by increasing or decreasing the strength of the BAC solution so that 2 ml = 1 ml of STPB solution (keeping 1 ml STPB = 1 percent K_2O).
- STPB solution: Dissolve 2.5 g of KH_2PO_4 in about 150 ml of water in a 250 ml volumetric flask, add 50 ml of 4 percent ammonium oxalate solution, dilute to volume with water, and mix. Transfer 15 ml of aliquot (51.92 mg of K_2O or 43.10 mg of K) to a 100 ml volumetric flask, add 2 ml of 20 percent NaOH, 5 ml of HCHO and 43 ml of STPB solution. Dilute to volume (100 ml) with water, and mix thoroughly. Let it stand for 5–10 minutes, and then pass through dry No. 42 filter paper. Transfer 50 ml of aliquot of filtrate to a 250 ml Erlenmeyer flask, add 6–8 drops of Clayton yellow indicator, and titrate excess STPB with BAC solution to pink end point. Calculate factor (f) by: f = percent K_2O/ml of STPB solution

$$= \frac{34.61}{43 - \text{ml of BAC used for standardization}}$$

where, 34.61 = % K_2O present in standard KH_2PO_4.

The procedure is:

1. K extraction/preparation of sample solution: Dissolve a known weight (2.5 g) of straight K fertilizer (MOP, SOP, potassium magnesium sulphate) in 200 ml of distilled water, and make the volume up to 250 ml for estimation. For NPK complex fertilizers or NPK fertilizer mixtures, dissolve the sample in 125 ml of water, add 50 ml of 4 percent ammonium oxalate solution, and boil for 30 minutes; after cooling, filter through dry No. 12 filter paper, and make the volume up to 250 ml for further estimation.
2. Transfer 15 ml of aliquot of sample solution to a 100 ml volumetric flask and add 2 ml of 20 percent NaOH and 5 ml of HCHO.
3. Add 1 ml of standard STPB solution for each 1 percent of K_2O expected in the sample plus an additional 8 ml in excess in order to ensure complete precipitation.
4. Dilute to volume (100 ml) with water, mix thoroughly, let it stand for 5–10 minutes, and pass it through No. 12 filter paper (or equivalent).
5. Transfer 50 ml of filtrate to a 250 ml Erlenmeyer flask, add 6–8 drops of Clayton yellow indicator, and titrate excess STPB with standard BAC solution to pink end point.

The relevant calculation is:

$$\% K_2O = (\text{ml STPB used - ml BAC used}) \times f$$

where, f = % K_2O/ml of STPB solution. This factor applies to all fertilizers where 2.5 g of sample is diluted to 250 ml, and 15 ml of aliquot is taken for analysis. To express the results as K rather than K_2O, substitute 28.73 for 34.61 in calculating the value of f.

Micronutrients

Chapter 3 has described the methods for estimating micronutrients in soils using an AAS. In the case of soils, the available micronutrient content is first extracted with the help of appropriate extractants such as DTPA. After standardization of the AAS, the micronutrient content in the extract is estimated. For mineral and organic fertilizers, it is the total content of micronutrients that is relevant. Therefore, organic fertilizers are first acid digested as per the di-acid digestion method described in Chapter 4 (wet digestion method) in order to bring them into solution. From this solution, micronutrient estimations are carried out using an AAS as described in Chapter 3. For mineral fertilizers that are in 100 percent water-soluble form, the estimation of micronutrients is carried out using water-dissolved samples. The NPK complex/compound fertilizers and fertilizer mixtures with water-insoluble ingredients need to be acid digested (similar to organic fertilizers) before analysis is carried out.

Standard curves are prepared for the relevant micronutrients in the same manner as described in Chapter 3. The water-soluble or acid-digested fertilizer samples are used in appropriate quantities so that the content is within the range of concentration used in the standard curve. The nutrient content is calculated taking into account the weight of the sample, the volume made and the aliquot used for estimation.

Organic fertilizers

Unlike soil analysis where available nutrients are estimated, in the case of fertilizers (both mineral and organic) the total contents of nutrients are estimated. In the case of organic fertilizers, the C content and the total content of nutrients are considered relevant and not their forms as they are low-analysis materials. The method for C content estimation is the same as described in Chapter 3. The methods for estimation of total N, P and K in organic fertilizers are the same as described above for mineral fertilizers. With organic fertilizers, the sample always needs to be prepared using the wet-digestion method as described in Chapter 4. The sample size should be 1.0 g (to be weighed exactly).

Chapter 7
Biofertilizer assay and production

Soils are considered dynamic living systems that contain a variety of microbes such as bacteria, actinomycetes, fungi and algae. Bacteria are more numerous than the other three groups combined. They have also been more widely studied and exploited for greater agricultural use than the others.

Maintaining a favourable population of useful microflora is important from a fertility standpoint. The most commonly exploited microbes are those that help in fixing atmospheric N for plant uptake or in solubilizing/mobilizing soil nutrients such as unavailable P into plant-available forms, in addition to secreting growth-promoting substances for enhancing crop yield.

As a group, such microbes are called "biofertilizers" or "microbial inoculants". They can be generally defined as: preparations containing live or latent cells of efficient strains of N-fixing, phosphate-solubilizing or cellulolytic micro-organisms used for application to seed or soil with the objective of increasing the numbers of such micro-organisms and accelerating certain microbial processes to augment the availability of nutrients in a form that plants can assimilate readily.

Table 19 lists the most commonly accepted specifications of important biofertilizers, when isolated and multiplied for agricultural use.

TABLE 19
Specifications of commonly used biofertilizers

Parameters	*Rhizobium*	*Azotobacter*	*Azospirillum*	Phosphate-solubilizing microbes
Base	Carrier-based			
Cell number at the time of preparation	10^7 cells/g of carrier material or 10^7 cells/ml of liquid material			
Contamination	Nil at 10^5 dilution			
pH	6.5–7.5			
Particle size of carrier material (µm)	150–212			
Moisture (%)	30–40			
Type of carrier	Peat/lignite/charcoal			
Gram stain test	Negative	Negative	Negative	Negative for Pseudomonas and positive for *Bacillus*
Other indicators	Nodule test positive	Minimum amount of N fixed not less than 10 mg/g of sucrose utilized	Should produce white pellicle in N-free bromothymol blue at 10^7 dilution	Should show phosphate-solubilizing zone of 10 mm in tricalcium phosphate medium at 10^6 dilution

Biofertilizers should always be stored in a cool place or at room temperature (25–28 °C), and away from direct heat and sunlight for a longer shelf-life. Shelf-life-expired biofertilizers should not be used. Biofertilizers should not come into direct contact with mineral fertilizers and pesticides.

In order to facilitate understanding of the terms used in this chapter, their descriptions (as per: Motsara, Bhattacharyya and Srivastava, 1995; Bhattacharyya and Tandon, 2002; FAO, 2004), are provided in Annex 13.

TYPES OF MICROSCOPES AND THEIR USE IN THE LABORATORY

The use of microscopes is crucial for biofertilizer identification. Depending on the type of micro-organism and the purpose of study, microscopes of different capacities are used. Microscopes are generally equipped with three objectives: a low-power objective (10×); a high-power objective (40×); and an oil immersion objective (100×). The desired objective is rotated into place. The total magnification obtained with the objectives is as follows:

- The 10× objective with a 10× eyepiece gives a total magnification of 100.
- The 40× objective with a 10× eyepiece gives a total magnification of 400.
- The 100× objective with a 10× eyepiece gives a total magnification of 1 000.

The procedure for using a microscope is:

1. Place the microscope in a position facing the source of light being used, and adjust the mirror to reflect good light into the body tube.
2. Place the slide containing the specimen on the stage.
3. Keeping the eye close to the eyepiece, turn the coarse adjustment slowly to raise the body tube until the slide comes into focus.
4. Turn the fine adjustment slowly until the focus is perfect and the object being examined is seen clearly.
5. Focusing with the oil immersion objective should be done very carefully. For this, first use the low-power objective to locate the portion of the specimen to be examined. Care should be taken to locate the portion in the exact centre of the low-power field as the field diameter is much smaller with the oil immersion objective than with either of the other objectives.
6. Raise the body tube and then rotate the nose-piece until the oil immersion objective clicks into position. Place an oil immersion drop on the portion of the slide directly under the objective. Watch the objective from the side and lower it carefully into the oil. Do not allow the objective to touch the slide. Then study/examine the specimen.

Various types of microscopes used in biofertilizer study are:

- Compound microscope: This microscope consists of two sets of lenses, the objective and the eyepiece. The main functions of the objective are to gather the light rays coming from any point of the object, to unite them in a point on the image, and to magnify the image. The eyepiece magnifies the image further. Most microscope manufacturers have adopted 160 mm as the standard tube length.

- Phase-contrast microscope: In this microscope, light rays passing through an object of a high refractive index are retarded in comparison with light rays passing through a surrounding medium with a lower refractive index. The retardation or phase change for a given light ray is a function of the thickness and the refraction index of the material through which it passes. This microscope has become an extremely important tool in a microbiology laboratory because it intensifies the contrast between translucent objects in unstained living specimens.
- Dark-field microscope: This microscope is so designed that the entering centre light rays are blacked out and the peripheral rays are directed against the object from the side. As a result, the object being viewed appears bright against a dark background. Many micro-organisms that are not visible under bright-field or phase-contrast microscopes can be detected because they reflect light in a dark field and, thus, appear larger than they really are.
- Fluorescent microscope: This microscope is similar to the dark-field microscope except that invisible ultraviolet (UV) light is used to illuminate the object. When exposed to UV radiation, certain substances absorb it and release it almost immediately as visible light of a longer wavelength. The emissions are known as fluorescence and the material as fluorescent.
- Electron microscope: This microscope has a magnifying system that uses a beam of electrons focused in a vacuum by a series of magnetic lenses with a very high detection power. Different types of electron microscopes are available, such as the transmission type (where the image is formed by electrons that pass through the specimen) and the scanning type (where the image formation is based on electrons reflected back from the specimen). The electron microscope has enabled microbiologists to study structures that are too small to be detected by optical microscopes.

EXAMINATION OF MICROBES BY STAINING TECHNIQUES

The morphology of bacteria is examined in two ways: (i) by observing the living, unstained organisms; and (ii) by observing dead cells stained with dyes. The stained preparations of micro-organisms are used to obtain information on the shape, anatomy and taxonomic characters of the cells, which cannot be observed easily in unstained material. The staining can be positive or negative in nature. Positive staining is further classified into simple and differential stains. Positive staining refers to the stain combining with the cell components. In negative staining, the stain does not combine with the cell but forms a deposit on the slide around the cell. The unstained cell appears bright against the darker background of the slide. By this procedure, one can observe the cell components and capsule, which are not easily stained by positive methods.

Before staining, the cells are placed on a microscope glass slide. This preparation is known as a microbiological smear. The slide should be free from grease, otherwise it will interfere with the clarity of the stained preparation. Slides are washed with dichromate-sulphuric acid solution or with detergent to make them grease-free. They are then washed with water, dried, and stored in industrial methylated spirit.

Preparation of smear for staining microbes

A smear is prepared on a microscope glass slide as also on cover glass. The procedure is:

1. Remove the slide from alcohol with the help of forceps. Pass through a Bunsen burner to burn off the alcohol. Allow it to cool.
2. Make a circle about 20 mm in diameter on the slide with the help of a glass marker.
3. Shake the culture tube well and then use a wire loop to take out a drop of the culture, place it on the slide and spread it within the marked circle.
4. Fix the smear by passing the slide through the tip of the blue portion of the flame 4–5 times, and then allow it to cool. The smear is now ready for staining.

Staining

Simple staining

This technique requires only one type of dye for the coloration of a bacterial smear. Methylene blue, crystal violet and carbolfuchsin are the basic dyes. However, they differ in the rate and degree to which they stain a cell. Methylene blue reacts with the negatively charged cells at the slowest rate, taking 30–60 seconds to stain a bacterial cell. Crystal violet is more reactive and a powerful dye. It needs only 10 seconds to stain a cell. Carbolfuchsin is similar to crystal violet in effectiveness.

The reagents required are:

- Methylene blue: This is also known as the Levowitz–Weber modification of the Newman–Lampert stain. Add 0.6 g of certified methylated blue chloride slowly to 52 ml of 95 percent ethyl alcohol and 44 ml of tetrachlorethane (technical grade) in a 200-ml flask. Swirl to dissolve. Let it stand for 12–24 hours at 5–7 °C, then add 4 ml of glacial acetic acid. Filter through No. 42 filter paper (or equivalent) and store in a clean, tightly closed container with a plastic wrapping bag. Traces of water may cause problems with this stain.
- Crystal violet: Dissolve 2 g of crystal violet chloride salt in 20 ml of 90 percent ethyl alcohol and mix with 80 ml of 1 percent aqueous ammonium oxalate.

The procedure is:

1. Prepare smears of two broth cultures. It will save time to make both smears on the same slide, and each should be labelled using a glass marker.
2. Flood the smears with dye (methylene blue or crystal violet solution), and leave on the slide for 2 minutes.
3. Wash the slide to free from stain under a gently running tap, and blot dry with a piece of clean blotting paper.
4. Examine the stained preparation under the oil immersion objective of the microscope (1.8 mm), and make a drawing on a sheet.
5. Observe closely for significant differences in cell size and arrangements.

Differential staining

Differential staining is based on the principle that micro-organisms differ from one another chemically and physically, thus, they react differently to a given staining procedure. This helps in differentiating different types of bacteria. The most common differential stain is Gram stain.

With this technique, when bacteria are stained with certain basic dyes, some species can be decolorized easily with organic solvent such as ethanol or acetone. These are called Gram-negative species. *Rhizobium*, *Azotobacter*, *Azospirillum* and *Pseudomonas* are Gram-negative. Others that resist decolorization are called Gram-positive species (e.g. *Bacillus*). The most commonly used dye is crystal violet.

Negative staining

In negative staining (also known as indirect staining), bacterial cells are not stained but made visible against a dark background. This techniques uses acidic dyes such as eosine and nigrosine. Eosine is used as a soluble salt (sodium eosinate, which ionizes into sodium and eosinate) with the colouring power of dye in the negatively charged eosinate ion. It forms a deposit around the cell, making the bacterial cell appear colourless against a dark background. The negative staining method is not so common because it leaves the cell colourless. However, its advantage over direct staining is that it gives a more accurate view of the bacterial cell.

The reagents required are:

- Eosine solution: Dissolve 2 g of sodium eosinate salt in 50 ml of hot water.
- Nigrosine solution: Dissolve 10 g of nigrosine salt in 100 ml of hot water.

The procedure is:

1. Place a loopful of bacterial suspension on a glass slide, and put a drop of 1 percent aqueous solution of the dye. Mix thoroughly with the help of a sterile wire loop.
2. Spread the mixture uniformly on the slide.
3. Allow the slide to air dry. Do not heat it. Examine it under the oil immersion objective, and make drawing on a sheet. The area around the cells will appear dark blue, and the cells themselves will be colourless.

The layering of the staining solution should be thin. Otherwise, it will not allow light to pass through the object, and the dye will crack on drying. However, if the layer (film) is too thin, it will not give a good contrast.

CULTURE MEDIA

In nature, microbes are found as a mixed population. The types that encounter the most favourable environmental and other growth conditions emerge in abundance while others that find the environment less favourable are fewer in number. However, for any kind of study on them or for their use as a pure species, they have to be separated from other species. The nutritional media or culture media are different for different species. The culture media may be of two types: liquid, without the use of agar; and solid, containing agar. The cultures may be: pure

(contain only one species); mixed (contain two or more species); or contaminated (contain unwanted species along with the intended species).

As the medium is specific for certain species, it checks or restricts the growth of other microbes for which it may not be suitable. However, some growth of and contamination by unwanted species may occur. Therefore, isolation of specific microbes is necessary for their greater efficiency in use.

Types of culture media

The media may be either empirical or synthetic.

Empirical media are natural media (usually meat extract) that provide all the nutritive substances such as N, C, proteins, amino acids, and mineral salts. Along with the meat extract, peptone, sodium chloride (for correct osmotic pressure) and phosphate salts (as buffer to stabilize pH) are also added to the medium. The exact chemical composition of the media remains unknown.

Synthetic media are prepared with known types and quantities of the chemical compounds. They contain a source of C and N together with other nutrients such as sodium and potassium phosphates, magnesium sulphate and calcium chloride. Molybdenum is also usually added. Treptophan (an amino acid) is also used as an N source. The absence of a particular nutrient source restricts the growth and multiplication of the micro-organisms.

Preparation of culture media

There are specific media for specific types of microbes. No single medium can help grow all types of microbes.

In preparing the culture medium, the contents specified for the purpose are weighed and mixed in a conical flask. Water is added and then heated to dissolve the contents, and the solution is stirred. The pH value is adjusted to the desired level. Precipitation is avoided during the preparation.

