III. SOIL CHEMICAL ANALYSES

Section Editors: Y.K. Soon and W.H. Hendershot
Chapter 16
Soil Reaction and Exchangeable Acidity

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16.1 INTRODUCTION

Soil pH is one of the most common and important measurements in standard soil analyses. Many soil chemical and biological reactions are controlled by the pH of the soil solution in equilibrium with the soil particle surfaces.

Soil pH is measured in an aqueous matrix such as water or a dilute salt solution. Soil pH measured in water is the pH closest to the pH of soil solution in the field (this is true for soils with low electrical conductivity and for soils that are not fertilized), but is dependent on the degree of dilution (the soil to solution ratio). Measuring soil pH in a matrix of 0.01 M CaCl₂, as opposed to water, has certain advantages, but the addition of the salt does lower the pH by about 0.5 pH units compared to soil pH in water (Schofield and Taylor 1955; Courchesne et al. 1995). In soil correlation work, the use of pH in CaCl₂ is preferred because the measurement will be less dependent on the recent fertilizer history. Other methods for soil pH measurement, such as pH in 1 M KCl, are presented elsewhere (Peech 1965); these methods are not commonly used in Canada for routine analysis and are not included in this chapter.

16.2 SOIL pH IN WATER

When measuring soil pH in water, the main concern is that an increase in the amount of water added will cause an increase in pH; it is therefore important to keep the ratio constant and as low as possible. However, the supernatant solution must be sufficient to immerse the
electrode properly without causing too much stress when inserting the tip of the electrode into the soil and to allow the porous pin on the electrode to remain in the solution above the soil.

16.2.1 MATERIALS AND REAGENTS

1 pH meter: an appropriate instrument provided with two calibration points should be used.

2 Combined electrode: since the volume of soil is generally limited and the soil to solution ratio kept as low as possible, a combination electrode is a valuable asset.

3 30 mL long form beakers (Pyrex or disposable plastic): beakers that have a narrow shape help to immerse the electrode in the supernatant without introducing the tip into the soil.

4 Stirrers: disposable plastic stirrers or glass rods can be used.

16.2.2 PROCEDURE

1 Weigh 10 g of air-dried mineral soil (<2 mm) into a beaker and add 20 mL of double deionized (d.d.) water. For organic samples, use 2 g of soil in 20 mL of d.d. water. Record the soil to solution ratio used. Include duplicate quality control samples in each batch.

2 Stir the suspension intermittently for 30 min.

3 Let stand for about 1 h.

4 Immerse the electrode into the clear supernatant and record the pH once the reading is constant. Note: Both the glass membrane and the porous salt bridge must be immersed.

16.2.3 COMMENTS

Soil samples containing high amounts of organic matter tend to form a thick dry paste when the ratio is kept the same as for mineral samples; therefore, a decreased ratio of sample to water must be accepted (1:5 or 1:10).

Two pH standards should be used to calibrate the pH meter; they must be chosen in accordance with the pH range expected for the soils analyzed (pH 4.0 and 7.0 or pH 7.0 and 10.0).

A large amount of a soil similar to the samples being analyzed should be kept as an indicator of the variability of pH results over time; duplicate subsamples of this quality control (QC) sample should be run with each batch of samples measured. Failure of the QC to fall within acceptable limits means that the whole batch should be reanalyzed.
16.3 SOIL pH IN 0.01 M CaCl₂

Standard measurement of soil pH in CaCl₂ is probably the most commonly used method to characterize soil pH. As mentioned by Peech (1965), Davey and Conyers (1988), and Conyers and Davey (1988), the use of CaCl₂ has some advantages for pH measurement: (1) the pH is not affected within a range of the soil to solution ratios used, (2) the pH is almost independent of the soluble salt concentration for nonsaline soils, (3) this method is a fairly good approximation of the field pH for agricultural soils, (4) because the suspension remains flocculated, errors due to the liquid junction potential are minimized, (5) no significant differences in soil pH determination are observed for moist or air-dried soil, and (6) one year of storage of air-dried soil does not affect the pH.

16.3.1 MATERIAL AND REAGENTS

1. pH meter: an appropriate instrument provided with two calibration points should be used.

2. Combined electrode: since the volume of soil is generally limited and the soil to solution ratio kept to a minimum, a combination electrode is a valuable asset.

3. 30 mL long form beakers (Pyrex or disposable plastic): beakers that have a narrow shape help to immerse the electrode in the supernatant without introducing the tip of the electrode in the soil thus avoiding breakage.

4. Stirrers: disposable plastic stirrers or glass rods can be used.

5. Calcium chloride, 0.01 M: dissolve 2.940 g of calcium chloride dihydrate (CaCl₂·2H₂O) with d.d. water in a 2 L volumetric flask. The electrical conductivity of the CaCl₂ solution must be between 2.24 and 2.40 mS cm⁻¹ at 25°C.

16.3.2 PROCEDURE

1. Weigh 10 g of air-dried mineral soil (<2 mm) or 2 g of organic soil into a 30 mL beaker and add 20 mL of 0.01 M CaCl₂. Note the soil to solution ratio used. Include duplicate quality control samples in each batch.

2. Stir the suspension intermittently for 30 min.

3. Let stand for about 1 h.

4. Immerse a combination electrode into the clear supernatant and record the pH once the reading is constant. Note: Both the glass membrane and the porous salt bridge must be immersed.

16.3.3 COMMENTS

The pH and electrical conductivity of the CaCl₂ should be fairly constant, i.e., pH in the range of 5.5–6.5 and the electrical conductivity around 2.3 mS cm⁻¹ at 25°C. If the pH is outside this range, it should be adjusted with HCl or Ca(OH)₂ solution. If the electrical conductivity is not within the acceptable range, a new solution must be prepared.
16.4 EXCHANGEABLE ACIDITY (EXPERT PANEL ON SOIL 2003)

In addition to bases (e.g., Ca, Mg, K, Na) there is also an amount of acidity that can be displaced from the exchange complex of a soil. The amount of this acidity is largely a function of soil pH and the exchange capacity. In most soils the exchangeable acidity will be composed of (i) exchangeable H\(^+\), (ii) exchangeable Al as either Al\(^{3+}\) or partially neutralized Al-OH compounds such as AlOH\(^{2+}\) or Al(OH)\(^{3+}\), and (iii) weak organic acids.

When a soil is limed, the exchangeable acidity is neutralized as the pH rises. Hence, exchangeable acidity is one measure of the amount of lime that will be needed to correct soil pH.

The method of Thomas (1982) used 1 M KCl as the displacing salt solution, whereas the Expert Panel on Soil (2003) proposes 0.1 M BaCl\(_2\). Since the method proposed in this manual for measuring exchangeable cations uses 0.1 M BaCl\(_2\), it seems more appropriate to use the same salt solution for measuring exchangeable acidity. Due to the lower concentration of the BaCl\(_2\) solution, the amounts of some cations are lower than when the extraction is done with KCl; however, Jonsson et al. (2002) have determined regression equations that could be used to estimate the difference between the two extraction procedures.

16.4.1 MATERIALS AND REAGENTS

1. 50 mL centrifuge tubes, a centrifuge capable of generating 5000 g and an end-over-end shaker (15 rpm).
2. Replacing solution, barium chloride 0.1 M: dissolve 24.43 g of BaCl\(_2\)-2H\(_2\)O with distilled deionized (d.d.) water and make to volume in a 1 L volumetric flask.
3. Aluminum complexing solution, 1 M sodium fluoride: dissolve 41.99 g of NaF in about 900 mL of d.d. water in a 1 L beaker and then titrate to the phenolphthalein endpoint with sodium hydroxide (NaOH). Transfer to a 1 L volumetric flask and make to volume.
4. Sodium hydroxide (NaOH), approximately 0.05 M, standardized.
5. Phenolphthalein solution: dissolve 1 g of phenolphthalein in 100 mL of ethanol.

16.4.2 PROCEDURES

1. Weigh a 2.5 g sample of mineral soil or 2.0 g of organic soil into a 50 mL centrifuge tube, add 30 mL of 1 M BaCl\(_2\) solution, and shake for 1 h. Centrifuge at 5000 g for 10 min. Transfer supernatant liquid to a 100 mL volumetric flask. Repeat by adding 30 mL aliquots of BaCl\(_2\) solution, shaking, centrifuging, and decanting two more times, collecting all the supernatant in the same 100 mL volumetric. Make up to volume with BaCl\(_2\) solution and mix. Filter the extract (Whatman No. 42 or equivalent) into a plastic bottle and store in a refrigerator prior to analysis.
To obtain exchangeable acidity, pipette 25 mL of the extract into a 100 mL polyethylene beaker, add 4 or 5 drops of phenolphthalein, and titrate with 0.05 M NaOH to the first permanent pink endpoint; record the volume of NaOH used as VA. (Note: A deep pink is too far.) Titrate a blank (25 mL of BaCl₂ solution) to the endpoint and record the amount VB. Centimoles of BaCl₂-extracted acidity per kg of soil (cmol(+) kg⁻¹) are calculated as shown below.

To determine exchangeable H⁺ acidity, pipette 25 mL of the extract into a 100 mL polyethylene beaker, then add 2.5 mL of 1 M NaF, and titrate with 0.05 M NaOH to the first permanent pink endpoint (Va). Repeat with a blank sample of BaCl₂ (Vb).

### 16.4.3 Calculation

\[
cmol(+) \text{ kg}^{-1} \text{ exchangeable acidity} = \frac{(VA - VB) \times M(\text{NaOH}) \times 100 \times V}{g \text{ sample} \times Vs}
\]  \hspace{1cm} (16.1)

cmol(+) kg⁻¹ H⁺ acidity is calculated using the same equation replacing VA by Va and VB by Vb, where VA or Va are the volumes of titrant used for the determination of exchangeable acidity and H⁺, Vs is the volume of extract titrated and V is the total volume of extract collected, M(\text{NaOH}) is the concentration of the titrant, and g sample is the mass of soil extracted.

### 16.4.4 Comments

1. The procedure has been written using a pH indicator solution, which is our preference for manual titrations. However, if an automated titrator is used, the endpoint should be set at pH 7.8.

2. Exchangeable cations and exchangeable acidity (including H⁺) can all be determined on the extracts obtained by this multiple washing procedure; this is the procedure recommended by the Expert Panel on Soil (2003). Although this extraction procedure is somewhat more complicated than the 0.1 M BaCl₂ method proposed in Chapter 18 (Section 18.2), it should give similar results.

### REFERENCES


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Chapter 17
Collection and Characterization of Soil Solutions

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17.1 INTRODUCTION

The soil solution plays a dominant role in the uptake of nutrients by plants, has direct impacts on other soil-living organisms, and under certain conditions it is the vector for the migration of dissolved and suspended materials through soils. We are defining the soil solution as the liquid phase of the soil present in the field. This definition precludes all methods in which salt solutions, or water, are added to a soil sample in the laboratory to simulate soil solutions as these procedures may be more accurately defined as soil extractions.

Studies of atmospheric deposition on soils and watersheds often incorporate monitoring of soil solution chemistry. In many cases lysimeters are used to collect soil solution on a regular basis to assess the ability of soils to absorb atmospherically deposited material and to control the release of nutrients and contaminants to ground and surface waters. Studies of macro-nutrient availability in forest soils may also use lysimeters as a means of measuring available nutrients. Examples of these types of studies are frequently found in the literature (Haines et al. 1982; Beier et al. 1992; Foster et al. 1992; Hendershot et al. 1992; MacDonald et al. 2003; Bélanger et al. 2004).
Field soil solution collectors fall into two main categories: zero-tension lysimeters and tension lysimeters. Both forms of lysimeters consist of an apparatus that is inserted into the soil column and collects water either moving through or held within soil capillaries. Recently, tension lysimetry has been further refined to include microlysimeters that can be used in the study of microenvironments in the soil. The development of microlysimeters shows potential for the study of the heterogeneity of soil solution chemistry at the microscale that is known to occur in soils, yet is poorly understood.

Methods of obtaining soil solution from fresh soil in the laboratory or directly in the field have been developed over the years (Heinrichs et al. 1995; Lawrence and David 1996). These techniques typically result in soil solutions with much higher concentrations of many ions, including dissolved organic matter, than those collected using field lysimeters (Ludwig et al. 1999) and some inconsistencies are observed in results depending on soil moisture content (Jones and Edwards 1993). Nonetheless, laboratory methods for sampling soil solutions from freshly sampled soils, e.g., centrifugation, miscible displacement, and syringe pressure, are useful tools for investigating plant nutrition and worthy of examination (Smethurst 2000).

We include a centrifugation method in this chapter; however, due to concerns raised about the influence of the force of extraction used to extract these solutions, we also propose a simple method developed by Ross and Bartlett (1990) that extracts solutions by applying pressure to moist soils packed in syringes in the field. Like the proposed centrifugation method, the syringe compression method is rapid and simple, and provides an alternative approach in cases where there is concern that centrifugation may overestimate concentrations of certain elements in solutions.

It is not always possible to carry out field studies. To approximate soil solution chemistry in the laboratory using bulk soil samples, we suggest a weak CaCl$_2$ (0.01 $M$) shake and centrifuge extraction (Quevauviller 1998) due to its simplicity and because it is an approach that is easily standardized. We also propose a column leaching extraction method that has been developed for bulk soil samples and has been shown to provide results similar to those obtained from zero-tension lysimeters (MacDonald et al. 2004a,b).

In this chapter, we provide five different procedures to separate the solution phase from the solid phase of soils. For in situ studies we propose zero-tension and tension lysimeters, as well as microlysimeters. To acquire solutions from fresh soil samples in the laboratory, we suggest centrifugation and syringe pressure techniques. The preferred methods of laboratory approximations of soil solutions, the column leaching method and weak CaCl$_2$ (0.01 $M$) extraction, can be found in Chapter 10. The applications, advantages, and disadvantages of the different methods of acquiring the soil solution from soils and the solution extractions described in Chapter 10 are summarized in Table 17.1.

17.2 ZERO-TENSION LYSIMETERS

Zero-tension lysimeters collect water moving through the soil profile only when the soil moisture content is greater than the field capacity. Several different designs of zero-tension lysimeters have been proposed. These include simple plates inserted in the soil, models with pierced plates installed in funnels, fiberglass wick-type collectors, and funnels filled with quartz sand. Our preferred design uses a plastic funnel filled with 2 mm quartz sand. Quartz sand is relatively unreactive and when the lysimeter is installed with a good contact between...
TABLE 17.1 Advantages, Disadvantages, and Potential Applications of Different Sample Methods and Sampler Types Proposed in the Present Chapter and Chapter 10

<table>
<thead>
<tr>
<th>Type</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Potential applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-tension lysimeters</td>
<td>Samples can be collected from same soil column over many years</td>
<td>Does not collect solution except under wet conditions</td>
<td>Sample solutions that move through the soil with only gravitational potential</td>
</tr>
<tr>
<td></td>
<td>Simple system with low maintenance</td>
<td>Requires long equilibration (many months to a year) period before samples are representative</td>
<td>Long-term solution chemistry monitoring</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mobility of elements and fine particles in soils</td>
</tr>
<tr>
<td>Tension lysimeters</td>
<td>Samples can be collected from same soil column over many years</td>
<td>Requires long equilibration (many months to a year) period before samples are representative</td>
<td>Sample solutions held at potentials greater than gravitational potential</td>
</tr>
<tr>
<td></td>
<td>Low maintenance</td>
<td>Variable results with soil moisture conditions</td>
<td>Long-term soil solution monitoring</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not efficient in surface organic horizons</td>
<td>Availability of elements in soils</td>
</tr>
<tr>
<td>Microlysimeters</td>
<td>Samples can be collected at very precise locations in soils</td>
<td>High maintenance, fragile devices that are difficult to maintain under field conditions</td>
<td>Sample solutions in soil microenvironment, e.g., in the rhizosphere, the outer layer of peds, or in the coatings lining macropores</td>
</tr>
<tr>
<td></td>
<td>Very high spatial resolution</td>
<td>Requires equilibration period</td>
<td>Microscale heterogeneity of soil solutions, nutrient uptake, biogeochemical processes</td>
</tr>
<tr>
<td></td>
<td>Samples can be repeatedly collected from the same location</td>
<td>Very small (sub mL) solution volumes, hence need adapted analytical procedures and systems</td>
<td></td>
</tr>
<tr>
<td>Centrifugation (low-speed)</td>
<td>Samples can be extracted rapidly from fresh moist soils</td>
<td>Lack of reproducibility of results as solution concentrations are dependent on moisture content of soils and time of sampling</td>
<td>Provides a single point in time extraction of capillary solutions present in soils</td>
</tr>
<tr>
<td></td>
<td>Reasonable alternative when lysimetry is not available or feasible</td>
<td>Small solution volumes and potential overestimations of certain anions and dissolved organic carbon (DOC)</td>
<td>Provides estimates of nutrient availability in soils</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Type</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Potential applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe pressure</td>
<td>Samples can be extracted rapidly from fresh, moist soils</td>
<td>Lack of reproducibility of results as solution concentrations are dependent on moisture content of soils and time of sampling</td>
<td>Provides a single point in time extraction of capillary solutions present in soils</td>
</tr>
<tr>
<td></td>
<td>Minimum disturbance of the soil during solution acquisition</td>
<td></td>
<td>Provides estimates of nutrient availability in soils</td>
</tr>
<tr>
<td></td>
<td>Reasonable alternative when lysimetry is not available or feasible</td>
<td>May yield small amounts of solution during dry periods, especially in coarse soil horizons (e.g., sandy loam) with low organic matter content</td>
<td>May avoid overestimation of certain elements associated with the force of extraction used in centrifugation</td>
</tr>
<tr>
<td>Column leaching</td>
<td>Soil samples can be collected and extracted relatively quickly</td>
<td>Estimates of field partitioning, but the method does not reproduce true field conditions</td>
<td>Estimation of field partitioning coefficients of divalent metals</td>
</tr>
<tr>
<td></td>
<td>Solution samples can be replicated from the same bulk soil sample</td>
<td>Does not provide information on nutrients availability, N, or P</td>
<td>Adsorption–desorption studies</td>
</tr>
<tr>
<td>0.01 M CaCl₂ salt extraction, shake, and centrifuge</td>
<td>Solution samples can be replicated from the same bulk soil sample</td>
<td>Solutions are often very different from field solutions collected from the same soil at the same site</td>
<td>Information about the chemistry of soil surfaces</td>
</tr>
<tr>
<td></td>
<td>Easily standardized methodology</td>
<td>Cannot measure Ca²⁺ and Cl⁻</td>
<td>Adsorption–desorption and bioavailability studies</td>
</tr>
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</table>
the quartz sand and the soil column above it, the soil–lysimeter interface has a large contact area. Capillary flow is not as likely to be interrupted and consequently the quartz sand design will tend to collect water under circumstances where the plate lysimeters and pierced plate lysimeters do not. The material is inexpensive relative to commercially available lysimeters. It is better to avoid using the fiberglass wick collectors since the fiberglass is more reactive than the quartz sand, and wick samplers have been suggested to cause modifications to soil solution chemistry (Goyne et al. 2000; Brahy and Delvaux 2001).

### 17.2.1 Materials

1. 180 mm diameter polyethylene or polypropylene funnels
2. 2 mm quartz sand
3. 75 mm diameter (3”) or 50 mm (2”) acrylonitrile–butadiene–styrene (ABS) drain pipe about 2 m long
4. ABS end cap, and flexible cap for ABS drain pipe
5. 19 mm (3/4”) plastic hose pipe fittings, one L-shaped and one straight
6. 19 mm (3/4”) clear plastic hose with 3 mm (1/8”) thick wall and about 70 cm long
7. Epoxy two component cement and ABS solvent glue
8. Nylon screening 25 × 50 mm
9. 5% (v/v) HCl

### 17.2.2 Preparation (Figure 17.1)

1. The funnels are prepared by gluing the L pipe fitting into the bottom with epoxy.
2. Cut the ABS drain pipe so that once it is buried it extends above the ground by at least 45 cm (to avoid rain-splash), or in areas of snow, at least the depth of normal snow cover. Attach the hard plastic ABS end cap to one end with either epoxy or ABS glue. Clearly label (paint or engrave) each pipe near the top.
3. Drill a hole in the ABS pipe and insert the straight hose fitting by coating with epoxy and hammering into place. The distance of the hose fitting from the base of the pipe is dependent on the desired volume of the lysimeter reservoir. For 75 mm ABS and a reservoir volume of 1 L, the hose fitting is installed at 23 cm from the bottom, whereas for 2 L, the distance is 45 cm. With 50 mm ABS, the distance for a 1 L reservoir volume is 51 cm. In shallow or rocky soil the 75 mm ABS is easier to install.
4. Acid wash the ABS pipe, the funnel, the clear plastic tube, and enough quartz sand to fill the funnel with 5% HCl. Rinse with deionized water until the electrical conductivity (EC) of wash water is equal or close to that of deionized water.
Place the quartz sand and the funnels with the clear tubing attached into separate plastic bags for transport to the field. The ABS tubes should have plastic taped over the hose fitting and the flexible plastic cap placed on the upper end.

17.2.3 INSTALLATION PROCEDURE

1. Cordon off the location of the lysimeter station taking care to avoid walking on the area or contaminating it with soil or other debris.

2. Dig a pit downslope, if the site is not flat, approximately 1 m² to a depth greater than that at which the lowest lysimeter is to be installed. Separate the surface layers from the underlying soil so that they can be replaced in such a way that the site is disturbed as little as possible at the end of the installation.

3. Starting with the deepest lysimeter, dig a tunnel into the side of the pit under the delineated area. Use a spare funnel to make sure the tunnel is cut to the correct size to avoid contaminating the acid-washed funnels.

4. Insert the nylon screen into the L fitting, fill the funnel with the quartz sand, and carefully slide it into place making sure that it makes good contact with the soil above it. Press it into place, pack rocks under the L fitting at the bottom, and backfill carefully all around the under surface of the funnel. Ensure that the soil has been well packed around the lysimeter such that contact between the silica sand and the soil column is solidly maintained. Attach the clear tube to the hose fitting in the ABS tube.
Several lysimeters at different depths can be installed in the same pit; however, it must be ensured that funnels are placed such that each has an undisturbed cone of soil above it (often requiring a larger pit than anticipated). When all the lysimeters have been installed in the face of the pit, make sure that the clear tube slopes downward from the funnel to the ABS tube. Record the placement of the collectors. Carefully refill the pit.

17.3 TENSION LYSIMETERS

Porous cup tension lysimeters are inserted into the soil such that the porous surface is in contact with the capillaries of the soil column. When a vacuum is applied to the porous cup, solution is drawn out of the capillaries into the lysimeter reservoir. Tension lysimeters extract soil solutions that are maintained within the micropores of the soil and consequently may be immobile. The solutions that they extract have been observed to differ significantly from zero-tension lysimeters (Haines et al. 1982; Hendershot and Courchesne 1991).

Various types of tension lysimeters are available, differing in the type of porous cup that is inserted into the soil. The most commonly used tension lysimeters observed in the literature are the ceramic cup lysimeters that are installed from the surface. Recently, porous poly(tetrafluoroethene) or Teflon® cups have been developed for tension lysimeters to avoid the impact that the exchange capacity of ceramic cups can have on solution chemistry (Swenson 1997; Russell et al. 2004). We have used ceramic cups in the past; and we feel that after adequate stabilization periods in the soil, the ceramic cups are representative of macroelements in soil solution. However, the new Teflon-treated cups appear to be less reactive and are therefore a more reliable method to extract solutions under tension.

17.3.1 PREPARATION

The lysimeters should be cleaned following the manufacturer’s recommendation or using the following procedure. Place the lysimeters in a container with 5% HCl and draw the solution through the porous cup and into the lysimeters using suction. Repeat this procedure three times and ensure that the PVC shaft above the porous cup is also effectively acid washed. Rinse with deionized water until the EC is close or equal to that of deionized water and is constant (may take up to 10 washings). When clean, place the lysimeters in clean plastic bags ready to go into the field.

17.3.2 INSTALLATION PROCEDURE

1. Cordon off the location of the lysimeter station and take care to avoid walking on the area or contaminating it with soil or other debris.

2a. Surface installation: place a plastic sheet with a hole the same diameter as the lysimeters on the soil surface to trap soil as it is excavated. Using an auger the same size as the lysimeters, dig a hole to the required depth.

   i. Install the lysimeters and refill the hole around the lysimeter shaft with soil from the same soil horizon in which the lysimeters are installed. Carefully reconstruct the soil horizons above the lysimeters until the hole is filled.
ii. Ensure that the soil is tightly sealed around the lysimeter shaft so that preferential flow does not occur. In soils where good soil-to-lysimeter contact is difficult to establish, a slurry can be prepared using soil taken from the same depth as that of the lysimeter. A small amount of slurry is poured into the auger hole before installation of the lysimeter.

2b Pit installation: dig a pit approximately 1 m² to a depth greater than that at which the lowest lysimeter is to be installed.

i. Separate the surface layers from the underlying soil so that they can be replaced in such a way that the site is disturbed as little as possible at the end of the installation.

ii. Starting with the deepest lysimeter, dig a tunnel into the side of the pit under the delineated area equal in diameter to the porous cup. Carefully insert the porous cup ensuring good contact with the tunnel walls. Repeat for all lysimeter depths.

iii. Connect the vacuum and sample tubes. Record the position of the lysimeters and carefully refill the pit and replace the surface layers.

3 Apply a vacuum of 30–60 kPa to the lysimeter. It is recommended that a constant vacuum be maintained in the lysimeter. Constant vacuum systems will provide cumulative samples over periods between sample collections; however, systems that maintain a constant vacuum between sampling periods are expensive. It is also possible to use discontinuous systems and apply a vacuum for a period of several days before sample collection. It should be noted that discontinuous vacuum systems will provide samples that are representative of the short time period over which the vacuum is maintained.

17.4 SAMPLING SOIL SOLUTIONS FROM LYSIMETERS

Soil solutions can be extracted from lysimeter reservoirs using handheld vacuum pumps or peristaltic pumps ensuring that solutions are not cross-contaminated during collection.

1 Lysimeters should be completely emptied each time they are sampled. Record the total volume of solution removed from the lysimeter.

2 Solutions should be transferred immediately to coolers and maintained at 4°C in the dark for transport to the laboratory.

3 Once solutions are in the laboratory, set aside a small subsample of soil solutions (10–20 mL) and filter the rest of the solution using low vacuum through 0.4 μm polycarbonate filters. Solutions intended for analysis of elements that could be modified through contact with the air (nitrogen species for example) should be sealed in polycarbonate vials immediately after filtration, leaving little to no air space. A subsample for metal analysis should be acidified (0.2% HNO₃ v/v); trace metal-grade acid should be used if trace elements are to be analyzed.

4 Filtration will modify solution pH, therefore take the pH and EC of unfiltered subsamples of solutions immediately at room temperature.
17.4.1 COMMENTS

1 Solutions should be drawn from the lysimeter reservoirs on a regular sampling schedule. Typically, lysimeter monitoring is carried out on a weekly, biweekly, or monthly schedule. Solutions that remain in the reservoir for long time periods may be modified, due to decomposition of dissolved organic carbon or the dissolution of suspended colloidal materials. Furthermore, it should be noted that lysimeter solutions, once separated from the soil, do not preserve in situ gas partial pressures and their associated chemistry.

2 The installation of lysimeters causes significant disturbance to the soil. Ensure that the lysimeters have stabilized before beginning a sampling regime. After installation, the pH and EC of lysimeter solutions should be monitored. Solutions cannot be considered representative of the soil chemistry until the pH and EC of the solution have stabilized. Stabilization periods for lysimeters can be long (6 months to 1 year). The pH and EC are good indicators of the stabilization point of soil solutions, but the initial data produced from lysimeters should be examined to ensure that stabilization of all elements of interest has occurred, particularly for nitrogen species.

17.5 MICROLYSIMETERS

The investigation of the microscale heterogeneity of soil materials, in particular the spatial variability in the liquid phase, requires a lysimeter system that is adapted to the characteristic small scale of the soil environment of interest. Göttlein et al. (1996) described a system for microscale lysimetry that allowed the monitoring of soil solution at a high spatial resolution to study gradients in concentrations of elements in the root–soil interface. The lysimeter unit consists of a 1 mm diameter ceramic cell with 1 μm pore size attached to 1.59 mm capillary tubing and connected to a vacuum device to extract the solution from the soil matrix. At a suction of 35 kPa, these cylindrical cups can sample solution in the volume of soil extending to a distance of ≥1 cm from their surface (Göttlein et al. 1996) and sample volumes range from 50 to 300 μL collected on a weekly basis at a suction of 40 kPa. Other microlysimeter designs have been proposed, but the cylindrical microlysimeters developed by Göttillein et al. (1996) are presented in this chapter because their design has been the most widely tested.

17.5.1 MATERIALS

1 Ceramic capillaries with porosity of about 48%, 1 mm wide, and a suggested maximum pore size of 1 μm.

2 Polyetheretherketone (PEEK) tubing 1.59 mm (1/16”) wide, 50 mm long with an inside diameter (ID) of 0.75 mm; this tubing, used for high-pressure liquid chromatography (HPLC), is widely available (see Section 17.5.4).

3 Epoxy, two component cement.

4 PEEK tubing with an ID of 0.25 mm.
17.5.2 CONSTRUCTION AND PREPARATION (Figure 17.2)

1. Cut the ceramic capillary into 12 mm long segments.
2. Seal the tip (exterior end) of the ceramic capillary by melting over a Bunsen burner to obtain a microceramic cup 10 mm long with a glass tip.
3. Cut the 0.75 mm ID PEEK tubing into 50 mm lengths.
4. Insert the 10 mm long ceramic cup 5 mm into the 0.75 mm ID PEEK tube.
5. Glue the ceramic cup to the PEEK tube using a two-part cement to complete assembly of the microlysimeter (Figure 17.2).
6. Clean the microlysimeters by drawing 5% HCl through the porous cup and into the tubing using suction. Repeat three times and then rinse with deionized water until the EC is close or equal to that of deionized water and is constant (may take up to 10 washings).
7. Fix a 0.25 mm ID PEEK tubing of the appropriate length to each of the microlysimeters.
8. Construct a vacuum chamber made of transparent Plexiglas and connected to a vacuum pump, as in Figure 17.2.
9. Install the vial rack and vials with caps in the vacuum chamber. Pierce holes in the caps.
10. Connect the tubing fixed to the microlysimeters to the vial through the hole pierced in the cap to avoid contamination and limit evaporation.
11. When clean, place all equipment in clean plastic bags ready to go into the field.
17.5.3 INSTALLATION PROCEDURE (FIGURE 17.3)

1. Determine the location where the microlysimeters are to be installed in the soil, either on the face of a natural profile or in soil materials contained in a rhizotron.

2. At that point, make a hole in the soil having the dimension of the microlysimeters using the stainless steel rod.

FIGURE 17.2. Schematic of microlysimeter suction device, support plate, and sample collection chamber for solutions from microlysimeters for microlysimeter installation. (From Göttlein, A., Hell, U., and Blasek, R., Geoderma, 69, 147, 1996. With permission.)
Remove the rod from the channel and insert the microlysimeter in the soil to the desired depth. Determine the exact position of the tip of the suction cup.

Use the Plexiglas plate, with holes the size of the microlysimeters (or one of the faces of the rhizotron), to support individual microlysimeters and to ensure their precise and constant position in the soil (Figure 17.3).

Apply a vacuum of 30–40 kPa to the microlysimeter. It is recommended that a constant vacuum be maintained in the lysimeter. It is also possible to use discontinuous systems and apply a vacuum for short time periods.

Like any lysimeter, microlysimeters should be allowed to equilibrate with the surrounding soil and the pH and EC of solutions should be monitored. Once pH and EC are stable, data from the microlysimeters can be considered to be representative of soil solution chemistry.

Solutions should be transferred immediately to coolers and maintained at 4°C in the dark for transport to the laboratory.

17.5.4 Comments

Microlysimeters solution volumes are small and can easily be contaminated, so the selection of tubing and container types is crucial to limit the adsorption of major ions, trace metals, or organic acids to surfaces during sampling and storage. Nylon or Teflon is recommended to reduce the sorption of trace metals whereas glass materials are suggested for dissolved organic substances.

The solution volumes collected with microlysimeters are in the range 50–300 µL. Therefore, analytical methods adapted to very small solution volumes are needed; for example, capillary electrophoresis (CE) (Göttlein and Blasek 1996) and other methods based on high-resolution inductively coupled plasma–mass...
spectrometry (Puschenreiter et al. 2005) have been used to analyze major anions and cations in very small sample volumes.

17.6 SEPARATION OF SOIL SOLUTION IN THE LABORATORY

A variety of methods to obtain soil solutions in the laboratory from freshly sampled soils have been proposed. These methods include low- and high-speed centrifugation (Gillman 1976; Reynolds 1984) displacement methods with miscible (Adams 1974; Wolt and Graveel 1986) and immiscible liquids (Kinniburgh and Miles 1983) and positive air pressure in sealed cylinders (Lawrence and David 1996). These methods have been compared and generally produce similar results (Adams et al. 1980; Wolt and Graveel 1986; Elkhatib et al. 1987). In all cases, the key to obtaining minimally altered results is the processing of the sample shortly after collection. Centrifugation is recognized as a rapid and simple method. The method that we propose is the classic Davies and Davies (1963) method outlined in the previous edition of this book with the exception that we propose the use of high-density polyethylene (HDPE) frits to contain the soil in the syringe as opposed to glass wool.

Although centrifugation is probably the most commonly used method to separate the soil solution from the solid phase in the laboratory, Ross and Bartlett (1990), when comparing high-speed centrifugation with miscible displacement and syringe compression on forest floor and Bsh horizons, came to the conclusion that high-speed centrifugation should be avoided as it yields high H⁺ and F⁻ concentrations as well as occasionally high Cl⁻, SO₄²⁻, and NO₃⁻ levels. The miscible displacement method, though it yielded large amounts of solution, was tedious and time-consuming. Since increased processing time inevitably results in increased alteration of soil solutions, we feel that the simple and relatively rapid syringe pressure technique is a good alternative for extracting solutions from moist soils. The syringe technique yielded solutions with similar chemistry to that of the miscible displacement method and the precision of analyses on duplicated samples was as good, or better, than the displacement or centrifugation methods.

17.6.1 CENTRIFUGATION (DAVIES AND DAVIES 1963)

Material and Equipment

1 The centrifuge apparatus is a 60 mL syringe that has been cut to 55 mm and is used to contain the fresh moist soil sample and a solution cup that can be made by cutting the top of a 50 mL HDPE centrifuge tube (see Figure 17.4).

2 Centrifuge with horizontal rotors and 50 mL centrifuge shields or adaptors, preferably with refrigeration.

3 HDPE frits, 27 mm in diameter.

4 Small solution bottles (HDPE).

5 Parafilm.

6 0.4 µm polycarbonate membrane filters.
Method

1. All plasticware in contact with soil samples and solutions should be acid washed (5% HCl) and rinsed with deionized water until the EC of the rinse water is close or equal to that of deionized water and is constant. If trace elements are of interest, plasticware should be prepared according to procedures outlined in Chapter 10.

2. Insert an HDPE frit into the base of the modified 60 mL syringe.

3. Place about 25 g of moist soil in the soil container (10 g if the soil is organic) and cover with parafilm to avoid evaporation during the centrifugation procedure. A subsample of each soil may be kept to determine the moisture.

4. Place the solution collecting cup under the syringe containing the soil in the centrifuge shield.

5. Centrifuge at a relative centrifugal force (RCF) of 1500 g at the bottom of the soil column for 30 min.

6. Set aside a portion of the solutions for analysis of pH and EC. Transfer the rest of the solution to clean storage bottles. Solutions may be further filtered using low vacuum through 0.4 μm polycarbonate filters before storage and analysis. A subsample for metal analysis should be acidified (0.2% HNO₃ v/v); trace metal-grade acid should be used if trace elements are to be analyzed.

7. Replicate all samples and include blanks.
17.6.2 Syringe Pressure Method (Ross and Bartlett 1990)

Materials
1. 60 mL polyethylene syringes
2. HDPE frits, 27 mm in diameter
3. Deionized H₂O
4. Compression apparatus (see Figure 17.5)
5. 0.4 μm polycarbonate membrane filters

Method
1. Wash HDPE frits with deionized H₂O.
2. Fit the HDPE frits into the bottom of the syringes.
3. Pack fresh soil samples (ideally within 12 h of sampling) into the polyethylene syringes.
5. Set aside a portion of the solutions for analysis of pH and EC. Transfer the rest of the solution to clean storage bottles. Solutions may be further filtered using low vacuum through 0.4 μm polycarbonate filters before storage and analysis.

FIGURE 17.5. The compression device for the syringe pressure extraction method (photo courtesy of Don Ross).
A subsample for metal analysis should be acidified (0.2% HNO₃ v/v); trace metal-grade acid should be used if trace elements are to be analyzed.

6 Replicate all samples and include blanks.

17.6.3 COMMENTS

1 In both methods, soil solutions should be separated from the soils as rapidly as possible after sampling. Soil samples should be kept cool (4°C in the dark but not frozen) before solutions are extracted. The time taken to separate the soil solution from the soil solid phase after the disturbance of taking the soil out of its natural environment is important in reducing sampling artifacts (Qian and Wolt 1990; Ross and Bartlett 1990).

2 The force of extraction during centrifugation can be calculated as the RCF:

\[
RCF = \frac{(2\pi n)^2 r}{g}
\]

where \( n \) is the number of revolutions per second, \( r \) the distance from the center of rotation in centimeters, and \( g \) is 981 cm s\(^{-2}\). The RCF is related to the size of pores (assumed to be capillary pores) drained by the centrifugal force. For example, pores of 1 μm diameter are drained at an RCF of roughly 1000 g (Edmunds and Bath 1976; Soon and Warren 1993). The force of extraction used in the syringe pressure method should also be measured and recorded to ensure comparable and consistent results.

3 Both methods will produce low volumes of solution (1–3 mL) and may require several replicates bulked together to produce enough solution for a range of solution analyses. Bulked solutions should also be replicated to provide a clear idea of the reproducibility of the procedure (i.e., if three extracted solutions are bulked together to produce a 5–10 mL sample; six solutions should be extracted to produce a replicate).

REFERENCES


Chapter 18
Ion Exchange and Exchangeable Cations

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18.1 INTRODUCTION

Soils possess electrostatic charge as a result of atomic substitution in the lattices of soil minerals (permanent charge) and because of hydrolysis reactions on broken edges of the lattices and the surfaces of oxides, hydroxides, hydrous oxides, and organic matter (pH-dependent charge). These charges attract counterions (exchangeable ions) and form the exchange complex. The principle of the methods used to measure exchangeable ions is to saturate the exchange complex with some ion that forces the exchangeable ions already present on the charged surfaces into solution (law of mass action). Exchange capacity can then be calculated as the sum of the individual cations displaced from the soil (summation method); or the ion used to saturate the exchange complex, termed the index ion, can be displaced with a concentrated solution of a different salt and the exchange capacity calculated as the amount of the index ion displaced (displacement method).

The cation-exchange capacity (CEC) is a measure of the amount of ions that can be adsorbed, in an exchangeable fashion, on the negative charge sites of the soil (Bache 1976). The results are commonly expressed in centimoles of positive charge per kilogram of soil (cmol(+) kg\(^{-1}\)). Anion-exchange capacity (AEC) is expressed in terms of negative charge (cmol(−) kg\(^{-1}\)). In most Canadian soils, CEC is much greater than AEC; as a result, in most routine soil analysis, only CEC and exchangeable cations are measured.

The measurement of CEC is complicated by (1) errors due to the dissolution of soluble salts, CaCO\(_3\), and gypsum (CaSO\(_4\) · H\(_2\)O); (2) specific adsorption of K and NH\(_4\) in the interlayer position in vermiculites and micas (including illite or hydrous mica); and (3) the specific adsorption of trivalent cations such as Al\(^{3+}\) or Fe\(^{3+}\) on the surface of soil particles.
In general, the errors can be reduced by using a method of CEC determination that employs reagents of similar concentration and pH to those of the soil to be analyzed. For this reason a method buffered at pH 7.0 or 8.2 using relatively high concentrations of saturating and extracting solutions will decrease errors due to dissolution of CaCO₃ and gypsum in soils from arid regions (Thomas 1982). In acidic soils, solutions buffered at pH 7.0 or 8.2 are less effective in replacing trivalent cations and an unbuffered method will provide a better estimate of the CEC and exchangeable cations.

Methods using a solution at a buffered pH are commonly used with agricultural soils providing a measurement that is independent of recent fertilization and liming practices. For forest soils and other low pH soils, it is often preferable to measure CEC at the pH of the soil (see Section 18.2), thus providing a more accurate measure of exchangeable cations and CEC under field conditions.

Soils containing appreciable amounts of amorphous materials (e.g. podzols, some brunisols, and soils containing volcanic ash) will show order of magnitude changes in CEC and AEC as a result of acidification or liming. The method for measuring pH-dependent CEC and AEC (see Section 18.3) is provided for those who wish to study the variation in charge properties as a function of pH. The method provides more useful information than does the potentiometric titration method. Although both can be used to give an estimate of the point of zero charge (PZC), the pH-dependent CEC and AEC method also provides a measure of the absolute amount of exchange capacity at any pH.

**18.2 EXCHANGEABLE CATIONS AND EFFECTIVE CEC BY THE BaCl₂ METHOD (HENDERSHOT AND DUQUETTE 1986)**

The BaCl₂ method provides a rapid means of determining the exchangeable cations and the “effective” CEC of a wide range of soil types. In this method CEC is calculated as the sum of exchangeable cations (Ca, Mg, K, Na, Al, Fe, and Mn). The method is particularly applicable in forestry or studies of environmental problems related to soils where information on the CEC at the pH of the soil in the field is of prime importance. In soils with large amounts of pH-dependent cation-exchange sites, the value measured at pH 7 will be considerably higher than that measured by this method. Problems may arise if this method is used with saline soils containing very high levels of SO₄ since BaSO₄ will precipitate.

This method has been compared to other methods of determining the CEC at the soil pH and provides comparable results (Hendershot and Duquette 1986; Ngewoh et al. 1989). Barium is a good flocculant and is able to displace trivalent cations. The relatively low ionic strength of the equilibrating solution causes a smaller change in pH than do more concentrated salt solutions. This method is simple and rapid; however, it is recommended that exchangeable iron and manganese be measured since they may be more abundant in some acidic soils than other commonly considered cations such as potassium and sodium.

The Expert Panel on Soil (2003) proposes an alternative method that involves three successive additions of 0.1 M BaCl₂. The soil:solution ratio of 1:60 and the successive shaking and decanting steps result in higher measured values of exchangeable cation. However, the more complicated procedure is less suitable for routine laboratory analysis. Since the method proposed in this chapter for measuring exchangeable cations uses 0.1 M BaCl₂, it seems more appropriate to use the same salt solution for measuring exchangeable acidity.
Jonsson et al. (2002) have determined regression equations that could be used to estimate the difference between the two extraction procedures.

The results of this method are dependent on the soil:solution ratio used, with higher values of exchangeable cations obtained with smaller amounts of soil. The suggested weights of soil are a reasonable compromise. We have decreased the maximum amount of soil to be used from 3.0 to 1.5 g compared to the previously published methodology (Hendershot et al. 1993). If results are to be compared over time, or between sites, it is important that standard weights of sample be used.

### 18.2.1 Materials and Reagents

1. Centrifuge tubes (50 mL) with screw caps and low-speed centrifuge.
2. End-over-end shaker.
3. Barium chloride, 0.1 M: dissolve 24.43 g of BaCl$_2$·2H$_2$O with double deionized (d.d.) water and make to volume in a 1 L volumetric flask.
4. Standards of Ca, Mg, K, Na, Al, Fe, and Mn are prepared using atomic absorption reagent-grade liquid standards of 1000 mg L$^{-1}$. The matrix in the standards must correspond to the BaCl$_2$ concentration of the analyzed sample (diluted or non-diluted matrix).
5. Lanthanum solution, 100 mg L$^{-1}$: dissolve 53.5 g of LaCl$_3$·7H$_2$O in a 200 mL volumetric flask and make to volume (for analysis by atomic absorption spectrophotometry [AAS]).
6. Cesium solution, 100 g L$^{-1}$: dissolve 25.2 g CsCl in a 200 mL volumetric flask and make to volume (for analysis by AAS).

### 18.2.2 Procedure

1. Weigh out about 0.5 g of air-dry (<2 mm) organic soil or fine-textured soil to 1.5 g of coarse-textured soil into a 50 mL centrifuge tube. Record the exact weight of soil used to the nearest 0.001 g. Include blanks, duplicates, and quality control samples.
2. Add 30.0 mL of 0.1 M BaCl$_2$ to each tube and shake slowly on an end-over-end shaker (15 rpm) for 2 h.
3. Centrifuge (15 min, 700 g) and filter the supernatant (SN) with Whatman No. 41 filter paper.
4. Analyze the following cations in the SN solution with an AAS or any other suitable instrument: Ca, Mg, K, Na, Al, Fe, and Mn. Dilution (10- or 100-fold) is usually required for Ca, K, and Mg. The addition of 0.1 mL of La solution and 0.1 mL of Cs solution to a 10 mL aliquot of diluted extract is required for the determination of Ca, Mg, and K by AAS. (For detailed instructions on this and other aspects of analysis refer to the manual for your AAS.) Preservation
of samples by acidifying to 0.2% HNO₃ will prevent the loss of metals, such as Fe and Al.

5. If desired, the pH of the equilibrating solution can be measured on a separate aliquot of the BaCl₂ solution before filtering. Leakage of K from the KCl salt bridge of the pH electrode is significant and therefore the same aliquot cannot be used for K analysis and pH measurement.

### 18.2.3 Calculations

1. Exchangeable cations

   \[ M^+ \text{ cmol(+)} \text{ kg}^{-1} = C \text{ cmol(+)} \text{ L}^{-1} \times (0.03 \text{ L/wt. soil g}) \times 1000 \text{ g kg}^{-1} \times DF \]  
   \[ (18.1) \]

   where \( M^+ \) is the concentration of an adsorbed cation, \( \text{cmol(+)} \text{ kg}^{-1} \), \( C \) is the concentration of the same cation measured in the BaCl₂ extract (\( \text{cmol(+)} \text{ L}^{-1} \)), and \( DF \) is the dilution factor, if applicable.

2. Effective CEC

   \[ \text{Effective CEC} \text{ cmol(+)} \text{ kg}^{-1} = \Sigma M^+ \text{ cmol(+)} \text{ kg}^{-1} \]  
   \[ (18.2) \]

3. Percent base saturation

   \[ \% \text{ BS} = (\Sigma \text{Ca} + \text{Mg} + \text{Na} + \text{K}/\text{Effective CEC}) \times 100 \]  
   \[ (18.3) \]

### 18.2.4 Comments

1. A large amount of a soil similar to the samples being analyzed should be kept as an indicator of the variability of results over time; duplicate subsamples of this quality control (QC) sample should be run with each batch of samples measured. Failure of the QC to fall within acceptable limits means that the whole batch should be reanalyzed. Analysis of QC samples is also useful to verify that samples analyzed by different people in the same laboratory are comparable, and that results do not change from one year to another or from one batch of chemicals to another.

2. For the sake of simplicity AAS standards are usually made up by diluting 1000 mg L⁻¹ concentrate to lower concentration values suitable for the range of the instrument being used. Calibrate the machine using the corresponding cmol(+) L⁻¹ value; the conversion values are as follows:

   - 1 mg L⁻¹ Ca = 5.00 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ Mg = 8.23 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ K = 2.56 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ Na = 4.35 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ Al = 11.11 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ Fe = 1.79 × 10⁻³ cmol(+) L⁻¹;
   - 1 mg L⁻¹ Mn = 3.64 × 10⁻³ cmol(+) L⁻¹.
18.3 pH-DEPENDENT AEC–CEC (FEY AND LeROUX 1976)

In the literature, the method of Fey and LeRoux (1976) is often cited in research on pH-dependent CEC and AEC. The method is time-consuming because of the multiple saturation and pH adjustment steps. An alternative is to add different amounts of acid or base to the soil suspensions and measure the resulting pH. This method is preferred because there are fewer steps, and therefore it is faster with less chance of errors due to contamination or loss of soil. The only disadvantage with the modified procedure is that it is more difficult to obtain an even distribution of pH values than with the method of Fey and LeRoux, but this can be corrected by rerunning the analysis and adjusting the amounts of HNO₃ or Ca(OH)₂ added.

18.3.1 MATERIALS AND REAGENTS

1. Centrifuge tubes (50 mL) with screw caps and low-speed centrifuge.
2. Vortex centrifuge tube mixer and end-over-end shaker.
3. Calcium nitrate, 0.05 M: dissolve 23.62 g of calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O) with d.d. water in a 2 L volumetric flask.
4. Nitric acid, 0.1 M: dilute 6.3 mL of concentrated nitric acid (HNO₃) with d.d. water in a 1 L volumetric flask.
5. Calcium hydroxide, 0.05 M: dissolve 3.70 g of calcium hydroxide (Ca(OH)₂) with d.d. water in a 1 L volumetric flask, and filter through a Whatman No. 41 filter (a prefiltration step can be done using a glass microfiber filter [Whatman GF/C]).
6. Calcium nitrate, 0.005 M: dilute 200 mL of 0.05 M Ca(NO₃)₂ solution with d.d. water in a 2 L volumetric flask.
7. Potassium chloride, 1.0 M: dissolve 149.12 g of potassium chloride (KCl) with d.d. water in a 2 L volumetric flask.
8. Lanthanum solution, 100 mg L⁻¹: dissolve 53.5 g of LaCl₃·7H₂O in a 200 mL volumetric flask and make to volume (for analysis by AAS).
9. Cesium solution, 100 g L⁻¹: dissolve 25.2 g CsCl in a 200 mL volumetric flask and make to volume (for analysis by AAS).

18.3.2 PROCEDURE

1. Weigh 20 empty 50 mL centrifuge tubes to the nearest 0.001 g (one set of 20 tubes for each soil sample to be analyzed).
2. Add 1.0 g subsamples of air-dry <2 mm soil to each tube and record the weight of the tube plus soil to the nearest 0.001 g. The analysis is done in duplicate for each targeted pH and corresponds to one pair of quality control samples per batch. If moist soil is used, start by weighing out four additional samples into small beakers and air-dry to determine the weight of moist soil equivalent to 1 g of air-dried soil.
Add 25 mL 0.05 M Ca(NO₃)₂ solution, cap the tubes, and shake for 1 h using an end-over-end shaker (15 rpm).

Centrifuge (10 min, 700 g) and discard SN by decantation. Be careful to avoid loss of soil during decantation.

Add a new 25 mL aliquot of 0.05 M Ca(NO₃)₂ solution to each tube. Then add 0, 0.25, 0.5, 1.0, or 2.5 mL of 0.1 M HNO₃ to tubes in duplicate, and finally add 0.25, 0.5, 1.0, or 2.5 mL of 0.05 M Ca(OH)₂ to the remaining tubes in duplicate. Add 1.0 mL of 0.1 M HNO₃ or 0.05 M Ca(OH)₂ to the quality control sample. A vortex mixer is useful to resuspend the soil after addition of the solution.

Cap and shake overnight on an end-over-end shaker.

Centrifuge (10 min, 700 g) and discard SN.

Resuspend the soil in 25 mL of 0.005 M Ca(NO₃)₂, centrifuge (10 min, 700 g), and discard SN.

Repeat step 8, but measure pH in a separate aliquot of the SN and keep the remaining SN for the analysis of Ca and NO₃ (after 100-fold dilution with d.d. water). Weigh tubes plus the soil and the interstitial soil solution.

Add 25 mL of 1.0 M KCl, shake for 1 h, and centrifuge (10 min, 700 g).

Keep this SN for determination of displaced Ca and NO₃. Dilute this KCl extract 10-fold with d.d. water.

Measure Ca by AAS in the 10-fold diluted KCl extract (saved in step 11) and in the 0.005 M Ca(NO₃)₂ equilibration solution (saved in step 9). The addition of 0.1 mL of La solution and 0.1 mL of Cs solution to a 10 mL aliquot of diluted extract is required for the determination of Ca by AAS. (For detailed instructions on this and other aspects of analysis refer to the AAS manual.)

Measure NO₃ in the undiluted KCl extract (saved in step 11) and in the diluted 0.005 M Ca(NO₃)₂ equilibration solution (saved in step 9).

**18.3.3 Calculations**

1. Residual Ca and NO₃

   a. Volume of interstitial solution

   Subtract the weight of the empty tube with the soil (step 2) from weight measured in step 9 to calculate weight of residual 0.005 M Ca(NO₃)₂ solution (Volres). Assume 1 g equals 1 mL.

   b. Residual amount of Ca and NO₃(Cares and NO₃ res): 

      \[ \text{Ca}_{\text{res}} \text{ (mol)} = \text{Vol}_{\text{res}} \text{ (mL)} \times \text{Ca}_{\text{sol}} \text{ (mM)} \times 0.001 \text{ (L mL}^{-1}) \times \text{DF} \]
\[ \text{NO}_3_{\text{res}} \text{(mol)} = \text{Vol}_{\text{res}} \text{(mL)} \times \text{NO}_3_{\text{sol}} \text{(mM)} \times 0.001 \text{ (L mL}^{-1}) \times \text{DF} \quad (18.5) \]

where \( \text{Ca}_{\text{sol}} \) and \( \text{NO}_3_{\text{sol}} \) are the measured concentrations of calcium and nitrate in the 0.005 \( \text{M Ca(NO}_3)_2 \) wash solution saved in step 9 (units in mM) and \( \text{DF} \) is the dilution factor if applicable.

2 Total amount of calcium and nitrate (\( \text{Ca}_t \text{NO}_3_{t} \)) in the KCl extract (including the residual):

\[ \begin{align*}
\text{Ca}_t \text{(mmol)} &= \text{Ca}_{\text{KCl}} \text{(mM)} \times 25 \text{ (mL)} \times 0.001 \text{ (L mL}^{-1}) \times \text{DF} \\
\text{NO}_3_{t} \text{(mmol)} &= \text{NO}_3_{\text{KCl}} \text{(mM)} \times 25 \text{ (mL)} \times 0.001 \text{ (L mL}^{-1}) \times \text{DF} 
\end{align*} \quad (18.6) \]

where \( \text{Ca}_{\text{KCl}} \) and \( \text{NO}_3_{\text{KCl}} \) are the calcium and nitrate concentrations (mM) in the KCl extract saved in step 11; and \( \text{DF} \) is the dilution factor if applicable.

3 Calculation of the CEC and AEC:

\[ \begin{align*}
\text{CEC cmol(±) kg}^{-1} &= (\text{Ca}_t - \text{Ca}_{\text{res}}) \text{(mmol)} \times 0.2 \text{ (cmol(±) mmol}^{-1}) \\
&\quad \times 1000 \text{ (g kg}^{-1})/\text{wt.soil (g)} \\
\text{AEC cmol(±) kg}^{-1} &= (\text{NO}_3_{t} - \text{NO}_3_{\text{res}}) \text{(mmol)} \times 0.1 \text{ (cmol(±) mmol}^{-1}) \\
&\quad \times 1000 \text{ (g kg}^{-1})/\text{wt.soil (g)}
\end{align*} \quad (18.7) \]

4 Plot CEC and AEC as a function of final equilibrium pH measure in step 9 of Section 18.3.2.

**18.4 EXCHANGEABLE CATIONS AND TOTAL EXCHANGE CAPACITY BY THE AMMONIUM ACETATE METHOD AT pH 7.0 (LAVKULICH 1981)**

The method described here was developed by Lavkulich (1981) for standard analysis of a wide range of soil types. It involves fewer steps than some other similar methods such as that of McKeague (1978). Problems with this approach to measuring exchangeable cations and CEC have been discussed extensively in the literature (Chapman 1965; Bache 1976; Rhoades 1982; Thomas 1982) but we agree with the conclusion of Thomas (1982) that “there is no evidence at the present time that cations other than \( \text{NH}_4^+ \) give results that are less arbitrary than those obtained using \( \text{NH}_4^+ \).”

Errors due to the dissolution of CaCO\(_3\) and gypsum will result in an excess of Ca\(^{2+}\) being extracted by \( \text{NH}_4^+ \) and a decrease in the amount of \( \text{NH}_4^+ \) retained due to competition between Ca\(^{2+}\) and \( \text{NH}_4^+ \) during equilibration in the saturating step. In soils containing these minerals, exchangeable Ca will be too high and total CEC too low. The former problem can not easily be corrected (Thomas 1982); however, more accurate measurement of CEC in this type of soil can be obtained by using the method described by Rhoades (1982).

Fixation of \( \text{K}^+ \) and \( \text{NH}_4^+ \) in phyllosilicates can result in either an over- or underestimation of exchangeable \( \text{K}^+ \) when \( \text{NH}_4^+ \) is used as an extractant depending on whether the \( \text{NH}_4^+ \) moves through the interlayer positions replacing the \( \text{K}^+ \) or whether it causes the collapse of the edges preventing further exchange.
Compared to the other methods presented in this chapter, this method uses a larger sample size, which helps to decrease the sample to sample variability. Another advantage of this procedure is that there are no decantation steps that can cause the loss of sample, particularly in the case of organic soils.

The method described below can be used to measure either exchangeable cations and CEC or just exchangeable cations. In the latter case, the sum of exchangeable cations (including Al) could be used as an estimate of CEC. Due to the high pH of the extracting solution, the amount of Al measured will usually be lower than that displaced by BaCl₂ or KCl.

18.4.1 MATERIALS AND REAGENTS

1. Centrifuge tubes: 100 mL centrifuge tubes and stoppers.
2. Reciprocal shaker.
3. Buchner funnels (55 mm diameter) and 500 mL filtering flasks connected to low-pressure vacuum line.
4. Ammonium acetate, 1 M: dissolve 77.08 g of NH₄OAc with d.d. water and make to volume in a 1 L volumetric flask. Adjust pH to 7.0 with ammonium hydroxide or acetic acid.
5. Isopropanol.
6. Potassium chloride, 1 M: dissolve 74.6 g of KCl with d.d. water and make to volume in a 1 L volumetric flask.
7. Standard ammonium solution, 200 mg L⁻¹ N: dissolve 0.238 g of (NH₄)₂SO₄ (dried for 3–4 h at 40°C) in about 100 mL of d.d. water and then dilute to volume in a 250 mL volumetric flask. Prepare diluted standards of 10, 20, 40, and 80 mg L⁻¹ from the 200 mg L⁻¹ stock.
8. Prepare Ca, Mg, K, and Na standards using 1 M NH₄OAc as the matrix.

18.4.2 PROCEDURES

For Exchangeable Cations

1. a. For samples low in organic matter: weigh out 10.000 g of soil into a 100 mL centrifuge tube.
   b. For samples high in organic matter: weigh out 5.000 or 2.000 g.
   c. Prepare a blank and include a quality control sample.