In the case of a solid medium (one containing agar), the container is placed in such a way that the maximum surface area is available for the growth of micro-organisms. For this purpose, slanting tubes, conical flasks and Petri dishes are considered suitable.

The culture medium and the glassware are sterilized in an autoclave in which the steam under pressure is maintained at 1–1.25 kg/cm^2 and the heating is done for 15–30 minutes at a temperature of 121 °C.

For a medium containing heat-sensitive materials (e.g. sugars, amino acids and vitamins), sterilization is done by passing the medium through different types of filters (asbestos or sintered glass) that are capable of retaining bacteria (where present as a contaminant).

Certain chemicals, such as salts of heavy metals ($AgNO_3$, $HgCl_2$, and $KMnO_4$), halogens (chlorine, bromine, iodine and their salts) and organic compounds are also used for sterilization of the medium.

Table 20 lists the most commonly used media for various micro-organisms.

TABLE 20
Micro-organism-specific media

Micro-organism	Media	Author
Bacteria (general)	Thornton's agar medium	Thornton (1922)
Rhizobium	CRYEMA (Congo red yeast extract mannitol agar)	Vincent (1970)
	Norris and Date medium	Norris & Date (1976)
Azotobacter	Jensen's N-free medium	Jensen (1942)
	Ashby's medium	Ashby (1907)
	Beijerinckia's medium	Becking (1959)
Azospirillum	Semi solid malate medium	Baldini & Dobereiner (1980)
	Nitrogen free bromothymol blue medium	Dobereiner, Marriel & Nery (1976)
	Okon's modified medium	Okon, Albrecht & Burris (1977)
PSMs	Pikovskaya medium	Pikovskaya (1948)

The composition and method of preparation of the media listed in Table 20 are described below.

Thornton's agar media

The composition of the medium is:

- mannitol: 1.0 g;
- asparagine: 0.5 g;
- K_2HPO_4: 1.0 g;
- KNO_3: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- $CaCl_2$: 0.1 g;
- NaCl: 0.1 g;
- $FeCl_3$: 0.002 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve phosphate, nitrate and asparagine in distilled water, add magnesium sulphate, calcium chloride, sodium chloride and ferric chloride. Add agar and dissolve, heat at 100 °C, and filter. Add mannitol, and cool to 60 °C. Adjust the pH to 7.4 with bromothymol blue, and autoclave at 121 °C for 30 minutes at 1–1.25 kg/cm^2 pressure. Cool, and store for use.

Congo red yeast extract mannitol agar medium

The composition of the medium is:

- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- NaCl: 0.1 g;
- mannitol: 10 g;
- yeast extract: 1 g;

- agar: 15.0 g;
- distilled water: 1 litre;
- 1 percent aqueous Congo red: 2.5 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Glucose peptone agar medium

The composition of the medium is:

- glucose: 5.0 g;
- peptone: 10.0 g;
- agar: 15.0 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Norris and Date liquid medium

The composition of the medium is:

- mannitol: 10.0 g;
- yeast extract: 1 g;
- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.8 g;
- NaCl: 0.2 g;
- $FeCl_3.6H_2O$: 0.01 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, adjust the pH to 7.0, and autoclave (as above).

Jensen's N-free medium

The composition of the medium is:

- sucrose: 20.0 g;
- K_2HPO_4: 1.0 g;
- $MgSO_4.7H_2O$: 0.5 g;
- NaCl: 0.5 g;
- $FeSO_4$: 0.1 g;
- $CaCO_3$: 2.0 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Ashby's medium

The composition of the medium is:

- mannitol: 20.0 g;
- K_2HPO_4: 0.2 g;

- $MgSO_4$: 0.2 g;
- NaCl: 0.2 g;
- K_2SO_4: 0.1 g;
- $CaCO_3$: 5.0 g;
- agar: 15.0 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 7.0, and autoclave (as above).

Beijerinckia medium

The composition of the medium is:

- sucrose: 20.0 g;
- KH_2PO_4: 0.8 g;
- K_2HPO_4: 0.2 g;
- $M_gSO_4.7H_2O$: 0.5 g;
- $FeCl_3$: 0.1 g;
- Na_2MoO_4: 0.005 g;
- agar: 15 g;
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 6.5, and autoclave (as above).

Semi-solid malate medium

The composition of the medium is:

- malic acid: 5.0 g;
- K_2HPO_4: 0.5 g;
- $MgSO_4.7H_2O$: 0.2 g;
- NaCl: 0.1 g;
- $CaCl_2$: 0.02 g;
- $Na_2MoO_4.2H_2O$: 0.002 g;
- $MnSO_4.H_2O$: 0.01 g;
- KOH: 4.5 g;
- biotin: 0.1 mg;
- Fe-EDTA (1.64 percent): 4.0 ml;
- bromothymol blue: 3.0 ml (0.5 percent alcoholic solution);
- distilled water: 1 000 ml.

To prepare the medium, dissolve the salts in water, adjust the pH to 6.8, and autoclave (as above).

Nitrogen-free bromothymol blue medium

The composition of the medium is:

- malic acid: 5.0 g;
- KOH: 4.0 g;
- K_2HPO_4: 0.5 g;

- $FeSO_4.7H_2O$: 0.05 g;
- $MnSO_4.H_2O$: 0.01 g;
- $MgSO_4.7H_2O$: 0.01 g;
- NaCl: 0.02 g;
- $CaCl_2$: 0.01 g;
- Na_2MoO_4: 0.002 g;
- distilled water: 1 litre;
- bromothymol blue: 2.0 ml (0.5 percent alcoholic solution);
- agar: 1.75 g.

To prepare the medium, dissolve the salts in water, add agar, adjust the pH to 6.6–7.0, and autoclave (as above).

Okon's modified liquid medium

The composition of the medium is:

- Part (i):
  - K_2HPO_4: 6.0 g;
  - KH_2PO_4: 4.0 g;
  - distilled water: 500 ml.
- Part (ii):
  - $MgSO_4$: 0.2 g;
  - NaCl: 0.1 g;
  - $CaCl_2$: 0.02 g;
  - NH_4Cl: 1.0 g;
  - malic Acid: 5.0 g;
  - NaOH: 3.0 g;
  - yeast extract: 0.05 g;
  - Na_2MoO_4: 0.002 g;
  - $MnSO_4$: 0.001 g;
  - H_3BO_3: 0.0014 g;
  - $Cu(NO_3)_2$: 0.0004 g;
  - $ZnSO_4$: 0.0021 g;
  - $FeCl_3$: 0.002 g;
  - distilled water: 500 ml;
  - bromothymol blue: 2 ml (0.5 percent alcoholic solution).

To prepare the medium, dissolve and sterilize parts (i) and (ii) separately, and mix aseptically while hot.

Pikovskaya medium

The composition of the medium is:

- glucose: 10.0 g;
- $Ca_3(PO_4)_2$: 5.0 g;
- $(NH_4)_2SO_4$: 0.5 g;
- NaCl: 0.2 g;
- KCl: 0.2 g;

- $MgSO_4.7H_2O$: 0.1 g;
- $MnSO_4$: traces;
- $FeSO_4$: traces;
- yeast extract: 0.5 g;
- agar: 15.0 g;
- distilled water: 1 litre.

To prepare the medium, dissolve the salts in water, add agar, and autoclave (as above).

ISOLATION AND IDENTIFICATION OF IMPORTANT MICROBES

Rhizobium

A well-established symbiotic relationship exists between *Rhizobium* species and leguminous plants, as a result of which elemental N is fixed or converted to ammonia. A specific *Rhizobium* culture for a specific legume crop that has a high ability for infection, nodulation, N_2-fixation and antibiotic resistance is needed. *Rhizobium* it is an aerobic and gram-negative microbe, and abundant in the root nodules of leguminous crops.

Isolation

Rhizobium strains can be isolated from the root nodules of legumes as follows.

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagents required are:

- Ethyl alcohol (95 percent).
- Mercuric chloride (0.1 percent weight/volume).
- Congo red yeast extract mannitol agar (CRYEMA) plate.

The procedure consists of two parts:

- Nodule selection and surface sterilization:
  1. Uproot a healthy legume plant from which a *Rhizobium* strain is required.
  2. Wash the roots of the plant gently under tap-water.
  3. Select pink-coloured healthy nodules, and separate them from the root with the help of a sharp clean blade.
  4. Put the nodules in a test-tube, and wash them thoroughly 4–5 times with tap-water so that the surface of the nodules becomes soil-free.
  5. Immerse undamaged nodules in 95 percent ethyl alcohol for 5–10 seconds, and rinse them with sterilized water.

6. Transfer the nodules to a sterile test-tube, and keep the nodules immersed in 0.1 percent mercuric chloride solution for 2–3 minutes.
7. Shake the test-tube from time to time in order to remove air bubbles from the surface of the nodules and to bring fresh sterilant in contact with the surface of the nodules.
8. Decant off the mercuric chloride solution, and flood the nodules with sterilized water 5–8 times in order to remove all the sterilant.

- Plating of nodule suspension:

1. Add a few drops of sterilized water to the test-tube, and crush the nodules in the tube with the help of a sterile glass rod.
2. The suspension so formed has a very high count of bacteria. Dilute this suspension by adding 2–4 ml of sterilized water to the tube.
3. Take one drop or 0.1 ml of the suspension, and put it on a plate containing CRYEMA medium.
4. Spread the suspension over the plate gently with the help of a sterile glass spreader. Repeat this spreading for three different plates containing the same medium.
5. Invert the plates, and keep them for incubation at 28–30 °C until small, elevated, round-shaped colonies develop. If the colonies are white, translucent, glistening and elevated, they may be expected to be colonies of *Rhizobium*. Any colony absorbing the red colour of the Congo red indicator may be assumed to be contaminated.

After proper identification, *Rhizobium* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

The following culture tests need to be carried out in order to check whether the *Rhizobium* colony as developed in the above incubation is pure or contaminated with common contaminants such as *Agrobacterium*.

- Growth in alkaline medium: *Agrobacterium radiobacter* can be detected by drawing streaks on Hoffer's alkaline medium (pH 11.0) where *Rhizobium* does not grow, while *A. radiobacter* does.
- Growth in glucose peptone agar: *Rhizobium* shows little or no growth on glucose.
- Gram test: It should test negative.

Azotobacter

Azotobacter is a non-symbiotic, aerobic, free-living bacteria. It is capable of fixing N and synthesizing growth-promoting substances and vitamins. It is found mainly near the rhizosphere. In soil, its population varies but rarely exceeds 10^2–10^3/g soil.

Isolation

The apparatus required consists of:

- an incubator;
- some test-tubes;
- some Petri dishes;
- some glass rods;
- some inoculation needles;
- some pipettes;
- some conical flasks.

The reagents required are:

- sterile water;
- Jensen's medium.

The microbes are isolated from the soil sample (collected from the rhizosphere) by the soil dilution method and plating on an N-free agar medium. The procedure is:

1. Collect moist soil from the rhizosphere, and make a suspension of 10 g of this soil in 90 ml of sterile water in a conical flask.
2. Shake the suspension for about 5 minutes.
3. Dilute this suspension serially as follows:
   - Arrange at least 6 test-tubes, each containing 9 ml of sterile water, in a test-tube stand.
   - Take 1 ml of the suspension from the conical flask aseptically and add to the first test-tube containing 9 ml of sterile water. Total volume will be 10 ml. Shake the test-tube vigorously.
   - Pipette out 1 ml of the suspension from this test-tube and add it to the second test-tube and shake.
   - Repeat this process serially until the last test-tube.
4. Pipette out 1 ml of liquid aseptically from the third, fourth, fifth and sixth test-tubes (to start with a minimum dilution of 1 000 times), and pour onto separate N-free agar medium (Jensen's medium) plates. Spread the aliquot on the plates, and mark the plates.
5. Invert the plates, and incubate them at about 28 ± 2 °C for at least three days.
6. After incubation, soft, flat, transparent or milky, mucoid colonies of *Azotobacte*r will develop on the plates.
7. Pick up a single colony aseptically with the help of a sterilized inoculation needle and streak it on an N-free agar media plate.
8. Invert and incubate the plate at 28 ± 2 °C. In this way, pure colonies of *Azotobacter* will be formed.

After proper identification, *Azotobacter* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Azotobacter has the ability to produce pigment. Different species of *Azotobacter* produce different pigments, thus facilitating their identification. The pigments produced by important species are:

- *Azotobacter chroococcum* – brown;
- *Azotobacter beijerinckii* – light brown;
- *Azotobacter vinelandi* – greenish yellow;
- *Azotobacter insignis* – light brown;
- *Azotobacter agilis* – green;
- *Azotobacter macrocytogenes* – purple.

Although pigment production does not give a confirmation of the species, it is a good indication of their presence.

The secretion of gum or polysaccharide is another important characteristic, as is the formation of cysts by all the species (for withstanding adverse conditions).

Azospirillum

Azospirillum is a spiral-shaped N-fixing bacteria. It also produces hormones and vitamins. Important species are *Azospirillum brasilense* and *Azospirillum lipoferum*. It is widely distributed in soils and grass roots.

Isolation

As *Azospirillum* bacteria occur inside as well as outside plant roots, plant roots are taken in order to isolate *Azospirillum*.

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagents required are:

- Ethyl alcohol.
- 0.1 percent mercuric chloride.
- Phosphate buffer.

The procedure is:

1. Take roots of any field crop plants.
2. Wash the roots first under tap-water, and then surface sterilize with 95 percent ethyl alcohol for about 5 seconds.
3. Cut the roots into small pieces (0.5 cm long), and wash them with 0.1 percent mercuric chloride for 1 minute, followed by washing with sterile water and then with phosphate buffer.
4. Place the pieces of roots in screw-capped tubes containing semi-solid sodium or calcium malate medium, and incubate at 28–30 °C for 3–4 days.

5. After proper incubation, a white pellicle of *Azospirillum* will develop 1–2 cm below the upper surface of the medium.
6. Transfer the isolate (white pellicle) 3–4 times to semi-solid calcium malate medium contained in screw-capped tubes.
7. Thereafter, make serial dilutions up to 10^{-10}, and then incubate 0.1 ml from the last dilution to a fresh tube/plate containing semi-solid calcium malate medium for growth.
8. Incubate the tube/plate for 3–4 days. Observe the growth of *Azospirillum*.

After proper identification, *Azospirillum* from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Azospirillum organisms are Gram-negative, curved, and rod-shaped of varying size. They contain poly-B-hydroxybutyrate granules. *Azospirillum* shows spiral movements. The formation of white pellicles on semi-solid calcium malate medium is a characteristic of *Azospirillum*. *Azospirillum* forms round-shaped colonies on the solid malate medium. *Azospirillum* micro-organisms are producers of strong bases. Hence, when they grow in a medium containing bromothymol blue indicator, they change the colour of the medium to blue.

Phosphate-solubilizing micro-organisms

Soil bacteria of the genera *Pseudomonas*, *Bacillus* and certain fungi of the genera *Penicillium* and *Aspergillus* possess the ability to solubilize insoluble forms of P owing to their secretion of organic acids. These microbes help in the solubilization of P from phosphate rock and other sparingly soluble forms of soil P by reducing their particle to nearly amorphous forms. They are present in abundance in the rhizosphere. The phosphate-solubilizing micro-organisms (PSMs) are isolated directly from the soil by the soil serial dilution method.

Isolation

The apparatus required consists of:

- an incubator;
- sterilized water;
- a Petri dish;
- a glass rod;
- a test-tube (sterilized);
- an inoculation needle.

The reagent required is sterile water.

The procedure is:

1. Take 10 g of soil, and dilute it serially by the same method as described for *Azotobacter* (above).
2. Take 4–5 plates containing Pikovskaya medium, which contains insoluble phosphate.

3. Take 1 ml of liquid soil suspension from 3rd, 4th, 5th and 6th test-tubes and pour aseptically onto the plates/Petri dishes containing the medium. Soil suspensions with different dilutions will ensure the growth of micro-organisms depending on their population and, thus, could be taken for further purification and multiplication from the appropriate tube.
4. Spread the aliquots (soil suspension) of different dilutions on the plates.
5. Invert the plates, and incubate them at 28–30 °C for 3–4 days.
6. Microbial colonies with a transparent zone will develop.
7. Take a single colony and streak it on phosphate-containing medium plates, and incubate at 28 °C for 3–4 days.
8. Take the single colony from the above plates having clear zones of solubilization, and maintain them on slants, as this provides a larger surface area for growth.

After proper identification, PSMs from the colony so developed can be taken and multiplied in order to obtain a pure culture through the inoculation and fermentation process.

Identification

Phosphate-solubilizing micro-organisms can be either bacteria or fungi. The following species are more effective:

- bacteria:
  - *Bacillus megaterium*,
  - *Bacillus polymyxa*,
  - *Bacillus pulvijaciens*,
  - *Pseudomonas striata*,
  - *Pseudomonas rathonis*;
- fungi:
  - *Aspergillus niger*,
  - *Aspergillus awamori*,
  - *Penicillium digitatum*.