2. Add 40 mL of 1 M NH₄OAc to the centrifuge tube. Stopper the tube and shake for 5 min on a reciprocal shaker (115 rpm). Remove tubes from shaker, agitate to rinse down soil adhering to the sides of the tube, and let stand overnight.
Shake tube again for 15 min. Prepare Buchner funnels with Whatman No. 42 filter paper and place them above 500 mL filtering flasks.

Transfer contents of the tube to the funnel with suction applied. Rinse the tube and the stopper with 1 M NH₄OAc from a wash bottle.

Wash the soil in the Buchner funnel with four 30 mL portions of 1 M NH₄OAc. Let each portion drain completely before adding the next, but do not allow the soil to become dry or cracked.

Transfer the leachate to a 250 mL volumetric flask; rinse the filtering flask with 1 M NH₄OAc and make up to volume with 1 M NH₄OAc. Mix well and save a portion of the extract for analysis of Al, Ca, Mg, K, and Na. Keep samples refrigerated prior to analysis.

**For Total-Exchange Capacity (CEC)**

Replace the funnels containing the ammonium-saturated soil onto the filtering flasks. To remove the residual NH₄OAc from the soil, wash the soil in the Buchner funnel with three 40 mL portions of isopropanol, again letting each portion drain completely before adding the next (turn off the suction after the last washing before the soil dries out). Discard the isopropanol washings and rinse the flask well with tap water followed by d.d. water.

Replace the funnels onto the flasks and leach the soil with four 50 mL portions of 1 M KCl, again letting each portion drain completely before adding the next. Transfer the leachate to a 250 mL volumetric flask. Rinse the filtering flask into the volumetric flask with d.d. water and make up to volume with d.d. water. Mix well and save a portion of the extract for analysis of NH₄ by auto analyzer.

### 18.4.3 Calculations

1. **Exchangeable cations:**

   \[ M^+ \text{ cmol}(+) \text{ kg}^{-1} = C \text{ cmol}(+) \text{ L}^{-1} \times (0.25 \text{ L/wt soil g}) \times 1000 \text{ g kg}^{-1} \]  
   \[ (18.9) \]

   where \( M^+ \) is the concentration of adsorbed cation, cmol(+) kg⁻¹; and \( C \) is the concentration of cation in the NH₄OAc extract (cmol(+) L⁻¹).

   Note: see Section 18.2.4 for conversion of mg L⁻¹ to cmol(+) L⁻¹.

2. **CEC:**

   \[ \text{CEC cmol}(+) \text{ kg}^{-1} = (\text{mg L}^{-1} \times (1 \text{ cmol}(+)/140 \text{ mg})) \times (0.25 \text{ L/wt.soil g}) \times 1000 \text{ g kg}^{-1} \]  
   \[ (18.10) \]
REFERENCES


Chapter 19
Nonexchangeable Ammonium

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19.1 INTRODUCTION

It has been known since the early part of the twentieth century that some types of soils have the ability to bind ammonium (to certain types of clay minerals, predominantly vermiculite and mica types) such that it is not readily recovered by extraction with dilute acid or alkali (McBeth 1917). This form of ammonium is referred to as fixed or nonexchangeable ammonium (NEA). Barshad (1951) proposed that fixed ammonium should be defined as ammonium that is not displaceable with prolonged extraction or leaching of soil with potassium salt solution. The proportion of soil N as NEA usually does not exceed 10% in surface soils, but it can increase with depth of soil to over 50% in some subsoil horizons (Hinman 1964; Bremner 1965). Sources of NEA in the soil include (i) \( \text{NH}_4^+ \) produced by mineralization of organic matter, and added through ammoniacal-N fertilizer material, and (ii) indigenous or native fixed ammonium found in parent rock materials. There is considerable interest in quantifying the NEA pool because the amount in the soil through the rooting depth can be considerable, and its availability to plants and microorganisms has been demonstrated in many studies (Kudeyarov 1981; Scherer 1993; Green et al. 1994; Scherer and Werner 1996; Soon 1998). Soderland and Svensson (1976) estimated that there is as much fixed \( \text{NH}_4^+ \)-N as there is plant biomass N in the global soil–plant system. The NEA pool in the soil has been found to be a slow-release reservoir of available ammonium when the exchangeable \( \text{NH}_4^+ \) levels become depleted (Drury and Beauchamp 1991). Ammonium fixation and release must be characterized and quantified especially in soils with a high ammonium fixation capacity (i.e., soils with a high vermiculite or mica content) in order to efficiently manage N use in soils for agronomic and environmental reasons.

Several procedures have been developed for the determination of NEA (Young and Aldag 1982); however, the most widely accepted method is that of Silva and Bremner (1966). Bremner et al. (1967) evaluated several methods and found that all except the Silva and Bremner method have defects: (i) the pretreatments used to eliminate interference by organic...
N compounds were either inefficient, or led to gain or loss of NEA, and (ii) the procedures used to release NEA were not quantitative, or led to formation of NH$_4$-N from organic N compounds. According to Keeney and Nelson (1982), the Silva–Bremner method enjoys widespread use because of its apparent lack of defects; however, they cautioned that “there is no way of establishing that the KOBr–HF method is accurate . . .”. Bremner et al. (1967) mentioned two possible problems associated with the determination of NEA: intercalated organic materials containing N that are released by the HF treatment and the presence of metal ammonium phosphates that are not soluble in KOBr or KCl but soluble in HF. Although either one will result in an overestimation of NEA, under normal conditions the contribution of either one would be remote or very slight. The Silva–Bremner procedure involves and comprises three basic steps: (i) removal of exchangeable NH$_4^+$ cations, (ii) oxidation and removal of organic matter including organic N, and (iii) extraction of NEA with HF and HCl, and determination of the released NH$_4^+$. A slightly and a substantially modified version of the method will be described below.

Zhang and Scherer (1998) proposed a simplified version (method A) of the Silva–Bremner method, which reduced the time involved and the amount of reagents used. A more substantial modification that eliminated entirely the HF extraction step (method B) was proposed by Nieder et al. (1996) and Liang et al. (1999): here, NEA in the soil residue left from the KOBr and KCl extractions is determined directly by dry combustion in an automated N-analyzer. This is a major advancement for the procedure because it eliminates the hazardous HF extraction step and the subsequent disposal of the HF, saving time in the process by reducing the number of steps in the procedure. Nitrogen isotope ratios can also be very conveniently determined when the N-analyzer is connected by a continuous flow linkage to a $^{15}$N/$^{14}$N isotope ratio mass spectrometer.

### 19.2 POTASSIUM HYPOBROMITE–HYDROFLUORIC ACID EXTRACTION

The procedure described below is an adaptation of the Silva and Bremner (1966) method by Zhang and Scherer (1998). In this variation of the method, organic matter in the sample is oxidized in a centrifuge tube immersed in a boiling water bath (instead of a beaker heated with a hot plate) and subsequent extraction steps are carried out without having to transfer the residual soil to a centrifuge tube. Zhang and Scherer (1998) also found that heating in a microwave oven (1150 watt) at 50% of full power for 10 min gave similar NEA values to heating in a boiling water bath. Use of microwave ovens of different power would likely require adjustments by trial and error. The method using a boiling water bath is described.

#### 19.2.1 MATERIALS AND REAGENTS

1. 50 mL polypropylene or polyethylene centrifuge tubes with lined screw caps.

2. Potassium hydroxide (KOH) solution, 2 M: Dissolve 112.2 g of KOH in approximately 600 mL of distilled deionized water and, after cooling, dilute to 1 L volume.

3. Potassium hypobromite (KOBr) solution, prepared immediately before use: Add 6 mL of Br to 200 mL of 2 M KOH solution. Add the Br slowly (approximately 0.5 mL min$^{-1}$) with constant stirring, keeping the KOH solution cool in an ice-bath during the addition.
4 0.5 M Potassium chloride (KCl) solution: Dissolve 149 g of KCl in 600 mL of deionized water and make up to 4 L.

5 Hydrofluoric acid–hydrochloric acid solution (approximately 5 M HF–1 M HCl): With a 1 L measuring cylinder, transfer 1.5 L of deionized water to a 2 L graduated polypropylene or polyethylene conical flask. Add slowly, with continuous stirring, 167 mL of conc. HCl (specific gravity 1.19) followed by 325 mL of approximately 52% HF (approximately 31 M). Dilute with deionized water up to the 2 L mark and mix well.

6 Boiling water bath.

7 Reciprocal shaker.

19.2.2 EXTRACTION PROCEDURE

1 Weigh 0.5 g of finely ground soil (<60 mesh) in a 50 mL centrifuge tube. Record the weight of tube and soil. Add 10 mL of KOBr solution, and screw the cap on. Invert the centrifuge tube several times to mix up the contents, loosen screw cap, and leave it standing for 2 h. In the mean time, heat up the water bath.

2 Place the centrifuge tubes in a rack and then immerse the rack in a boiling water-bath so that the water level in the water bath exceeds the level of the KOBr solution in the centrifuge tube. Once the solution in the centrifuge tubes starts to boil, allow it to continue boiling for 10 min.

3 Remove the tubes and allow the contents to cool and settle. If necessary, centrifuge at 1000 g for 5 min.

4 Decant and discard the clear supernatant solution.

5 Add 30 mL of 0.5 M KCl, suspend the soil by shaking for 5 min, and centrifuge at 1000 g for 5 min. Decant the clear supernatant solution.

6 Repeat step 5 two more times.

7 Weigh the centrifuge tube and soil. The increase over the initial weight (in step 1) is taken to represent the volume of KCl retained by the soil. This liquid volume has to be added to the acid reagent volume added in step 8 when calculating mg kg⁻¹ soil of NEA.

8 Add 10 mL of 5 M HF–1 M HCl working solution and shake for 24 h on a reciprocal shaker at 120 cycles min⁻¹. If the sample contains carbonates, allow the evolved CO₂ to escape before starting the overnight shaking.

9 Centrifuge at 1000 g for 5 min. Decant the clear supernatant solution into a plastic vial for subsequent NH₄-N determination.
19.2.3 Determination of Extracted NH$_4$-N

The original Silva–Bremner method determines nonexchangeable NH$_4$-N in the acid extractant by steam distillation and subsequent titrimetry. However, colorimetric determination using the development of indophenol has been used by Doram and Evans (1983) and Soon (1998). A manual procedure is outlined below, which is easily adaptable for automated analysis. The autoanalyzer method outlined in Chapter 6 for exchangeable NH$_4$-N determination or the procedure described by Kempers and Zweers (1986) can be readily adapted for analysis of NEA.

Reagents

Unless specified otherwise, all reagents used must be of analytical grade.

1. Trisodium citrate solution: Dissolve 20.0 g of Na$_3$C$_6$H$_8$O$_7$·2H$_2$O in 700 mL of deionized water. Dissolve 10.0 g of NaOH in deionized water and dilute to 700 mL. Combine the citrate and NaOH solution (reagent A).

2. Salicylate–nitroprusside reagent: Dissolve 18.0 g of sodium salicylate (HOC$_6$H$_4$CO$_2$Na, 2-hydroxybenzoic acid, sodium salt) in 250 mL of water. Dissolve 0.20 g of sodium nitroprusside (Na$_2$Fe(CN)$_5$NO·2H$_2$O) in 250 mL of water. Combine the two reagents and store in a brown bottle (reagent B).

3. Alkaline hypochlorite solution: Dissolve 1.5 g of NaOH in 50 mL of deionized water, add 8 mL of sodium hypochlorite (5%–5.25% NaOCl), and dilute to 100 mL (reagent C). Prepare fresh as needed.

4. Ammonium standard solution: 0, 5, 10, 15, 20, and 25 µg NH$_4$-N mL$^{-1}$ in 5 M HF–1 M HCl prepared by dilution of 1000 mg N L$^{-1}$ stock solution.

Procedure

1. Pipette 0.2 mL of the HF–HCl soil extract or ammonium standard solutions (containing up to 5 µg NH$_4$-N) into a 16 mm × 125 mm culture tube.

2. Add 7 mL of reagent A and mix immediately.

3. Add 2 mL of reagent B and mix immediately.

4. Add 0.5 mL of reagent C and mix immediately.

5. Immediately cover with a dark colored plastic sheet and leave for 60 min for color to develop.

6. Measure absorbance of standard and test solutions at 660 nm using 1 cm cuvette.

7. The concentrations of NH$_4$-N in the test solutions are read off the calibration curve, either manually or by the processor in the spectrophotometer.
Results can be calculated as follows:

\[
\text{mg NEA kg}^{-1}\text{soil} = \mu\text{g NH}_4^-\text{N per mL extract} \times (10 + \text{increase in weight in step 7 of Section 19.2.2}) \\
\times F/\text{weight of soil} \tag{19.1}
\]

where \( F \) is the dilution factor if dilution of the extract is required, and weight is measured in grams.

Comments

1. A final solution pH of about 13 will result in maximum color development. The advantages of salicylate as a substitute for phenol are increased sensitivity, lower toxicity, and increased stability (Kempers and Zweers 1986). Sodium citrate was found to be a better complexing agent for removing interfering elements than either EDTA or potassium sodium tartrate (Willis et al. 1993).

2. If the automated procedure is to be used, the following steps should be taken to minimize the slow corrosion of glass elements of the analytical cartridge. An analytical cartridge with dialyzer is used to further dilute the fluoride concentration in the test solution. The wash solution used need not contain hydrofluoric acid, and this does not influence the baseline: its acidity is maintained using 6 M HCl.

19.3 POTASSIUM HYPOBROMITE–DRY SOIL COMBUSTION METHOD

This major modification of the Silva and Bremner (1966) method was proposed by Nieder et al. (1996). However, the procedure gained greater prominence only after more extensive testing and validation by Liang et al. (1999). The method follows the Silva–Bremner method from the oxidation and removal of organic materials through the removal of exchangeable NH\(_4^+\) cations. It is assumed that any NH\(_4^+\) not removed from the soil at this stage would be nonexchangeable or fixed. This N fraction is then determined by dry (Dumas) combustion of the sample using an automated N-analyzer. Liang et al. (1999) showed that dry combustion recovered 100% of fixed NH\(_4^+\) and gave results similar to those obtained using the full Silva–Bremner method. The coefficient of variation for 17 soils was 6.4% for the full Silva–Bremner method and 2.0% for the modified version.

19.3.1 MATERIALS AND REAGENTS

1. 50 mL polypropylene or polyethylene centrifuge tubes with lined screw caps.

2. Potassium hydroxide (KOH) solution, 2 M: Dissolve 112.2 g of KOH in approximately 600 mL of distilled deionized water and, after cooling, dilute to 1 L volume.

3. Potassium hypobromite (KOBr) solution, prepared immediately before use: Add 6 mL of Br to 200 mL of 2 M KOH solution. Add the Br slowly (approximately 0.5 mL min\(^{-1}\)) with constant stirring, keeping the KOH cool in an ice-bath during the addition.
0.5 M Potassium chloride (KCl) solution: Dissolve 149 g KCl in 600 mL of deionized water and make up to 4 L.

Boiling water bath.

Reciprocal shaker.

19.3.2 Procedure

1. Weigh 0.5 g of finely ground soil (100 mesh) into a centrifuge tube. Record the weight of tube and soil. Add 10 mL of KOBr solution and screw the cap on. Invert centrifuge tube several times to mix up contents, loosen screw cap, and allow to stand for 2 h. In the mean time, heat the water bath.

2. Place the centrifuge tubes in a rack and then immerse the rack in a boiling water bath so that the water level in the water bath exceeds the level of the KOBr solution in the centrifuge tube. Once the solution in the centrifuge tubes starts to boil, allow it to continue boiling for 10 min.

3. Remove the tubes and allow the contents to cool and settle. Centrifuge at 1000 g for 5 min if necessary.

4. Decant and discard the clear supernatant solution.

5. Add 30 mL of 0.5 M KCl, suspend the soil by shaking for 5 min, centrifuge at 1000 g for 5 min. Decant the clear supernatant solution.

6. Repeat step 5 two more times.

7. Dry residue overnight in a drying oven at 105°C or freeze-dry the residue.

8. Weigh dry residue. (Let this weight be $Y$ g.)

19.3.3 Determination of NEA in the Residue

Follow the procedure for the elemental N-analyzer that is to be used. Samples (normally 50–100 mg) are weighed in tin foil sample cups which are loaded into autosamplers. Combustion of the samples with oxygen at 1030°C converts NEA to N$_2$ and NO$_x$ gases (with other combustion products). These gases are routed to a reduction furnace containing heated Cu, which removes excess oxygen and converts NO$_x$ to N$_2$, which is separated by gas chromatography and the concentration of N$_2$ is measured using a thermal conductivity detector (for more on dry combustion for N determination see Chapter 22). The analyzer is calibrated with certified standards.

Since the determination is done on a sample that is free of organic matter, a correction is needed to convert the analytical result to a whole soil basis. Suppose that (i) the instrument determines NEA to be Z% of residue, and (ii) the weight of the dry residue (in grams) as determined in step 8 is $Y$. Multiply Z% by 10 to convert percent to milligram per gram or gram per kilogram basis. Since the weight of the original whole soil samples is 0.5 g, the corrected value of NEA in the original soil (in milligram per kilogram) = $Z \times 10 \times Y/0.5$. 

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19.3.4 Comments

The method has several advantages of the original Silva and Bremner (1966) method. The use of an automated N-analyzer for the determination of NEA in the soil residue (i) eliminates the use and handling of hydrofluoric acid, (ii) increases the precision of the method, and (iii) simplifies and simultaneously allows the determination of N isotopic ratios by linking an isotopic ratio mass spectrometer to the elemental analyzer. It also shortens the time required for analysis.

REFERENCES


Chapter 20
Carbonates

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20.1 INTRODUCTION

Inorganic carbon occurs in soils commonly as the carbonate minerals calcite (CaCO₃), dolomite (CaMg(CO₃)₂), and magnesian calcites (Ca₁₋ₓMgₓCO₃). Other less common forms are aragonite (CaCO₃) and siderite (FeCO₃). Carbonate in soils can be of primary (inherited from parent material) or secondary (pedogenic) origin. Secondary carbonates are usually aggregates of silt- and clay-sized calcite crystals that are easily identified in grain mounts. Larger crystals of calcite or dolomite are of primary origin (Doner and Lynn 1989). Once routinely reported by sedimentologists, the qualitative and quantitative determination, especially of Ca and Mg carbonates, is useful in studies of soil genesis and classification, and micronutrient and phosphorus sorption. Furthermore, soil carbonates affect root and water movement, soil pH (Nelson 1982), and the nature of the exchange complex (St. Arnaud and Herbillon 1973). The variability in topsoil carbonate content due to incorporation of subsoil calcite and dolomite has been used successfully to explain differences in crop yield in eroded landscapes (Papiernik et al. 2005).

A variety of methods can be used for the determination of calcite, dolomite, and magnesian calcite in soils. Chemical determinations of carbonates include the use of empirical standard curves relating pH to known carbonate content as well as the measurement of CO₂ evolved when treated with acid. These permit a measurement of inorganic C from carbonates in soil. Most procedures express the carbonate content as the calcium carbonate equivalent. Further analysis of the Ca and Mg content provides a means of estimating the kind of inorganic carbonate in soil. The largest source of error is in apportioning the cations between the carbonate minerals and the soluble cations from the exchange complex of the soil.

In instances where the carbonates are of primary origin, and hence consist of larger crystals, it may first be useful to separate them by density fractionation techniques (Jackson 1985; Laird and Dowdy 1994) before further attempting to distinguish between calcite and dolomite.
20.2 CARBONATE CONTENT BY USE OF EMPIRICAL STANDARD CURVE (LOEPPERT ET AL. 1984)

The analysis is suitable for rapid and routine analysis of large numbers of samples. A known quantity of acetic acid is consumed by reaction with carbonates, and the final pH following complete dissolution of CaCO$_3$ is recorded for each sample. Calcium carbonate content is determined empirically from a standard curve relating pH to weight of CaCO$_3$ according to the equation

$$\text{pH} = K + n \log \left[ \frac{\text{CaCO}_3}{(T - \text{CaCO}_3)} \right]$$  \hspace{1cm} (20.1)

where $K$ and $n$ are constants and $T$ is the total amount of CaCO$_3$ that could be completely neutralized by the quantity of acetic acid used.

20.2.1 REAGENTS AND EQUIPMENT

1. Calcite standard: Pure calcite such as Iceland spar calcite ground to <270 mesh in size is suitable.
2. Acetic acid, 0.4 $M$: Dilute 400 mL of 1 $M$ CH$_3$COOH to the mark in a 1 L volumetric flask with deionized distilled water.
3. pH meter: A digital pH meter is recommended.
4. Ultrasonic probe: A suitable model with a probe that can be inserted into a 50 mL centrifuge tube.

20.2.2 PROCEDURE

Standard Curve

1. Weigh accurately, Iceland spar calcite, ranging from 5 to 500 mg into separate 50 mL polypropylene centrifuge tubes.

2. Add 25 mL of 0.4 $M$ acetic acid, which is sufficient to exactly neutralize all the CaCO$_3$ in the largest sample of the standard (500 mg CaCO$_3$), according to the reaction:

$$\text{CaCO}_3 + 2\text{CH}_3\text{COOH} \rightarrow \text{Ca}^{2+} + 2\text{CH}_3\text{COO}^- + \text{H}_2\text{O} + \text{CO}_2$$

3. Shake tubes intermittently for 8 h. At approximately hourly intervals, swirl the contents for a few minutes to allow for adequate mixing and degassing. Allow tubes to stand overnight with caps loosened to allow escape of CO$_2$.

4. A final degassing is carried out for approximately 30 s using an ultrasonic probe at low setting to prevent excessive splashing.

5. Centrifuge and record pH of the supernatant to two decimal places after 4 min.
6 Plot standard curve of pH versus log \( \frac{[\text{CaCO}_3]}{(T - \text{CaCO}_3)} \). Note: \( T \) is the weight of \( \text{CaCO}_3 \) (mg) used to exactly neutralize the volume of acetic acid used and will vary if either the volume or concentration of acetic acid is changed.

**Calcium Carbonate Content of Soil Samples**

1 Weigh accurately, up to 2 g soil (<100 mesh size) containing up to 400 mg \( \text{CaCO}_3 \). Reduce the soil sample weight if the carbonate content exceeds 20%.

2 Repeat steps (2) through (5) as for the standard curve above.

**20.2.3 Calculations**

From the pH value recorded, determine the value of log \( \frac{[\text{CaCO}_3]}{(T - \text{CaCO}_3)} \) using the standard curve and calculate the weight of \( \text{CaCO}_3 \) (mg) in the soil sample. The total carbonate content so determined is expressed as percent calcium carbonate equivalent.

\[
\% \text{ CaCO}_3 \text{ equivalent} = \frac{\text{mg CaCO}_3}{\text{mg sample}} \times 100
\]  

(20.2)

**20.2.4 Comments**

If dolomite is present in the soil sample, increased reaction times may be required for the dissolution to go to completion. The accuracy of results is influenced by (i) proton consumption by soil constituents, (ii) acid-generating hydrolysis reactions during mineral decomposition, (iii) high PCO\(_2\), (iv) volatilization of acetic acid, and (v) errors in pH determination. These can be minimized by standard additions of Ca\(^{2+}\), from a solution of CaCl\(_2\), to all samples and standards; grinding of samples to increase reactivity of sand-sized carbonates and reduction of reaction time between acetic acid and other minerals; use of covers to reduce loss of acetic acid; degassing CO\(_2\); and reduction of suspension effects in pH reading (Loeppert et al. 1984).

**20.3 Approximate Gravimetric Method**

*(ALLISON AND MOODIE 1965; RAAD 1978)*

A preweighed soil sample containing carbonates is reacted with acid. The resultant loss in weight from CO\(_2\) released is used to calculate the calcium carbonate content. Calcite and dolomite cannot be accurately distinguished, but a fair estimate of the proportion of dolomite in the sample can be obtained by checking the weight loss with time.

**20.3.1 Reagents**

1 Hydrochloric acid (HCl), 4 \( M \).

2 Hydrochloric acid (HCl)–ferrous chloride (FeCl\(_2\) · 4H\(_2\)O) reagent: Dissolve 3 g of FeCl\(_2\) · 4H\(_2\)O per 100 mL of 4 \( M \) HCl immediately before use.

**20.3.2 Procedure**

1 Weigh a stoppered, 50 mL Erlenmeyer flask containing 10 mL of the HCl–FeCl\(_2\) reagent.
2 Transfer a 1–10 g soil sample containing between 100 and 300 mg of carbonate to the flask gradually to avoid excessive frothing.

3 After effervescence has subsided, replace the stopper loosely and allow the carbonate to decompose further in the mixture for about 30 min with occasional swirling to displace any accumulated CO₂. Replace the stopper and weigh the flask with its contents.

4 Repeat step (3) until the change in weight of the flask and its contents is no more than 2–3 mg. The reaction is usually complete within 2 h.

20.3.3 Calculations

Weight of CO₂ lost from carbonates = difference in initial and final weights of (flask + stopper contents)

\[
\% \text{ CaCO}_3 \text{ equivalent} = \frac{\text{g CO}_2 \text{ lost}}{\text{g soil}} \times 227.3
\]  

(20.3)

20.3.4 Comments

When dolomite is present, it is considerably less reactive to cold HCl. Therefore, if the weight is observed to decrease markedly after 30 min, some dolomite is present. The use of acid containing FeCl₂ as an antioxidant eliminates errors caused by oxidizing interferences due to MnO₂ in soil. The accuracy of this method depends upon the accuracy of weighing and the degree to which CO₂ retained in solution is compensated for by loss of water vapor.

20.4 Quantitative Gravimetric Method

(USDA Soil Conservation Service 1967)

The loss in weight of a soil sample is measured accurately after reaction between carbonates in the soil and acid. In this method, the loss of water vapor evolved with CO₂ is eliminated by a trap containing anhydron. The addition of a CO₂ trap to the apparatus is an alternative to the method, by measuring the gain rather than the loss in weight, and provides a check against any leaks in the connections to the glassware. With several units in operation, the method is quite rapid and accurate.

20.4.1 Apparatus

The apparatus is assembled as depicted in Figure 20.1 for the weight loss method. A polyethylene drying tube packed with Ascarite IIR to trap CO₂ can also be attached to the end of the gas train after stopcock D in the weight gain method.

20.4.2 Reagents

1 Hydrochloric acid (HCl), 6 M.

2 HCl–ferrous chloride (FeCl₂ · 4H₂O) reagent: Dissolve 3 g of FeCl₂ · 4H₂O per 100 mL of 6 M HCl immediately before use.
Anhydrone (Mg(ClO₄)₂), drying agent.

Ascarite IIR: 20–30 mesh, optional.

20.4.3 Procedure

Weight Loss Method

1. Weigh a 1–10 g sample of oven-dry soil (<100 mesh) containing <1 g CaCO₃ equivalent in a 125 mL Erlenmeyer flask.

2. Wash down the sides of the flask with 10 mL of deionized distilled water.

3. Transfer 7 mL of HCl–FeCl₂ reagent into vial C (Figure 20.1), and place the vial upright in the flask without spilling any acid.

4. Moisten stopper G with glycerin, sprinkle it with a small amount of 180 mesh abrasive to overcome slipperiness, and assemble the apparatus as in Figure 20.1 without connecting the U-tube to stopcock E. Close stopcocks D and E.

5. Place the apparatus beside the balance and allow the temperature in the flask to equilibrate with that of the air in the balance.

6. Using tongs, place the apparatus on the weighing pan, open stopcock D, and record weight to the nearest 0.1 mg. Close stopcock D immediately. Weigh again after 10 min to ensure that weight has stabilized.

Open stopcock D and shake the flask to upset vial C, thus allowing the acid to react with the soil.

After 10 min, attach the U-tube H to the apparatus, open stopcock E, and apply gentle suction at stopcock D at a rate of 5–10 bubbles per second at tube J to sweep out CO₂ with dry air. Shake the flask at 10 min intervals.

Stop the suction when the reaction is complete (usually 30 min; 1 h if dolomite is present). Close stopcocks D and E. Disconnect the U-tube H. Wait for 1 h and weigh the apparatus and its contents with stopcock D open. Check the weight after 10 min.

Calculate as follows:

\[
\% \text{ CaCO}_3 \text{ equivalent} = \frac{(\text{Initial weight, g} - \text{Final weight, g})}{\text{Sample weight, g}} \times 227.3
\]  

Weight Gain Method

Weigh drying tube containing Ascarite II⁺ to the nearest 0.1 mg. Attach to the apparatus depicted in Figure 20.1 at stopcock D.

Proceed as described in the weight loss method but apply suction at the end of the polyethylene drying tube so that the gas train passes through the CO₂ trap.

Disconnect from the CO₂ trap and weigh drying tube and its contents.

Calculate as follows:

\[
\% \text{ CaCO}_3 \text{ equivalent} = \frac{(\text{Final weight, g} - \text{Initial weight, g})}{\text{Sample weight, g}} \times 227.3
\]

20.4.4 COMMENTS

The results obtained by the two methods should agree within the limits of weighing error. Larger discrepancies may indicate leaks in the connections of the apparatus.

20.5 QUANTIFICATION OF CALCITE AND DOLOMITE

(PETERSON ET AL. 1966)

The citrate buffer method described by Raad (1978) is presented here. Calcite and dolomite are selectively dissolved in a citrate buffer solution, Ca and Mg in solution are determined, and the calcite content of the sample is calculated. It is assumed that dolomite has a Ca:Mg molar ratio of 1:1 and the only sources of Ca and Mg in the solution are calcite and dolomite (i.e., no magnesian calcite is present), and exchangeable calcium and magnesium have been removed first or otherwise accounted for (Hesse 1971). The portion of dolomite
dissolved in the citrate buffer is calculated from the Mg in solution; an equivalent amount of Ca is assigned to it; and the remaining Ca determines the calcite content of the sample. The total dolomite content of the sample is obtained by the difference between the total carbonate content previously determined in another subsample and the portion of carbonate from calcite. As a check of accuracy, the dolomite content of the sample can also be calculated from the Mg in solution. The method is useful if clay-sized dolomite is present in the sample.

20.5.1 REAGENTS

1. Citrate buffer: Dissolve 64 g citric acid (C₆H₈O₇) in 1 L of deionized water. Titrate to pH 5.85 with concentrated NH₄OH.

2. Sodium chloride–ethanol: Dissolve 58.5 g NaCl in 30% (v/v) ethanol and bring to 1 L with deionized water.

3. Sodium dithionite.

20.5.2 PROCEDURE

1. Weigh 50–500 mg oven-dry soil ground to pass a 100 mesh sieve into a 50 mL centrifuge tube.

2. Wash twice with NaCl–ethanol solution and discard washing.

3. Add 25 mL of citrate buffer solution, and heat in a water bath at 80°C. Add approximately 0.5 g of sodium dithionite and continue heating with stirring for about 15 min.

4. Centrifuge and collect supernatant in a 250 mL volumetric flask. Wash the residue once with 25 mL of citrate buffer, centrifuge, and collect the supernatant. Make to volume with deionized water.

5. Determine Ca and Mg in solution by atomic absorption spectroscopy using standards made up in the same concentrations of citrate buffer and dithionite. Standards and sample solutions should contain 1 mg La mL⁻¹ to minimize interference effects.

6. The total carbonate content is determined in another subsample by other quantitative methods (Section 20.2).

20.5.3 CALCULATIONS

The molecular formula of calcite is CaCO₃, and the molecular formula of dolomite is CaMg(CO₃)₂. If total citrate-soluble Ca = X mmol, and citrate-soluble dolomite Mg = Y mmol, then citrate-soluble dolomite-Ca = Y mmol, and the mmol calcite-Ca = X – Y. Since 1 mmol of Ca is contained in 1 mmol of calcite, and since 1 mmol calcite weighs 100 mg, then:
\[
\% \text{ calcite in sample} = \frac{(X - Y) \text{ mmol}}{\text{mg sample}} \times \frac{100 \text{ mg}}{\text{mmol}} \times 100 \quad (20.6)
\]

The dolomite content of the sample can be calculated if total carbonate in sample = Z mmol. Since total carbonate = calcite carbonate + dolomite carbonate, the mmol dolomite-CO\(_3\) = \(Z - (X - Y)\), and since 2 mmol of carbonate is contained in 1 mmol of dolomite, then, the mmol dolomite = \(\frac{1}{2}\) (mmol dolomite-CO\(_3\)) = \(\frac{1}{2}[Z - (X - Y)]\), and since 1 mmol dolomite weighs 184 mg, then

\[
\% \text{ dolomite in sample} = \frac{\frac{1}{2}[Z - (X - Y)] \text{ mmol}}{\text{mg sample}} \times \frac{184 \text{ mg}}{\text{mmol}} \times 100 \quad (20.7)
\]

Alternatively, the dolomite content can be calculated by the mmol dolomite-Mg = Y:

\[
\% \text{ dolomite in sample} = \frac{Y \text{ mmol}}{\text{mg sample}} \times \frac{184 \text{ mg}}{\text{mmol}} \times 100 \quad (20.8)
\]

The \% CaCO\(_3\) equivalent of the dolomite present

\[
= \frac{[Z - (X - Y)] \text{ mmol}}{\text{mg sample}} \times \frac{100 \text{ mg}}{\text{mmol}} \times 100 \quad (20.9)
\]

### 20.5.4 COMMENTS

The dolomite content calculated from dolomite-CO\(_3\) should agree with that calculated from the Mg in solution unless some source of citrate-soluble Mg other than dolomite, or magnesian calcite is present. If magnesian calcite is present, the calcite content of the sample will be underestimated. Therefore, identification and quantification of magnesian calcite (e.g., St. Arnaud et al. 1993) should be conducted for more specialized research.

### REFERENCES


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Chapter 21
Total and Organic Carbon

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21.1 INTRODUCTION

Carbon in soils exists in both organic and inorganic forms. Carbonate, in a variety of forms, makes up the inorganic component of total carbon (TC), whereas a range of organic moieties make up the organic carbon (OC) component. The terms OC or organic matter associated with soil have been defined in various ways. Stevenson (1994) and Baldock and Nelson (1998) defined OC as the total of all organic materials existing within and on soil, whereas Oades (1988) excluded charcoal and charred materials and MacCarthy et al. (1990) excluded nondecayed plant and animal tissues, their partial decomposition products, and the living soil biomass. In reality, however, the methods used to determine both TC and OC do not discriminate between the various fractions described above, and consequently, the all encompassing definition of OC used by Stevenson (1994) and Baldock and Nelson (1998) is also used in this chapter.

There are a number of approaches available for the determination of TC and OC in soils. These are broadly based on either the chemical or thermal oxidation of soil OC. Chemical or wet oxidation is followed by the measurement of expelled CO\textsubscript{2} (Snyder and Trofymow 1984) or the consumption of oxidant required to quantitatively oxidize the OC (Walkley and Black 1934). Under acidic conditions, any chemical or wet oxidation methods that measure expelled CO\textsubscript{2} will also include carbonate C and will be a measure of TC. In dry combustion methods, samples are heated to high temperatures, usually exceeding 1000°C in the presence of excess O\textsubscript{2}. Under these conditions, all C present in OC and carbonate is quantitatively converted to CO\textsubscript{2}. Liberated CO\textsubscript{2} may be determined gravimetrically (Allison et al. 1965), volumetrically (Rayment and Higginson 1992), titrimetrically (Snyder and Trofymow 1984), or spectrometrically (Merry and Spouncer 1988). If thermal oxidation (dry combustion) at temperatures exceeding 1100°C is used, all carbon in the sample including carbonates will be determined (Giovannini et al. 1975). For both chemical and thermal oxidation methods where CO\textsubscript{2} is measured, a correction for carbonate can be made from a separate carbonate measurement or the carbonate may be removed with acid before carbon analysis.
A comparison of several titrimetric and gravimetric methods was made by Kalembasa and Jenkinson (1973) and showed that dry combustion methods were the more precise. Loss on ignition at various temperatures has also been used as a simple predictor of soil organic matter in soil types where clay contents are low (Ball 1964; Lowther et al. 1980) but these methods are not recommended if alternative methods are available.

21.2 DRY COMBUSTION METHODS

Currently, there are a large number of instruments available commercially for the determination of TC. Some instruments will simultaneously determine C and one or more of the following additional elements: N, H, and S. For some instruments, an induction furnace is used to heat the sample rather than the more commonly available resistance furnace. These methods rely on the addition of Fe chips and catalysts to the sample to produce high temperatures of up to 1400°C (Rayment and Higginson 1992) or the use of a quartz enclosed graphite crucible to heat the sample externally (Allison et al. 1965).

For all instruments, combustion is usually carried out at high temperatures (>1000°C) and in a stream of O₂. This is to ensure that all C species are quantitatively converted to CO₂. At lower temperatures, combustion may not be complete, resulting in the generation of some CO or incomplete decomposition of carbonate species. Unless the CO is converted to CO₂, losses will be recorded due to the inability of detectors tuned to CO₂ to detect CO. This can be overcome by mixing a catalyst such as V₂O₅ (Morris and Schnitzer 1967) with the sample, or by the inclusion of a catalytic conversion furnace (usually CuO) in the train prior to detection. For some instruments, samples are loaded into Sn or Al cups and the sample and cup are ignited. The exothermic reaction that takes place as the Sn or Al cups ignite increases the combustion temperature significantly, even though the furnace may operate at 1000°C or less.

Carbonates will be decomposed at elevated temperatures (500°C–1000°C) to also produce CO₂; and so for OC measurements, carbonates must be either removed prior to combustion or a correction must be made. Some authors have suggested using low combustion temperatures to confine the carbon measured to only that contained within organic materials. However, this requires temperatures <720°C, since dolomite starts to decompose above this temperature. At temperatures <720°C, not only is the production of CO an issue, but also a substantial amount of charcoal can be generated and may not fully oxidize if the combustion duration is short.

Carbonate is removed through the addition of acid before analysis. Sulfurous acid (H₂SO₃) is the only suitable acid because its reducing properties minimize the oxidation of OC during the process (Piper 1944). This can be accomplished in two ways: either by destruction of the carbonate within the combustion vessel or before subsampling for OC analysis. The former approach is preferable because it minimizes sample handling, but the combustion vessel needs to be large enough in relation to the sample size to enable the addition of excess acid and the potential effervescence that may result. Three methods are described here; addition of acid directly to the combustion vessels at two different scales and a method for removing carbonate before subsampling.

A range of modern instruments with several levels of automation are commercially available for dry combustion methods. The two most common detection systems are based on infrared or thermal conductivity measurement. All instruments are provided with comprehensive
instructions on the setting up, standardization, and use of the equipment for routine analysis. For the remainder of this section, we will not describe TC and OC analysis based around one or more specific instruments but will discuss the issues that arise at different parts of the carbon analysis train and how these may be dealt with.

21.2.1 REMOVAL OF CARBONATE—LARGE COMBUSTION VESSEL

This method is directed toward instruments such as those manufactured by Laboratory Equipment Corporation (LECO), which utilize large ceramic combustion boats with a 5–6 mL capacity. These boats are porous and so acid treatment cannot be performed directly within the boat, since some leakage will occur and OC rendered soluble by the treatment will be lost. The sample is therefore weighed into a commercially available Ni liner placed within the combustion boat.

Reagents

6% (w/v) H₂SO₃ solution.

Procedure

1. Weigh 0.1–1.0 g of soil (<0.15 mm) into a Ni liner placed within a ceramic combustion boat, and place on a hot plate. Larger samples are not recommended unless the samples are known to contain only very small concentrations of carbonate and low concentrations of OC.

2. Moisten samples with a little distilled or deionized water and add 1.0 mL of 6% H₂SO₃ to each boat and allow to stand. Meanwhile, turn on the heating plate and set the temperature to ensure that the samples do not exceed 70°C. Because of the insulating properties of the ceramic boat and the generally large loss of heat from the hot plate between samples, the hot plate temperature may need to be set as high as 120°C.

3. When the samples have stopped reacting, add a further 1.0 mL of 6% H₂SO₃. It is important that the samples are not allowed to dry until the treatment is completed, since this will lead to deterioration in the Ni liner and ultimately leakage. Some evaporation will be necessary, however, to allow the addition of sufficient acid to complete the carbonate removal.

4. When addition of acid no longer promotes a reaction, allow the samples to dry.

5. Analyze samples using an OC determinator as described by the manufacturer, but ensure that the initial weight of the sample (prior to 6% H₂SO₃ treatment) is entered into the calculation and not the final weight after treatment.

Comments

14 mL of 6% H₂SO₃ is required to neutralize 1.0 g of CaCO₃ · H₂SO₃ solutions will slowly deteriorate with time, losing SO₂; and more acid may be needed depending on the age of the H₂SO₃ solution.
21.2.2 REMOVAL OF CARBONATE—SMALL COMBUSTION VESSEL

This method is directed toward instruments such as those manufactured by Carlo Erba, which utilize small samples placed in metal combustion cups. For pretreatment for carbonate, Ag cups are recommended instead of the normal Sn or Al cups, since Ag has a much greater resistance to 6% $\text{H}_2\text{SO}_3$. The reaction is carried out in a small Al block that has been drilled out to hold the capsules with a reasonably tight fit similar to that described by Verardo et al. (1990).

Reagents

6% (w/v) $\text{H}_2\text{SO}_3$ solution.

Procedure

1. Place Ag cups into a small Al block. Weigh up to 20 mg of the sample (<0.02 mm) into the cups and place the small block onto a heating plate.

2. Add 10 µL distilled or deionized water to each sample. This slows the initial reaction when 6% $\text{H}_2\text{SO}_3$ is added and minimizes the risk of losing sample due to a strong effervescence. Add 10 µL of 6% $\text{H}_2\text{SO}_3$ to each sample and allow to stand. Meanwhile, turn on the heating plate and set temperature to 70°C.

3. When reaction has ceased, add another 10 µL of acid. It is important that the samples are not allowed to dry until the treatment is complete, because this will lead to deterioration in the Ag cups and they may crumble during the balling process. Some evaporation will be necessary, however, to allow the addition of sufficient acid to complete the carbonate removal.

4. When addition of acid no longer promotes a reaction, allow the samples to dry and analyze samples using an OC determinator as described by the manufacturer ensuring that the initial weight of the untreated soil is used in the calculations.

21.2.3 REMOVAL OF CARBONATE PRIOR TO SUBSAMPLING

Reagents

6% (w/v) $\text{H}_2\text{SO}_3$ solution.

Procedure

1. Weigh 1.0–2.0 g of sample into a preweighed test tube or beaker and place in a digestion block or on a hot plate.

2. Add 1.0 mL of 6% $\text{H}_2\text{SO}_3$ and allow the reaction to subside. Meanwhile, turn on the block/hot plate and set temperature to 70°C.

3. Continue to add 6% $\text{H}_2\text{SO}_3$ in 1.0 mL aliquots until further addition no longer yields a reaction and allow samples to dry.
When dry, remove samples from block, place in a desiccator or cabinet with silica gel, and allow to cool. Weigh tube or beaker, and quantitatively remove treated sample. Maintain samples in an oven dry state (105°C) under desiccation for OC analysis.

Take 10 g of soil and place in a preweighed beaker or silica dish. Dry for 24 h at 105°C in an oven. Cool in a desiccator and reweigh.

**Calculations**

This pretreatment modifies the sample in two ways. First, because the sample is heated, the water content of the sample is changed; and second, because SO$_3^{2-}$ has more mass than CO$_3^{2-}$, the mass of the sample will increase. These changes need to be taken into consideration when calculating the OC content of the sample.

1. Oven dry factor (ODF) is calculated as
   
   \[
   ODF = \frac{\text{weight air dry soil}}{\text{weight oven dry soil}} \quad (21.1)
   \]

2. Soil OC content (105°C) g kg$^{-1}$ = (OC content of treated sample g kg$^{-1}$) \[\times\] (weight of sample post treatment at 105°C)/(weight of sample taken for treatment/ODF) \[\quad (21.2)\]

**21.2.4 CORRECTION FOR CARBONATE**

For analyzers that rely on a continuous flow of exhaust gases for the determination of CO$_2$, and the temperature of the combustion is high (>1000°C), a correction for carbonate content can be made mathematically. Some analyzers, however, rely on the collection of a given volume of exhaust gases that limits the time over which the sample is heated to high temperature. This is common in instruments that analyze for C and N simultaneously. Under these conditions, carbonates may not be fully decomposed and the TC may be underestimated. This can often be overcome by taking much smaller samples, but such analyzers need to be tested to determine whether a TC measurement in the presence of carbonate is quantitative. If not, then a simple correction is not quantitative and carbonates will need to be removed before analysis as described by one of the procedures given above.

For those analyzers that quantitatively determine TC, the following correction can be made:

\[
\text{OC} \ g \ \text{kg}^{-1} = \text{TC} \ g \ \text{kg}^{-1} - 0.12 \times \text{CaCO}_3 \ g \ \text{kg}^{-1} \quad (21.3)
\]

**21.2.5 STANDARDS**

Preignition of combustion vessels may be necessary to eliminate contamination. For metal cups, this can be achieved at 550°C and for ceramic boats at 1000°C for 16 h.

A wide range of materials can be used as OC standards for these instruments. These include CaCO$_3$, EDTA, sucrose, glucose, potassium hydrogen phthalate, and urea.
Standard materials of given C content can also be purchased from companies such as LECO. It is recommended, but not essential, that the standards used exhibit similar combustion characteristics to the samples. This becomes an issue when highly organic standards or samples are used. For continuously monitoring instruments, the linear operating range of the detector may be exceeded if the standards or samples “flash” and an intense pulse of CO₂ passes through the detector within a short time. This can be overcome by reducing the rate of combustion by covering the sample with a layer of ignited sand. The sand used should be < 1 mm and ignited at > 1000°C over at least 24 h with frequent stirring of the sand to ensure any C present is totally combusted to CO₂.

The linear operating range of the detector can be determined by analyzing different weights of standards and samples over a range that encompasses expected TC and OC contents.

21.3 DICHROMATE REDOX METHODS

With these methods, dichromate (Cr₂O₇²⁻) solution in combination with sulfuric acid (H₂SO₄) is used to oxidize OC to CO₂. The orange dichromate is reduced to the green Cr³⁺ form according to the following equation:

\[2\text{H}_2\text{Cr}_2\text{O}_7 + 6\text{H}_2\text{SO}_4 + 3\text{C} \rightarrow 2\text{Cr}_2(\text{SO}_4)_3 + 3\text{CO}_2 + 8\text{H}_2\text{O}\]  (21.4)

The oxidation state (Baldock et al. 2004) of the C in the organic matter can influence the consumption of oxidant. Molecules with a high H/C ratio, such as lipids, give higher recoveries than molecules with high O/C ratio (Skjemstad 1992). Because soil organic matter is highly diverse in chemistry, these two effects tend to cancel one another when the whole soil is considered; however, variations in oxidation state of OC with increasing extent of decomposition have been documented (Baldock et al. 2004). This issue, therefore, may be more serious if specific soil OC fractions are being considered.

If consumption of oxidant is to be used, the analysis can be performed with heating (Heanes 1984) or without external heating (Walkley and Black 1934). The consumption of oxidant can be determined either by titration using an indicator or platinum–calomel electrode or colorimetrically. If only the heat of reaction is used with no applied external heating, as in the case of the Walkley and Black (1934) method, then a 75%–80% recovery of carbon is usually obtained and a conversion factor of 1.3 is commonly used to equate the OC value to the thermal oxidation (dry combustion) methods. This factor will vary among soil types and with depth and must be applied with caution.

Any material that can be oxidized by the dichromate will be measured as OC. Chlorides are quantitatively oxidized to free chlorine by chromic acid. Thus, where consumption of dichromate is used to determine OC, the presence of Cl can result in erroneously high OC contents. This methodological error can be corrected when the Cl content of the sample is known. Four Cl ions have the same reducing power as 1 C atom (4Cl ≡ 2O ≡ 1C) and hence 11.83 g of Cl is equivalent to 1 g of C. Several workers have suggested that the addition of Ag₂SO₄ can suppress Cl interference (Walkley 1947). Heanes (1984), however, demonstrated that the addition of Ag₂SO₄ either as a solid or in solution with the H₂SO₄ was ineffective. For saline soils therefore, it is recommended that the correction for measured Cl be used rather than additions of Ag₂SO₄.
In this chapter, we detail two methods. One based on the Schollenberger (1945) method uses external heating and titration with an indicator. The alternative method uses external heating and colorimetric determination of Cr\textsuperscript{III} (Heanes 1984). Reduced forms of Fe and Mn may also interfere with these methods. These interferences are rare but can be overcome by the procedures described in Jackson (1958). The use of steel or iron mills should also be avoided since these can act as a source of Fe metal, which is readily oxidized under the conditions of the reaction.

21.3.1 DICHROMATE REDOX COLORIMETRIC METHOD (HEANES 1984)

The dichromate redox colorimetric method utilizes the formation of the green Cr\textsuperscript{III} species resulting from the reduction of the orange dichromate (Cr\textsuperscript{VI}) species. The amount of dichromate consumed is determined against a set of standards and measured on a spectrophotometer in the visual range. Carbonates are not determined by this procedure but Cl\textsuperscript{−} will interfere. Because the dichromate solution is not used as the primary standard in this method, we describe here the use of the more soluble Na\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} salt rather than the K salt.

Reagents

1. Na dichromate solution: dissolve 50 g of Na\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} in distilled or deionized water and dilute to 1 L.
2. Sulfuric acid: 98% conc. H\textsubscript{2}SO\textsubscript{4}.
3. Standards: dissolve 1.376 g of glucose monohydrate in distilled or deionized water and dilute to 250 mL. A small crystal of HgCl\textsubscript{2} can be added to preserve the standard against microbial decomposition. 1.0 mL of this solution = 2.0 mg of OC.

Procedure

1. Prepare standards by adding a range of aliquots of the glucose solution to borosilicate tubes (25 mm OD) marked at 100 mL. A convenient range is 1–12 mL of standard that equates to 2–24 mg of OC. Tubes containing glucose solution and a blank are dried in an oven at a temperature not exceeding 60°C.
2. Weigh 0.1–2.0 g of air-dried soil (<0.15 mm) containing <20 mg of OC into digestion tubes.
3. Add 10.0 mL of Na\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} solution, and while agitating add 20.0 mL of 98% H\textsubscript{2}SO\textsubscript{4} cautiously so that the reaction is confined to the bottom of the tube. Agitate for a further 30 s before inserting into a preheated (135°C) digestion block. Agitate tubes occasionally to ensure all of the soil material is exposed to the chromic acid mixture.
4. After 45 min, remove tubes from the block and allow to cool. Add 50 mL of distilled or deionized water to digest and agitate with a thick-walled glass capillary tube that has a stream of air passing through it so that the samples are thoroughly mixed. After removal from the block, the samples still contain H\textsubscript{2}SO\textsubscript{4} at a strong enough concentration to cause heating when water is added.
If the tubes are inverted after the addition of water, enough heat is generated to potentially cause hot chromic acid to be lost. Agitation with the assistance of a stream of air prevents any losses. When cool, make the tubes up to 100 mL with distilled or deionized water and invert to mix using a rubber bung.

5 Decant diluted chromic acid mixture into 15 mL centrifuge tubes and centrifuge at 2000 rpm for 15 min. Measure the absorbance of the centrifuged samples at 600 nm in a 10 mm cell.

Calculations

Construct a standard curve plotting absorbance at 600 nm against mg C present in the standards. Using this curve, estimate the mg C in the unknown samples.

\[ \text{g C kg}^{-1}\text{soil} = \text{mg C in digest/weight soil in grams} \]  

(21.5)

If the mg C content of samples is <2 or >20, analysis should be repeated with more or less weight to bring them within the optimum range of the determination.

Modification for Saline Soils

For saline soils, a separate determination of the chloride content of the soil is required and expressed as g Cl kg\(^{-1}\) soil. The OC content of the soil is then corrected for the Cl content:

\[ \text{g C kg}^{-1}\text{soil} = \text{apparent g C kg}^{-1}\text{soil} - (\text{g Cl kg}^{-1}\text{soil}/12) \]  

(21.6)

21.3.2 DICHROMATE REDOX TITRATION METHOD

This procedure is similar to the spectroscopic method but utilizes the unreacted dichromate (Cr\(^{VI}\)) that remains following the reaction of OC with acid dichromate. Back titration with Fe\(^{II}\) solution is used to determine the remaining dichromate. The procedure described here is based on that described by Schollenberger (1945) with the modification by Jackson (1962) for the o-phenanthroline indicator. N-phenantranilic acid (Nelson and Sommers 1982) or diphenylamine (Piper 1944) can be substituted. Carbonates do not interfere but Cl does and a correction must be made if Cl levels are high.

Reagents

1 Digestion mixture: dissolve 39.22 g of K\(_2\)Cr\(_2\)O\(_7\) (dried at 90\(^\circ\)C) in 800–900 mL of distilled or deionized water in a large glass beaker. Carefully add 1 L of 98% H\(_2\)SO\(_4\). As the acid is added, the mixture will become very hot and will boil. When cool, make to 2 L with distilled or deionized water. This solution is 0.067 \(M\) (0.4 \(N\)) in dichromate and 9 \(M\) in H\(_2\)SO\(_4\) and is the primary standard for the OC determination.

2 Ferrous ammonium sulphate: dissolve 157 g of Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) \cdot 6H\(_2\)O in about 1 L of distilled or deionized water containing 100 mL of 98% H\(_2\)SO\(_4\). Make to 2 L to give a \(\sim 0.2 \, M\) (\(\sim 0.2 \, N\)) solution. The solution does not store well and must be standardized against the dichromate solution at each use.

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Phosphoric acid: 85% H₃PO₄.

Indicator solution: dissolve 3.00 g of o-phenanthroline monohydrate (Ferroin) and 1.40 g of FeSO₄·7H₂O in distilled or deionized water and dilute to 200 mL. Alternatively, dissolve 0.1 g of N-phenanthranilic acid and 0.1 g of Na₂CO₃ in 100 mL of distilled or deionized water or dissolve 0.5 g of diphenylamine in 100 mL of 98% H₂SO₄ containing 20 mL of distilled or deionized water.

Procedure

1. Weigh samples (<0.15 mm) up to 1.0 g that contain between 1 and 10 mg of OC into 100 mL digestion tubes. Add 15 mL of digestion mixture and place on digestion block preheated to 150°C.

2. After 45 min, remove samples from the block and allow to cool before quantitatively transferring solution and sample to a titration vessel with approximately 50 mL of distilled or deionized water. Add 5 mL of 85% H₃PO₄ and four drops of indicator. The H₃PO₄ eliminates interference from Fe³⁺.

3. Titrate with Fe(NH₄)₂(SO₄)₂ solution to a color change from green to reddish brown for the o-phenanthroline, dark violet–green to light green for the N-phenanthranilic acid, and violet–blue to green for the diphenylamine.

4. Two unheated blanks are also titrated to standardize the Fe(NH₄)₂(SO₄)₂ solution.

Calculations

1. Calculate the molarity (normality) of the Fe(NH₄)₂(SO₄)₂ solution as

   \[
   \text{Molarity of Fe(NH₄)₂(SO₄)₂ solution} = \frac{(15 \times 0.4)}{T₁}
   \]

   where \( T₁ \) is the titer of the Fe(NH₄)₂(SO₄)₂ solution in mL.

2. Calculate the OC concentration in the sample as

   \[
   \text{g OC kg}^{-1}\text{soil} = \frac{(B - T₂) \times M \times 3}{W}
   \]

   where \( B \) and \( T₂ \) are titers in mL of heated blank and sample, respectively, \( M \) is the molarity of the Fe(NH₄)₂(SO₄)₂ solution, and \( W \) is the weight of sample in grams.

21.4 DICHROMATE OXIDATION CO₂ TRAP METHOD
(SNYDER AND TROFYMOW 1984)

With this method, the sample is oxidized with a H₂SO₄–dichromate mixture and the evolved CO₂ is captured in NaOH solution and determined by titration using either an indicator or pH meter. This approach is more complex than the redox approach but most of the interferences encountered with the redox methods are eliminated. A further advantage of this method is that the trapped CO₂ can also be used to determine isotopic composition (Amato 1983). Various vessels have been used to contain the reaction and collect the CO₂. Snyder and

Because of the acidic conditions under which the reaction progresses, any carbonates present will also be quantitatively converted to CO₂ and determined. If carbonates are present, OC is determined by either first removing the carbonates in the reaction vessel or correcting for their presence. Carbonate content can either be determined using the same reaction vessel or can be determined by another suitable method of analysis.

The method as described by Snyder and Trofymow (1984) can handle solid or liquid samples and uses a temperature of 120°C. If only solid samples are processed, the digestion can be performed at a higher temperature, provided it remains below the boiling point of the acid mixture. If boiling occurs, the vessels may leak or even break. For the determination of OC only in the presence of carbonates, an acid pretreatment is required or the TC can be corrected for carbonate as outlined in Section 21.2.4.

### 21.4.1 Preparation of Reaction Tubes

Standard culture tubes capped with screw caps containing a conical polyseal are modified with three indentations near the top capable of supporting an inserted glass vial (15 × 45 mm). Alternatively, a glass rod bent at one end can be inserted into the tube so that the bend in the rod supports the vial at an appropriate distance above the reaction mixture (Amato 1983). Amato (1983) also suggests the use of regular digestion tubes sealed with subaseals. Either approach is satisfactory.

### 21.4.2 Reagents

1. Pretreatment acid mixture (for elimination of carbonates): dilute 57 mL of 98% H₂SO₄ in 600 mL of distilled or deionized water and add 92 g of FeSO₄·7H₂O. Dissolve and make to 1 L to give approximately 1 M H₂SO₄ containing 5% antioxidant.

2. Digestion mixture: these are kept separate and only combined in the reaction tube: (a) K₂Cr₂O₇ and (b) a mixture of three parts 98% H₂SO₄ and two parts 85% H₃PO₄.

3. CO₂ absorption solution: dissolve 16.0 g of NaOH and bring to 200 mL with distilled or deionized water to give a ~2 M solution. This should be kept in an airtight flask or under a CO₂ trap.

4. Indicator solution: dissolve 0.4 g of thymolphthalein in 100 mL in a mixture of 1:1 ethanol:distilled or deionized water.

5. Barium chloride solution: dissolve 41.66 g of BaCl₂ (48.86 g of BaCl₂·H₂O) in distilled or deionized water and make to 200 mL to give a ~1 M solution.

6. Titrant: use exactly 1.000 M HCl.

The following reagents are for use with the two endpoint titration procedure in combination with a pH meter or autotitrator.
7 Tris standard solution: dissolve 2.8000 g of Tris (hydroxy-methyl)-amino-methane (MW = 121.14) in distilled or deionized water and make to 100 mL.

8 ~0.5 M HCl: dilute 100 mL of conc. HCl to 2 L with distilled or deionized water.