The bacterial species are aerobic and heterotrophic. Cell size is 1.1–2.2 µm, and the cells are rod-shaped. The transparent zone around microbial colonies indicates the extent of phosphate solubilization and the effectiveness of the microbes.

INOCULATION OF CULTURE MEDIUM

This is a method by which micro-organisms are transferred from any source (purified culture) to the medium for their multiplication in a laboratory. To avoid any contamination, inoculation is done in a laminar air-flow chamber aseptically as follows:

1. Select a pure culture of the micro-organism to be multiplied.
2. Take a specific culture medium in a sterilized flask/tube (already prepared and kept ready).
3. Hold the pure culture tube between thumb and forefinger in such a way that the cotton plug of the tube is towards the body of the worker.

4. Remove the cotton plug from the tube/flask containing the culture, take the pure culture from the tube using a sterilized platinum needle and transfer it to the medium in the flask/tube. Plug the inoculated tube/flask, keep it in the incubator at 28 ± 2 °C for 3–5 days to allow the microbes to grow. When a large volume of culture medium is required for mass production of biofertilizer, multiplication is done in large flasks (2–5 litres) in a rotary shaker or in fermenters.

FERMENTATION

The sterilized medium is inoculated with a pure culture of the desired micro-organism as described above. This is called a broth, and it will be used for the further multiplication of micro-organisms and their commercial production. The broth is put in fermenters of the requisite size depending on the amount of broth required to be used in the production of biofertilizer. The broth is aerated continuously by forcing sterile air through a porous stainless steel tube at 10–12 litres of air per hour. The aeration requirement of microbes varies from species to species. When the number of microbes reaches 10^8–10^9 cells/ml, it is considered ready for mixing with the carrier. A large amount of inoculum reduces the time required to reach maximum viable numbers and, therefore, reduces the risk and effects of contamination. Inoculum levels generally vary from 0.1 to 1 percent (sometimes 5 percent). Generally, the process should provide 10^6–10^7 bacteria per millilitre of culture medium at the beginning of fermentation. The fermentation time also varies from 6 to 18 hours depending on species, growth conditions and the initial amount of microbes taken from the mother culture.

Apart from large-capacity steel fermenters, broth can also be fermented/incubated in large conical flasks (2–5 litres), which are mounted and shaken continuously on a rotary shaker for 12–36 hours in order to achieve the same population of microbes as in steel fermenters.

MEASUREMENT OF MICROBIAL GROWTH

Microbial cells are usually counted using a Petroff-Hauser bacterial counter (after placing it under a phase-contrast or dark-field microscope). This counter consists of a thick slide containing one block, which is divided into ten sub-blocks with grooves. The depth of each groove is 0.2 mm. Each sub-block has the capacity to retain a definite number of micro-organisms. Thus, the number of microbes in a block can be determined. It is observed that 1 ml of liquid pure culture of bacterium medium may contain about 5 000 million bacteria.

The growth of any micro-organism (e.g. bacteria) can also be measured by counting the number of colonies developed on Petri dishes. Colony counting is a commonly practised method in microbiological laboratories.

There are many techniques for counting viable cells that are able to divide and form offspring. The usual method is to determine the number of cells in a given sample capable of forming colonies on a suitable agar media. The method involves:

- serial dilution;

- spreading of diluted suspension on plates, and counting of colony-forming units on plates.

Serial dilution

A laminar air-flow chamber is used in order to achieve serial dilution of the broth culture of the strain or biofertilizer sample suspension. For plate counts, the countable range is generally 30–300 cells/ml. To achieve this concentration, the procedure is:

1. Set out 8 tubes, each containing 9 ml of sterile water.
2. Dilute 1 ml of broth culture or biofertilizer sample suspension (1 g of sample in 9 ml of water) in steps (10^{-1} to 10^{-8}) with a sterilized 1-ml serological pipette equipped with a rubber bulb of 1 ml capacity.
3. Suck up broth culture or sample suspension from tube 1 to the 1-ml mark.
4. Immediately expel the broth culture or sample suspension back into the tube with sufficient vigour to effect a thorough mixing.
5. Repeat sucking up and expelling 5 times, and then transfer 1 ml to tube 2.
6. Take a new sterile pipette, attach the rubber bulb, and remove 1 ml to tube 3.
7. Repeat this procedure using a fresh sterile pipette each time until the dilution series is completed.
8. After completion of serial dilution, put an aliquot of the diluted sample on a pour plate, spread plate or drop plate with specified nutrient agar media (as described below). After incubation at 28 ± 2 °C for 3–5 days, each viable cell will give rise to one distinct colony, which is counted and calculated for the number of viable cells per millilitre of suspension. The microbes so grown are taken for further multiplication and testing for their efficiency/quality.

Spreading of diluted suspension on plates and colony counting

Pour plate method

In this method, sterilized molten medium is kept ready in conical flasks placed in a water-bath at a constant temperature of 48 °C. The procedure is:

1. Remove dry sterilized Petri dishes from paper packs and stack them in a laminar air-flow chamber.
2. For each dilution, three Petri dishes will be required. Stack the plates in sets of three each and label them with the help of a glass marker pen.
3. Using a fresh sterile pipette, pour 1 ml of aliquot from the last dilution (say, 10^{-8}) into each of the three Petri dishes.
4. Using the same pipette, pour similar aliquots from the next two dilutions (say, from 10^{-7} and 10^{-6}) into three Petri dishes for each dilution.
5. Pour about same volume of molten medium into each of the plates.
6. Immediately after pouring, move the plates gently in a whirling motion to mix the contents.
7. Allow the medium to solidify, and incubate at 28 ± 2 °C for 3–5 days.
8. Count the colonies after 3–5 days.

9. Multiply the average number of colonies by the dilution factor. If the average number of colonies at 10^{-8} dilution is 60, then the sample had a concentration of $60 \times 10^8 = 6 \times 10^9$ cells/ml.

Spread plate method

Using the same serially diluted samples prepared for the previously described pour plate method, the procedure is:

1. Begin with the 10^{-7} dilution, and deliver 0.1 ml of the sample into each of 4 plates of yeast extract mannitol agar (YEMA) medium previously dried at 37 °C for about 2 hours.
2. Using the same pipette, dispense 0.1-ml samples from the 10^{-6} and 10^{-5} dilutions, in that order.
3. Prepare a glass spreader by bending a 20-cm glass rod of 4 mm diameter to the shape of a hockey stick, dip it into alcohol and hold on flame, then cool the spreader by touching it on the surface of a separate YEMA plate.
4. Lift the cover of each Petri dish just enough to introduce the spreader, and place it in position on the agar surface.
5. Spread the sample evenly over the agar surface, sterilizing and cooling the spreader between samples.
6. Incubate as before.
7. Calculate the number of viable cells as outlined for the pour plate method, adjusting for the smaller volume plated (0.1 ml instead of 1.0 ml). For example, if 60 colonies were counted on a plate inoculated with 0.1 ml of a 10^{-7} dilution, the results should be $60 \times 10 \times 10^7 = 6 \times 10^9$ cells/ml.

Drop plate method

For the drop plate method, the procedure is:

1. Select three-day-old agar plates, which have been dried enough to absorb some moisture, or dry the agar plates in a bacteriological incubator at 37 °C for 2 hours.
2. Take a fixed-volume or variable-volume microlitre pipette and set the volume 30 µl.
3. Sterilize appropriate-sized microtips in a microtip box and keep them ready.
4. Take two Petri dishes containing solidified and dried agar media and divide the bottom of each plate into eight equal parts.
5. Using a microlitre pipette, deliver one drop (30 µl) of diluted suspension in one part.
6. From the last dilution (say, 10^{-8}), deliver four aliquots of 30 µl in each of the four parts of the Petri dish. Use the remaining four parts for the next dilution (say, 10^{-7}). Repeat the process for two further dilutions.
7. Incubate the plates in incubator. In this case, as the area used is very small, observations are to be recorded at the earliest. Otherwise, overlapping of colonies will make counting difficult.

Other methods in brief

Roll-tube method
In this method, the dilutions are mixed with molten agar in a test-tube or bijou bottle, and rolled mechanically while setting, so that the agar forms a film. Aerobes may then be incubated as usual. Anaerobic counts usually require pyrogallol plugs to be inserted in the tube, or some other device for nursing in an anaerobic atmosphere.

Surface-spread method
This is the plating method for aerobes. The agar plate should be dried for 5 minutes at 55 °C or for 45 minutes at 37 °C. The next step is to add 0.2-ml portions from each dilution where the culture is diluted to different levels, say, 1/10, 1/20, and spread evenly over each surface in turn with a wire or a glass spreader in the shape of an L in order to avoid the passage of bacteria under the agar through the plate edge. The disadvantage of this method is that some organisms are taken up by the glass rod or wire, hence, decreasing the count.

Shake-culture method
This method is used to count anaerobes. It involves taking 5 narrow tubes (known as anaerobic or vanilla tubes), adding 0.2 ml of 1/10 dilution of culture and then about 7 ml of molten agar medium (heated at 40 °C or less). While the agar is still molten, the tube is inverted once. Here, some organisms are lost by adhesion to the cotton wool or capsule of the tube. When set, more sterile molten agar is added in order to exclude air. This procedure is not suitable for strongly aerogenic anaerobes. Such organisms (e.g. *Clostridium welchii*) can be counted by a modified roll-tube method. Anaerobes that are not exacting may often be counted on ordinary Petri dishes (as described for the surface-spread method) if these are incubated in an atmosphere of N_2 or H_2 containing anaerobic jar.

Membrane counts
This method involves filtering a known volume of sample through a sterile membrane of a pore size suitable to retain all the required microbes, then transferring the membrane to a nutrient pad, and counting the colonies that grow after incubation. For reasons that are not yet clear, this procedure is not very successful with very exacting anaerobes, but it can be used for aerobes.

Other methods for determining the total number of bacteria
The following methods are also used in order to determine the total number of bacteria (dead or alive):

- Turbidimetry method and nephalometery method: These methods depend on the estimation of turbidity by scattered light from a suspension as well as a transmitted beam. The turbidity is caused by the extent of content of microbes in a given sample. The estimation is done spectrophotometrically.

- Dry-weight method: All the cells harvested from a culture are weighed. This method is not very accurate.
- Cell packing by centrifugation: A sample of cell suspension is centrifuged in a special tube, and the packed cell volume is calculated from the height of the column of solid material.
- Analysis of cellular components such as N, C, H, P, protein, DNA and RNA.

QUALITY CONTROL OF BIOFERTILIZERS

In addition to the counting of viable cells (as detailed above, but described in brief here), the methods for testing the efficiency (as a measure of quality) of different biofertilizers are described below.

Rhizobium

The apparatus required consists of:

- some graduated pipettes – 1 and 10 ml;
- some dilution bottles or flasks;
- some Petri dishes – uniform, flat-bottomed;
- a hot-air oven;
- an autoclave;
- an incubator;
- a hand tally or mechanical counting device;
- a pH meter.

The reagents required are:

- Congo red – 1 percent aqueous solution;
- CREYMA medium.

The procedure is:

1. Put 1.0 g of the biofertilizer sample in a test-tube. Add 9 ml of water and make a suspension. Serially dilute this suspension (as described above). Use the diluted suspension to grow the bacteria by any of the plating techniques described above.
2. Label the plates and incubate at 28 ± 2 °C for 3–5 days for fast-growing Rhizobia and 5–10 days for slow-growing ones.
3. Count the colonies with the aid of a magnifying lens under uniform and properly controlled artificial illumination. Use a colony counter, equipped with a guide plate and rules in square centimetres. Record the total number of colonies with the hand tally.
4. Count all plates, but for the purpose of calculation consider plates showing more than 30 and fewer than 300 colonies per plate. Disregard colonies that absorb Congo red and stand out as reddish colonies. *Rhizobium* stands out as white, translucent, glistening and elevated colonies. Count such colony numbers, and calculate the figure in terms of per millilitre of suspension used for plating. Relate it to the original sample (1 g), taking into account the dilution factor. Also check for being free from contamination at 10^{-6} dilution.
5. Take uncontaminated *Rhizobium* cells and multiply them further for use in nodulation tests in pot culture trials.

TABLE 21
Plant nutrient solution

Composition	g/litre
Potassium chloride	0.0745
Potassium hydrogen phosphate (K_2HPO_4)	0.175
Calcium sulphate ($CaSO_4.2H_2O$)	0.344
Magnesium sulphate ($MgSO_4.7H_2O$)	0.246
Trace elements solution	
Copper sulphate ($CuSO_4.5H_2O$)	0.78
Zinc sulphate ($ZnS0_4.7H_2O$)	2.22
Manganese sulphate ($MnSO_4.4H_2O$)	2.03
Ammonium molybdate $[(NH_4)_6MO_7O_{24}.4H_2O]$	0.01
Boric acid (H_3BO_4)	1.43
Iron solution	
Ferrous sulphate	50.00
Citric acid	50.00

Pot culture test for nodulation and N fixation

A microbiological laboratory normally has a growth room with a growth chamber for carrying out studies/tests through pot culture, etc.

Prepare the nutrient solution by dissolving potassium chloride (0.0745 g), potassium hydrogen phosphate (0.175 g) and magnesium sulphate (0.246 g) in 1 litre of water. To this solution, add 0.5 ml of trace elements solution (respective amounts of micronutrient salts as given in Table 21 are dissolved in 1 litre of water) and 0.5 ml of iron solution (respective amounts are dissolved in 1 litre). In a mortar, grind 0.344 g of calcium sulphate to a fine consistency and add it to the final nutrient solution. Autoclave the nutrient solution at 120 °C for 20 minutes. Keep the pH of the solution at 6.0.

The procedure for pot culture is:

1. Immerse legume seeds in 95 percent alcohol, and wash with chlorine water and then with 0.1 percent mercuric chloride solution for 2–3 minutes. Wash the seeds with sterile water in order to remove the sterilant.
2. Fill glazed pots of 2 kg capacity with soil (2 parts soil and 1 part sand), and autoclave for 2 hours at 120 °C.
3. Inoculate the surface of the sterilized seeds with a water slurry of inoculant taken from a culture packet (biofertilizer sample). Depending on the size of the seeds, 1 ml of inoculant inoculates 15–100 g of seeds.
4. Keep a set of pots sown with non-inoculated seeds as control, another set with inoculated seeds and a third set with ammonium nitrate at 100 kg N/ha. Take four replications of each treatment.
5. Keep the pots in the growth room.
6. Add nutrient solutions to the pots at the start to attain WHC and subsequently with sterile water to keep the soil moist.
7. After 2–3 weeks of growth, thin down the number of plants in each pot to four uniform plants.
8. After 6–8 weeks, harvest the plants separately from each set, and separate the plants carefully from the soil under slow running water. Observe the number, colour and mass of nodules for each treatment.
9. If good effective pink nodulation is obtained in inoculated plants together with absence (or sometimes presence) of stray nodules in controls, and if there is at least a 50-percent increase in the dry matter yield of plants compared

with non-inoculated controls, it may be concluded that the culture is of the required quality. The growth and dry matter yield with ammonium nitrate treatment enables a comparison between the N used through fertilizers and the N fixed through inoculation, i.e. the extent of contribution in terms of N fixation by *Rhizobium*.

Azotobacter

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is Beijerinckia medium (composition given above).

The procedure is the same as that for *Rhizobium* above.

Azotobacter chroococcum colonies are gummy, raised and often sticky. The pigmentation varies from very light brown to black. Count the number of colonies, observe the cyst formation, and calculate the number per gram of the carrier material.

The uncontaminated cells can then be taken and multiplied further for use in the test for N fixation in pure culture.

Test for nitrogen fixation by pure culture

With pure culture medium, the procedure is:

1. Prepare Beijerinckia medium as per the composition given above.
2. Take *Azotobacter* from a suitable colony. Multiply and use this culture for inoculating the broth for N fixation.
3. For this purpose, put 50-ml aliquots of broth in a 250-ml conical flask, and inoculate with *Azotobacter*. Keep a non-inoculated flask as a control. After 12 days' growth at 28 °C, test the contents of the flask for purity by streaking on fresh medium and concentrating over a water-bath (50–60 °C) to dryness. Wash the dried culture, and take it as a sample. Process the contents of the flask in the non-inoculated controlled series in the same manner.
4. Determine the N in the sample using the Kjeldahl method (Chapter 3). The difference in the N content between the control and the inoculated flasks will indicate the N-fixing capacity of *Azotobacter*.

Azospirillum

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is N-free bromothymol blue medium (as described above).

The procedure is the same as that for *Rhizobium* (above).

For colony counting, count the tubes or plates that have turned blue in colour after inoculation and ascertain the presence of pellicles in undisturbed medium. Distinct subsurface pellicle formation in the malic acid medium is the confirmatory test of the presence of pure *Azospirillum* in the inoculant. However, the test for N fixation by pure culture as described for *Azotobacter* (above) is also valid for testing the purity of *Azospirillum*. Count the colony numbers, and calculate the number per gram of the carrier material.

Phosphate-solubilizing bacteria

The apparatus required is the same as that for *Rhizobium* (above).

The reagent required is Pikovskaya medium as per composition given under media preparation (above).

The procedure is the same as that for *Rhizobium* (above).

For colony counting, count the total number of colonies on the plates including colonies with a solubilization zone with the help of a colony counter. Measure the diameter of the solubilization zone. The minimum acceptable zone is 10 mm in diameter. The greater the solubilization zone is, the higher is the efficiency of the bacteria.

COMMERCIAL PRODUCTION OF BIOFERTILIZERS

In addition to quality control of biofertilizers/bioinoculants, service laboratories may be able to produce quality biofertilizers on a commercially viable scale.

Rhizobium

The types and composition of various media suitable for growing *Rhizobium* species have been described above. For commercial production, a liquid medium (without agar), such as Norris and Date (1976), is used.

Non-sterile production system

This system uses an unsterilized carrier.

The procedure is:

1. Prepare liquid media in conical flasks as described above.
2. Sterilize the media in an autoclave.
3. Add pure culture at 10 ml/litre of sterilized medium in each flask. This is called broth.
4. Incubate the flasks on a rotary shaker at 28–30 °C for 36–48 hours. There should be a population of 10^8–10^9 cells/ml at the end of the incubation. Broth can also be incubated in a fermenter of suitable size.
5. Neutralize the carrier (lignite) with a suitable chemical (lime), and add gum acacia at 4 g per 100 g of carrier.
6. Mix the broth culture with the carrier to attain a moisture content of about 30–40 percent, and incubate at 28 °C for 24 hours. Leave for curing for 2–3 days.
7. Dispense the cured broth-mixed carrier in polyethylene pouches. Pouches of 250 g, 500 g or 1 kg are prepared to meet the requirement of different quantities of seed to be treated for sowing in a given area.
8. Seal the pouches by heat sealer.
9. Put the pouches in printed polyethylene packets.
10. Store the packets at less than 20 °C until dispatch.

Sterile production system

This system uses a sterile carrier.

The procedure is:

1. Prepare liquid media in conical flasks.
2. Sterilize the medium in an autoclave.
3. Add pure culture at 10 ml/litre of sterilized media in each flask. This is called broth.
4. Incubate the flasks on a rotary shaker at 28–30 °C for 36–48 hours. There should be a population of 10^8–10^9 cells/ml at the end of incubation. Broth can also be incubated in a fermenter of suitable size.
5. Neutralize the carrier with a suitable chemical (lime), and add gum acacia at 4 g per 100 g of carrier.
6. Put the carrier in polypropylene bags, and autoclave for 4–6 hours.
7. Seal the pouches after autoclaving and keep them for 2 days before inoculation.
8. Wipe the pouches with alcohol.
9. Dispense the desired broth culture aseptically with an automatic dispenser by making holes in the polypropylene pouches or packets.
10. Seal the hole immediately with tape.
11. Blend the packets only manually to ensure that broth mixes with the carrier adequately (the sealed pouches/packets have to be manually manipulated to ensure mixing).
12. Incubate the pouches for further multiplication of microbes by keeping the pouches in the incubator at 28 °C for 24 hours. Leave for 2–3 days for curing.
13. Put the pouches in printed polyethylene packets.
14. Store the packets at less than 20 °C until dispatch.

Table 22 lists the quantities of chemicals required for the production of 25, 50 and 100 tonnes of *Rhizobium* biofertilizer.

TABLE 22
Chemicals required for the production of *Rhizobium* biofertilizer

Chemicals required	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
Mannitol	87.50	175.00	350.00
Yeast extract	8.50	17.50	34.80
K_2HPO_4	4.50	8.50	17.50
$MgSO_4.7H_2O$	7.00	14.00	28.00
NaCl	1.75	3.50	7.00
$FeCl_3.6H_2O$	0.10	0.20	0.50

Note: Calculated on the basis of Norris and Date (1976) medium.

Azotobacter

The production process is similar to that for *Rhizobium* production. However, *Azotobacter*-specific broth is prepared. This is the same medium as that for laboratory use (Jensen's or Ashby's media) except that agar is not added in the preparation of broth to be used for carrier-based commercial production. Table 23 lists the quantities of chemicals required for the production of 25, 50 and 100 tonnes of *Azotobacter* biofertilizer.

Azospirillum

The procedure is same as for *Rhizobium* and *Azotobacter* (above). The broth is prepared in N-free bromothymol blue medium (excluding the use of agar) and/or Okon's modified liquid medium (excluding bromothymol blue because bromothymol blue is only for identification/confirmation – once it is confirmed, there is no need to add it again).

Table 24 lists the quantities of chemicals/reagents required for the production of 25, 50 and 100 tonnes of *Azospirillum*.

Phosphate-solubilizing micro-organisms

Isolated and purified culture of PSM is further multiplied by using Pikovskaya medium for commercial production. The same procedure as followed for production of *Rhizobium*, *Azotobacter* and *Azospirillum* (above) can be adopted. As in the other cases, agar is not used in the medium. Liquid broth and charcoal/lignite as carrier are used by maintaining a moisture content of the product at about 30–40 percent.

Table 25 lists the chemicals/reagents required for the production of 25, 50 and 100 tonnes of PSMs.

TABLE 23
Chemicals required for the production of *Azotobacter* biofertilizer

Chemicals	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
Sucrose	150.00	300.00	600.00
K_2HPO_4	7.50	15.00	30.00
$MgSO_4.7H_2O$	3.75	7.50	15.00
NaCl	3.75	7.50	15.00
$FeSO_4$	0.75	1.50	3.00
$CaCO_3$	15.00	30.00	60.00

Note: Calculated on the basis of Jensen's medium.

TABLE 24
Chemicals required for the production of *Azospirillum* biofertilizer

Chemicals	Biofertilizer (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
K_2HPO_4	45.00	90.00	180.00
KH_2PO_4	30.00	60.00	120.00
$MgSO_4$	1.50	3.00	6.00
NaCl	0.75	1.50	3.00
$CaCl_2$	0.15	0.30	0.60
NH_4Cl	7.50	15.00	30.00
Malic acid	37.50	75.00	150.00
NaOH	22.50	45.00	90.00
Yeast extract	0.40	0.80	1.50
Na_2MoO_4	0.015	0.03	0.06
$MnSO_4$	0.01	0.02	0.04
H_3BO_4	0.01	0.02	0.04
$Cu(NO_3)_2$	0.005	0.01	0.02
$ZnSO_4$	0.015	0.03	0.06
$FeCl_3$	0.015	0.03	0.06
Bromothymol blue (0.5% alcoholic)	15 litres	30 litres	60 litres

Note: Calculated on the basis of Okon's medium.

TABLE 25
Chemicals required for the production of PSMs

Chemicals	Biofertilizers (30–40% moisture)		
	25 tonnes	*50 tonnes*	*100 tonnes*
		(kg)	
$Ca_3(PO_4)_2$	37.50	75.00	150.00
Sucrose	75.00	150.00	300.00
$(NH_4)_2SO_4$	3.75	7.50	15.00
NaCl	1.50	3.00	6.00
$MgSO_4.7H_2O$	0.75	1.50	3.00
KCl	1.50	3.00	6.00
Yeast extract	3.75	7.50	15.00
$MnSO_4$	0.01	0.02	0.04
$FeSO_4$	0.01	0.02	0.04

Note: Calculated on the basis of Pikovskaya medium.

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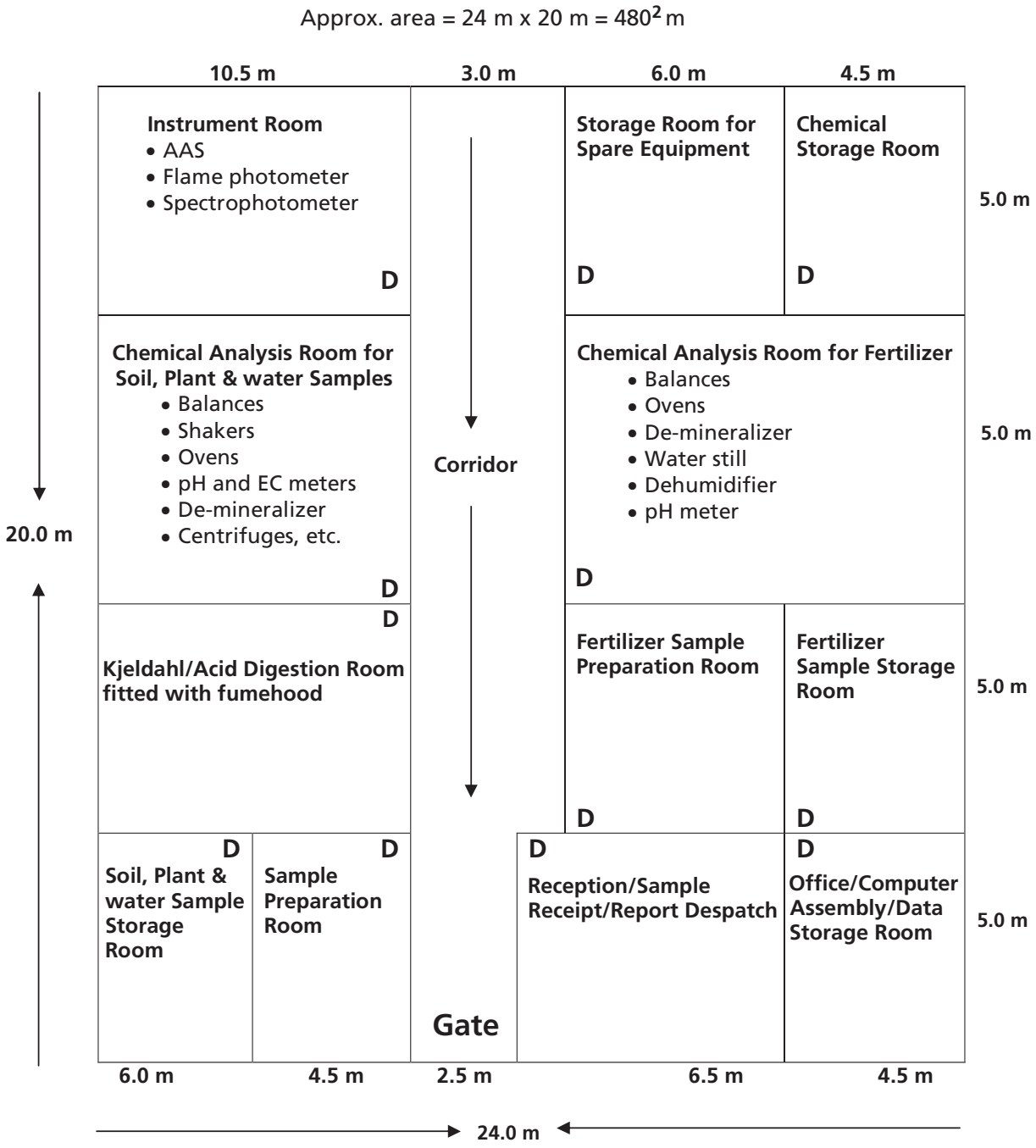
Annex 1

Floor plan of a soil, plant, water and fertilizer analysis laboratory

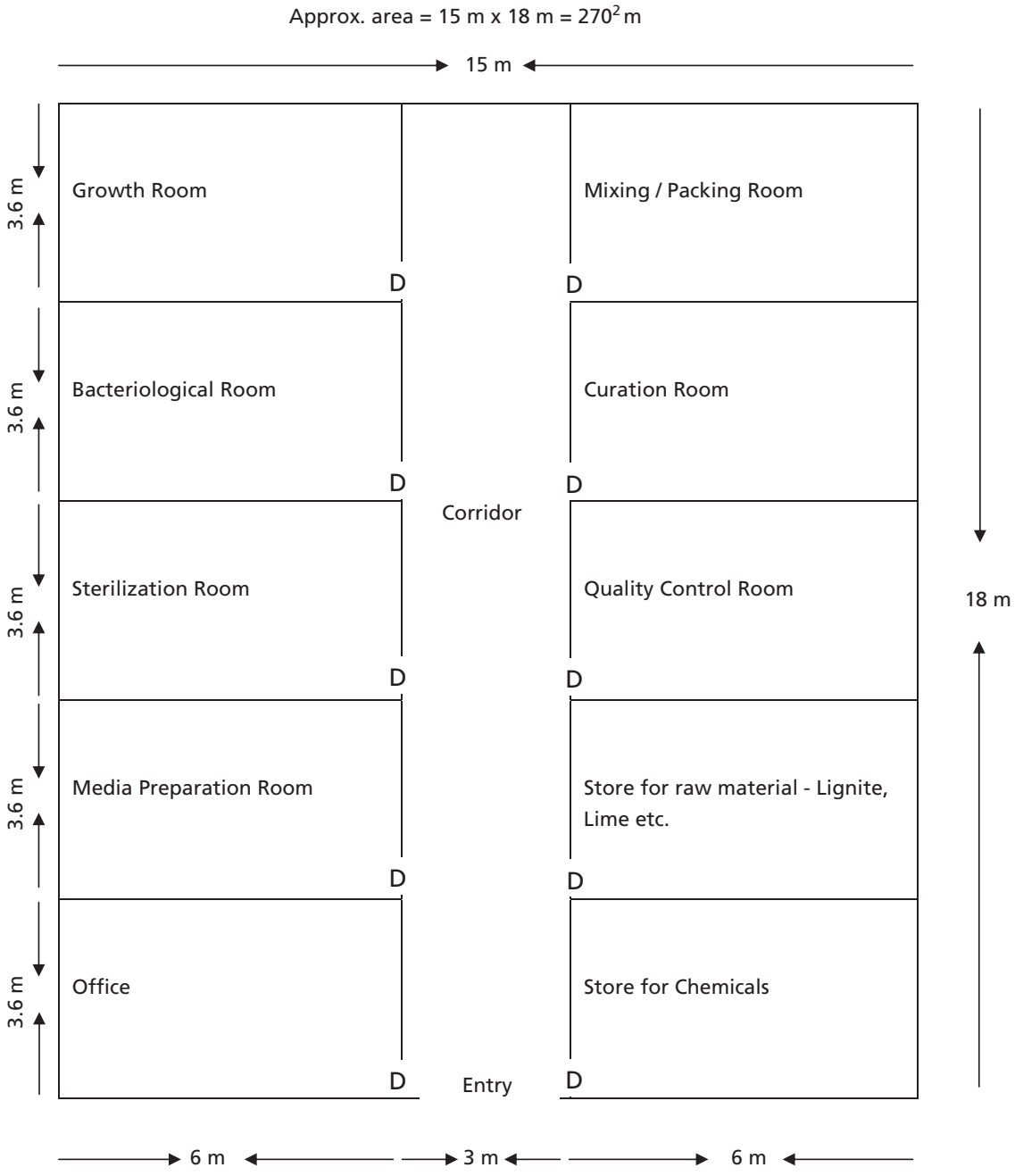
FIGURE A1.1
Laboratory floor plan for soil, plant, water and fertilizer analysis



Annex 2

Floor plan of a biofertilizer laboratory and production unit

FIGURE A2.1
Floor plan of a biofertilizer laboratory and production unit



Annex 3
Items required for a soil, plant and water analysis laboratory

Tables A3.1–A3.3 list the equipment, chemicals and glassware required for a laboratory with a capacity to analyse 10 000 soil samples, 1 000 plant samples and 500 water samples annually.