21.4.3 Oxidation Procedure

Soil sample (ground to <0.15 mm) weights are limited to 2.0 g. Liquid samples up to 5 mL can be digested without pretreatment; larger samples must be evaporated to <5 mL in the digestion tube at 100°C. When liquid samples are processed, the temperature of the digest must be limited to 120°C.

For samples containing up to 10% carbonates, 3 mL of pretreatment acid is added per gram of soil. The pretreatment is done in the digestion tube by shaking them uncapped for 60 min on a reciprocal or orbital shaker set at slow speed. The water added with the acid limits the digestion temperature to 120°C.

1 Place samples into the bottom of the digestion tubes with a long spatula and then pretreat to remove carbonates.

2 Approximately 1 g of K₂Cr₂O₇ is added using a long glass funnel. Add 25 mL of the digestion acid mixture and quickly insert the CO₂ trap (vial containing NaOH).

3 Tightly cap the tubes and place in a digestion block preheated to 150°C (120°C for wet samples) for 2 h.

4 Remove the tubes from the block and after 12 h, remove and titrate contents of the CO₂ trap.

21.4.4 Comments

The amount of NaOH in the trap limits the amount of CO₂ that can be absorbed. Using 1 mL of 2 M NaOH in a 6 mL capacity vial allows the titration to be made directly in the vial. 1 mL of 2 M NaOH will trap 12 mg of CO₂-C, but absorption efficiency drops before this maximum is approached.

21.4.5 Titration Procedure

Carbonic acid trapped in the NaOH can be titrated by the direct, two endpoint method, or by back titration.

Back Titration Procedure

1 Add 2 mL of 1 M BaCl₂ solution to the NaOH to precipitate BaCO₃.

2 Add approximately five drops of the thymolphthalein indicator solution and titrate the NaOH with 1.000 M HCl using a microburette accurate to 0.001 mL. Four blanks per 40 tube digestion batch should be included.
Calculation

The OC content of the soil or plant material is calculated as

\[ g \text{ OC kg}^{-1}\text{sample} = (\text{mL HCl blank} - \text{mL HCl sample}) \times 6/\text{weight sample} \quad (21.9) \]

since 2 mol of OH\(^\text{-}\) are equivalent to 1 mol (12 g) of C and molarity of the acid is 1.00.

**Two Endpoint Titration Procedure**

1. Pipette accurately 20.0 mL of Tris standard into a titration vessel and titrate with \(~0.5\) M acid to pH 4.7. Perform standardization of acid at least three times.

2. Three blanks of the NaOH solution and NaOH traps are then titrated against standardized 0.5 M HCl as follows.

3. Titrate each solution slowly using standardized 0.5 M HCl to pH 8.3 and note volume \((T_1)\). Continue titration to pH 3.8 and note volume \((T_2)\).

If using an auto burette, the speed of the titrations and endpoints will need to be optimized for the burette and strength of acid to ensure the endpoints at pH 8.3 and 3.8 are not overshot.

Calculation

\[ \text{Molarity of HCl} (M_{\text{HCl}}) = 0.23114 \times 20/\text{mean titre HCl} \quad (21.10) \]

\[ g \text{ C kg}^{-1}\text{sample} = (T_1 - T_2) \times M_{\text{HCl}} \times 6/\text{weight sample} \quad (21.11) \]

**REFERENCES**


Chapter 22
Total Nitrogen

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22.1 INTRODUCTION

Total soil N includes all forms of inorganic and organic soil N. Inorganic N includes soluble forms (e.g., NO\textsubscript{2} and NO\textsubscript{3}), exchangeable NH\textsubscript{4}\textsuperscript{+}, and clay-fixed nonexchangeable NH\textsubscript{4}\textsuperscript{+}. Organic N content includes numerous identifiable and nonidentifiable forms (Stevenson 1986) and can be determined by the difference between total soil N and inorganic soil N content. Total N analyses may be divided into two main types: (i) wet digestion (e.g., Kjeldahl method) or (ii) dry combustion (e.g., Dumas method). Wet digestion techniques involve conversion of organic and inorganic N to NH\textsubscript{4}\textsuperscript{+} in acid and its subsequent measurement. Salts (e.g., K\textsubscript{2}SO\textsubscript{4}) and catalysts (e.g., Cu) are usually added to increase digestion temperatures and accelerate oxidation of organic matter (Bremner 1996). The dry combustion method normally involves an initial oxidation step followed by passage of the gases through a reduction furnace to reduce NO\textsubscript{x} to N\textsubscript{2}. The quantity of N\textsubscript{2} is usually determined using a thermal conductivity detector. Near-infrared reflectance spectrometry has recently been used for the determination of total soil N (Chang and Laird 2002), but the method will not be described here.

The Dumas method is becoming increasingly common due to greater availability and simplicity of modern automated instruments, which can determine C, H, N, or S on the same sample, and O with a simple modification. Modern systems are available in various configurations and have improved accuracy and precision for total N determination over earlier models (Bremner 1996). Dry combustion instruments for total N may be connected in-line with an isotope ratio mass spectrometer for simultaneous analyses of \textsuperscript{15}N (Minagawa et al. 1984; Marshall and Wheway 1985; Kirsten and Jansson 1986). Bellomonte et al. (1987) concluded that the automated Dumas procedure was comparable to Kjeldahl analysis for heterogeneous substrates. They reported, using a commercial Dumas system for total N analysis, coefficients of variation of 0.79\% for cereal flour and 1.08\% for meat. Reports since then confirm total N in plant materials, feeds, excreta, carcasses, and other agricultural...
materials determined by dry combustion to be comparable to or slightly greater than by Kjeldahl digestion (Matejovic 1995; Etheridge et al. 1998; Schindler and Knighton 1999; Marcó et al. 2002). Results are slightly more variable with soils. While results for total soil N have been reported as comparable for Kjeldahl and dry combustion methods in some studies (Artiola 1990; Yeomans and Bremner 1991), total N determined by dry combustion was found to be slightly lower but proportional to Kjeldahl determination by Kowalenko (2001). Others have found total soil N by dry combustion to be slightly greater than conventional Kjeldahl digestion (McGeehan and Naylor 1988; Vittori Antisari and Sequi 1988). High NO$_3^-$ concentrations in sample materials contribute to lower total N by the Kjeldahl method if pretreatments to include NO$_3^-$ (see below) are not used (Matejovic 1995; Watson and Galliher 2001); however, NO$_3^-$ alone may not account for lower Kjeldahl values (Simonne et al. 1998).

Kjeldahl procedures are still widely used for total N determination. Although some fixed or intercalary NH$_4^+$ is normally included in Kjeldahl digestion, it may not be quantitatively extracted from soils with a high proportion of their N constituted as fixed NH$_4^+$. In such cases, an HF–HCl pretreatment, as described by Bremner (1996), may be necessary to free intercalary NH$_4^+$. Corti et al. (1999) reported a method to measure fixed NH$_4^+$ using a Kjeldahl digestion followed by distillation, and a digestion of the residue with 5 M HF:1 M HCl and a second distillation to quantify strongly fixed NH$_4^+$. Vittori Antisari and Sequi (1988) reported that both dry combustion and microwave digestion with HF–HCl, H$_3$BO$_3$, and H$_2$O$_2$ followed by micro-Kjeldahl distillation were effective at including fixed NH$_4^+$ in total N analysis.

Given the time involved in Kjeldahl analyses, efforts have been made to speed up digestions. One such example is the peroxy method, which replaces K$_2$SO$_4$ and metal catalyst with peroxymonosulfuric acid (H$_2$SO$_5$), and involves carbonizing the sample in H$_2$SO$_4$ before adding the peroxy reagent (viz. H$_2$SO$_4$ + H$_2$O$_2$). It is 25 times faster than conventional Kjeldahl procedures and fully recovers the N of a variety of plant materials and one of the most refractory organic compounds, nicotinic acid (Hach et al. 1985). To enhance safety and improve speed, Hach et al. (1987) developed a system using a Vigreux fractionation head to simplify the addition of the peroxy reagent, and to maintain constant residual H$_2$O$_2$ in the digestion solution. A procedure for soils described by Christianson and Holt (1989) takes only 38 min; however, N recovery from six soils ranged from 89% to 98% compared to Kjeldahl digestion. Further investigation on soil materials would be warranted. Mason et al. (1999) used microwave heating to accelerate digestion using only H$_2$SO$_4$ and CuSO$_4$ for twofold reduction in time for soil samples. Their system can perform six digests simultaneously. Although microwave-assisted digestion has been widely adopted for sample preparation for metal analyses (Smith and Arsenault 1996), it has not received wide application for digestion of soils for total N analysis.

The total time required for Kjeldahl determination of N can be reduced considerably by using automated colorimetric analysis of NH$_4^+$. The most common method uses the Berthelot or indophenol reaction, which is specific for ammonia, and is well-documented (Searle 1984). The method has similar results and precision as the distillation procedure (Schuman et al. 1973). Mason et al. (1999) used the method following microwave-assisted digestion of soil. A manual version of the Berthelot reaction has been used to quantify NH$_4^+$ from Kjeldahl digestion of soils (Wang and Oien 1986).

The presence of NO$_3^-$ can be a concern because unmodified Kjeldahl digestion recovers some, but not all, NO$_3^-$, thereby precluding the addition of NO$_3^-$ from a separate analysis to
Selection of the most suitable combination of variables for the Kjeldahl method must be based on local requirements and facilities. Digestion options include H₂SO₄ with or without H₂O₂, heating mantles or digestion blocks, macro- or semimicro digestion, inclusion or omission of NO₂⁻ plus NO₃⁻. Subsequent measurement of NH₄⁺ may use the Berthelot reaction, NH₄⁺ electrode, diffusion in digestion tubes or Conway dishes, steam distillation directly from digestion tubes or from standard taper flasks, macro- or semimicro distillation, titration with an indicator or using automated titrators. Several semiautomatic to fully automatic distillation systems are now commercially available and allow for very rapid analysis. Digestion in tubes using block heaters with the temperature controlled electronically is now common. Several modern infrared digestion systems are available and have much faster heat-up and cool-down times than traditional aluminum block systems.

The choice here has been to present details of modern methods that could be widely used, to introduce special purpose methods by way of comments, and to provide citations for older or classical methods. Micro-Kjeldahl digestion procedures are given with and without steps to include NO₂⁻ and NO₃⁻. Usually NO₂⁻ plus NO₃⁻ is a negligible component of soil total N, and procedure given in Section 22.2 should be satisfactory. Macro-Kjeldahl procedures are little used nowadays because of the cost and disposal of chemicals used in the digestion, and the high precision of micro-Kjeldahl and Dumas procedures.

22.2 MICRO-KJELDAHL DIGESTION FOLLOWED BY STEAM DISTILLATION: WITHOUT PRETREATMENT TO INCLUDE NO₂⁻ AND NO₃⁻ QUANTITATIVELY

This method is appropriate for total N determination on samples of surface soil horizons in which the NO₃⁻ and NO₂⁻ contents are negligible. If used with samples containing significant amounts of NO₃⁻ or NO₂⁻, the results will be higher than for the fixed NH₄⁺ plus organic N content alone, but lower than for total N including NO₃⁻ and NO₂⁻. This method is not recommended for analysis of total N in soil samples from ¹⁵N tracer studies because of the significant influence of highly labeled NO₂⁻ or NO₃⁻ on ¹⁵N analyses. The method outlined in Section 22.3 is recommended for such samples.

22.2.1 MATERIALS AND REAGENTS: DIGESTION

Heating block with digestion tubes, timer, and temperature controller (see Section 22.2.5). The block must be capable of maintaining a temperature of 360°C for up to 5 h. Blocks holding 40 tubes (20 mm OD × 350 mm long) calibrated to hold 0.1 L are commonly used for micro-Kjeldahl digestions.
Air condenser designed to fit over the digestion tubes in the block (see Section 22.2.5).

Concentrated (18 M) H$_2$SO$_4$.

Low N content K$_2$SO$_4$, CuSO$_4$: mixed in mass ratio of 8.8:1 (K$_2$SO$_4$: CuSO$_4$ · 5H$_2$O). Approximately 3.5 g of mix is required per sample.

Hengar granules, both selenized and nonselenized.

### 22.2.2 Materials and Reagents: Distillation and Titration

1. Micro-Kjeldahl steam distillation apparatus (Figure 22.1). See Section 22.2.5.

2. Steam distillation flasks: 0.5 L round bottom, with 19/38 standard taper ground glass joint.

3. NaOH: 10 M and 0.1 M, prepared in CO$_2$-free deionized water.

![FIGURE 22.1. Steam distillation apparatus.](image-url)
Boric acid (2% w/v) plus indicator: place 80 g of boric acid (H₃BO₃) powder into a 0.25 L beaker. Add ~20–40 mL of H₂O and mix with a glass rod to wet all the H₃BO₃. Pour into ~3 L of H₂O in a 4 L flask and stir with an electric stir rod. Once wet, the H₃BO₃ dissolves readily. Add 80 mL of mixed indicator prepared as follows: 0.099 g bromocresol green and 0.066 g methyl red dissolved in 100 mL ethanol. Add 0.1 M NaOH cautiously until the solution turns reddish-purple (pH 4.8–5.0). Make up to 4 L with deionized H₂O and mix thoroughly.

Graduated beakers: 100 mL.

Burette: 10 mL graduated at 0.02 or 0.01 mL intervals. A magnetic stirrer is desirable.

H₂SO₄: 0.01 M (standardized).

**22.2.3 Procedure: Digestion, Distillation, and Titration**

1. Place sample, containing about 1 mg N, in a dry digestion tube. This will usually vary from 0.25 to 2.0 g.
2. Add 2 mL deionized H₂O (3 mL if using 2 g soil) and swirl to wet all the soil.
3. To each tube add 3.5 g of K₂SO₄:CuSO₄, mix.
4. Add one selenized and one nonselenized Hengar granule.
5. Add 10 mL concentrated H₂SO₄.
6. Place the digestion tubes into the digestion block.
7. Program the block to raise the temperature to 220°C and maintain it there for 1.5 h. Digestion will start and water will be removed during this time.
8. After 1.5 h of digestion at 220°C, put the air condensers onto the digestion tubes in the block.
9. Program the block to raise the temperature to 360°C and maintain it there for 3.5 h.
10. After digestion is complete, cool the samples overnight in the block or on a fiberglass pad.
11. Remove the air condenser and rinse with water.
12. Slowly and with swirling, add 25 mL deionized water to each cooled digestion tube. Vortex the sample to dissolve salts that may have solidified during cooling. If all the material does not enter into suspension, warm gently until it does. Transfer the sample quantitatively, with three washes of deionized water, to a 0.5 L round-bottom distillation flask.
13. With the condensers on, connect the distillation flask to the steam distillation apparatus; secure with a clamp.
Open the steam supply to the distillation head to allow steam into the tubing. Be sure the drain line is already open so that steam can exit to the drain.

Place a 100 mL graduated beaker with 5 mL of 2% H₃BO₃ under the condenser so that the tip of the condenser is immersed in the H₃BO₃.

Very slowly add an excess (usually 30 mL; see Section 22.2.5) of 10 M NaOH through the distillation head. Do not completely empty the NaOH reservoir, otherwise NH₃ may be lost through the stopcock.

Close the pinch clamp, or stopcock, going to the steam drain; this directs steam into the distillation flask. The steam generation rate should be such that the distillate is collected at about 6 mL min⁻¹. Collect 40 mL of distillate.

Open the pinch clamp to the steam drain and remove the distillation flask then close the pinch clamp to the distillation head. This sequence is important to prevent steam burns and drawback of fluid from the distillation flask into the steam line.

Wash the tip of the condenser into the beaker.

Titrate the distillate with 0.01 M H₂SO₄. The color change at the endpoint is from green to pink (pH ≈ 5.4).

22.2.4 CALCULATIONS

One mL of 1 M H₂SO₄ is equivalent to 28.01 mg of N.

\[
\text{Total N, g kg}^{-1} = \frac{(\text{mL sample} - \text{mL blank}) \times M \times 28.01}{\text{oven-dry mass of soil sample (g)}}
\]  

(22.1)

where \(M\) is the molarity of standard H₂SO₄, mL sample is the volume of standard H₂SO₄ used during titration of sample, and mL blank is the volume of standard H₂SO₄ used during titration of blank.

The blank is included from the digestion step onward; it contains all materials excluding a soil sample.

22.2.5 COMMENTS

Soil samples should be dried (usually air-drying) and ground to pass a 100 mesh (150 μm) sieve.

Heating blocks and tubes supplied by Tecator or Technicon have been found satisfactory for Kjeldahl digestions in our laboratory. The air condenser described by Panasiuk and Redshaw (1977) can be constructed by a competent glassblower. Equivalent devices are available from Tecator. A variety of sophisticated digestion systems are also available from VELP, Gernhart, and other manufacturers (e.g., Fisher, VWR, Cole Parmer, or other suppliers). Some of these units use infrared
digestion to greatly decrease heating and cooling times over traditional aluminum block systems.

3 The K$_2$SO$_4$:CuSO$_4$ mix can be prepared in the laboratory, obtained as a loose mix or in appropriately sized packages (~3.5 g) from commercial suppliers (e.g., Kjeltabs—trademark of Tecator, Inc.). Bulk mixes should use low N materials and be kept tightly sealed during storage to avoid absorption of moisture and caking.

4 A variety of manual, semiautomatic to fully automatic distillation systems are available from a variety of manufacturers (e.g., Labconco, Gerhardt, VELP). These systems are quite rapid, often reducing distillation times to ~2 min from ~7 to 8 min for the setup shown in Figure 22.1. Distillation systems similar to those in Figure 22.1 are described by Bremner (1996) and Bremner and Breitenbeck (1983). Commercial systems specifically designed for use with digestion tubes are available. We have used two distillation heads, each supplied with steam from a 5 L round-bottom boiling flask heated by a 600 W heating mantle. Several dozen Hengar granules are placed in each flask. Concentrated phosphoric acid (about 2 mL) is added to each boiling flask to absorb NH$_3$.

5 We have eliminated sample transfer from the digestion tube to a distillation flask by doing digestions in a 0.25 L digestion tube designed for a block that holds 20, rather than 40, tubes. The distillation head was modified by attaching a rubber stopper with a hole through which the standard taper joint of the distillation head fits. The 0.25 L digestion tube is attached to the distillation unit by fitting the end over the rubber stopper. It is held securely in place with a clamp, allowing distillation directly from the digestion tube. The distillation system described by Bremner and Breitenbeck (1983) uses tubes designed for the 40 tube blocks.

6 The NaOH must be added slowly and carefully to avoid violent bubbling that would force the liquid into the condenser and contaminate the distillation head. The amount of NaOH needed varies with the amount of H$_2$SO$_4$ consumed during digestion of the sample. Consumption of H$_2$SO$_4$ varies with the amount of soil organic matter and reduced minerals present; e.g., 1 g of C consumes 10 mL of H$_2$SO$_4$ (Bremner 1996).

7 Distillation can be replaced by autoanalyzer analysis of the digested sample. When we use this approach, the digested sample is diluted to 0.1 L followed by an autoanalyzer method for colorimetric measurement of NH$_4^+$ (Smith and Scott 1991).

8 Use of an automatic titrator can improve consistency and eliminate the need to mix an indicator into the boric acid solution.

9 The above Kjeldahl digestion method does not quantitatively recover fixed NH$_4^+$ in most soils. For total N analysis of soils with a high proportion of their N as fixed NH$_4^+$, an HF–HCl modification as described by Bremner (1996) may be necessary to release fixed NH$_4^+$.
22.3 MICRO-KJELDAHL DIGESTION FOLLOWED BY STEAM DISTILLATION: NO$_2^-$ AND NO$_3^-$ INCLUDED QUANTITATIVELY

This is the method of choice for total N analysis of samples of surface soil horizons containing an appreciable quantity of N as NO$_2^-$ or NO$_3^-$. Because of the significant influence of highly labeled NO$_2^-$ or NO$_3^-$ on $^{15}$N analyses, this method is recommended for analysis of total N in all soil samples from $^{15}$N tracer studies. This method is the same as in Section 22.2, except for the addition of a pretreatment to oxidize NO$_2^-$ to NO$_3^-$ and then to reduce the NO$_3^-$ to NH$_4^+$.

22.3.1 MATERIALS AND REAGENTS: PRETREATMENT AND DIGESTION

1. All items from Section 22.2.1, plus the following:
   2. Potassium permanganate solution: dissolve 50 g KMnO$_4$ in 1 L deionized H$_2$O; store in an amber bottle.
   3. H$_2$SO$_4$, 9 M: dilute concentrated H$_2$SO$_4$ to twice its volume with deionized H$_2$O.
   4. Fe powder; finer than 100 mesh sieve.

22.3.2 MATERIALS AND REAGENTS: DISTILLATION AND TITRATION

All items from Section 22.2.2.

22.3.3 PROCEDURE: PRETREATMENT TO REDUCE NO$_2^-$ AND NO$_3^-$ TO NH$_4^+$

1. Place sample, containing about 1 mg N, in a dry digestion tube. Usually this is 0.25–2.0 g of soil.
2. Add 2 mL deionized H$_2$O (3 mL if using 2 g soil) and swirl to wet all the soil.
3. Add 1 mL KMnO$_4$ and swirl for 30 s.
4. Hold the digestion tube at a 45° angle and very slowly pipette 2 mL dilute H$_2$SO$_4$.
5. Allow to stand for 5 min.
6. Add one drop N-octyl alcohol (to control frothing).
7. Add 0.5 g reduced Fe using a scoop, through a dry, long-stemmed funnel or thistle funnel tube.
8. Immediately cover the digestion tube with an inverted 25 mL beaker or 50 mL Erlenmeyer flask inverted to prevent loss of water.
9. Swirl to bring the Fe into contact with the acid.
Allow to stand (about 15 min) until strong effervescence has ceased.

Place digestion tubes into the digestion block and program it to raise the temperature to 100°C and hold it there for 1 h.

Cool the tubes before proceeding to digestion.

**22.3.4 Procedure: Digestion, Distillation, and Titration**

Follow steps 3 to 20 inclusive, of Section 22.2.3.

**22.3.5 Calculations**

Use Equation 22.1, described in Section 22.2.4.

**22.3.6 Comments**

1. The KMnO₄ oxidizes NO₂⁻ to NO₃⁻, which is reduced to NH₄⁺ by reduced Fe.
2. N-octyl alcohol is added to reduce frothing.
3. Goh (1972) concluded that it was not necessary to include the permanganate pretreatment when reduced iron is used as a reductant in the procedure to include NO₃⁻.
4. See Section 22.2.5 for additional important information.

**22.4 Dumas Methods**

Several automated Dumas systems are available (Kirsten and Jansson 1986; Tabatabai and Bremner 1991; Bremner 1996). Many systems combust the sample in a stream of pure O₂ at high temperatures, producing NOₓ and N₂. An aliquot of gas is carried by pure He into a reduction zone where elemental Cu reduces NOₓ to N₂, which is subsequently measured by a thermal conductivity detector. Other systems convert total N to N₂ by fusing the sample in a graphite crucible at very high temperatures in a He atmosphere, followed by determination of N₂ by gas chromatography (Bremner 1996). Numerous configurations are available depending on what other elements (e.g., C, H, S, O) are also to be determined. Since Dumas procedures are quite variable and instrument dependent, it is not possible to provide a generic methodology here.

Compared to Kjeldahl, Dumas techniques have the advantage of requiring less laboratory space, provide rapid analysis, require less chemical reactants, do not produce noxious fumes or hazardous chemical wastes, and include all forms of N without lengthy pretreatments (Bellomonte et al. 1987; Vittori Antisari and Sequi 1988). They are suitable for ¹⁵N tracer studies when linked by a continuous flow interface from the nitrogen analyzer to an isotope ratio mass spectrometer (Fiedler and Proksch 1975; Minagawa et al. 1984; Marshall and Whiteway 1985). For tracer studies, they avoid digestion, distillation, titration, evaporation, and subsequent oxidation of NH₃ to N₂.

Sample variability is a concern with combustion techniques because of the small sample size required in some instruments (<50 mg). Schepers et al. (1989) recommended that plant and
soil samples should be ball milled before combustion analysis. Arnold and Schepers (2004) reported on a simple roller-mill grinding procedure as an alternative to ball milling plant and soil samples. See Bremner (1996), Kowalenko (2001), Pérez et al. (2001), and Wang et al. (1993) for further information on grinding and sample preparation.

We recommend that soil samples be air-dried and passed through a 2 mm (10 mesh) sieve. Subsamples are then finely ground using a ball-mill such as the Brinkmann, Mixer Mill, model MM2. With the grinder set to its maximum setting, soils take 1.5–2 min to grind to a fine powder (<100 mesh). Ensure grinding capsules and balls are well cleaned before attempting the next sample. If samples are resinous, grind a small scoop of pure silica sand between samples (for ≈30 s) to help clean the resin from the ball and capsules; vacuum and wipe capsules and balls before grinding the next sample. After fine grinding, samples should be dried overnight at 60°C–70°C and cooled in desiccators before weighing for analysis.

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Chapter 23
Chemical Characterization of Soil Sulfur

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23.1 INTRODUCTION

Sulfur is relatively abundant in the terrestrial environment and assumed to be the 15th most abundant element (Arnhold and Stoeppler 2004). It is present in the soil in a variety of forms, both organic and inorganic, and various valence states (Blanchar 1986), each having different chemical, biological, and environmental significance. Various chemical analyses have been proposed to measure the various forms of sulfur in soils, for different purposes (e.g., soil genesis, plant availability, or environmental assessment). The relative success for measuring different forms, however, is limited by available chemical quantification methods. The most common basic measurements of soil sulfur forms are total, organic, inorganic, and extractable (i.e., plant-available) sulfur.

23.1.1 TOTAL SULFUR

Measurement of total sulfur is an important measurement on its own but it is often also involved in quantifying specific forms by difference calculation (e.g., total organic $S = total S$ minus total inorganic $S$). Numerous methods have been proposed for determining total sulfur, but none are universally accepted (Tabatabai 1982; Blanchar 1986). Almost all methods for measuring total soil require two steps: (1) conversion of all sulfur to one form and (2) quantification of the resulting form. Methods available for the conversion step include ashing (or dry combustion) and wet digestion (Tabatabai 1982; Blanchar 1986). Dry ashing includes use of ovens, heating elements, open flames (e.g., fusion), enclosed flames (e.g., oxygen flask), or high-temperature combustion using induction or resistance furnaces. Wet digestion may be either alkaline or acidic. Numerous sulfur quantification methods are available for gases and solutions in oxidized or reduced forms. Gases can be
quantified directly by infrared, chemiluminescent, coulometric, flame photometric, and other methods. Solutions (either absorbed/dissolved gases, liquid digests, or solubilized solids) are usually analyzed as sulfide or sulfate by spectrometry (colorimetry, flame emission, atomic absorption, etc.), ion-selective electrode, titration, gravimetry, and chromatography. Both or either of the conversion and the quantification steps can be automated. X-ray fluorescence (Jenkins 1984) measures sulfur in one step.

Comparisons of methods for determining total sulfur in soils have been conducted (e.g., Gerzabek and Schaffer 1986); however, the comparisons have been limited with respect to the scope of soils analyzed and the type of methods compared (Tabatabai and Bremner 1970b; Matrai 1989; Kowalenko 2001). Variable results occurred and no one method could clearly be said to give a true estimate of total sulfur (Hogan and Maynard 1984; Kowalenko 2000). The highest value cannot necessarily be taken as the true value.

Several studies have found that high-temperature combustion has not resulted in particularly satisfactory results for some soil samples, but more recent instrumentation has shown good results (Kowalenko 2001). Many combustion units have relatively high detection limits for analysis of materials, such as coal, that have substantial concentrations of sulfur. X-ray fluorescence requires an adjustment for the organic matter content in the sample (Brown and Kanaris-Sotiriou 1969). The success of low-temperature ashing has been variable (Tabatabai and Bremner 1970a; Killham and Wainwright 1981). Acid digestion should be used with caution, as gaseous losses of sulfur are possible (Randall and Spencer 1980). Dry ashing with sodium bicarbonate and silver oxide followed by ion chromatography (IC) or hydriodic acid reduction has shown variable results (Tabatabai and Bremner 1970b; Tabatabai et al. 1988). A fusion technique proposed for geological samples using sodium peroxide followed by IC was not completely satisfactory (Stallings et al. 1988). Hordijk et al. (1989) found good agreement for total sulfur measurement in freshwater sediments by IC with inductively coupled plasma (ICP) and roentgen fluorescence methods after Na₂CO₃/KNO₃ fusion.

### 23.1.2 Organic Sulfur

Organic sulfur accounts for most of the sulfur present in the surface horizons of soils (Tabatabai 1982; Blanchar 1986). A number of methods have been attempted to directly determine organic sulfur compounds in soils (Kowalenko 1978), but none have been universally accepted. Measurement of sulfur-containing amino acids such as methionine, cystine, etc., requires special precautions and these compounds do not appear to account for a very large portion of the total sulfur present. There has been some success in determining the sulfur content of lipid extracts (Chae and Lowe 1981; Chae and Tabatabai 1981), but this fraction also accounts for only a small portion of the sulfur in the soil. Sulfur that is present in microorganisms does not constitute a specific organic compound and may include inorganic as well as various organic forms. This fraction comprises only a small portion of the total sulfur (Strick and Nakas 1984; Chapman 1987) but may have considerable biological significance. In order to estimate microbial sulfur, the method requires the measurement of extractable inorganic sulfate.