TABLE A3.1
Equipment

Serial No.	Name/specification/description	No.
1.	Analytical / semi-micro balance Capacity = 300 g Resolution = 0.1 mg Pan size = 100 mm	2 (1 of each type)
2.	Two-pan balance Capacity = 500 g Resolution = 0.2%	1
3.	pH meter: range 0–14 pH with accuracy of ±0.05 pH, complete with combination electrode, mains operated; to work on 220 V, 50 cycles	2
4.	Conductivity bridge: single range 0–15 mS/cm directly calibrated with temperature compensation and cell constant adjuster, complete with pipette-type conductivity cell having platinum electrodes duly coated with platinum black and having a cell constant of 1.00±0.01%; mains operated with electronic "eye" null indicator; to work on 220 V, 50 cycles	1
5.	Photoelectric colorimeter: with dual-barrier-type matched photocells with sensitive galvanometer for null adjustment and logarithmically calibrated potentiometer to read directly optical density on dial, complete with optical glass filters for maximum transmission at 420, 540 and 660 nm wavelengths; mains operated; to work on 220 V, 50 cycles	1
	Or spectrophotometer	
6(i).	Shaking machine: reciprocating type, variable speed of 70–300 strokes per minute, with box-type platform carrier (size: 79 cm long × 43 cm wide × 8 cm high) fitted with heavy-duty electric motor for continuous operation and built-in 0–60 minutes timeswitch; to work on 220 V, 50 cycles	1
6(ii).	Water-bath shaker with 50 cm × 38 cm water-bath size mounted on the shaking machine as 6(i)	1
7.	Centrifuge: clinical type with head to take 12 tubes of 15 ml capacity, complete with metal shields, rubber cushions and 15 ml centrifuge polyethylene tubes; to work on 220 V, 50 cycles	1
8.	Voltage stabilizers (constant voltage transformers): input 170–250 V, AC output 220 V Capacity: 1, 2 & 5 kW	1 each
9.	Voltage stabilizers (constant voltage transformers): Input 170–250 V, AC output 220 + 5 V	2
10.	Pressure vacuum pump: to deliver 0.04 m^3 of air per minute at a maximum pressure of 1.1 kg/cm^2 and create vacuum of 28 ° mercury column, complete with pressure gauge and ballast; to work on 220 V, 50 cycles (SI unit)	2

Serial No.	Name/specification/description	No.
11.	Automatic pipetting machine: Brewer-type, adjustable volume and frequency of cycling, to deliver up to 50 ml solution; syringe, plunger cylinder, valves and intake and delivery tubes made of neutral hard glass or resistant plastic material; to work on 220 V, 50 cycles	2
12.	Demineralizer plant: For obtaining deionized water, regenerating type, separate or mixed-bed resin columns treated water to have pH 6.8–7.0, portable model, capacity – 75 litres of deionized silica free water per hour	1
13.	Oven: laboratory model, made of stainless steel inside and outside, maximum temperature 180 °C, thermostatically controlled, ±1.0 °C accuracy, inside chamber – 30 cm × 30 cm × 30 cm	1
14.	Trolleys (push carts) made of tubular frame mounted on rubber casters, with mild steel top; size 60 cm × 75 cm and with a height of 90 cm	4
15.	Flask stand: To hold eleven 100 ml conical flasks, with adjustable base and collar – total length of stand 75 cm, width 8 cm, overall height 12 cm, distance between flasks 6.5 cm to fit multiple dispensing equipment (wood)	10
16.	Funnel stand: To hold eleven 5–7 cm diameter glass funnels, to correspond to flask stand	10
17(i).	Beaker stand: To hold eleven 50 ml beakers – to fit multiple dispensing equipment (wood)	10
17(ii).	Burette stand with clamps	4
18.	Test-tube stand: To hold 11 test-tubes of 25 mm × 150 mm, distance between test-tubes 6.5 cm, overall height 16 cm (wood)	10
19.	Multiple dispensing equipment: with 11 units each 50 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm, overall height of tray 12 cm	2
20.	Multiple dispensing equipment: with 11 units each 25 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm, overall height of tray 12 cm	2
21.	Multiple dispensing equipment: With 11 units each 20 ml, to fit 75 cm tray, distance between flasks from centre to centre 6.5 cm, width of tray 8 cm, distance from centre of outer flasks and end of tray 4.5 cm overall, height of tray 12 cm	2
22.	Washing assembly: for washing glassware used with multiple dispensing equipment, pipe and jet made of PVC, combined unit for both tap-water washing and distilled water rinsing, complete with brackets for mounting in sink	1
23.	Scoops: for soil sampling, made of brass with wooden handle. A set of 5 scoops to measure 1 , 2.5 , 5.0 , 10 and 12.5 g of soil	2
24.	Mortar and pestle: heavy cast-iron mortar or porcelain mortar, glazed outside only, size 160 mm diameter with wooden rubber-tipped pestle	2
25(i).	Sieves: 20 cm in diameter, 5 cm high with 2 mm round holes, preferably made of stainless steel, complete with cover lid and receiver pan	3
25(ii).	1 mm sieve with similar specifications	1
26.	Trays: for drying soil samples, made of aluminium sheet, 22 cm × 22 cm × 8 cm	100
27.	Hotplate: rectangular 45 cm × 60 cm with three positions: low, medium and high, heavy-duty rotary switch, 1 kW to work on 220 V, 50 cycles	2
28.	Kjeldahl assembly, both as digestion and distillation set, having a capacity to hold 6 round-bottomed flask of 300 ml capacity each complete with condenser and connecting tubes Heating capacity of 500 W of each heater	1
29.	Fumehood – digestion chamber to hold Kjeldahl assembly of 6 sets	1
30.	Atomic absorption spectrophotometer: double-beam with spare hollow cathode lamps for zinc, copper, manganese, iron, boron and molybdenum	1
31.	Muffle furnace with temperature 1 000 °C ± 5 °C, size of furnace 10 cm × 15 cm	1

TABLE A3.2
Chemicals

Chemical / item	Estimated annual requirement
Acetic acid (glacial)	10 litres
Activated charcoal – P-free	10 kg
Ammonium ferrous sulphate (CP)	10 kg
Ammonia solution (CP)	10 litres
Ammonium acetate (CP)	40 kg
Ammonium bicarbamate (AR)	1 kg
Ammonium metavanadate (AR)	1 kg
Ammonium molybdate (AR)	3 kg
Ammonium vanadate (AR)	500 g
Ascorbic acid	100 g
Barium chloride (CP and AR)	1 kg each
Boric acid (CP)	10 kg
Bromocresol green	500 g
Buffer solutions (pH 4.0, 7.0 & 9.2)	2 bottles each
Calcium acetate (CP)	500 g
Calcium carbonate (CP)	0.5 kg
Calcium chloride (CP)	0.5 kg
Calcium sulphate (CP)	500 g
Copper sulphate (AR)	500 g
Copper sulphate (CP)	1 kg
Diethylene triamine pentaacetic acid	2 kg
Digestion mixture containing potassium sulphate and copper sulphate	10 kg
Diphenyl amine indicator	100 g
EDTA – disodium salt	1 kg
Eriochrome black – T	50 g
Ethyl alcohol	5 litres
Ferrous sulphate (AR)	500 g
Ferrous sulphate (CP)	500 g
Filter paper, No. 1 (460 mm × 570 mm)	2 500 sheets
Filter paper, 110 mm, No. 40	5 packets
Filter paper, 110 mm, No. 42	5 packets
Gum acacia	0.5 kg
Hydrochloric acid (CP)	100 litres
Hydrogen peroxide	5 litres
Hydroxylamine hydrochloride	1 kg
Magnesium chloride (AR)	500 g
Manganese sulphate (AR)	500 g
Manganese chloride (CP)	1 kg

Chemical / item	Estimated annual requirement
Methyl orange	25 g
Methyl red	25 g
Methylene blue	50 g
Monocalcium phosphate (AR)	500 g
Mureoxide indicator	100 g
Nitric acid (CP)	10 litres
Nitric acid (AR)	10 litres
Nitrophenol (CP)	500 g
Paranitrophenol (CP)	1 kg
Perchloric acid (CP)	10 litres
pH indicator papers (full pH range)	10 books
Phenolphthalein indicator	100 g
Phosphoric acid (LR)	5 litres
Potassium dihydrogen phosphate (KH_2PO_4) (AR)	500 g
Potassium chloride (AR)	2 kg
Potassium chromate (CP)	500 g
Potassium dichromate (AR)	2 kg
Potassium dihydrogen orthophosphate	500 g
Potassium hydrogen phthalate (AR)	500 g
Potassium permanganate (CP)	2 kg
Potassium sulphate (AR)	1 kg
Salicylic acid (CP)	1 kg
Silver sulphate	1 kg
Sodium bicarbonate (LR)	30 kg
Sodium carbonate (AR)	500 g
Sodium carbonate (CP)	1 kg
Sodium cyanide	100 g
Sodium diethyl dithiocarbamate	500 g
Sodium fluoride (CP)	500 g
Sodium hydroxide (CP)	50 kg
Sodium thiosulphate (CP)	500 g
Stannous chloride (AR)	500 g
Sucrose (AR)	500 g
Sucrose (CP)	1 kg
Sulphuric acid (conc./CP)	50 litres
Toluene	500 ml
Tri-ethanol amine granule (CP)	500 g
Tri-ethanol amine	500 ml
Universal indicator	100 ml
Zinc sulphate (AR)	500 g

TABLE A3.3
Glassware

Serial No.	Item	Size/specification	Quantity (No.)
1.	Bottle (polyethylene)	20 litres	5
2.	Bottle (polyethylene)	10 litres	5
3.	Bottle (glass) for reagents with glass stoppers	Glass-stoppered	
		125 ml	10
		250 ml	10
		500 ml	20
		1 000 ml	5
		2 000 ml	5
4.	Bottle (glass), amber	250 ml	5
		500 ml	5
5.	Bottle (polyethylene) – wash bottle	250 ml	6
		500 ml	6
6. (i)	Burettes fitted with screw-thread stopcocks		
	Graduation interval (ml)		
	0.05	10 ml	2
	0.05	25 ml	2
	0.1	10 ml	2
	0.1	25 ml	2
6. (ii)	Burette (automatic)		
	(mounted on reservoir)		
	Graduation interval (ml)		
	0.1	25 ml	2
	0.1	50 ml	2
7.	Cylinder (glass) graduated with an interval of:		
	0.5 ml	10 ml	2
	1 ml	25 ml	2
	2 ml	50 ml	2
	2 ml	100 ml	2
	5 ml	500 ml	2
8.	Crucible (silica)	30 ml	10
9.	Desiccator with approx. I.D. of ground flange as 200 mm		2
10.	Dishes, evaporating flat-bottom with pour out, having outer diameter as 150 mm and height as 80 mm		20
11.	Water distilling unit, mounted with borosilicate condenser, with a capacity (output) to distil 2.5 litre/hour		2
12.	Flask distilling/Kjeldahl, round-bottom, long-neck		
	Capacity	O.D. × height (mm)	
	100 ml	64 x 210	12
	250 ml	85 x 226	12
13.	Flask (conical)		
	100 ml cap., 64 × 105 mm (O.D. × height) with approx. neck O.D. as 25 mm		50
	250 ml cap., 85 × 140 mm (O.D. × height) with app. neck O.D. as 34 mm		50

Serial No.	Item		Size/specification	Quantity (No.)
	500 ml cap., 104 × 180 mm (O.D. × height) with app. neck O.D. as 34 mm			10
	1 000 ml cap., 131 × 225 mm (O.D. × height) with app. neck O.D. as 34 mm			10
14.	Flask (volumetric)			
	Capacity (ml)	Tolerance (± ml)		
	25	0.04		50
	50	0.06		50
	100	0.10		100
	250	0.15		25
	500	0.25		25
	1000	0.40		10
15.	Funnel, plain, 60-degree angle			
	Diameter			
	50 mm			20
	65 mm			20
	75 mm			20
	100 mm			20
16.	Pipette (measuring)			
	Capacity (ml)	Graduation interval (ml)	Tolerance (± ml)	
	1.0	0.1	0.006	2
	2.0	0.1	0.01	2
	5.0	0.1	0.05	2
	10.0	0.1	0.05	5
	25.0	0.2	0.1	5
	50.0	0.5	0.1	2
17.	Porcelain dish – 100 and 150 ml			6 each
18.	Test-tube			
	App. O.D. × height (mm)			
	12 × 10			60
	15 × 125			60
	18 × 150			60
19.	Watch glass			
	App. diameter (mm)			
	100			60
	200			60
20.	Rubber stopper			
	15, 18, 20, 25 & 30 mm diameter			12 each
21.	Spatula (stainless) with wooden handle, blade length 100 mm			12
22.	Wire gauge with asbestos centre 150 × 150 mm			20

Notes:
Twenty percent of glassware may need annual replacement because of breakage or changed requirement.
O.D. = outer diameter.
I.D. = internal diameter.
When estimating boron, boron-free glassware must be used. Commonly required glassware includes beakers, flasks, pipettes, funnels and water distilling sets. Generally, marketed glassware is made of borosilicates, which contains traces of boron, hence, not suitable for boron estimation.

Annex 4
Items required for a fertilizer testing laboratory

Tables A4.1 and A4.2 list the equipment, chemicals and glassware required for a fertilizer testing laboratory with a capacity to analyse 2 000 samples (1 000 mineral fertilizer samples and 500 organic fertilizer samples) annually. The glassware requirements are similar in terms of number and type to those for the soil testing laboratory (Annex 3).

TABLE A4.1
Equipment

Name of equipment / specification (details in Annex 3)	No.
Analytical balance (semi-micro) with 0.1 mg resolution	1
Micro balance with 0.01 mg resolution	1
Two-pan balance, 500 g capacity	1
pH meter	1
Spectrophotometer	1
Shaking machine	1
Water-bath shaker	1
Centrifuge	1
Atomic absorption spectrophotometer	1
Voltage stabilizer (1, 2 & 5 kW)	1 each
Pressure vacuum pump	1
Demineralizer plant	1
Oven	1
Hotplate	2
Kjeldhal digestion and distillation unit to hold 6 flasks each	1 each
Fumehood	1
Muffle furnace	1
Automatic pipetting machine	1
Trolleys	4
Flask stand	4
Beaker stand	4
Test-tube stand	4
Pestle and mortar	2
Sieves: 0.25, 0.5, 1, 2 & 4 mm	2 sets each
Trays	6

TABLE A4.2
Chemicals

Chemical/grade	Estimated annual requirement
Acetic acid (glacial) (AR)	1 litre
Ammonium hydroxide (CP)	10 litres
Ammonium metavanadate (AR)	1 kg
Ammonium molybdate (AR)	1 kg
Ammonium oxalate (CP)	2 kg
Ammonium vanadate (CP)	500 g
Barium chloride (AR)	500 g
Benzalkonium chloride (AR)	1 kg
Boric acid (CP)	5 kg
Bromocresol green	100 g
Buffer solution (pH 4, 7 & 9.2)	500 ml each
Citric acid (CP)	2 kg
Clayton yellow	100 g
Copper sulphate (AR)	500 g
Copper sulphate (CP)	5 kg
Devarda's alloy	1 kg
Diphenylamine indicator	100 g
Ferrous sulphate (AR)	500 g
Filter paper	
No. 1 (460 mm × 570 mm)	1 000 sheets
No. 12 (11 cm diameter)	10 packets
No. 44 (11 cm diameter)	20 packets
No. 42 (11 cm diameter)	20 packets
Formaldehyde (CP)	5 litres
Hydrochloride acid (AR)	5 litres
Hydrochloride acid (CP)	20 litres
Magnesium nitrate (CP)	2 kg
Magnesium oxide (CP)	2 kg
Manganese sulphate (AR)	500 g
Methyl orange indicator	100 g
Methyl red indicator	100 g
Monocalcium phosphate (AR)	500 g
Mureoxide indicator	100 g
Nitric acid (AR)	5 litres
Nitric acid (CP)	20 litres
Perchloric acid (CP)	10 litres
Phenolphthalein indicator	100 g
Phosphoric acid (CP)	10 litres
Potassium chloride (AR)	1 kg
Potassium chloride (CP)	2 kg

Chemical/grade	Estimated annual requirement
Potassium chromate (CP)	500 g
Potassium dichromate (CP)	2 kg
Potassium dichromate (AR)	500 g
Potassium ferricyanide (AR)	500 g
Potassium ferrocyanide (AR)	500 g
Potassium hydrogen phosphate (AR)	500 g
Potassium hydrogen phthalate (AR)	1 kg
Potassium permanganate (CP)	1 kg
Potassium sulphate (CP)	5 kg
Quinolinium phosphomolybdate	1 kg
Salicylic acid (CP)	1 kg
Sodium chloride (CP)	1 kg
Sodium cyanide	100 g
Sodium hydroxide (CP)	50 kg
Sodium molybdate (AR)	2 kg
Sodium nitrate (AR)	1 kg
Sodium oxalate (CP)	1 kg
Sodium tetraphenyl boron (AR)	2 kg
Sodium thiosulphate (CP)	500 g
Sucrose (AR)	500 g
Sulphuric acid (AR)	5 litres
Sulphuric acid (CP)	20 litres
Universal indicator	100 ml
Zinc granules (AR)	500 g
Zinc sulphate (AR)	500 g

Annex 5
Items required for a microbiological laboratory

Tables A5.1–A5.3 list the equipment, chemicals and glassware required for a microbiological laboratory with a capacity to analyse 1 000 samples and produce 25–100 tonnes of biofertilizer per year.