Lowe and DeLong (1963) proposed the determination of carbon-bonded sulfur using a digestion with Raney nickel in sodium hydroxide. Although this method was shown to be quite specific for carbon-bonded sulfur, and hence most of the organic sulfur in soils, the method is not quantitative due to interference problems in soils and soil extracts (Freney et al. 1970; Scott et al. 1981). The difference between total sulfur and hydriodic acid-reducible sulfur appears to provide a better estimate of carbon-bonded sulfur than direct determination.
by Raney nickel digestion. The success of this or other difference approaches to quantifying organic sulfur in soils depends on accurate measurements of all fractions involved in the calculation. Likewise, determining organic sulfur by subtracting inorganic from total sulfur requires accurate measurement of all inorganic forms that are present in the sample.

### 23.1.3 Inorganic Sulfur

Inorganic sulfur is largely in oxidized (sulfate) form in aerobic soils, and in reduced forms (sulfide, elemental sulfur, etc.) in anaerobic soils (Tabatabai 1982; Blanchard 1986). No single method has been developed to measure total inorganic sulfur that includes reduced and oxidized forms. Highly reduced forms of sulfur are not very soluble; therefore, cannot be readily extracted for quantification. Methods for directly determining reduced inorganic sulfur have been proposed, but not thoroughly evaluated (Barrow 1970; Watkinson et al. 1987) because these forms are limited in agricultural soils. Zinc–hydrochloric acid distillation has been used to measure reduced inorganic sulfur in soils (David et al. 1983; Roberts and Bettany 1985), but would not include all inorganic forms (Aspiras et al. 1972). Digestion of soil with tin and hydrochloric or phosphoric acid has been proposed for measuring sulfide, but this method is not specific to inorganic sulfur (Melville et al. 1971; Pirela and Tabatabai 1988).

Elemental sulfur can occur naturally in some soils such as those that are anaerobic and associated with marine or marsh situations, or from aerial depositions (e.g., industrial pollution) and fertilizer applications. Measurement of elemental sulfur in soils usually requires extraction with an organic solvent (e.g., chloroform, acetone, toluene) followed by colorimetry, liquid or gas chromatography, or ICP (Maynard and Addison 1985; Clark and Lesage 1989; O’Donnell et al. 1992; Zhao et al. 1996).

Inorganic sulfate may be present in water in the soil, bound or adsorbed on soil surfaces, as relatively insoluble compounds such as gypsum (Nelson 1982), or in association with calcium carbonate (Robert and Bettany 1985). Sulfate is adsorbed on positive charges that occur in acidic soils (Tabatabai 1982), although a recent study has shown that sulfate-binding mechanisms are complex (Kowalenko 2005). Solution and adsorbed inorganic sulfate are assumed to be immediately available for plant uptake. To measure total inorganic sulfate in soils, all of these forms would need to be measured. Although there is a good theoretical basis for solution and adsorbed pools being present in the soil, there are practical limitations in their extraction and subsequent quantification. The choice of the extractant will depend on analytical equipment available, form of sulfate (e.g., solution, sorbed) to be examined, and type of soil to be analyzed. Numerous solutions have been used for extracting combined soluble and adsorbed sulfate including acetates, carbonates, chlorides, phosphates, citrates, and oxalates (Beaton et al. 1968; Jones 1986). Most of these studies have focused on measurement of plant-available rather than total inorganic sulfate. If only soil solution sulfate is to be measured, water would theoretically be sufficient. However, weak calcium chloride is often preferred, since it depresses clay and organic matter during extraction (Tabatabai 1982). Lithium chloride is also used, since lithium would inhibit microbial activity that may mineralize organic sulfur during and after extraction (Tabatabai 1982). Adsorbed sulfate (together with solution sulfate) is usually extracted with sodium, potassium, or preferably, calcium phosphate (Beaton et al. 1968). A concentration of 500 mg P L$^{-1}$ is usually adequate to displace sulfate in most soils; however, for soils that fix considerable phosphate, 2000 mg P L$^{-1}$ may be required. Alkaline solutions theoretically are effective for extracting adsorbed sulfate, since the adsorption mechanism is pH dependent, but would extract additional, highly colored organic materials that cause...
problems for some sulfate quantification methods. Acidic extractants may extract portions of gypsum- or carbonate-associated sulfate that may be present in some soils. Buffered extractants may result in more consistent results. Preextraction treatment on the sample such as air drying will also influence the results (Kowalenko and Lowe 1975; Tabatabai 1982). Specialized methods are required to measure insoluble sulfate in gypsiferous (Khan and Webster 1968; Nelson 1982) or acid-sulfate (Begheijn et al. 1978) soils. It is possible that oxidized forms other than sulfate such as thiosulfate, tetrathionate, or sulfite (Nor and Tabatabai 1968; Wainwright and Johnson 1980) may be found in soils, but are probably present only as intermediates during oxidation or reduction of sulfur.

There are a number of methods for quantifying sulfate (Patterson and Pappenhagen 1978; Tabatabai 1982), but not all are compatible with all soil extract solutions. The method should be quantitative, adequately sensitive, free from interferences, and specific for sulfate. Unfortunately, there does not appear to be a specific, direct colorimetric method to determine sulfate. The most frequently used sulfate quantification methods applied to soil extracts include precipitation with barium or sulfide analysis after hydriodic acid reduction. There are numerous variations for barium-precipitation methods, including titrimetric, turbidimetric, gravimetric, and colorimetric methods (Beaton et al. 1968), but all are subject to interferences. The hydriodic acid reduction method is quite sensitive and relatively free from interferences, but the reduction procedure is time consuming, difficult to automate, and the chemicals are costly. The hydriodic acid reagent (Johnson and Nishita 1952; Beaton et al. 1968) reduces both organic and inorganic sulfate to sulfide. This method has been used extensively for soil analyses. It is quite specific for sulfate (Tabatabai 1982), whether organic or inorganic, therefore, is not specific to inorganic sulfate. Various pretreatments have been attempted to make the methods specific to inorganic sulfate, but each has distinct limitations (Kowalenko and Lowe 1975). Pirela and Tabatabai (1988) have shown that the hydriodic reagent will decompose some elemental sulfur, thus, the resulting values should be interpreted accordingly. More recently, IC, ICP, and x-ray fluorescence have been used for sulfur analysis of soil extracts (Gibson and Giltrap 1979; Tabatabai 1982; Maynard et al. 1987). Although IC is specific for inorganic sulfate analysis, it is quite sensitive and not affected significantly by interferences; specialized instrumentation and attention to the choice of the extraction salts (concentration and types) are required. Inductively coupled plasma spectrophotometry and x-ray fluorescence provide fast quantification; however, they include all forms (organic and inorganic) of sulfur.

**23.2 DIGESTION FOR TOTAL SULFUR DETERMINATION (TABATABAI AND BREMNER 1970a; KOWALENKO 1985)**

Since there is potential for sulfur to be lost by volatilization from hot, acidic solutions, an alkaline solution is preferred for digesting soil samples for total sulfur determination. A method that uses sodium hypobromite for the digestion developed by Tabatabai and Bremner (1970a) is described here. The method is modified from the original by the use of a different custom-built reduction/distillation apparatus (Kowalenko 1985) that allows the measurement of sulfur as sulfide using bismuth to quantify the resulting sulfide rather than methylene blue reaction (Kowalenko and Lowe 1972). The bismuth method combined with the modified glassware apparatus has the advantages of short analysis time, versatility for types of sulfur analyses, and the equipment requirements are small. The digestion and quantification must be done in the same vessel; therefore, an alternate sulfur quantification method (e.g., ICP or IC) should not be substituted for the hydriodic acid method without assessment of effectiveness and making appropriate modifications.
23.2.1 MATERIALS AND REAGENTS

1 Sodium hypobromite digestion reagent: in a fume hood, add slowly with constant stirring 3 mL of bromine to 100 mL 2 M sodium hydroxide. This reagent has limited stability, and should be prepared immediately before use or at least daily.

2 Formic acid (90%).

3 Temperature-controlled digestion block: the block heater (commercially available or can be custom-built) must accommodate the sample vessel and be capable of maintaining a temperature of 250°C–260°C.

23.2.2 APPARATUS

The recommended apparatus (Kowalenko 1985) for the sample digestion and subsequent sulfur determination by hydriodic acid reduction is custom-built glassware and includes two components, the vessel into which the sample is placed (capable of being heated in a block) and a part that fits onto this vessel to form an airtight unit that includes the ability to dispensing the reducing solution for nitrogen gas flushing (Figure 23.1). The inlet of the gas flush is attached to a steady and controllable source of nitrogen gas, and the outlet should include an arm with a capillary dropper attached by flexible inert tubing such that the exiting gas is bubbled through the sodium hydroxide absorbing solution. The hydriodic acid reduction procedure is described in Section 23.3. The apparatus must be supported to allow it to be lowered to and lifted from a block to heat the sample vessel at 110°C–115°C during the hydriodic acid reduction phase.

23.2.3 PROCEDURE

1 Weigh a finely ground (<100 mesh) soil sample directly into the dry sample vessel. The sample size (an oven-dry basis is recommended) should be adjusted so that the sulfur content is within the range of the calibration standards. For example, digest 0.1–0.4 g of Ah horizon of mineral soil or 0.1 g or less of organic horizon with a 30 mL bismuth sulfide final volume that provides a 0–200 mg S range of analysis.

2 Add 3 mL of the sodium hypobromite digestion reagent and thoroughly wet the soil sample with the reagent by swirling the tube. After letting the sample stand for 5 min, evaporate the mixture to dryness in the digestion block at 250°C–260°C, then continue heating for an additional 30 min. Remove from heat and allow the tube to cool for about 5 min.

3 Resuspend the digested sample in 1 mL of water by swirling and heating briefly. After cooling, add 1 mL of formic acid to eliminate any excess bromine that may be present.

4 Quantify the sulfur content with the hydriodic acid reduction reagent method (as described in Section 23.3).
23.2.4 CALCULATIONS

Calculate the sulfur content (as mg S kg\(^{-1}\)) of the sample by taking into consideration the oven-dry weight of the sample and using the standard curve produced by the hydriodic acid determination of sulfur in the sample (see Section 23.3.3).

23.2.5 COMMENTS

1. The sulfur content of soil samples containing a large amount of organic material may be underestimated by this method as described, and sequential digestions, and/or longer heating times may be required (Guthrie and Lowe 1984).

2. This digestion method can also be used to determine total sulfur content in solutions, but the aliquot of the solution should be dried before the digestion is conducted (Kowalenko and Lowe 1972). However, an ICP instrument capable of
sulfur determination could be used as an alternative direct analysis since the instrument operates on liquid samples and measures total sulfur. Ion chromatography which also works with liquids, measures a specific form of sulfur (e.g., sulfate); therefore, would have to be applied to a digested sample to determine total sulfur.

**23.3 SULFATE DETERMINATION BY HYDRIODIC ACID REAGENT REDUCTION**

The quantification of sulfate in soil studies has been limited by the lack of an accurate, suitable, and direct colorimetric analysis method. As noted earlier, sulfate colorimetric methods that have been used are essentially based on precipitation with barium, and, hence, all are subject to interference and most lack sufficient sensitivity. The other method that has been used widely in soil studies is based on the reduction of sulfate with hydriodic acid reagent and sulfur measured on the resulting hydrogen sulfide. More recently, several new sulfur analytical methods have been developed and used for soil studies with ICP and IC being predominant. Both of these methods require specialized and expensive instrumentation. Since their operation is specific to the instrument and defined by the manufacturer, they will not be described here. Although instruments for these methods are now widely available, there have been few assessments of their effectiveness or comparisons with values using traditional methods in soil studies. It is generally accepted that interference is limited for ICP measurements, but the sulfur measurement includes all forms rather than sulfate specifically. Ion chromatography can be specific for sulfate measurements, but is subject to interference by a wide array of anions and cations including those that are a part of traditional digestion and extraction solutions. These potential interferences must be addressed in each specific case. These influences can be circumvented in a variety of ways and depend on the specific instrument that is used. The hydriodic acid method is, therefore, outlined here because it does not require specialized instrumentation and procedures, and has a long history of application to soils. The method involves hydriodic acid reagent described by Johnson and Nishita (1952) used in a modified apparatus (Kowalenko 1985) and bismuth sulfide (Kowalenko and Lowe 1972) rather than methylene blue quantification of the sulfur evolved as hydrogen sulfide.

**23.3.1 MATERIALS AND REAGENTS**

1. Custom-built reduction/distillation apparatus and heating block (Kowalenko 1985) as outlined in Section 23.2.2.

2. Nitrogen gas: the gas must be relatively pure and free from sulfides in particular. The gas may be purified by bubbling it through a solution containing 5 to 10 g mercuric chloride in 100 mL of 2% (w/v) potassium permanganate. The flow of the nitrogen gas to the reduction/distillation apparatus should be regulated to approximately 200 mL min^{-1}. This can be done by commercially available flow meters (e.g., Rotometer [Kowalenko 1985]) or forcing the gas through an appropriate length (e.g., 30 cm) of capillary glass tubing (Kowalenko and Lowe 1972).

3. Hydriodic acid reducing reagent: mix 4 volumes (e.g., 400 mL) of hydriodic acid (e.g., 57% with 1%–2.5% hypophosphorus acid preservative), 1 volume (e.g., 100 mL) of hypophosphorus acid (50%), and 2 volumes (e.g., 200 mL) of
formic acid (90%) and, while bubbling purified nitrogen gas through it, heat for 10 min at 115°C–117°C. Continue the nitrogen gas flow through the reagent while cooling. The heating should be done in a well-ventilated hood; refluxing or a special gas-trapping apparatus (Tabatabai 1982) is recommended. Since this reagent is not very stable, only sufficient reagent for several days of sample and standard analyses should be prepared. Storage in a brown bottle and refrigeration will extend its stability.

4. 1 M sodium hydroxide: for absorbing hydrogen sulfide.

5. Bismuth reagent: dissolve 3.4 g bismuth nitrate pentahydrate in 230 mL glacial acetic acid. Also, dissolve 30 g gelatin in 500 mL water and mix thoroughly. Both solutions will require gentle heating for dissolution. Filter the bismuth solution if it is not clear. The final bismuth reagent is prepared by combining the bismuth and gelatin solutions and diluting to 1 L. This reagent is quite stable at room temperature.

6. Sulfate-S standards: prepare a 1000 mg S L⁻¹ stock solution by dissolving 5.435 g dried reagent-grade potassium sulfate and diluting to 1 L. Working standards are made by appropriate dilutions.

7. Spectrophotometer: the instrument should be suitable for measurement at 400 nm and capable of accommodating small (e.g., 7.5 mL) volumes, including provision for rinsing the cuvette or analysis chamber.

23.3.2 Procedure

1. Weigh a portion of dry, whole soil or pipette an appropriate volume (e.g., 2–5 mL) of the filtered extract into the modified Taylor tube for the hydriodic acid reduction/distillation apparatus and evaporate (up to 100°C) the aliquot of the extract solution to dryness. The weight of soil or volume of extract solution should be adjusted in size such that the amount of sulfur in the apparatus will be within the range of calibration standards that is conducted.

2. Assemble the custom-built reduction/distillation apparatus above the small heating block in such a way that the modified Taylor tube can be easily installed on or removed from the dispenser portion. This can be accomplished by either having the heater in a fixed position and the dispenser portion with the Taylor tube easily raised and lowered, or the dispenser plus Taylor tube fixed and the heater on a jack assembly. A tube (50 mL test tube or larger, depending on the range of the standard curve) containing the sodium hydroxide solution for absorbing the hydrogen sulfide should be fixed in a position such that the nitrogen gas from the outlet of the apparatus will bubble through several centimeters of the absorbing solution. The volume of the absorbing solution is adjusted for the concentration range of sulfate to be analyzed. Adjust the nitrogen gas at the appropriate rate and fill the burette with reducing reagent. As each distillation is completed and the Taylor tube is removed, a watch glass should be placed above the heater to intercept any drops of reducing reagent. The entire apparatus should be adequately ventilated.

3. Condition the reduction/distillation apparatus by attaching a modified Taylor tube containing a high (e.g., 200 mg S L⁻¹) sulfate standard to the reduction/distillation
apparatus, place the apparatus in heating position, adjust the nitrogen gas flow, and dispense 4 mL of reducing reagent into the attached Taylor tube. The apparatus requires conditioning at the beginning of each new session with high sulfate–sulfur standard to ensure quantitative initial distillation. Distill until all the sulfate has been reduced and transferred into the absorbing solution. The time required for this process will vary with the flow rate of the nitrogen and the ‘‘dead’’ volume within the apparatus. About 8 to 10 min should be adequate, but calibration under specific conditions is recommended (Kowalenko 1985). After distillation of the hydrogen sulfide is complete, remove the tube containing the absorption solution from the apparatus and check that the distillation process is functioning by immediately adding an appropriate volume of bismuth reagent and mix thoroughly. This volume should correspond to the volume of the absorbing solution (2:1 absorbing solution:bismuth reagent) depending on the range of the standard sulfate–sulfur required. For example, 20 mL of absorbing solution is suitable for a 1–200 mg S L\(^{-1}\) range and 5 mL for a 1–40 mg S L\(^{-1}\) range.

After the initial setup and conditioning of the apparatus, digested soil samples, whole soil sample or dried soil extract aliquot, and dried standards are distilled into appropriate volumes of absorption solution, and bismuth reagent is immediately added in preparation for quantitative measurements of the bismuth sulfide produced. Measurements are best conducted in batches and adequate standards included in each batch. Measure absorbance of the sample and standard solutions at 400 nm.

### 23.3.3 Calculations

For measurements of sulfate in a soil sample placed directly into the sample vessel of the hydriodic acid analysis apparatus, prepare the standard curve for the bismuth colorimetric determination as an appropriate range of a quantity (e.g., mg S) of sulfur distilled in a single analysis. Then the concentration of sulfur in the soil sample is calculated as the quantity of sulfur relative to the standard curve divided by the weight of the soil (oven-dry basis) in the vessel during the analysis. Also, for measurements on solution samples, standardize the apparatus on an appropriate range of a quantity of sulfur in a single analysis, but calculate the concentration of the sulfur on an oven-dry basis taking into account the aliquot size of the analyzed solution that was dried in the apparatus sample vessel and the ratio of the analyzed solution to the weight of the soil.

### 23.3.4 Comments

1. Filter paper has been found to contain variable amounts of sulfate which will be leached during the filtration. Washing the filter paper with some of the extractant prior to filtration is recommended.

2. Water has been shown to reduce the efficiency of hydriodic acid reagent to reduce sulfate to sulfide (Kowalenko and Lowe 1975); therefore, the standard and sample solution aliquot volumes should either be the same throughout or all the liquid of the aliquot evaporated to dryness. Although evaporating the sample to dryness is time consuming, it does provide an opportunity for altering the sensitivity of the analysis (i.e., evaporate a small volume for soils with a significant sulfate–sulfur content or a large volume for soils with a low sulfate–sulfur content).
The methylene blue color reaction is subject to interference; therefore, passing the nitrogen gas through a pyrogallol–sodium phosphate wash just prior to the hydrogen sulfide absorption is recommended (Johnson and Nishita 1952). The bismuth sulfide method is much less sensitive to interference; therefore, the pyrogallol–sodium phosphate wash can be eliminated (Kowalenko and Lowe 1972). Use of a Taylor tube rather than a condenser to provide refluxing can shorten reduction/distillation times from 60 to 10 min (Kowalenko 1985). The apparatus is also simpler to fabricate and is versatile for different types of analyses. Although the sensitivity of the bismuth reaction is considerably lower than the methylene blue reaction, it can be adequately enhanced for most soil studies by decreasing the volume into which the hydrogen sulfide is absorbed and/or by increasing the size of the original sample being analyzed. However, as the volumes of the absorbing solution and bismuth reagent are decreased to increase the sensitivity, increased attention must be given to the precision and reproducibility of absorbing solution and bismuth reagent volume measurements, particularly relative to the standard samples. The spectrophotometer should be capable of accommodating the small sample sizes involved, including appropriate rinsing between samples.

The original sulfate determination procedure (Johnson and Nishita 1952) recommended that the nitrogen gas should be purified before use. Currently available sources of nitrogen are more uniform and free from impurities; therefore, purification of the gas may be omitted. The purity of the gas for analysis purposes can be evaluated by examining blanks. There should also be fairly good control of the flow rate of the nitrogen gas with a high enough rate to transfer the hydrogen sulfide produced into the receiver solution quickly, but slow enough that the sulfide gas can be absorbed by the sodium hydroxide.

Sources of contamination, such as rubber connectors or lubricants for sealing connections, should be considered, particularly in the reduction/distillation procedure. A small amount of water is adequate to seal the Taylor tube to the rest of the apparatus during the reduction/distillation.

Hydriodic acid is available in concentrations ranging from 48% to 66% and with or without preservative. Although these products contain varying quantities of sulfate contamination, the sulfate is removed by heating the mixed reagent. The 57% hydriodic acid with preservative has been found to be acceptable. If other products are used, the proportion of hydriodic acid to the other acids may need adjustment and the final reagent tested for effectiveness. Adequate time should be allowed for acquisition of hydriodic acid, as stocks are often limited.

Although the hydriodic acid reduction procedure is not influenced by a wide variety of salts, it is recommended that the standards should be similar to the sample’s matrix (e.g., water for whole soil or the solution used for extraction). For the alkaline digested soil, standards should be included through the digestion process. This precaution will also provide a check on sulfate or sulfide contamination that may be present in the extract or digestion solutions.

The hydriodic acid method, although relatively specific for sulfate, includes both inorganic and organic forms. This should not be neglected when the results are being interpreted. When this method is used to determine total organic sulfate...
(or total carbon-bonded sulfur by difference) the capability of accurately determining total inorganic sulfate or total sulfur must be considered, particularly when unusual samples (e.g., subsurface, anaerobic, organic, etc. samples) are being examined. The difference value will involve the error or variability associated with two analyses rather than one.

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Chapter 24
Total and Organic Phosphorus

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24.1 INTRODUCTION

This chapter describes several methods used for the determination and characterization of total phosphorus (P\textsubscript{t}) and organic phosphorus (P\textsubscript{o}) in soils.

Determination of the P\textsubscript{t} in soil requires the solubilization of P through the decomposition or destruction of mineral and P\textsubscript{o} containing materials in the soil. Historically, the two most widely recognized procedures for the determination of soil P\textsubscript{t} are the sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}) fusion method and the perchloric acid (HClO\textsubscript{4}) digestion method (Olsen and Sommers 1982). Currently, neither method is widely used in studies involving the determination of soil P\textsubscript{t}. Although the Na\textsubscript{2}CO\textsubscript{3} fusion method is considered the most reliable procedure for quantitative determination of P\textsubscript{t} in soils, it is laborious, tedious, and generally unsuitable for the analyses of large numbers of samples. Digestion with HClO\textsubscript{4}, although more adaptable as a routine laboratory procedure, requires the use of fume hoods specifically designed for the HClO\textsubscript{4} digestion. The potential danger of explosions, due to HClO\textsubscript{4} buildup or reaction of HClO\textsubscript{4} with organic materials, has led many institutions and laboratories to discontinue the use of HClO\textsubscript{4} digestions. Given the greater applicability of the HClO\textsubscript{4} digestion method as a routine laboratory procedure for the analysis of a large number of samples, this procedure has been included in the current text. The reader is referred to the previous edition of this text (O’Halloran 1993) or Olsen and Sommers (1982) for details on the Na\textsubscript{2}CO\textsubscript{3} fusion method. Three additional methods for the determination of P\textsubscript{t} in soil are presented in this chapter. One involves the alkaline oxidation of the sample using sodium hypobromite (NaOBr)/sodium hydroxide (NaOH) (Dick and Tabatabai 1977) and the other two are wet acid digestion procedures using either sulfuric acid (H\textsubscript{2}SO\textsubscript{4})/hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})/hydrofluoric acid (HF) (Bowman 1988) or H\textsubscript{2}SO\textsubscript{4}/H\textsubscript{2}O\textsubscript{2}/lithium sulfate (Li\textsubscript{2}SO\textsubscript{4})/selenium (Se) (Parkinson and Allen 1975).

Soil P\textsubscript{o} methods can be divided into methods that attempt to measure the total P\textsubscript{o} and methods that attempt to characterize both the amount and the forms of P\textsubscript{o} in the soil.
The first group consists of extraction (Mehta et al. 1954; Bowman and Moir 1993) or ignition techniques (Saunders and Williams 1955). In each case, total soil P\textsubscript{o} is not determined directly, but rather is calculated as the increase in inorganic P (P\textsubscript{i}) measured after the digestion of a soil extract or ignition of a soil sample. The ignition technique is less laborious than the extraction techniques, but it is also subject to a greater number of errors.

Characterization of soil P\textsubscript{o} forms can be done either by sequential fractionation techniques that provide operationally defined pools of P\textsubscript{o} or techniques that identify specific groups or forms of P\textsubscript{o} materials in soils. Sequential fractionation techniques, such as the modified procedure of Hedley et al. (1982) that is presented in Chapter 25 of this manual, characterize soil P\textsubscript{o} forms by measuring P\textsubscript{i} and soluble-reactive P (srP) in each fraction and then assuming that the difference between P\textsubscript{i} and srP is P\textsubscript{o}. However, caution should be used when interpreting P\textsubscript{o} results by sequential fractionation: first, because there is the potential for P\textsubscript{o} forms to be altered by previous extractants within any sequential fractionation procedure; and second, because srP measures only orthophosphate (HPO\textsubscript{4}\textsuperscript{2-}, H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}), the “P\textsubscript{o}” measured by the difference between P\textsubscript{i} and srP in a solution may also include complex P\textsubscript{i} forms such as pyrophosphate or polyphosphates.

In the identification of specific P\textsubscript{o} compounds, four groups of soil P\textsubscript{o} compounds have been detected in soils. Orthophosphate monoesters are esters of phosphoric acid, with one C moiety per P, and include inositol phosphates, sugar phosphates, phosphoproteins, and mononucleotides. Orthophosphate diesters are also esters of phosphoric acid, but have two C moieties per P. These include DNA, RNA, phospholipids, teichoic acid, and aromatic compounds. Phosphonates contain C–P bonds rather than ester linkages, and occur as phosphonic acids and phosphonolipids. The final group of soil P\textsubscript{o} compounds is orthophosphate anhydrides. Although most of these are complex P\textsubscript{i} compounds such as pyrophosphate and polyphosphate, this grouping also includes important organic orthophosphate anhydrides such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP). More details on soil P\textsubscript{o} compounds can be found in Condron et al. (2005).

A range of techniques is available to examine specific soil P\textsubscript{o} compounds. Most involve extraction with a reagent specific to the recovery of a particular P compound, followed by analysis using techniques such as \textsuperscript{31}P nuclear magnetic resonance (NMR) spectroscopy, enzyme hydrolysis, thin-layer chromatography, high-performance liquid chromatography (HPLC), and mass spectroscopy. These methods are often laborious, may require complex and expensive instrumentation, and may be specific to only one P compound or group of P compounds. In addition, the interpretation of results is limited by incomplete extraction or poor detection. Presented in this chapter are two common procedures to characterize P\textsubscript{o} compounds in soil extracts: \textsuperscript{31}P NMR spectroscopy and enzyme hydrolysis. Both allow the determination of the relative proportions of a range of P compounds in soil samples.

### 24.2 TOTAL PHOSPHORUS

Currently, the most popular approaches to soil P\textsubscript{i} determinations involve either alkali or acid oxidation or digestion of the soil samples. Each of the following methods has an advantage over the Na\textsubscript{2}CO\textsubscript{3} fusion method in that they are more adaptable for the routine analysis of a greater number of soil samples. As mentioned previously, digestion with HClO\textsubscript{4} requires special HClO\textsubscript{4} fume hoods, and care must be taken to avoid explosions. It has been reported that the HClO\textsubscript{4} digestion method gives relatively low P\textsubscript{i} values in highly weathered materials and with samples containing apatite inclusions (Syers et al. 1967, 1968, 1969). Each of the
remaining three methods has been reported to produce similar to slightly greater results to those obtained with the HClO$_4$ digestion procedure (Dick and Tabatabai 1977; Bowman 1988; Rowland and Grimshaw 1985) without the need for a special fume hood to carry out the oxidation or digestion. The H$_2$SO$_4$/H$_2$O$_2$/HF digestion method is relatively fast and is ideally suited for small numbers of samples. This procedure, however, requires HF-resistant materials, since HF attacks glass. The NaOBr/NaOH and H$_2$SO$_4$/H$_2$O$_2$/Li$_2$SO$_4$/Se methods are well suited for the analysis of large numbers of samples.

The reader is also referred to two additional procedures for the determination of soil P, that may be of interest. The first involves a fusion method using NaOH that melts at a lower temperature than Na$_2$CO$_3$ (325°C versus 850°C), and therefore allows the use of nickel rather than platinum crucibles (Smith and Bain 1982). Although this procedure was initially reported to give similar soil P$_i$ values as the Na$_2$CO$_3$ fusion method for 10 Scottish soils (Smith and Bain 1982), problems with low recoveries of soil P$_i$ in New Zealand soils with high organic matter content (soils that have >80% weight loss on ignition) have been identified (Taylor 2000). The second method is based on the Thomas et al. (1967) method for plant tissue digestion using H$_2$SO$_4$/H$_2$O$_2$ to digest a soil sample in an aluminum block digestor. This procedure is presented as the final step (i.e., digestion of soil residue) in the sequential fractionation procedure described in Chapter 25. Agbenin and Tiessen (1994) found for semiarid tropical soils in Brazil that the H$_2$SO$_4$/H$_2$O$_2$ method gave comparable to slightly higher soil P$_i$ values than the Na$_2$CO$_3$ fusion method. Gasparatos and Haidouti (2001) studying 15 soils varying in extractable P levels reported that this method gave soil P$_i$ values that were 95%–105% of those obtained with HClO$_4$ digestion.