TABLE A5.1
Equipment

Serial Number	Item	No.
1.	Binocular microscope for phase-contrast interchangeable inclined binocular body, rotatable through 360 °, high-quality eyepiece in pairs of 7×, 10×, 15× and parfocal, achromatic objectives of 4×, 10×, 40× and 100×.	1 set
2.	Trinocular microscope with CCD specification, nano CAM 2:	1 set
	- CCD chip: 0.8 cm	
	- video system: PAL	
	- resolution: 460 lines horizontal	
	- minimum luminance: 2.5 lux.	
	- autoshutter: 1/50 – 1/30 000	
	- video signal: composite	
3.	Autoclave, bench-top:	1
	- chamber capacity: 30 litres	
	- chamber dimensions (h × w × d): 265 × 225 × 450 mm	
	- bench space required (w × d): 483 × 610 mm	
	- mass: 110 kg	
	- power: 2 600 W	
	- temperature range: 100–140 °C	
	- pressure range: 0–3 kg/cm^2	
	- power supply: 220/240 V, 50 Hz	
	Accessories:	
	- basket, stainless steel mesh of 40 × 215 × 155 mm	
4.	Centrifuge:	1
	- maximum speed: 14 000 rpm	
	- maximum rpm: 20 800	
	- maximum load: 4 × 250 ml	
	- power supply: 230 V, 50–60 Hz	
	- maximum power required: 1 100 W	

Serial Number	Item	No.
	- dimensions (w × d × h): 700 × 608 × 345 mm	
	- weight: 99 kg	
	Accessories:	
	- swing-out rotor 4 × 250 ml including four 250 ml rectangular buckets	
	- aerosol tight cups for 250 ml buckets	
	- adapters for 250 ml buckets	
	- 4 buckets for swing-out rotor each holding 4 microtitre plates	
	- centrifuge tube of 5 ml	
	- centrifuge tube of 15 ml	
5.	Digital bacteriological and cell culture incubator:	1
	- capacity: 400 litres	
	- internal dimensions (h × w × d): 100 × 80 × 50 cm	
	- external dimensions (h × w × d): 130 × 114 × 75 cm	
	- shelf positions: 10	
	- power: 1 000 W	
	- weight: 160 kg	
	Accessories:	
	- shelves	
6.	CO_2 incubator for anaerobic cell and tissue culture:	1
	- capacity: 136 litres	
	- internal dimensions (h × w × d): 62 × 44 × 50 cm	
	- external dimensions (h × w × d): 100 × 60 × 60 cm	
	- shelf positions: 6	
	- power: 550 W	
	- weight: 70 kg	
7.	Drying and sterilizing oven:	1
	- capacity: 150 litres	
	- internal dimensions (h × w × d): 50 × 60 × 50 cm	
	- external dimensions (h × w × d): 70 × 95 × 68 cm	
	- shelf positions: 8	
	- power: 2000 W	
	- weight: 68 kg	
	Accessories:	
	- guides	
	- shelves	
8.	Laminar air flow:	1
	Cabinet: horizontal type with a workspace of 120 × 60 × 60 cm provided with pre-filters and HEPA filters with a capacity to retain particles of 0.2–0.3 micron (0.0002 – 0.0003 mm) from the air. Workhood covered with perspex sheets and provided with fluorescent illumination system and UV germicide tubes.	
9.	Analytical / semi-micro balance:	1
	- capacity: 310 g	
	- resolution: 0.1 mg	

Serial Number	Item	No.
	- pan size: 90 mm diameter	
10.	Multifunctional precision balance:	1
	- capacity: 410 g	
	- resolution: 0.001 g (1 mg)	
	- pan size: 120 mm	
11.	Thermostatic water-bath/circulator:	1
	- temperature range: -15 °C to +150 °C	
	- stability: 0.004 °C	
	- uniformity: ±0.001 °C	
	- tank capacity: 38 litres	
	- tank dimensions (l × w × d): 690 × 300 × 200 mm	
	- top opening (l × w): 580 × 300 mm	
	- liquid depth (min./max.): 120/180 mm	
	- overall (l × w): 720 × 325 mm	
	- height to tank rim: 225 mm	
	- overall height: 320 mm	
	- pump, maximum head: 1.7 m	
	- maximum flow of water: 8.5 litres/minute, with adjustable cutout	
	- head power: 1.4 kW	
	- total consumption: 1.5 kW	
	Accessories:	
	- lid for bath: 38 litres	
	- raised shelves	
	- lid hinge	
12.	Micropipettes:	5 each
	- adjustable: 0.5–10 µl (microlitres)	
	- adjustable: 10–50 µl	
	- adjustable: 50–200 µl	
	- adjustable: 200–1 000 µl	
	Pipette tips:	
	- tips: 5–200 µl	
	- tips: 201–1 000 µl	
	- adapter volume: 0.5–10 µl	
	- adapter volume: 30–300 µl	
13.	Stand for pipettes	3
14.	Hotplate, circular:	1
	General purpose with thermocouple control from ambient to 350 °C, epoxy-finished case is acid and impact resistant. The 600 W silumin hotplate diameter is 135 mm, 220 V, 50 Hz	
15.	Microscope slide box: polished, wooden box with slots to hold up to 100 slides	2
16.	Dissecting instruments kit:	2
	Dissecting kit in a wooden case. Contains: 1 fine-point needle, 1 needle (lancet-type), 1 set of blunt-tip forceps, 1 set of fine-point forceps, 1 spatula handle No. 4 with 3 sterile blades (Nos. 20, 22 and 23) and 1 pair of scissors	

Serial Number	Item	No.
17.	Jar, anaerobic, 2.5 litres	2
18.	Colony counter:	1
	For use with standard Petri dishes up to 100 mm in diameter; the counter has circular glare-free illumination and a choice of dark or light background, solid-state electronics with pressure-operated counting adjustable sensitivity and audible indication of count. Digital readout (to 999) and reset button mounted on the top panel, main switch and sensitivity adjustment at rear. For use on 220/240 V, 50 Hz	
19.	Mortar and pestle: porcelain, non-glazed with spout	3 sets
20.	Water distilling unit, mounted with borosilicate condenser with a capacity (output) to distil 2.5 litres/hour	2

TABLE A5.2
Chemicals – for analysing 1 000 samples, CP-grade

Chemical/item	Estimated annual requirement
Agar powder	2 kg
Ammonium chloride	1 kg
Ammonium oxalate $(COONH_2)_2.H_2O$	500 g
Aniline blue (water-soluble) for microscopical staining	25 g
Boric acid	500 g
Bromothymol blue	100 ml
Calcium carbonate	5 kg
Calcium chloride	1 kg
Calcium sulphate precipitated ($CaSO_4.2H_2O$)	500 g
Congo red	100 g
Congo red for microscopical staining	20 × 25 g
Copper sulphate, anhydrous ($CuSO_4$)	500 g
Dipotassium hydrogen orthophosphate heptahydrate, anhydrous (K_2HPO_4)	500 g
Ethanol 96% V/V (C_2H_5OH)	4 × 500 ml
Ethanol 99.7–100% V/V (absolute) (C_2H_5OH)	500 ml
Ethylenediamine tetraacetic acid (ferric monosodium salt) $[(CH_2N(CH_2.COOH)_2)_2FeNa]$	250 g
Ferric chloride	500 g
Fuchsin (acid) for microscopical staining	25 g
Fuchsin (basic) for microscopical staining	5 × 100 g
Glucose	2 × 500 g
Iodine resublimed	500 g
Lactophenol	25 g
Lactophenol blue	25 g
Magnesium sulphate - dried ($MgSO_4.H_2O$)	500 g
Magnesium sulphate heptahydrate - molecular biology grade ($MgSO_4.7H_2O$)	500 g
Malic acid	2 × 500 g
Manganese chloride ($MnCl_2.4H_2O$)	500 g
Mannitol	2 × 500 g
Molybdenum trioxide - AR (MoO_3)	100 g
Orthoboric acid powder (H_3BO_3)	500 g
Peptone	2 × 500 g
Phenol (redistilled molecular biology grade) (C_6H_5OH)	500 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	500 g
Potassium hydroxide	500 g
Potassium iodide (KI)	500 g
Potassium sulphate (K_2SO_4)	500 g
Saframin O for microscopical staining	10 × 25 g
Sodium chloride (NaCl)	20 × 25 g
Sodium molybdate	500 g
Sucrose	2 × 500 g
Yeast extract	500 g
Zinc sulphate ($ZnSO_4.7H_2O$)	500 g

TABLE A5.3
Glassware

Item	No.
Pipette, disposable, pre-plugged with non-absorbent cotton wool including 8 PVC teats	1 Pack of 1 000
Beaker 2 000 ml	2
Beaker 1 000 ml	2
Beaker 500 ml	5
Beaker 250 ml	10
Beaker 100 ml	50
Beaker 50 ml	10
Beaker 25 ml	10
Beaker 10 ml	10
Beaker 5 ml	10
Cylinder: 10, 25, 50, 100, 250, 500 & 1 000 ml capacity	2 each
Microscope slide: - type: plain - grade: super premium - thickness number: 1	2 packs of 100 each
Cover-glass, square packed in plastic hinged-lid dispenser box: - size: 18 × 18 mm - thickness number: 1	2 packs of 200 each
Cover-glass, square packed in plastic hinged-lid dispenser box: - size: 24 × 24 mm - thickness number: 1	2 packs of 200 each
Pipette, graduated type 3 Class B: Calibrated to deliver from zero at the top to any graduation line down to the jet, capacity 5 ml	1 pack of 5
Test-tube, heavy-wall, heat-resistant glass without rim: Size: 150x16 mm	1 pack of 100
Petri dish, heat-resistant glass, complete with lid: - diameter of bottom dish: 94 mm - diameter of lid: 100 mm	1 pack of 200
Petri dish, shatterproof, transparent, chemically resistant, useful for biotechnology as resists cell adherence, autoclavable; diameter 100 mm, height 15 mm	20 packs of 10 each

Annex 6
Summary of plant nutrient estimation methods

TABLE A6.1
Plant nutrient estimation methods

Plant nutrients/forms	Soil	Mineral fertilizers	Plants
Total N	Kjeldahl method	Kjeldahl method	Kjeldahl method
Mineralizable N	Alkaline $KMnO_4$ method	-	-
NO_3-N	Phenoldisulphonic acid method	Estimation of NH_4+NO_3-N (below) - estimation of NH_4-N (below)	-
NH_4-N	Indophenol blue method	Distillation with MgO/alkali	-
NH_4+NO_3-N	-	Devarda's treatment followed by distillation with MgO/alkali	-
P	Available P:	Total P:	Total P:
	Bray's method No. 1 for acid soils	Gravimetric quinolinium phosphomolybdate method	Spectrophotometric vanadium phosphomolybdate method
	Olsen's method for alkali soils	Volumetric ammonium phosphomolybdate method	
K	Available K:	Total K:	Total K:
	Flame photometric method (neutral ammonium acetate extraction)	Sodium tetraphenyl boron (STPB) method	Estimation by AAS
S	Available S:	-	Total S:
	Spectrophotometric barium sulphate precipitation method		Spectrophotometric barium sulphate precipitation method
Ca/Mg	EDTA titration method	-	Estimation by AAS
			EDTA titration method
Zn, Cu, Fe & Mn	DTPA extraction and estimation by AAS	Estimation by AAS	Estimation by AAS
B	Hot water extraction and estimation by AAS	Estimation by AAS	Estimation by AAS
	Hot water extraction and determination by azomethine-H colorimetric method		Colorimetric method as in soils
Mo	Ammonium acetate extraction and estimation by AAS	Estimation by AAS	Estimation by AAS
			Colorimetric method as in soils
	Ammonium oxalate extraction and estimation by colorimetric method		

Annex 7

Automation of analytical procedures

In view of the accepted importance of analysis, the workload on soil, plant and water testing laboratories has increased considerably in recent years. Therefore, analytical methods have needed to be accelerated through the automation of instruments. Almost all instruments either contain inbuilt computers or can interface directly with microprocessors, thereby simplifying instrument operation and providing versatility for the analyst. Most operations can be performed through keyboard commands. This type of instrument automation enables:

- higher working speeds;
- lower labour requirements;
- added consistency and accuracy;
- flexible and extensive data processing;
- various modes of display;
- potential for unattended measurements.

Instrument-based methods that can be automated relatively easily are:

- atomic absorption spectrometry;
- inductively coupled plasma (ICP) emission spectrometry;
- mass spectrometry (MS);
- near infrared reflectance spectrometry (NIR);
- ion chromatography (IC);
- electrochemical methods (to a lesser extent).

In combination with the above techniques, continuous flow analysis (CFA) and flow injection analysis (FIA) are often used. An ICP emission spectrophotometer cannot be operated without the use of microprocessors. The microprocessor (with the help of software) enables the automatic sampling, setting of instrument operating parameters, calibration, result evaluation, data storage and retrieval, data transfer, etc. required for the operation of the instrument for the analysis of a particular element.

The entire laboratory operation from sample preparation to calculating and reporting of concentration of analyte can be automated with the help of robotic systems, which are available and undergoing continuous development. The type of robot that may work best in a soil testing laboratory is one that travels on a track, the length of which can be essentially unlimited (Munter, 1990).

Through the use of a laboratory information management system (LIMS), the automatic control of the functioning of the system, computations and data

management can be carried out efficiently. Skotnikov (1998) developed an automated workstation for soil analysis.

The methods and uses of some modern multi-element analysing are discussed below (adapted from Singh, Chhonkar and Pandey, 1999). Each item of equipment has an operational manual supplied by the manufacturers.

AUTOANALYSERS

Autoanalysers are versatile modularized instruments used for automatic chemical analysis of soil, plant, water and fertilizer samples. These systems are mainly of two types, i.e. CFA and FIA. They are designed to offer automatic simulation of operations used in manual procedures for the estimation of an element on a conveyer-belt principle. Usually, autoanalysers use the same reagents as those used in manual assays.

Principle

In autoanalysers based on the CFA mechanism, the samples are loaded into cups or test-tubes on the sampler. The samples and a number of streams of reagents are made to flow from one module to the next through plastic or glass tubings by the action of multiple-channel peristaltic pumps operating continuously. Each module automatically performs a different function in the analysis. Air bubbles are added to the flowing analytical stream to segment the streams of samples and reagents (Ferreira, Rangel and Lima, 1998). The samples and reagents are brought together under controlled conditions in the mixing coils that are part of the manifold, causing a chemical reaction that produces colour.

The FIA system is similar to the CFA system in its modules. However, there is no air segmentation and the sample is injected rapidly into the carrier stream via an injection valve or syringe as a "plug".

Operating procedure

The operating procedure is:

1. Prepare working standards of required concentrations, reagents and carrier solutions.
2. Switch on the sampler, analyser and spectrophotometer and allow at least 30 minutes to warm up.
3. Set the wavelength, gain factor, pump times, injection valve cycle, plotting parameter, etc. through keyboard commands of the modules as per the requirement of the analysis. Where the instrument interfaces with a microprocessor, the above parameters may be set automatically by the default choice.
4. Place the blank, working standards and samples in cups or test-tubes on circular tray of sampler in proper sequence.
5. Place the carrier and reagent solution bottle in the holder.
6. Loosen the tube holders of the pumps and insert the pump tubes. Connect the aspiration ends of all the pump tubes except one to a bottle containing

degassed water. Then press the ends of all the pump tubes except one, which is connected to the manifold (chemifold). Connect the aspiration end of the remaining pump tube to the injection valve outlet and its pressed end to the waste tube.

7. Attach the pump tube holders, and release the tension by turning the tension screws counter-clockwise.
8. Lubricate the pump tubes and the rollers with a small amount of silicon oil.
9. Assemble the manifold (chemifold) as per the requirement of the procedure and flow diagram. The reaction coils and tubes used for various connections should be checked carefully according to the prescribed colour code.
10. Connect the inlet stream of the flow cell to the manifold, and the outstream to the waste bottle.
11. Check the flow pattern of the carrier stream by running the pumps. Apply sufficient pressure on the pump tube holders by turning the tension screws clockwise until liquid starts flowing through the pump tubes. In the event of any leakage, stop the pump and correct it.
12. After checking the flow pattern, remove the aspiration end of the pump tube from degassed water bottle and connect it to the carrier and reagent solution bottles according to the requirement of the method (see application note). Now the instrument is ready for the actual run.
13. Start the pumps and set the baseline zero from the keyboard on the spectrophotometer by injecting the blank. Then, run the instrument for calibration and sample analysis.
14. Generate a calibration curve by recording absorbance from the display of the recorder of a series of standard solutions of increasing concentrations. From the absorbance of the test solution, determine the concentration using the calibration curve. With microprocessor-interfaced instruments, the sample peaks are compared automatically with the calibration curve after being corrected for baseline and sensitivity drift, and the results are displayed on the monitor screen.
15. If the instrument shows signs of "over" or "error", then dilute the samples considering the observed absorbance of the sample, and feed the sample again.

Switching off

The procedure for closing down the instrument is:

1. Before closing down, operate the instrument using degassed distilled water from all the channels until the detector reading returns to zero.
2. Loosen the pump tube holders immediately after the analysis.
3. Switch off the instrument in the following order: spectrophotometer, analyser, sampler and computer.

Precautions

It is important to observe the following precautions at all times:

- Never use any component of the instrument if it is in need of adjustment and/or repair.
- Always keep the equipment clean. It is important to clean the sampler and manifold immediately after the analysis is over.
- Clean the rollers and the pump tube holders every month. Lubricate the rollers and the pump tube holders with silicon oil regularly.
- Replace flattened and dirty pump tubes. While using new pump tubes, care should be taken for the change in the flow rate.
- Use all the tubings of prescribed diameter or colour code for a particular method as given in the operating manual.
- Check all the tubings before use for clogging of the passage.
- Develop different manifold (chemifold) units for different analytical methods in order to avoid sample contamination.
- Use solvent-resistant manifold for organic solvents. Never use organic solvent on perspex manifold, not even for cleaning stains.
- Check the manifold bores regularly for plugging. Use a nylon wire or steel to remove solid particles.
- Keep the external walls of the flow cell absolutely clean. Fingerprints, grease, etc. can be removed using tissue paper soaked in acetone or absolute ethanol.
- Care should be taken in handling cadmium during column preparation as it is toxic.

ATOMIC ABSORPTION SPECTROPHOTOMETER

Principle

In an analysis employing an atomic absorption spectrophotometer (AAS), the sample in the form of a homogeneous liquid is aspirated into a flame where "free" atoms of the element to be analysed are created. A light source (hollow cathode lamp) is used to excite the free atoms formed in the flame by the absorption of the electromagnetic radiation. The decrease in energy (absorption) is then measured. It follows the Lambert–Beer law, i.e. the absorbance is proportional to the number of free atoms in the ground state (Baker and Suhr, 1982).

Preparation of standards and sample solutions

The procedure is:

1. Prepare stock standards in concentrations of 1 000 mg/litre from pure metal wire, granules, foil, metal oxides or other suitable primary standard compounds of the elements.
2. Prepare a blank sample using the same reagents as used for standard samples but without the elements intended to be estimated.

3. Free the sample solution from interfering elements and suspended solids, which may cause clogging of the nebulizer.
4. Adjust both standards and unknowns to a concentration range that is compatible with the analytical range of the instrument. It should preferably be at least 5–10 times the detection limit of the instrument.

Instrument operation

The operating procedure is:

1. Check the instrument for the proper fitting of all the tubings, required type of burner (air acetylene or nitrous oxide acetylene) and hollow cathode lamps.
2. Fill the liquid trap with the solvent to be used for the analysis.
3. Align the hollow cathode lamp of the element to be analysed with the optical path of the instrument by rotating the lamp turret.
4. Switch on the instrument and allow at least 30 minutes for warming up.
5. Switch on the deuterium lamp for background correction, which is generally required when the wavelength of the resonance line of the element is less than 250 nm.
6. Use the lamp current recommended by the lamp manufacturer.
7. Select the desirable wavelength and the bandpass width or slit width.
8. Optimize the burner position by using the vertical, horizontal and rotational adjustment knobs until the burner slot is aligned with the beam and is just below the position from where it starts to block the light path.
9. Switch on the compressor in order to generate air supply in the case of an air acetylene flame. If an N_2O acetylene flame is used, turn on the N_2O supply cylinder. Select air with the support selector knob. Adjust the support flow (air) reading to between 6 and 9 flow units.
10. If a nitrous oxide acetylene flame is used, then first ignite an air acetylene flame and then change over to a nitrous oxide acetylene flame.
11. Turn on the gas supply from cylinder, use the fuel-control knob of the instrument and light the flame.
12. Adjust the fuel control (acetylene) and support control (air or nitrous oxide) knobs to produce the required kind of flame of air acetylene or nitrous oxide acetylene flame.
13. Set the instrument to zero by means of the "zero" control against a reagent "blank" solution.
14. Aspirate a standard (or sample) and optimize fuel, oxidant and sample flow rates by adjusting the fuel knob, fuel support knob and nebulizer so that a maximum signal (absorbance) is achieved.
15. Prepare a calibration curve by recording the absorbance of a series of working standards. The calibration must be done for each set of analysis.
16. If the instrument shows signs of "over" or "error", then dilute the samples depending on the absorbance of the sample, and feed the sample again.

17. If the instrument has been used in a higher concentration range, then operate the instrument using distilled water until the reading returns to zero, before closing down.

Switching off

The procedure for closing down the instrument is:

1. Turn off the gas from the cylinder.
2. Wait for extinction of the flame, and then turn off the fuel-control knob.
3. Turn off the air compressor and fuel-support knob.
4. The shutdown sequence for a nitrous oxide acetylene flame involves first changing over to an air acetylene flame and then extinguishing it.
5. Switch off the instrument.

Precautions

It is important to observe the following precautions at all times:

- Acetylene cylinders should always be used in a vertical position in order to prevent liquid acetone entering the gas line.
- Acetylene cylinders should not be run at a pressure lower than 500 kPa. Never operate acetylene lines at more than 100 kPa. At higher pressures, acetylene can decompose or explode spontaneously.
- Never run the nitrous oxide acetylene flame without the "red feather" visible, or with less than five flow units of acetylene.
- Do not leave uncovered containers of the volatile organic solvents near the uncovered flame.
- Do not look at flame without the aid of safety glasses or the flame shield.
- Do not leave the flame completely unattended.
- Do not ignite the flame if the air flow is below six flow units.
- Do not adjust the air (or N_2O) and gas supply to alter the sensitivity of the instrument after calibration of the instrument.

INDUCTIVELY COUPLED PLASMA – ATOMIC EMISSION SPECTROSCOPY

A new analytical technique called inductively coupled plasma – atomic emission spectroscopy (ICP–AES) is used for simultaneous multielement analysis of biological materials and soils. This technique offers advantages over an AAS and other multielement methods because matrix problems are eliminated or minimized through the use of the high-temperature argon plasma. Apart from multielement capability at all concentration levels, plasmas are noted for their relative freedom from the chemical and ionization interferences that are common with an AAS. The detection limits are equal to or better than an AAS (depending on the element analysed). Elements such as aluminium, phosphorus, sulphur and boron, which are either poorly measured at low concentrations or not possible with an AAS, are readily determined with higher sensitivity by ICP–AES.

Principle

The ICP–AES method is based on the observation of atomic emission spectra when samples in the form of an aerosol, thermally generated vapour or powder are injected into an ICP atomization and excitation source. By definition, plasma refers to a hot gas in which a significant fraction of the atoms or molecules is ionized. Plasmas are electrically conducted and have been referred to as electrical flames, as no combustion takes place. This is because instruments using a plasma source generally use inert argon gas.

The ICP is produced by passing initially ionized argon gas through a quartz torch located within an induction coil (copper coil) that is connected to a radio frequency (RF) generator. The RF generator produces 1.5–3 kW of power at a frequency of 27.1 MHz. An oscillating magnetic field is formed within the quartz torch in response to the RF energy passing through the coil. Electrons and ions passing through the oscillating electromagnetic field flow at high acceleration rates within the quartz torch space. As argon gas enters the magnetic field associated with the induction coil, its atoms collide with the accelerated ions and electrons, resulting in the ionization of the argon gas. These collisions give rise to ohmic heating, which produces plasma with temperatures ranging from 4 000 to 6 000 °C. The resultant plasma is contained within the torch by means of argon flow.

The method of presenting the sample to the plasma is similar to that used in flame atomic absorption. The liquid sample is aspirated into the plasma through a nebulizer system, using argon carrier gas at a rate of about 1 litre of argon per minute. The prevailing high temperature in the plasma leads to complete vaporization, atomization and excitation of the element to be analysed. The exited neutral atoms or ions of the sample emit radiation of characteristic wavelengths. The intensity of the emitted radiation is measured by the spectrophotometer component of the ICP–AES instrument.

Preparation of soil and plant samples

The digestion of soil and plant samples for total elemental analysis by ICP–AES is similar to that used for other emission instruments. Universal/multielement soil extractants are used for the extraction of soil samples. Recently, acidified AB-DTPA and Mehlich No. 1 extracts have been analysed by ICP–AES (Soltanpour *et al.*, 1998).

Preparation of standard solution

The procedure for the preparation of stock standard solution containing 1 000 mg/litre of an element from pure metal wire or suitable compounds of the element is similar to that described for AAS. However, multielement working standard solutions (secondary standards) should be made in such a way that these contain the maximum number of the elements compatible with stability considerations and match with the sample solutions in kind and strength of acids. In soil and plant analysis, one set of secondary standards is required for each multielement extracting solution, and one for each soil and plant digest. McQuaker, Kluckner

and Chang (1979) devised a calibration scheme for 30 elements that satisfies the needs of analysts engaged in soil, water, tissue, and particulate matter analysis.

Instrument operation

The general principles that allow the determination of the optimal analytical conditions for the operation of ICP–AES are similar to those for AAS. Microprocessors are used for the automatic control of instrument components, setup and optimization of required operational parameters, instrument calibration, and manipulation and storage of data through key commands. As the actual operation of different ICP spectrometers varies with the type, make and computer software of the instrument, the operation manual provided by the manufacturers should be consulted. Usually, when an ICP spectrophotometer is purchased, the manufacturer/dealer provides training to the analysts on operating procedures.

Precautions

It is important to observe the following precautions at all times:

- Filter soil extracts and soil and plant tissue digests with No. 42 filter paper to prevent clogging of the nebulizer.
- To prevent clogging of the nebulizer tip, use either a high salt nebulizer (Babington-type) or standards and samples with a very low salt content.
- Avoid mixing chemicals that cause precipitation during the preparation of multielement working standard solutions.

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Annex 8
Examples of laboratory registers

TABLE A8.1
Equipment register

Item	Description	Qty	Reference	Model	Price/unit	Total price
					(US$)	*(US$)*
1	Spectrophotometer	1	BDH/331/1042/04	1112	3 620	3 620
2	Flame photometer					
3						
4						
5						

TABLE A8.2
Glassware register

Item	Description	Qty	Reference	Model	Price/unit	Total price
					(US$)	*(US$)*
1	Beaker 5 ml	30	BDH/209/0310/01	Abc	5	150
2	Flask					
3						
4						
5						

TABLE A8.3
Chemicals register

Item	Description	Qty	Reference	Model	Price/unit	Total price
					(US$)	*(US$)*
1	Hydrochloric acid	2 litres	BDH-10125-5Y	AR	16	32
2	Sulphuric acid					
3						
4						
5						

TABLE A8.4
Register for miscellaneous items

Item	Description	Qty	Reference	Model	Price/unit	Total price
					(US$)	*(US$)*
1	Funnel stand	2	ABC	Wooden	5	10
2						
3						
4						
5						

Annex 9
Grades of chemicals and glassware

TABLE A9.1
Grades of chemicals

Grade	Purity	Notes
Technical or commercial	Indeterminate quality	For use in preparation of cleaning solution only.
Chemically pure (CP)/ Laboratory reagent (LR)	More refined, but still unknown quality	For general chemical use where very high purity is not required. It is also used in training and for laboratory practice.
Specially pure (SP)	Meets minimum purity standards	Conforms to tolerance set by the United States Pharmacopeia for contaminants dangerous to health.
Analytical reagent (AR)	High purity	Conforms to minimum specifications set by the Committee on Analytical Reagents of the American Chemical Society.
Primary standard	Highest purity	Required for accurate volumetric analysis (for standard solutions). Composition of primary standards does not undergo change. Sodium carbonate and borax are primary standards of bases. Potassium hydrogen phthalate is used as primary standard acid.

TABLE A9.2
Tolerances for volumetric glassware, Class A

Capacity	Tolerance		
(less than and Including)	Volumetric flask	Transfer pipette	Burette
(ml)		*(ml)*	
1 000	±0.30	-	-
500	±0.15	-	-
100	±0.08	±0.08	±0.10
50	±0.05	±0.05	±0.05
25	±0.03	±0.03	±0.03
10	±0.02	±0.02	±0.02
5	±0.02	±0.01	±0.01
2	-	±0.006	-

Annex 10
Equivalent and molecular weights of compounds

TABLE A10.1
Equivalent and molecular weights of some important compounds needed in chemical analysis

Compound	Formula	Molecular weight	Equivalent weight
		(g)	*(g)*
Ammonium acetate	CH_3COONH_4	77.08	77.08
Ammonium chloride	NH_4Cl	53.49	53.49
Ammonium fluoride	NH_4F	37.04	37.04
Ammonium nitrate	NH_4NO_3	80.04	80.04
Barium chloride	$BaCl_2.2H_2O$	244.28	122.14
Boric acid	H_3BO_3	61.83	20.61
Calcium acetate	$(CH_3COO)_2Ca$	158.00	79.00
Calcium carbonate	$CaCO_3$	100.09	50.05
Calcium chloride (dihydrate)	$CaCl_2.2H_2O$	147.02	73.51
Calcium hydroxide	$Ca(OH)_2$	74.00	37.00
Calcium nitrate	$Ca(NO_3)_2$	164.00	82.00
Calcium sulphate	$CaSO_4.2H_2O$	172.17	86.08
Ferrous ammonium sulphate	$FeSO_4.(NH_4)_2SO_4.H_2O$	392.13	392.13
Ferrous sulphate	$FeSO_4.7H_2O$	278.01	139.00
Magnesium chloride	$MgCl_2.6H_2O$	203.30	101.65
Magnesium nitrate	$Mg(NO_3)_2.6H_2O$	256.41	128.20
Potassium chloride	KCl	74.55	74.55
Potassium dichromate	$K_2Cr_2O_7$	294.19	49.04
Potassium hydroxide	KOH	56.10	56.10
Potassium permanganate	$KMnO_4$	158.03	31.60
Potassium nitrate	KNO_3	101.10	101.10
Potassium sulphate	K_2SO_4	174.27	87.13
Potassium hydrogen phthalate	$COOH\ C_6H_4\ COOK$	204.22	204.22
Oxalic acid	$C_2H_2O_4.2H_2O$	126.00	63.00
Silver nitrate	$AgNO_3$	169.87	169.87
Sodium acetate (anhydrous)	CH_3COONa	82.04	82.04
Sodium bicarbonate	$NaHCO_3$	84.01	84.01
Sodium carbonate	Na_2CO_3	106.00	53.00
Sodium chloride	NaCl	58.45	58.45
Sodium hydroxide	NaOH	40.00	40.00
Sodium nitrate	$NaNO_3$	84.99	84.99
Sodium oxalate	$Na_2C_2O_4$	134.00	67.00
Sodium sulphate	Na_2SO_4	142.04	71.02
Sodium thiosulphate	$Na_2S_2O_3.5H_2O$	248.18	248.18

Annex 11
Soil sample information sheet

FIGURE A11.1
Soil sample information sheet

Sample No. _____
Name of sample collector _____
Address _____ Date _____
Area _____ Location _____
Name of farmer _____ Farm size _____
Vegetative cover _____
Source of water _____ Water quality _____
Sample depth _____ Previous crop _____

Purpose of analysis:	Land capability assessment Fertility evaluation and fert. recommendation Salinity appraisal and causes of the source of salinity, if known Soil classification	Slope:	1–2 percent 2-5 percent 5-10 percent 10-25 percent > 25 percent
Irrigation method:	Flood Furrow Sprinkler Drip Rainfed	Years of irrigation:	Never irrigated 1–5 5–15
Years of cultivation:	Never cultivated 1–5 5–15 >15	Drainage:	Good Moderate Poor

Manure used in the previous crop and dose _____

Fertilizers used in the previous crop and dose _____

Annex 12
Colour change of solutions owing to pH change

TABLE A12.1
Colour changes owing to pH changes in the presence of pH indicators

pH indicator		pH transition intervals		
Name	Colour	pH	pH	Colour
Cersol red	Pink	0.2	1.8	Yellow
m-Cresol purple	Red	1.2	2.8	Yellow
Thymol blue	Red	1.2	2.8	Yellow
2,4-Dinitrophenol	Colourless	2.8	4.7	Yellow
Bromochlorophenol blue	Yellow	3.0	4.6	Purple
Bromophenol blue	Yellow	3.0	4.6	Purple
Methyl orange	Red	3.1	4.4	Yellow-orange
Bromocresol green	Yellow	3.8	5.4	Blue
2,5-Dinitrophenol	Colourless	4.0	5.8	Yellow
Methyl red	Red	4.4	6.2	Yellow-orange
Chlorophenol red	Yellow	4.8	6.4	Purple
Litmus extra pure	Red	5.0	8.0	Blue
Bromophenol red	Orange-yellow	5.2	6.8	Purple
Bromocresol purple	Yellow	5.2	6.8	Purple
4-Nitrophenol	Colourless	5.4	7.5	Yellow
Bromoxylenol blue	Yellow	5.7	7.4	Blue
Bromothymol blue	Yellow	6.0	7.6	Blue
Phenol red	Yellow	6.4	8.2	Red
3-Nitrophenol	Colourless	6.6	8.6	Yellow-orange
Cresol red	Orange	7.0	8.8	Purple
1-Naphtholphthalein	Brownish	7.1	8.3	Blue-green
Thymol blue	Yellow	8.0	9.6	Blue
Phenolphthalein	Colourless	8.2	9.8	Red-violet
Thymolphthalein	Colourless	9.3	10.5	Blue

Annex 13
Glossary of biofertilizer terms

Actinomycetes: A member of the bacterial order Actinomycetales, representing transition between bacteria and fungi, characterized by radiating arrangement of the mycelium and by small spores. They differ from fungi in that they are prokaryotic, have bacterial-type cell walls and are inhibited by antibacterial agents. Most actinomycetes are saprophytic and aerobic. Some (e.g. *Frankia*) are known to fix N in association with higher plants.