Regardless of the procedures selected, it is recommended that finely ground soil, 0.15–0.18 mm (100–80 mesh), be used to allow for efficient recovery or extraction of P from the soil material, and to improve the reproducibility. The moisture content of the soil should be known so as to allow expression of P content on an oven-dried basis. Blank samples containing no soil should also be included to assess the possibility of P contamination and to serve as a suitable reagent blank for the colorimetric determination of P. Inclusion of a reference sample with known P$_i$ and the use of duplicate samples are also encouraged.

### 24.2.1 PERCHLORIC ACID DIGESTION (OLSEN AND SOMMERS 1982)

As a safety precaution, samples should routinely be predigested in concentrated nitric acid (HNO$_3$) before proceeding with the HClO$_4$ digestion. This method is suitable for the determination of soil P$_i$ in a large number of samples, although the use of an HClO$_4$ fume hood is essential. The digestion can be carried out using 250 mL Erlenmeyer flasks and a hot plate, or by using an aluminum block digestor with 75, 100, or 250 mL digestion tubes (see comments on p. 268). The HClO$_4$ digestion typically recovers 92%–96% as much P as the Na$_2$CO$_3$ fusion method (Sommers and Nelson 1972; Dick and Tabatabai 1977; Bowman 1988), although the pretreatment of soil samples with HF can increase the recovery of P (Kara et al. 1997).

#### Materials and Reagents

1. HClO$_4$ fume hood

2. Hot plate (with 250 mL Erlenmeyer flasks) or aluminum block digestor (with 250 mL digestion tubes)
**Procedure**

1. Accurately weigh approximately 2.0 g of finely ground soil into a 250 mL volumetric or Erlenmeyer flask.

2. Add 20 mL concentrated HNO₃ to flask and mix well. Heat (approximately 130°C) to oxidize the organic matter in the sample. Organic matter oxidation is complete when the dark color due to the organic matter in the sample disappears.

3. Allow the soil–HNO₃ mixture to cool slightly. In the HClO₄ fume hood, add 30 mL of HClO₄ and digest the sample at the boiling temperature (approximately 200°C) for 20 min. During this time dense white fumes should appear and the insoluble solid material left in the bottom of the flask or digestion tube should appear like white sand. If necessary, use a little (less than 2 mL) extra HClO₄ to wash down any black particles that have stuck to the sides of the flask or digestion tube. Heat for another 10–15 min.

4. Allow the mixture to cool. With distilled/deionized water transfer the mixture to a 250 mL volumetric flask and make to volume with distilled/deionized water. Mix thoroughly.

5. Allow sediment to settle before taking an aliquot for analysis.

6. Determine P concentration in an aliquot of the clear supernatant as indicated in Section 24.5.

**Comments**

A 40 tube aluminum block digestion system with volumetric 75 or 100 mL digestion tubes can also be used in this procedure by using half the amounts of sample, HNO₃, and HClO₄ described above. The digested material is diluted to a final volume of 75 or 100 mL (step 4).

**24.2.2 SODIUM HYPOBROMITE/SODIUM HYDROXIDE ALKALINE OXIDATION METHOD (DICK AND TABATABAI 1977)**

This method involves boiling to dryness a mixture of soil and NaOBr–NaOH solution using a sand bath or, as modified by Cihacek and Lizotte (1990), an aluminum block digestor. Formic acid is added after completion of the NaOBr–NaOH treatment to destroy residual NaOBr remaining after oxidation of the sample. Soil Pᵡ is then extracted from the sample using 0.5 \( M \) \( H₂SO₄ \). The method permits the digestion of a large number of samples at one time, although more manipulation of the sample is required compared to the HClO₄ method. Dick and Tabatabai (1977) using a wide range of soils from the United States and Brazil found that for soil Pᵡ, this method removed about 96% as much P as the \( Na₂CO₃ \) fusion method, and was comparable (about 1% higher) to the P determined by HClO₄ digestion. Cihacek and Lizotte (1990), using soils from the Great Plains region of the United States, found that this procedure
removed significantly (about 3%) more P than the HClO₄ digestion. Kara et al. (1997) found this method to recover 93%–100% of the P determined by Na₂CO₃ fusion and 99%–102% of the P determined by HClO₄ digestion on soils from Scotland and Turkey.

**Materials and Reagents**

1. Sand bath for which the temperature of the sand can be regulated at 260°C–280°C or an aluminum block digestor (see Comment 3 on p. 270).
2. Fume hood.
3. Boiling flask (50 mL) with stoppers or digestion tubes for the aluminum block digestor.
4. Centrifuge and 50 mL centrifuge tubes.
5. NaOH, 2 M: dissolve 80 g NaOH in a 1 L volumetric flask containing 600 mL of distilled/deionized water. Allow to cool and make to volume with distilled/deionized water.
6. NaOBr/NaOH solution: prepare this solution in a fume hood by adding 3 mL of bromine slowly (0.5 mL min⁻¹) and with constant stirring to 100 mL of 2 M NaOH. Prepare the NaOBr–NaOH solution immediately before use.
7. Formic acid (HCOOH), 90%.
8. H₂SO₄, 0.5 M: add 28 mL concentrated H₂SO₄ to 600 mL distilled/deionized water in a 1 L volumetric flask. Mix, allow to cool, and make to volume using distilled/deionized water.
9. Color developing solutions (see Section 24.5.1).

**Procedure**

1. Accurately weigh between 0.10 and 0.20 g of finely ground soil into a dry 50 mL boiling flask.
2. Add 3 mL of NaOBr–NaOH solution to the boiling flask, and swirl the flask for a few seconds to mix the contents. Allow the flask to stand for 5 min, and then swirl the flask again for a few seconds.
3. Place the flask upright in a sand bath (temperature regulated between 260°C and 280°C) situated in a fume hood. Heat the flask for 10–15 min until its contents are evaporated to dryness, and continue heating for an additional 30 min.
4. Remove the flask from the sand bath, cool for about 5 min, add 4 mL of distilled/deionized water and 1 mL of 90% HCOOH. Mix the contents, and then add 25 mL of 0.5 M H₂SO₄. Stopper the flask and mix the contents.
5. Transfer the mixture to a 50 mL plastic centrifuge tube and centrifuge at 15,000 g for 1 min.
Determine P concentration in an aliquot of the clear supernatant as indicated in Section 24.5.

Comments
1. It is very important that the NaOBr–NaOH solution be prepared immediately before use. Dick and Tabatabai (1977) reported that storing the NaOBr–NaOH at 4°C for 24 h reduced Pt values by 2%–4%.

2. Sample sizes up to 0.5 g can be analyzed for most soils. However, samples containing high amounts of Fe show large decreases in the value of Pt when sample sizes are increased above 0.2 g.

3. A sand bath may be prepared by placing 3–4 cm of silica sand on a hot plate; however, even temperature regulation across the sand bath can be difficult to achieve. Cihacek and Lizotte (1990) found that the use of an aluminum block digester resulted in a more uniform heating of all samples and improved the precision of Pt determination.

24.2.3 SULFURIC ACID/HYDROGEN PEROXIDE/HYDROFLUORIC ACID DIGESTION (BOWMAN 1988)

This method involves the digestion of the soil sample by the sequential additions of concentrated H₂SO₄, H₂O₂, and HF. The precision and accuracy is similar to that of the HClO₄ method and gives soil Pt values that are approximately 94% of those obtained with Na₂CO₃ fusion (Bowman 1988). This method is suited for the analysis of a small number of samples at one time. The time required for manual additions of the H₂O₂ and HF is similar to the manipulations required for the NaOBr–NaOH method, which makes the procedure slightly more labor intensive than the HClO₄ or H₂SO₄/H₂O₂/Li₂SO₄/Se methods.

Materials and Reagents
1. Fluoropolymer beaker (100 mL) of known weight
2. Fume hood
3. Balance or 50 mL volumetric flask
4. Quantitative fine filter paper (e.g., Whatman No. 42)
5. H₂SO₄, concentrated
6. H₂O₂, 30%
7. HF, concentrated (see Comment 1, p. 271)
8. Color developing solutions (see Section 24.5.1)

Procedure
1. Accurately weigh 0.5 g of finely ground soil into a 100 mL fluoropolymer beaker of known weight. Use 0.25 g for soil high in organic matter.
In the fume hood add 5 mL (9.2 g) of H₂SO₄ to the soil and gently swirl to suspend solid materials adhering to the bottom of the beaker.

In the fume hood, slowly add 0.5 mL of H₂O₂ and mix vigorously to promote the oxidation of organic materials. Repeat this step until 3 mL of H₂O₂ has been added to the beaker. Let the sample sit until the reaction with H₂O₂ has subsided.

Add 0.5 mL of HF to the beaker and mix. Repeat this step again so that a total of 1 mL of HF has been added.

Place beaker on a preheated hot plate (approximately 150°C) for 10–12 min to eliminate excess H₂O₂.

Remove beaker, and while sample is still warm, wash down the sides of the beaker with 10–20 mL of distilled/deionized water. Mix and cool to room temperature.

Weigh the beaker and its contents and add sufficient distilled/deionized water to bring the final contents weight to 55 g (equivalent to 50 mL volume). Alternatively, the material in the beaker can be quantitatively transferred to a 50 mL volumetric flask and made to volume using distilled/deionized water.

Mix and filter the extract.

Determine P concentration in an aliquot of the clear filtrate as indicated in Section 24.5.

Comments

1. HF acid attacks glass and it is very important that HF-resistant materials such as polytetrafluoroethylene (PTFE) are used. An example of such material is Teflon. (The use of this trade name is provided for the benefit of the reader and does not imply endorsement by the CSSS.)

2. Excess H₂O₂ will interfere with the colorimetric determination of P in the digested sample. The formation of a yellow color instead of the blue color normally associated with the reduced molybdophosphate complex indicates the presence of excess H₂O₂.

24.2.4 Sulfuric Acid/Hydrogen Peroxide/Lithium Sulfate/Selenium Digestion (Parkinson and Allen 1975)

This method involves the digestion of the soil sample with H₂SO₄ and H₂O₂. The addition of the salt Li₂SO₄ allows for the use of a higher digestion temperature and Se is added as a catalyst in the oxidation of the organic material present in the sample. Rowland and Grimshaw (1985) studying 103 soils across eight major soil types in Britain found that this procedure has a similar accuracy, and on average removed slightly more P (103%) than the HClO₄ digestion. The digestion is usually completed after 2–2.5 h and is therefore somewhat longer than the other procedures. However, using an aluminum block digestor with either 75 or 100 mL tubes enables the analysis of a large number of samples at a time, with a relatively modest amount of sample manipulation. Samples may also be digested using 50 mL boiling flasks, although it is critical that the proper soil:digestion mixture ratio is maintained to ensure proper digestion of the sample.
Materials and Reagents

1. Aluminum digestion block with 100 mL volumetric digestion tubes having a suitable stopper or sealing device (i.e., silicon stopper). A hot plate is required if using 50 mL boiling flasks.

2. Fume hood.

3. Vortex mixer (optional).

4. Silicon stopper (or other device) for sealing digestion tubes.

5. \( \text{H}_2\text{SO}_4 \), concentrated.

6. \( \text{H}_2\text{O}_2 \), 30%.

7. Lithium sulfate monohydrate (\( \text{Li}_2\text{SO}_4 \cdot \text{H}_2\text{O} \)).

8. Selenium (Se) powder.

9. Color developing solutions (see Section 24.5.1).

Procedure

1. Digestion solution: the day before sample digestion, mix 175 mL of \( \text{H}_2\text{O}_2 \) with 0.21 g Se powder and 7 g \( \text{Li}_2\text{SO}_4 \cdot \text{H}_2\text{O} \) in a suitable container. A plastic, sealable bottle is preferred as some pressure may develop in the container. Store this solution in a refrigerator overnight; the Se powder should be dissolved by the following day. Do not heat the solution to dissolve the Se as this may severely decrease the effectiveness of the \( \text{H}_2\text{O}_2 \). This solution is stable for 2–3 weeks.

2. Accurately weigh 0.2–0.4 g of finely ground soil into a 100 mL digestion tube.

3. To reduce the risk of “bumping” an inert boiling stone, glass, or PTFE bead can be added to the digestion tube (see Comment 1, p. 273).

4. In a fume hood add 5 mL of concentrated \( \text{H}_2\text{SO}_4 \) to the digestion tube and swirl (or use vortex mixer) until soil is thoroughly mixed with the acid and turns a dark brown or black color.

5. Carefully and slowly add 1 mL of the digestion mixture to the digestion tube. The sample should react by foaming or spattering and due caution should be exercised. If there is no apparent reaction with the addition of the digestion mixture, gently tap the tube to facilitate the mixing of the digestion solution with the acid–soil mixture. Repeat this step three more times to add a total of 4 mL of digestion mixture.

6. Place the digestion tubes in a cold block digestor and gradually increase the temperature over a 1–1.5 h period until a temperature of 360°C is reached and maintained for 30 min. There should be evidence of \( \text{H}_2\text{SO}_4 \) vapors refluxing in the tubes.
7 Remove tubes from the block digester and allow to cool (5–10 min). Add 0.5 mL H₂O₂, washing down any soil particles that are stuck to the sides of the digestion tube, mix well and replace on block for 30 min.

8 Repeat step 7 until solution is a clear to milky-white color (usually requires two 0.5 mL additions of H₂O₂) indicating a complete digestion of the soil organic matter (SOM). Note, some samples with relatively high iron contents may have a yellowish tinge, which will not change with further additions of H₂O₂.

9 After the final heating for 30 min, remove the tubes from the block digester and allow to cool for 30 min. Slowly add 20–30 mL distilled/deionized water and mix (vortex) the sample to ensure the residue is easily suspended in the solution.

10 Add distilled/deionized water until liquid level is slightly below the volumetric mark on the tubes. Allow the solution to cool before making to final volume with distilled/deionized water.

11 Stopper or seal the tube and thoroughly mix the contents by slowly inverting the tubes several times.

12 Allow the contents to settle before decanting into storage containers. Let the samples sit overnight in a refrigerator or filter through quantitative fine filter paper before colorimetric analysis.

13 Determine P concentration in an aliquot of the clear filtrate as indicated in Section 24.5.

Comments

1 Digestions at high temperatures involving soil–acid mixtures can cause bumping resulting in the violent and dangerous ejection of materials from the digestion tube. The risk of bumping can be reduced through the use of inert boiling stones, glass, or PTFE beads that facilitate a consistent, smooth boiling of the acid. This is more likely to be a problem with blanks than with tubes containing the sample.

2 Alternatively, 210 mL of concentrated H₂SO₄ can be added to the digestion solution prepared in step 1, and the addition of concentrated H₂SO₄ directly to the sample (step 3) omitted. A total of 9 mL of the digestion mixture would be required for each sample, and should be added in careful and incremental additions to avoid too vigorous a reaction that may cause loss of material from the tube or flask. This digestion mixture should be refrigerated and is stable for 2–3 weeks.

24.3 TOTAL ORGANIC PHOSPHORUS

Total soil P₀ is not measured directly, but rather as the increase in Pᵢ resulting from the ignition of a soil sample or digestion of a soil extract. Differences among techniques or soil types may reflect a change in the efficiency of the procedure, rather than a true change in the amount of P₀ in the soil. The extraction techniques (Anderson 1960; Bowman 1989; Bowman and Moir 1993) involve the use of various acid and base treatments with the
The subsequent determination of the $P_i$ and $P_t$ in the extractants. The two major problems with these techniques are the incomplete extraction of soil $P_0$ and the possible hydrolysis of $P_0$ by the extractants. In general, these techniques tend to give the lower range of soil $P_0$ values. The ignition techniques use either high (550°C, Saunders and Williams 1955) or low (250°C, Legg and Black 1955) temperatures to oxidize soil $P_0$ to $P_i$. Matched ignited and unignited samples are then extracted with either weak or strong acids. The difference between $P_i$ (ignited sample) and $P_t$ (unignited sample) is considered $P_0$. This technique may result in erroneous estimates of $P_0$ due to incomplete oxidation of $P_0$ and changes in the solubilities of $P$ minerals by ignition at either high or low temperatures, while ignition at higher temperatures may also cause volatilization of $P$. Each technique has its advantages and disadvantages, depending on the situation and the purpose of the study in question. As indicated by Bowman (1989), the extraction techniques are more suited for comparisons of $P_0$ levels across different soil types, whereas ignition techniques are more suitable for comparisons among treatments within a soil type. Due to the errors that may be associated with $P_0$ determinations and since the $P_0$ is determined by difference, little significance can be given to treatments that differ by less than 20 μg P g$^{-1}$ soil (Olsen and Sommers 1982).

As indicated by Condron et al. (1990), several studies have reported good agreement between ignition and extraction techniques with ignition methods tending to give higher soil $P_0$ values, although studies have shown higher soil $P_0$ levels with extraction compared to ignition techniques (Condron et al. 1990; Agbenin et al. 1999). In addition, considerable differences between the two techniques have been noted for certain soil types. Further information regarding comparisons of various methods for the determination of $P_0$ in soils can be obtained by referring to Condron et al. (1990, 2005), Dormaar and Webster (1964), Steward and Oades (1972), and Turner et al. (2005).

For improved accuracy and precision of analysis, it is recommended that the soils used be air-dried and finely ground (0.15–0.18 mm; 100–80 mesh). Duplicate soil samples and blanks containing no sample should be used in each analysis. There are no certified reference materials for total organic $P$.

### 24.3.1 Hydrochloric Acid/Sodium Hydroxide Extraction Method (Anderson 1960 as Modified by Condron et al. 1990)

In this method, soils are sequentially extracted with 0.3 $M$ NaOH, concentrated HCl (hot and then at room temperature), 0.5 $M$ NaOH at room temperature, and 0.5 $M$ NaOH at 90°C. The $P_i$ in extracts is determined immediately after extraction and the $P_t$ is determined after the oxidation of the organic matter with persulfate digestion.

#### Materials and Reagents

1. Heat-resistant polypropylene screw-top centrifuge tubes (50 mL) with caps.
2. Water bath at 90°C.
3. Centrifuge.
4. Vortex mixer (optional).
5. End-over-end shaker.
Oven for NaOH extraction of samples at 90°C.

Autoclave.

Aluminum foil.

Volumetric flasks (50 and 100 mL).

H₂SO₄, concentrated.

H₂SO₄ 0.9 M: add 50 mL of concentrated H₂SO₄ to a 1 L volumetric flask containing 600 mL of distilled/deionized water. Mix and make to volume using distilled/deionized water.

HCl, concentrated.

NaOH, 0.3 M: dissolve 12 g NaOH in a 1 L volumetric flask containing approximately 700 mL of distilled/deionized water. Make to volume with distilled/deionized water.

NaOH, 0.5 M: dissolve 20 g NaOH in a 1 L volumetric flask containing approximately 700 mL of distilled/deionized water. Make to volume with distilled/deionized water.

Ammonium persulfate: (NH₄)₂S₂O₈.

Color developing solutions (see Section 24.5.1).

**Extraction Procedure**

1. Weigh 0.5 g of finely ground soil into a 50 mL polypropylene centrifuge tube.

2. 0.3 M NaOH extraction: add 30 mL of 0.3 M NaOH, cap the tube, and shake on an end-over-end shaker for 16 h at room temperature. After shaking, centrifuge (12,500 g) the soil suspension for 10 min and then carefully decant the supernatant into a 100 mL volumetric flask ensuring that the soil residue remains in the tube.

3. Concentrated HCl extraction: to the soil residue in the centrifuge tube add 10 mL of concentrated HCl, mix thoroughly, and then place the tube in an 82°C water bath for 10 min. Remove the tube from the water bath, add 5 mL concentrated HCl, and allow to stand at room temperature for 1 h with regular (approximately every 15 min) vortex shaking. Centrifuge (12,500 g) for 10 min, carefully decant the supernatant into a 50 mL volumetric flask, and make to volume using distilled/deionized water.

4. Room temperature 0.5 M NaOH extraction: to the soil residue in the centrifuge tube add 20 mL 0.5 M NaOH, mix well, and allow to stand for 1 h at room temperature with regular (approximately every 15 min) vortex shaking. Centrifuge (12,500 g) the soil suspension for 10 min, and carefully decant the supernatant into the 100 mL volumetric flask containing the previous 0.3 M NaOH extract.
Hot 0.5 M NaOH extraction: to the soil residue in the tube, add 30 mL of 0.5 M NaOH, shake to suspend the soil in solution. Loosely cover the tubes with an inverted 50 mL beaker or funnel and place in an 82°C oven for 8 h. Remove tubes from the oven, allow to cool, centrifuge (12,500 g), and decant the supernatant into the 100 mL volumetric flask containing the previous two NaOH extracts. Make the contents of the 100 mL volumetric flask to volume using distilled/deionized water.

**Determination of P<sub>i</sub> and Total P in the Extracts**

**Determination of P<sub>i</sub>**

To determine P<sub>i</sub> in the NaOH extract, pipette a suitable aliquot (usually ≤5 mL) into a 50 mL centrifuge tube. Acidify to precipitate organic material by adding 2.0 mL of 0.9 M H<sub>2</sub>SO<sub>4</sub> and set in a refrigerator for 30 min. Centrifuge at 25,000 g for 10 min at 0°C. Decant the supernatant into a 50 mL volumetric flask. Using a little acidified water, rinse the tube carefully so as not to dislodge any of the precipitated organic matter, and add the liquid to the contents of the flask (repeat two or three times). Develop color as described in Section 24.5.2 starting with pH adjustment (step 3).

To determine P<sub>i</sub> in the HCl extract, pipette a suitable aliquot (usually ≤5 mL) into a 50 mL volumetric flask. Develop color as described in Section 24.5.2 starting with pH adjustment (step 3).

**Determination of total P**

To colorimetrically determine total P in the extracts, pipette a suitable aliquot (usually ≤2 mL) of solution into a 50 mL volumetric flask.

To the NaOH extract add ≈0.5 g (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and 10 mL of 0.9 M H<sub>2</sub>SO<sub>4</sub>.

To HCl extract add ≈0.4 g (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and 10 mL deionized/distilled water.

Cover the mouth of the flask with aluminum foil (double layer for HCl extract) and autoclave (60 min for HCl extracts and 90 min for NaOH extracts).

Cool, add approximately 10 mL distilled/deionized water. Develop color as described in Section 24.5.2 starting with pH adjustment (step 3).

**Calculations**

Total P<sub>t</sub> in the soil sample is determined as the summation of the total P in the HCl and NaOH extracts minus the summation of the P<sub>i</sub> in the HCl and NaOH extracts. After determining the concentration of P in the digests and extracts and converting each to a soil weight basis (e.g., mg P kg<sup>-1</sup> soil), P<sub>t</sub> is calculated as

$$ P_t = (\text{HCl-P}_t + \text{NaOH-P}_t) - (\text{HCl-P}_i + \text{NaOH-P}_i) $$

(24.1)

**Comments**

P<sub>i</sub> should be determined in the extracts as soon as possible to reduce the chance of P hydrolysis resulting in an underestimation of soil P<sub>t</sub>.
Soils high in humic materials and metals, such as forest soils and wetland soils, may lose P\textsubscript{i} through the formation of P–metal–organic matter complexes during the precipitation of organic matter (Darke and Walbridge 2000), resulting in overestimation of P\textsubscript{o} concentrations. P\textsubscript{o} concentrations for these types of soils should be confirmed with a second method, such as ignition (Section 24.3.3).

Instead of acidifying an aliquot of the NaOH extract to precipitate organic matter, P\textsubscript{i} can be determined directly, provided a suitable blank is used to correct for absorbance by organic matter in solution. To do this, pipette equal aliquots of the same sample extract into two separate 50 mL volumetric flasks and adjust the pH. To one add 8 mL of the color developing solution as described in Section 24.5. To the other add 8 mL of the color developing solution without ascorbic acid added. Dilute to volume and measure absorbance. The absorbance of the solution without ascorbic acid is subtracted from the absorbance of the solution with ascorbic acid.

Total P in the extracts can be determined directly using ICP, although dilution may be required if samples are relatively high in organic matter.

Total P in the extracts can also be determined by other digestion techniques such as the procedure of Thomas et al. (1967) using H\textsubscript{2}SO\textsubscript{4}/H\textsubscript{2}O\textsubscript{2}, or the potassium persulfate digestion using a hotplate (Bowman 1989).

### 24.3.2 Basic EDTA Extraction Method (Bowman and Moir 1993)

In this procedure, P\textsubscript{o} is extracted from the soil using 0.25 \textit{M} NaOH and 0.05 \textit{M} disodium ethylene diamine tetraacetic acid (Na\textsubscript{2}EDTA). This method is simple, faster than either the HCl/NaOH extraction method of Anderson (1960) or the ignition method of Saunders and Williams (1955), and is equally efficient. Excessive amounts of EDTA in solution can interfere with the colorimetric determination of P. Acidification of the extracts to pH \textlesssim 1.5 will precipitate both extracted SOM and EDTA, and this precipitate can then be removed by centrifugation or filtration (Nnadi et al. 1975) allowing for the determination of srP in solution.

#### Materials and Reagents

1. Heat-resistant polypropylene screw-top centrifuge tubes (50 mL).
2. Centrifuge.
3. Incubator or oven set at 85°C.
4. Quantitative fine filter paper (e.g., Whatman No. 42).
5. NaOH, 0.5 \textit{M}: dissolve 10 g NaOH in a 500 mL volumetric flask containing 300 mL of distilled/deionized water. Make to volume with distilled/deionized water.
6. Na\textsubscript{2}EDTA, 0.1 \textit{M}: dissolve 18.6 g Na\textsubscript{2}EDTA in a 500 mL volumetric flask containing 300 mL of distilled/deionized water. Make to volume with distilled/deionized water.
7. NaOH–EDTA mixture: combine the 0.5 \textit{M} NaOH and 0.1 \textit{M} Na\textsubscript{2}EDTA solutions (final concentration 0.25 \textit{M} NaOH + 0.05 \textit{M} Na\textsubscript{2}EDTA).
Ammonium persulfate: \((\text{NH}_4)_2\text{S}_2\text{O}_8\).

\[ \text{H}_2\text{SO}_4 \ 5.5 \text{ M}: \text{add 306 mL of concentrated H}_2\text{SO}_4 \text{ to a 1 L volumetric flask containing 500 mL of distilled/deionized water. Mix, cool, and make to volume using distilled/deionized water.} \]

\[ \text{H}_2\text{SO}_4 \ 0.9 \text{ M}: \text{add 50 mL of concentrated H}_2\text{SO}_4 \text{ to a 1 L volumetric flask containing 600 mL of distilled/deionized water. Mix and make to volume using distilled/deionized water.} \]

Color developing solutions (see Section 24.5.1).

**Extraction Procedure**

1. Weigh 0.5 g of finely ground soil into a heat-resistant 50 mL centrifuge tube.
2. Add 25 mL of combined NaOH–EDTA solution to the tube, cap tightly, and shake briefly to mix.
3. Loosen caps. Place in incubator or oven (preheated to 85°C) for 10 min.
4. Cap tightly and incubate for 1 h 50 min (2 h incubation total).
5. Centrifuge at 25,000 g for 10 min.
6. Filter supernatant and keep the filtrate for analysis.

**Determination of \( \text{Pi} \) and Total \( \text{P} \) in the Extract**

1. Determination of \( \text{Pi} \): pipette \( \leq 5 \) mL of extract into a 50 mL centrifuge tube. Acidify by adding 0.5 mL 0.9 M \( \text{H}_2\text{SO}_4 \). Cool in refrigerator for 30 min. Centrifuge at 25,000 g for 10 min at 0°C. Decant the supernatant into a 50 mL volumetric flask. Using a little acidified water, rinse the tube carefully so as to not dislodge any of the precipitated organic matter and add the liquid to the contents of the flask (repeat two or three times). Develop color as described in Section 24.5.2 starting with pH adjustment (step 3).

2. Determination of total \( \text{P} \): pipette \( \leq 5 \) mL extract into a 25 mL volumetric flask. Add \( \approx 0.5 \) g \((\text{NH}_4)_2\text{S}_2\text{O}_8\) and 10 mL of 0.9 M \( \text{H}_2\text{SO}_4 \). Cover the mouth of the flask with aluminum foil and autoclave for 90 min. Cool, add approximately 10 mL distilled/deionized water, and develop color as described in Section 24.5.2 starting with pH adjustment (step 3).

**Calculations**

After determining the concentration of \( \text{P} \) in the digests and extracts and converting each to a soil weight basis (e.g., mg \( \text{P} \) kg\(^{-1}\) soil), \( P_0 \) is calculated as

\[ P_0 = P_t \text{ (digest or ICP)} - P_i \text{ (extract)} \]  \hspace{1cm} (24.2)
Comments

1. The initial 10 min period with loose caps is to minimize gas buildup.

2. The combination of EDTA and NaOH simultaneously eliminates the formation of cationic bridges with SOM and solubilized organic matter (Bowman and Moir 1993). This eliminates the need for acid pretreatment.

3. Extraction at room temperature for 16 h produced similar $P_o$ concentrations as extracting at 85°C for 2 h.

4. As discussed in Comments, pp. 276 and 277, the removal of organic matter by precipitation may produce inaccurate estimations of $P_o$ concentrations in soils high in humic materials and metals.

5. $P_i$ in the NaOH–EDTA extract can also be determined directly following the same procedure as outlined in the third point in Comments, p. 277. If this alternative procedure is used, the aliquot size should not exceed 4 mL, because excessive EDTA will retard color development.