Activated charcoal: Charcoal that has been treated to remove impurities and hydrocarbons in order to increase its purity and adsorptive capacity. Inhibitory substances in the nutrient medium may be adsorbed onto charcoal included in the medium. It acts by condensing and holding a gas or solute on its surface. It may be of different origin and variable composition.

Aerobe: An organism that grows best in the presence of free oxygen, e.g. *Rhizobium* and *Azotobacter*.

Agar or agar-agar: A gelatinous polysaccharide obtained from Ceylon moss (red algae, Rhodophyceae). It is a solidifying agent and, thus, when mixed with nutrient media (at 0.6–1.5 percent), it forms a gel. Its firmness is affected by pH and salt concentration of the medium. It is softer when the medium is more dilute and acidic. Agar gel solidifies at about 38 °C and melts at about 100 °C. It is a base for several solid and semi-solid media.

Agar plate count: The number of bacterial colonies that develop on an agar-containing medium in a Petri dish seeded with a known amount of inoculum. From the count, the concentration of bacteria per unit volume of inoculum can be determined.

Anaerobe: An organism that lives and grows in the absence of free oxygen. Anaerobes are either obligatory anaerobes, which grow only in the absence of oxygen, e.g. *Clostridium tetani*, or facultative anaerobes, which can grow either in the presence or absence of oxygen, e.g. *Escherichia coli*.

Anaerobic microbes: Microbes capable of obtaining oxygen for their growth by reducing oxygen-containing compounds in the soil. They are not dependent on a supply of free oxygen.

Autoclave: An enclosed chamber for heating substances above their boiling points under pressure using steam to sterilize liquids, glassware, etc.

Azotobacter: Free-living (non-symbiotic) N-fixing aerobic bacteria. They are plemorphic with ovoid cells, and are relatively large organisms measuring 2.0–7.0 × 1.0–2.5 µm. Cell size and shape vary considerably with species, strain, age of culture, and growth conditions. The seven species of *Azotobacter* on the basis of cell shape, pigmentation and mobility are *A. chroococcum*, *A. beijerinckii*, *A. agilis*,

A. macrocytogenes, *A. paspali, A. insignis* and *A. vinelandi*. Among the important species, *A. beijerinckii* is non-mobile and ovoid rod-shaped. Cells occur singly, in pairs and sometimes in chains with large capsules. As cultures age, the cells become coccoid, form cysts and turn yellow or cinnamon with a water-insoluble pigment.

Bacterial count: Number of bacteria per unit of volume of a substance.

Bacterial culture: Any medium enriched with any particular bacteria.

Bacterial population: A group of bacteria belonging to the same species and exchanging genetic material among one another but with little contact with other groups of the same species. The population is expressed as 10^4/g, 10^5/g, etc.

Batch fermentation: A fermentation system run as a batch culture in which the reactor is charged with the substrate, and microbial inoculum is added with the substrate. The fermentation process is allowed to continue for 4–10 days until completed. After completion of fermentation, the cells are removed from the broth medium.

Biological N_2 fixation (BNF): N fixation by biological process as distinguished from chemical N fixation in a fertilizer factory. It is mediated by several micro-organisms. *Rhizobium*, blue-green algae (BGA), *Azotobacter* and *Azospirillum* are important in BNF. In *Rhizobium*–legume symbiosis, atmospheric N makes its way through the soil to the nodules, where it is reduced to ammonia by the nitrogenase of the *Rhizobium* bacteroids. This ammonia is subsequently incorporated into carbon skeletons to produce amino acids and proteins.

Broth: A suspension of micro-organism culture. Broths are very common in the laboratory e.g. *Rhizobium* broth.

Carbon source: A source that provides C to organisms for their growth. For photosynthetic and autotrophic organisms, the C source is generally carbon dioxide. For many heterotrophic organisms, it is usually glucose or sucrose.

Carrier: Substances used to contain and carry the cultures of micro-organisms in a commercial biofertilizer product, e.g. peat, lignite, charcoal, rice husk, vermiculite, soil, coir dust, press mud, and polymer compounds. A good carrier should be: (i) chemically inert; (ii) able to support growth of organisms in storage; (iii) have a high moisture holding capacity; and (iv) have no toxic influence on the organisms it carries. Most biofertilizers except liquid biofertilizer are carrier-based. Carriers giving an acid reaction (e.g. peat and lignite), depending on the acidity, are mixed with powdered calcium carbonate to be neutralized. Carriers may be sterile or unsterile. Sterilized carriers are desirable because unsterile carriers contain more contaminants. Sterile carriers have been either irradiated or heat-sterilized. After growing the microbial broth in fermenters, the micro-organisms are mixed with the carrier. Good carriers can support 1 000 million (10^9) cells/g when the inoculant is fresh and maintain 100 million (10^8) cells/g for six months after production. Before use, the carrier should be dried and ground. After mixing, carriers should be allowed time for curing in order to dissipate the heat generated during mixing.

Cell count: The number of cells per unit of suspension. The cell numbers are estimated with a haemocytometer.
Cell counter: An automated device to count the number of cells in a sample. The simpler cell counters consist of a mechanism to draw a known volume of a suitably diluted cell culture through a detection device.
Charcoal: A variety of carbon. It is black, porous, imperfectly combusted OM, similar to burned wood. It has adsorbent and filtering qualities. It is used as a carrier for biofertilizers.
Colony: A contiguous group of single cells derived from a single ancestor and growing on a solid surface.
Congo red test: This test differentiates Rhizobia from other contaminants. An aliquot of 25 ml of 1-percent solution of Congo red (a basic dye) in water is added to 1 litre of yeast extract mannitol agar (YEMA). On this medium, when suspected strains of nodule bacteria are plated, Rhizobia stand out as white, translucent, glistening, elevated and comparatively small colonies with entire margins in contrast to strained colonies of other contaminants.
Contaminant: An undesirable bacterial, fungal or algal micro-organism accidentally introduced into a culture or culture medium. It may over grow the cells of interest or inhibit their growth by releasing toxic metabolites. In biofertilizer production, no contaminants are desirable.
Contamination: Accidental introduction of an undesirable substance or organism into a medium of culture.
Continuous fermentation: Process in which the cells are kept in a state of exponential growth, or in which stationary cells produce a secondary product continuously. In general, a suitable medium is fed into the fermenter at the same rate as the effluent is removed so that conditions remain constant.
Culture: A term referring to the growth of micro-organisms under artificial conditions, or the results of microbial growth on artificial culture media.
Culture medium: Any material in which micro-organisms find nourishment and in which they can reproduce (plural is media).
Culture room: A controlled-environment room (light, temperature and relative humidity) for incubation of cultures.
Dry cell weight: The weight of cells contained in an aliquot obtained after drying to constant weight in an oven at 105 °C. Dry weights are often expressed in terms of grams of material per litre or cubic metre of fermenter volume.
In vitro: Refers to biological experiments performed in test-tubes or other laboratory glassware.
In vivo: Refers to laboratory testing of agents within a living organism.
Incubation: Holding cultures of micro-organisms under conditions favourable for their growth. Specialized incubation permits control of humidity, light and temperature. Incubation is usually achieved within closed, head-insulated, thermostatically-controlled chambers.
Inoculant: Culture of microbes (*Rhizobium*, *Azotobacter*, BGA, phosphate-solubilizing microbes, etc.) used for inoculation at field level. Biofertilizers are

inoculants. Inoculants are made available to end users in many forms. They include agar-slope-based cultures, liquid-paraffin-covered agar cultures, lyophilized cultures, carrier-based cultures (peat, charcoal. etc.), and liquid cultures.

Inoculant quality: Describes the population and effectiveness of micro-organisms in an inoculant. A good-quality inoculant contains 10^8 viable cells per gram.

Inoculation: Artificial introduction of micro-organisms onto or into a medium. Inoculation is carried out using an aseptic technique.

Isolate: To set apart, place or separate from others and make a pure culture. The process is isolation.

Isolation medium: A medium suitable for the survival and development of micro-organisms.

Gram negative: Bacteria that lose the colour of the initial Gram stain (e.g. crystal violet) and take the colour of the final stain (safranin). Example: *Rhizobium*.

Gram positive: Bacteria that take the colour of the initial Gram stain and are not decolorized, and do not take the colour of the final stain, i.e. counter-stain. Example: *Bacillus*.

Growth: An irreversible increase in cell size and/or number resulting from cell division or enlargement, usually accompanied by differentiation and an increase in mass.

HEPA filter: An acronym for high-efficiency particulate air filter. A filter capable of screening out particles larger than 0.3 µm. HEPA filters are used in laminar air-flow cabinets (hoods) for sterile transfer work.

Hoffer's alkaline broth test: Test based on the fact that agrobacteria grow at higher pH levels (pH 11), while Rhizobia are unable to do so. The media with pH 11 is used to screen new isolates of nodule bacteria for the purpose.

Liquid medium: Medium not solidified with gelling agent such as agar.

Microbiological tests: Methods of examining specimen objects or materials to determine the presence or absence of micro-organisms, their taxonomic identification, and/or their relative frequency, types and activity.

Mixed culture: Mixture of two or more species of micro-organisms growing together.

Mother culture: Inoculant production starts with a pure slant culture containing the original strain. It may also be termed starter culture.

Plating: A technique used to obtain pure cultures of micro-organisms (bacteria, yeast and fungi) that produce a distinct colony when grown on a solid medium.

Population: Total number of organisms of one kind. The infinite group from which a sample might be taken.

Shelf-life: The period up to which a product (biofertilizer) contains a certain minimum specified number of viable organisms. Products are not useful beyond their shelf-life.

Staining: A method of preparing samples that enhances the contrast when examined under a microscope.

Starter culture: Pure culture or mixture of micro-organisms used for starting a fermentation process. It may also be termed mother culture.

Sterile: Free from infectious matter, agents or living micro-organisms.
Sterilization: The process of making materials sterile through killing or excluding micro-organisms or their spores with heat, filters, chemicals or other sterilants.
Stock culture: Known species of micro-organisms maintained in the laboratory for various tests and studies.
Strain: Pure culture of an organism composed of the descendants of a single isolation. The term also refers to a cell or population of cells that exhibit a particular named characteristic.
Substrate: The medium on which a fungus and bacteria grow, especially in culture. The substance or the object on which an organism lives and from which it obtains its nourishment.
Subculture: A culture derived from another culture or the aseptic division and transfer of a culture or a portion of that culture (inoculum) to a fresh nutrient medium. Subculturing is usually done at set time intervals, the length of which is called the subculture interval or passage time.
Viable count: A determination of the number of cells in a population that is capable of growth and reproduction.
Yield (bacterial): The yield is the difference between the initial and the maximum bacterial mass. It is expressed in grams of dry weight. The yield is also frequently related to the concentration of the substrate and calculated as the molar growth yield (grams of cells per mole of substrate).

Annex 14
Units and conversion factors

TABLE A14.1
Units and conversion factors

To convert unit in Column 1 to unit in Column 2 multiply by:	Column 1: SI unit	Column 2: non-SI unit	To convert unit in Column 2 to unit in Column 1 multiply by:
		Area	
2.47	hectare, ha	acre	0.405
247	square kilometre, km^2 ($(10^3 m)^2$)	acre	4.05×10^{-3}
0.386	square kilometre, km^2 ($(10^3 m)^2$)	square mile, mi^2	2.590
2.47×10^{-4}	square metre, m^2	acre	4.05×10^3
10.76	square metre, m^2	square foot, ft^2	9.29×10^{-2}
1.55×10^{-3}	square millimetre, mm^2 ($(10^{-3} m)^2$)	square inch, in^2	645
		Volume	
9.73×10^{-3}	cubic metre, m^3	acre-inch	102.8
35.3	cubic metre, m^3	cubic foot, ft^3	2.83×10^{-2}
6.10×10^4	cubic metre, m^3	cubic inch, in^3	1.64×10^{-5}
1.057	litre, L ($10^{-3} m^3$)	quart (liquid), qt	0.946
3.53×10^{-2}	litre, L ($10^{-3} m^3$)	cubic foot, ft^3	28.3
0.265	litre, L ($10^{-3} m^3$)	gallon	3.78
33.78	litre, L ($10^{-3} m^3$)	pint (fluid), pt	0.473
		Mass	
2.20×10^{-3}	gram, g (10^{-3} kg)	pound, lb	454
3.52×10^{-2}	gram, g (10^{-3} kg)	ounce (avdp), oz	28.4
2.205	kilogram, kg	pound, lb	0.454
0.01	kilogram, kg	quintal (metric), q	100
1.10×10^{-3}	kilogram, kg	ton (2 000 lb), ton	907
1.102	megagram, Mg (tonne)	ton (U.S.), ton	0.907
1.102	tonne, t	ton (U.S.), ton	0.907
		Length	
0.621	kilometre, km (10^3 m)	mile	1.609
1.094	metre, m	yard, yd	0.914
3.28	metre, m	foot, ft	0.304
3.94×10^{-2}	millimetre, mm (10^{-3} m)	inch, in	25.4
10	nanometre, nm (19^{-9} m)	angstrom, å	0.1
		Yield and rate	
0.893	kilogram per hectare, kg ha^{-1}	pound per acre, lb $acre^{-1}$	1.12
7.77×10^{-2}	kilogram per cubic metre, kg m^{-3}	pound per bushel, lb bu^{-1}	12.87

To convert unit in Column 1 to unit in Column 2 multiply by:	Column 1: SI unit	Column 2: non-SI unit	To convert unit in Column 2 to unit in Column 1 multiply by:
1.49x 10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 60 lb	67.19
1.59x 10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 56 lb	62.71
1.86x 10^{-2}	kilogram per hectare, kg ha^{-1}	bushel per acre, 48 lb	53.75
0.107	litre per hectare, L ha^{-1}	gallon per acre	9.35
893	tonne per hectare, t ha^{-1}	pound per acre, lb $acre^{-1}$	1.12x 10^{-3}
893	megagram per hectare, Mg ha^{-1}	pound per acre, lb $acre^{-1}$	1.12x 10^{-3}
0.446	megagram per hectare, Mg ha^{-1}	ton (2 000 lb) per acre, ton $acre^{-1}$	2.24
2.24	metre per second, m s^{-1}	mile per hour	0.447
		Pressure	
9.90	megaPascal, MPa (10^6 Pa)	atmosphere	0.101
10	megaPascal, MPa (10^6 Pa)	bar	0.1
1.00	megagram per cubic metre, Mg m^{-3}	gram per cubic centimetre, g cm^{-3}	1.00
2.09x 10^{-2}	Pascal, Pa	pound per square foot, lb ft^{-2}	47.9
1.45x 10^{-4}	Pascal, Pa	pound per square inch, lb in^{-2}	6.90x 10^3
		Temperature	
1.00 (K - 273)	Kelvin, K	Celsius, °C	1.00 (°C + 273)
(9/5 °C) + 32	Celsius, °C	Fahrenheit, °F	5/9 (°F - 32)
		Water measurement	
9.73x 10^{-3}	cubic metre, m^3	acre-inch, acre-in	102.8
9.81x 10^{-3}	cubic metre per hour, m^3 h^{-1}	cubic foot per second, ft^3 s^{-1}	101.9
4.40	cubic metre per hour, m^3 h^{-1}	U.S. gallons per minute, gal min^{-1}	0.227
8.11	hectare-metre, ha-m	acre-foot, acre-ft	0.123
97.28	hectare-metre, ha-m	acre-inch, acre-in	1.03x 10^{-2}
8.1x 10^{-2}	hectare-centimetre, ha-cm	acre-foot, acre-ft	12.33
		Concentration	
1	centimole per kilogram, cmol kg^{-1} (ion exchange capacity)	milli-equivalents per 100 grams, me 100 g^{-1}	1
0.1	gram per kilogram, g kg^{-1}	percent, %	10
1	milligram per kilogram, mg kg^{-1}	parts per million, ppm	1
		Plant nutrient conversion	
	Element	Oxide	
2.29	P	P_2O_5	0.437
1.20	K	K_2O	0.830
1.39	Ca	CaO	0.715
1.66	Mg	MgO	0.602
1.216	N	NH_3	0.777
4.426	N	NO_3	0.226
6.25	N	Protein	0.160
3.00	S	SO_4	0.330
2.5	S	SO_3	0.440
1.724	Organic C	Organic matter	0.580

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