6. Total $P$ in the extract can be determined using an ICP, or by other methods such as the procedure of Thomas et al. (1967) using $H_2SO_4/H_2O_2$, or potassium persulfate digestion using a hotplate (Bowman 1989).

24.3.3 Ignition Method (Saunders and Williams 1955, as Modified by Walker and Adams 1958)

In this method, $P_o$ is estimated by the difference between 0.5 $M$ $H_2SO_4$-extractable $P$ in a soil sample ignited at 550°C and an unignited sample. The method is suitable for the determination of soil $P_o$ in a large number of samples. Dormaar and Webster (1964) have indicated that significant volatile losses of $P$ may occur at temperatures above 400°C, especially with peat soils.

Materials and Reagents

1. Muffle furnace and porcelain crucibles for igniting soils at 550°C.

2. Polypropylene centrifuge tubes (100 mL) with caps or stoppers.

3. Shaker capable of holding the above tubes.


5. $H_2SO_4$, 0.5 $M$: add 28 mL concentrated $H_2SO_4$ to 600 mL distilled/deionized water in a 1 L volumetric flask. Allow to cool and make to volume using distilled/deionized water.

6. Color developing solutions (see Section 24.5.1).
**Procedure**

1. Weigh 1.0 g of finely ground soil in a porcelain crucible, and place the crucible in a cool muffle furnace.

2. Slowly raise the temperature of the muffle furnace to 550°C over a period of approximately 2 h. Continue to heat the samples at 550°C for 1 h, then remove the samples and allow them to cool.

3. Transfer the ignited soil to a 100 mL polypropylene centrifuge tube for extraction.

4. To a separate 100 mL polypropylene centrifuge tube, weigh 1.0 g of unignited soil for the extraction of P.

5. Add 50 mL of 0.5 M H₂SO₄ to both samples, mix well, and allow to sit lightly stoppered for a few minutes to relieve pressure from CO₂ released from any carbonates that may be present in the soil sample. Tightly stopper the tubes and place them on a shaker for 16 h. Blank samples containing only 0.5 M H₂SO₄ should also be included.

6. Centrifuge the samples at approximately 1500 g for 15 min. If the extract is not clear, filtration using acid-resistant filter paper may be required.

7. Determine P concentration in an aliquot of clear supernatant or filtrate as indicated in Section 24.5.

**Calculations**

After determining the concentration of P in the extracts and converting each to a soil weight basis (e.g., mg P kg⁻¹ soil), P₀ is calculated as

\[
P₀ = P₁ (\text{ignited sample}) - P₁ (\text{unignited sample})
\]  \hspace{1cm} (24.3)

**Comments**

To prevent volatilization of P from the sample, care must be taken to not allow temperatures in the muffle furnace to exceed 550°C when using mineral soils (Sommers et al. 1970; Williams et al. 1970).

**24.4 ORGANIC PHOSPHORUS CHARACTERIZATION**

There are no direct methods to speciate soil P₀. Although there have been attempts to characterize P₀ directly in soil using solid-state ³¹P NMR spectroscopy, results have generally been poor due to line broadening from the close association of soil P with paramagnetics such as Fe (Cade-Menun 2005). As such, soil P₀ must be extracted before speciation. Association with mineral components stabilizes much of the soil P₀, making it difficult to extract, and using strong acid or base extraction introduces the risk of P₀ hydrolysis. Thus, the ideal extractant for chemical characterization of soil P₀ should maximize recovery while minimizing alteration of chemical structure. Post extraction, the ideal speciation technique should allow the quantitative determination of the relative proportions of a range of P
compounds. Two techniques that best fit these criteria are solution $^{31}$P NMR spectroscopy and enzyme hydrolysis.

### 24.4.1 NaOH–EDTA EXTRACTION FOR SOLUTION $^{31}$P NMR SPECTROSCOPY

From its first use on soil extracts by Newman and Tate (1980), solution $^{31}$P NMR spectroscopy has substantially advanced our knowledge of $P_0$ compounds in soil and other environmental samples. Various extractants have been used including 0.5 $M$ NaOH alone or combined with either EDTA or the cation-exchange resin Chelex (Bio-Rad Laboratories). (The use of this trade name is provided for the benefit of the reader and does not imply endorsement by the CSSS.) The choice of extractant will influence both the recovery of $P_0$ from soil and the composition of extracted compounds (Cade-Menun and Preston 1996; Cade-Menun et al. 2002). The extractant most commonly used at present is a combination of NaOH–EDTA based on the Bowman and Moir (1993) extraction procedure for total $P_0$, described in Section 24.3.2.

$^{31}$P NMR spectroscopy allows the characterization of the relative abundances of both $P_0$ and $P_i$ forms in an extract. Figure 24.1 shows $^{31}$P NMR spectra for standard reference materials available from the National Institute of Standards and Testing (NIST) in the United States. The top sample is apple leaf reference, and the bottom is the San Joaquin soil reference. Note the differences in the relative abundances of $P_0$ and $P_i$ compounds.

It is beyond the scope of this chapter to fully describe the workings of a $^{31}$P NMR spectrometer, and exact analytical procedures will vary with each spectrometer. Please see Cade-Menun (2005) for important considerations on conducting a successful $^{31}$P NMR experiment on soil extracts. Included here is a protocol to extract soil samples for $^{31}$P NMR spectroscopy.

**Materials and Reagents**

1. Polypropylene screw-top 50 mL centrifuge tubes.
2. Mechanical shaker for the centrifuge tubes.
3. Vortex mixer (optional).
5. Freezer.
6. Freeze dryer.
7. NMR spectrometer with broadband probe. Ideally, a 500 MHz (for proton) spectrometer and a 10 mm probe (see Cade-Menun 2005).
8. NaOH, 10 $M$: dissolve 20 g NaOH in a 50 mL volumetric flask containing 30 mL of distilled/deionized water. Allow to cool and make to volume with distilled/deionized water.
9. NaOH, 0.5 $M$: dissolve 10 g NaOH in a 500 mL volumetric flask containing 300 mL of distilled/deionized water. Allow to cool and make to volume with distilled/deionized water.
Na₂EDTA, 0.1 M: dissolve 18.6 g Na₂EDTA in a 500 mL volumetric flask containing 300 mL of distilled/deionized water. Make to volume with distilled/deionized water.

NaOH–EDTA mixture: combine the 0.5 M NaOH and 0.1 M Na₂EDTA solutions (final concentration 0.25 M NaOH + 0.05 M Na₂EDTA).

Deuterium oxide (D₂O) suitable for NMR analyses.

Procedure

1 Weigh 1–2 g of soil into a 50 mL centrifuge tube. Use larger sample if soil is known to be low in Pt. Use smaller sample if high in Fe or organic matter.
Add 30 mL of combined NaOH–EDTA solution to the tube and cap tightly.

Shake at room temperature for 5–16 h. Longer extractions can increase the recovery of total P, particularly occluded P forms, but may also increase the risk of degradation of P forms such as RNA and phospholipids.

Centrifuge at 1500 g for 20 min. Decant supernatant into another 50 mL centrifuge tube. If the supernatant contains particulate material, samples should be filtered before decanting into the second centrifuge tube.

Remove 1 mL of supernatant. Dilute to 10 mL with distilled/deionized water, and analyze for P, Fe, and Mn.

Cap centrifuge tubes containing remainder of supernatant tightly, and freeze for 16–24 h, until completely frozen. Note: freeze tubes on a slant to maximize surface area.

Remove caps from tubes and cover loosely with Parafilm (poke small holes in Parafilm to allow air to circulate) or similar material. Place tubes upright in freeze-dryer flasks. Lyophilize for 24–48 h according to freeze-dryer instructions, until completely dry. Remove tubes from freeze-dryer flask. Cap tightly. Store at room temperature.

If using a spectrometer with a 10 mm probe, samples can be redissolved directly in the centrifuge tube by adding 1.6 mL of distilled/deionized water, 1 mL of D$_2$O, and 0.4 mL of 10 M NaOH (to adjust the pH to >12, for maximum peak separation). Let stand for 30 min, mixing or vortexing occasionally to dissolve all solids. Centrifuge at 1500 g for 20 min. Decant into NMR tube. If using a spectrometer with a 5 mm probe, adjust volumes accordingly.

See Cade-Menun (2005) for a discussion of suitable spectrometer parameters to conduct a successful $^{31}$P NMR experiment on soil extracts.

### 24.4.2 Organic Phosphorus Characterization by Enzyme Hydrolysis

Characterization of P$_o$ is based on the principle that substrate-specific phosphatase enzymes will release P$_i$ from specific P forms. Thus, by adding commercially available phosphatase enzymes to soil extracts and colorimetrically analyzing the P$_i$ released, the P forms within the extracts can be grouped into P compound categories. The specific classification of P forms will depend on the enzymes used in the assay. For example, acid phosphatase or alkaline phosphatase will hydrolyze orthophosphate monoesters in general, while phytase will hydrolyze one specific orthophosphate monoester, phytic acid (myo-inositol hexakisphosphate).

One aspect of enzyme hydrolysis is that it can be conducted on a number of different soil extracts, including water, sodium bicarbonate (NaHCO$_3$), NaOH, and HCl, and has been used with sequential extraction procedures (e.g., He and Honeycutt 2001). However, solutions should be adjusted to the suitable pH range for each enzyme before characterization with enzyme hydrolysis.

A number of different protocols exist for enzyme hydrolysis, including the universal buffer procedure recently developed by He et al. (2004). The following protocol was adapted from Turner et al. (2002, 2003) and Toor et al. (2003).
Materials and Reagents

1. Polypropylene screw-top 50 mL centrifuge tubes.
2. Shaker capable of holding the above tubes.
3. Calibrated disposable plastic centrifuge tubes (15 mL), 5 for each soil sample to be extracted.
4. Incubator or shaking water bath, set at 37°C.
5. Centrifuge.
6. 0.45 µm membrane filter and vacuum filtration apparatus.
7. NaOH, 1 M: dissolve 20 g NaOH in a 500 mL volumetric flask containing 300 mL of distilled/deionized water. Allow to cool and make to volume with distilled/deionized water.
8. NaHCO₃, 0.5 M pH 8.5: dissolve 21 g NaHCO₃ and 0.25 g of NaOH in a 500 mL beaker containing 300 mL of distilled/deionized water. Transfer to a 500 mL volumetric flask, and make to volume with distilled/deionized water.
9. Sodium azide (NaN₃), 25 mM: dissolve 0.163 g NaN₃ in a 100 mL volumetric flask containing 40 mL of distilled/deionized water. Make to volume with distilled/deionized water.
10. H₂SO₄, 3 M: add 83 mL of concentrated H₂SO₄ to 500 mL volumetric flask containing 300 mL of distilled/deionized water. Mix and allow to cool before making to final volume with distilled/deionized water.
11. Tris–HCl buffer, 2 M: dissolve 31.5 g Tris–HCl powder (Polysciences, Inc., Warrington, PA) and 0.041 g MgCl₂ ⋅ 6H₂O in a 100 mL beaker containing 60 mL of distilled/deionized water. Adjust to pH 8. Transfer to a 100 mL volumetric flask, and make to volume with distilled/deionized water.
12. Glycine–HCl buffer, 2 M: dissolve 0.041 g MgCl₂ ⋅ 6H₂O in a 100 mL beaker containing 60 mL of distilled/deionized water. Add 20 mL of 1 M Glycine–HCl buffer, 10× concentrate (Polysciences, Inc., Warrington, PA). Check pH, which should be 2.5. Transfer to a 100 mL volumetric flask, and make to volume with distilled/deionized water.
13. Enzymes: suitable enzymes can be obtained from a variety of sources. (All the following enzymes are available from Sigma Chemicals, St. Louis, MO: trade names are mentioned only for the benefit of the reader.)
   a. Alkaline phosphatase (EC 3.1.3.2), Type V-IIS, from bovine intestinal mucosa, activity of preparation 1 unit mL⁻¹: add 0.1 mL of alkaline phosphatase (2.2 mg protein per mL, 2420 units activity per mg protein) to 20 mL of 2 M Tris–HCl buffer, pH 8.
b. Phospholipase C (EC 3.1.4.3), Type XI, from *Bacillus cereus*, activity of preparation 1 unit mL$^{-1}$: add 24.94 mg of phospholipase (16.04 units activity mg$^{-1}$ solid) and 0.1 mL of alkaline phosphatase to 20 mL of 2 M Tris–HCl buffer, pH 8. See Comment 5, p. 286.

c. Phosphodiesterase (EC 3.1.4.1), Type IV, from *Crotalus atrox* venom, activity of preparation 0.03 units mL$^{-1}$: add 20 mg of phosphodiesterase (0.02 units activity mg$^{-1}$ solid) and 0.1 mL of alkaline phosphatase to 20 mL of 2 M Tris–HCl buffer, pH 8. See Comment 5, p. 286.

d. Phytase (EC 3.1.3.8), Type myo-inositol hexakisphosphate 3-phosphohydrolase, from *Aspergillus ficuum*, activity of preparation 1 unit mL$^{-1}$: add 23 mg of phytase (1.1 units activity mg$^{-1}$ solid) to 80 mL of 2 M Glycine–HCl buffer, pH 2.5. Centrifuge for 10 min at 1500 g.

14 Magnesium chloride (MgCl$_2$), 2 mM: dissolve 0.041 g MgCl$_2$·6H$_2$O in a 100 mL volumetric flask containing 60 mL of distilled/deionized water. Make to volume with distilled/deionized water.

15 Color developing solution: see Section 24.5.1.

Procedures

1 Weigh 1.5 g of soil into a 50 mL centrifuge tube. Blank samples containing no soil should also be analyzed.

2 Add 30 mL of 0.5 M NaHCO$_3$. Shake for 30 min.

3 Centrifuge at approximately 1500 g for 15 min. Filter supernatant through 0.45 μm filters.

4 Label five 15 mL centrifuge tubes for each extracted soil sample or blank. Four tubes should be labeled with the names of the enzymes (one per enzyme), while the fifth tube should be labeled “control.” Add 1 mL of the NaHCO$_3$ extract to five 15 mL centrifuge tubes for each extracted soil sample. Preacidify by adding 0.1 mL of 3 M H$_2$SO$_4$ and neutralize by adding 0.12 mL of 1 M NaOH (see Comment 2, p. 286).

5 Add 1 mL of 25 mM NaN$_3$ to prevent microbial activity.

6 Add 0.25 mL of each enzyme–buffer mixture to the appropriately labeled tube for each sample or blank. Add 0.25 mL of the MgCl$_2$ solution to the controls. Dilute to 5 mL with distilled/deionized water.

7 Incubate with shaking at 37°C for 16 h (incubator or shaking water bath).

8 Terminate enzyme reaction by adding 1 mL of color developing solution (see Section 24.5). Final volume for samples (and standards) is 6 mL.

9 Measure the absorbance after 12 min at 880 nm. Calculate Pi concentration in solution by comparison to a standard curve. Note that phosphodiesterase and
phytase cause slight interferences with the molybdate blue reaction. Prepare separate calibration curves from orthophosphate standards containing the enzymes.

Comments

1. All buffers contain 2 mM MgCl₂ because Mg²⁺ ions are natural activators of phosphatase enzymes (Dixon and Webb 1966).

2. The preacidification and neutralization steps are necessary to remove carbonates from bicarbonate extractions, to prevent foaming during subsequent colorimetric analysis.

3. “Activity of preparation” refers to the activity in the centrifuge tube with the soil extract and other reagents.

4. Commercial phytase is not purified, and contains other P-hydrolyzing enzymes. For a purification procedure, see Hayes et al. (2000).

5. Alkaline phosphatase was added to the phospholipase and phosphodiesterase preparations because phosphodiesterase and phospholipase hydrolyze only one ester-P bond on the diester molecule. This leaves an orthophosphate monoester, which requires alkaline phosphatase to completely release orthophosphate (Turner et al. 2002).

6. If working with acid extracts, use acid phosphatase (EC 3.1.3.2) rather than alkaline phosphatase.

7. Sodium azide and phosphodiesterase from Crotalus atrox venom are both poisons, and should be handled and disposed of accordingly.

Calculations

Functional classes of organic P compounds are calculated as

1. labile monoester P: hydrolyzed by alkaline phosphatase;

2. phospholipids: the difference between the P released by phospholipase + alkaline phosphatase and the P released by alkaline phosphatase alone;

3. nucleic acids: the difference between the P released by phosphodiesterase + alkaline phosphatase and the P released by alkaline phosphatase alone; and

4. inositol hexakisphosphate (phytic acid): the difference between the P released by phytase and all other treatments.

24.5 DETERMINATION OF PHOSPHORUS

The determination of P in solutions is usually conducted by colorimetric methods or by inductively coupled plasma (ICP) spectroscopy. Colorimetric methods for the determination of P in solution require that the desired pool of soil P that is extracted from the soil is
completely converted to orthophosphate, while total P in solution may be analyzed without prior digestion by ICP.

One of the most commonly used methods for the colorimetric determination of orthophosphate concentration in solutions is the method developed by Murphy and Riley (1962). This method uses the blue color developed by a phosphoantimonymolybdenum complex (Going and Eisenreich 1974; Drummond and Maher 1995) reduced by ascorbic acid to estimate the concentration of orthophosphate in solution. As the procedure was originally developed for seawater, Murphy and Riley (1962) only assessed adherence to Beer’s law up to a final solution concentration (i.e., solution in which the color has been developed) of 0.2 mg P L$^{-1}$. The procedure is suitable for final solution P concentrations of approximately 0.8 mg P L$^{-1}$ (Rodriguez et al. 1994) and subsequent modifications of the strength of the antimony (Sb) and ascorbic acid solutions have extended this to a final solution concentration of 3 mg P L$^{-1}$ (Harwood et al. 1969). This procedure is fairly simple, less susceptible to interferences than procedures using SnCl$_2$ as a reductant, and is capable of being used manually or adapted to automated systems (Drummond and Maher 1995). A manual method as modified by Watanabe and Olsen (1965) is present here, and is suitable for aliquots containing between 1 and 40 µg srP when made to a final volume of 50 mL for color determination.

### 24.5.1 Reagents

1. **Ammonium molybdate solution**: dissolve 12 g of ammonium molybdate tetrahydrate ($\left(\text{NH}_4\right)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) in 250 mL distilled/deionized water.

2. **Potassium antimony tartrate solution**: dissolve 0.2908 g of potassium antimony tartrate ($\text{K}_2\text{SbOC}_4\text{H}_4\text{O}_6$) in 100 mL distilled/deionized water.

3. **$\text{H}_2\text{SO}_4$ 2.5 M**: to a 1 L volumetric flask containing approximately 600 mL of distilled/deionized water slowly add 139 mL of concentrated (18 M) $\text{H}_2\text{SO}_4$. Mix by swirling the contents of the flask, allow to cool, and make to volume with distilled/deionized water.

4. **Reagent A**: combine the three solutions above in a 2 L volumetric flask, make to volume with distilled/deionized water and mix thoroughly. Store in a Pyrex glass bottle in a refrigerator.

5. **Color developing solution**: dissolve 1.056 g of ascorbic acid in 200 mL of reagent A and mix. Prepare this solution daily and do not use if more than 24 h old.

6. **$p$-Nitrophenol solution**: dissolve approximately 0.25 g of $p$-nitrophenol in 100 mL of distilled/deionized water.

7. **NaOH 4 M**: in a 1 L volumetric flask dissolve 160 g of NaOH in approximately 800 mL of distilled/deionized water. Allow to cool, make to volume using distilled/deionized water, and mix thoroughly.

8. **$\text{H}_2\text{SO}_4$ 0.25 M**: slowly add 14 mL of concentrated $\text{H}_2\text{SO}_4$ to 1 L volumetric flask containing approximately 800 mL of distilled/deionized water, make to volume with distilled/deionized water, and mix thoroughly.

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Standard P stock solution (100 mg P L\textsuperscript{-1}): dissolve 0.4394 g of potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}) in 1 L of distilled/deionized water. Prepare a working standard (10 mg P L\textsuperscript{-1}) by dilution with distilled/deionized water.

24.5.2 Procedure

1. Pipette an aliquot containing 1–40 µg of P into a 50 mL volumetric flask containing approximately 15 mL distilled/deionized water.

2. Pipette standard P solutions into a set of volumetric flasks so as to encompass the range of P concentrations anticipated in the extracts. To each flask containing a standard solution, pipette an aliquot of blank solution equal to the aliquot size of the sample.

3. Add 1–2 drops of p-nitrophenol and adjust the pH of the solution to ~5. If the sample aliquot has a pH <5, add 4 M NaOH drop wise until the solution turns yellow in color and then add 0.25 M H\textsubscript{2}SO\textsubscript{4} until colorless. If the sample aliquot has a pH >5, add 0.25 M H\textsubscript{2}SO\textsubscript{4} until colorless.

4. Add 8 mL of the color developing solution, make to volume with distilled/deionized water, and mix thoroughly. After 10 min read absorbance at either 882 or 712 nm (if solution is slightly colored due to the presence of organic matter).

5. Appropriate standards (final solution concentrations of 0–0.8 µg P mL\textsuperscript{-1}, or 0–40 µg P in the 50 mL volumetric flask) should be analyzed in the same manner as samples, and contain similar amounts of extracting or digestion solutions as the samples.

24.5.3 Comments

1. Many versions of the Murphy and Riley (1962) procedure have been published, and the reader is cautioned that deviations from proposed methodologies can lead to erroneous results. The development of a stable blue color that adheres to Beer’s law requires the proper adjustment of solution pH, as well as specific ranges of Mo, Sb, and ascorbic acid concentrations relative each other, to the amount of P in the sample, or both (Harwood et al. 1969; Going and Eisenreich 1974; Rodriguez et al. 1994; Drummond and Maher 1995). Any changes to proposed methods should be verified using samples and standards of known P content.

2. The original method described by Watanabe and Olsen (1965) for sodium bicarbonate extracts of soil P used 25 mL volumetric flasks, and therefore only 4 mL of color developing reagent and a sample aliquot containing a maximum of 20 µg P.

3. Arsenate (AsO\textsubscript{4}) will also form a blue color with the Murphy and Riley solution. Olsen and Sommers (1982) indicate that in most soils the average As concentration is 6 mg kg\textsuperscript{-1}, and as such would be a negligible amount compared to typical P concentrations in soils. However, if a soil has been contaminated with As, this could lead to substantial overestimation of P in the sample. In soils with high As contents, Olsen and Sommers (1982) recommend reducing AsO\textsubscript{4} to AsO\textsubscript{3} by
adding 5 mL of sodium hydrogen sulfite solution (5.2 g of NaHSO₃ dissolved in 100 mL of 0.5 M H₂SO₄) to the sample aliquot and either heating the mixture in a water bath for 30 min (20 min at 95°C) or letting it stand for 4 h before adjusting pH and developing the color.

REFERENCES


25.1 INTRODUCTION

Phosphate availability is a function of chemical equilibrium-controlled solubility and rate-limited processes. Most methods for available P determination attempt to quantify P solubility using different extractants, but few relate this to P supply rates that are relevant to plant uptake.

Soil test methods for P do not measure the quantity of P available to a crop, but extract a portion of soil P that is related to plant-available P. This relationship is usually established over years of agronomic experimentation and testing of fertilizer responses through regression equations. These equations relate plant performance to soil test P levels, or indicate fertilizer requirement for optimum crop production. Results obtained with this approach are not always transferable between crops or soil types, and different equations are established by soil testing services for varying crops and soils. The approach does not work when perennial plants or natural ecosystems are examined, because measurable pools are often small, and P cycling is the major determinant of P availability. Since any “immediately available” pool of P is constantly replenished through dissolution or desorption of “less-available” P, and through the mineralization of organic P, “plant-available” P is strongly time-dependent.

25.2 SOIL TEST METHODS FOR AVAILABLE P

Agronomic tests for available P are designed with several aims; they should:

1. Be simple enough for routine application.
2. Extract sufficient P to be easily measurable.
Extract P that represents a significant portion of potential plant uptake, so that plant supply is represented closely by the quantity measured rather than being dependent on P turnover and replenishment of the measured pool.

Not extract significant amounts of P that are not plant available over the growing period.

This is achieved with moderately acidic or alkaline solutions which release P associated with the soil mineral phase without solubilizing significant amounts of phosphatic minerals. Alternatively, or in combination with these pH changes, specific anions are introduced that bring P into solution by competing with P sorption sites or by lowering the solubility of cations that bind P in the soil. Based on these principles, numerous extraction methods exist, all of which have some merits and limitations and are used in various parts of the world, where their value relies on long-term correlation studies that establish the relationship between extractable P and crop response. An exhaustive review of extraction methods by a working group in Spain (Anon. 1982) listed 50 different methods and more than 50 publications comparing different extracts.

The most common methods are probably the alkaline bicarbonate method of Olsen et al. (1954) and the acid ammonium fluoride extraction (Bray and Kurtz 1945) with various modifications. An extraction using lactate (Egnér et al. 1960) is popular in Europe. The rationale for the use of bicarbonate or lactate for the extraction of available P is that plant roots produce CO\textsubscript{2} which forms bicarbonate in the soil solution as well as various organic acids similar to lactate that may solubilize soil P. It is proposed that these extractants somehow simulate the solubilizing action of plant roots and, thus, give a more appropriate measure of plant-available P. Chelating extracts (Onken et al. 1980) have been proposed for similar reasons. An advantage of chelating extracts is that the same extract can also be used for cation soil testing (micronutrients and K).

The bicarbonate extractant (Olsen et al. 1954) has been used successfully on a wide range of acid to alkaline soils. Available P is extracted with a solution of sodium bicarbonate of pH 8.5 for 30 min. Interference from organic matter dissolved in the solution has frequently been eliminated by sorbing the organic matter onto activated acid-washed charcoal (carbon black) added to the extract, but it is difficult to obtain P-free charcoal. An alternative was therefore developed which eliminates organic interference with polyacrylamide (Banderis et al. 1976). If organic matter content in the extract is low (as judged by its yellow coloring) a blank correction can be used. When the blue phosphomolybdate complex is measured at a wavelength of 712 nm, color interference from the yellow organic matter is negligible. However, using color correction with blanks will not work at high organic matter concentrations in the extract because the organic matter will precipitate upon acidification during the Murphy and Riley (1962) procedure and interfere with P colorimetry. The extraction time of 30 min has been designed for rapid routine soil testing. A more complete extraction is obtained by extracting for 16 h (Colwell 1963). For all applications that attempt to functionally evaluate the bicarbonate-extractable P pool, and that include organic P determination, the more complete 16 h extract should be used, because at 30 min the extraction is far from complete.

The acid ammonium fluoride extraction (Bray and Kurtz 1945) has been widely used on acid and neutral soils, and a large database exists. This is a purely chemical test that cannot be interpreted in terms of plant function like the bicarbonate or some of the organic acid or chelating extracts. Fluoride has been used to extract Al-associated P, but it is not obvious what the link to plant availability would be. In addition, Ca phosphates which are of low
plant availability in high-pH soils would be extracted by the acid and give excessive values for available P. The relatively low acid strength and importance of acidity for the extraction mechanism make the method unsuitable for calcareous or strongly alkaline soils, which would partially neutralize the acidity and eliminate the standard test conditions. However, buffered variations of this soil test have been reported to correlate well with bicarbonate-extracted P and plant response to P (van Lierop 1988; Soon 1990).

25.3 APPROACHES FOR CHARACTERIZING AVAILABLE P

Since available P is a functional concept rather than a measurable quantity, no simple direct measurements are available. Plant-available P is that P taken up by a plant during a specific period, such as a cropping season, year, or growth cycle. Since the plant obtains P from the soil solution through its roots or root symbionts, available P is composed of solution P plus P that enters the solution during the period used to define availability. Phosphorus may enter the solution by desorption or dissolution of inorganic P (P_i) associated with the soil’s solid phase, or by the mineralization of organic P (P_o). In some dystrophic rain forests, P may not even cycle through the soil, but can be taken up directly from plant litter.

It is difficult to resolve whether desorption or dissolution replenishes solution P from P_i forms. In one case the solubility product of the least-soluble P compound, and in the other, the saturation of sorbent surfaces would determine the P supply at equilibrium. Countless publications have fitted theoretical equations to the reverse of these reactions—precipitation and adsorption. Empirical data usually fit either process to some degree (Syers and Curtin 1989). There is an increasing realization, though, that solid-phase P is not static, and that sorption–desorption and precipitation–dissolution equilibria change with time due to secondary processes (Parfitt et al. 1989) such as recrystallization (Barrow 1983) or solid-state diffusion (Willett et al. 1988). A measurement of available P_i therefore needs to consider both the amounts and rates of release of P from the solid phase. Very few appropriate methods have been published. Among the approaches taken are repeated water extracts and sorption–desorption isotherms (Fox and Kamprath 1970; Bache and Williams 1971), possibly at elevated temperatures to substitute for impractically long reaction times (Barrow and Shaw 1975).

A simple and more realistic approach is the use of anion-exchange resin, a sink for solution P_i. The resin offsets the equilibrium between dissolved and soluble P_i, and “exchangeable” P_i as well as some of the more soluble precipitated P forms will enter the depleted solution and be absorbed by the resin. The P sorbed by the resin is subsequently measured. Several different methods have been developed and tested, using different anionic forms, ratios of soil:water:resin, times and methods of shaking, and enclosure in bags or mixing through the suspension (Sibbesen 1977, 1978; Barrow and Shaw 1977). By far the simplest method uses polyester- or Teflon-based anion-exchange membranes, which can be cut into strips and used repeatedly and easily (Saggar et al. 1990; Schoenau and Huang 1991). These ion-exchange membranes have also been used in situ, inserted or buried in soil where they integrate processes of nutrient release and diffusion (to the membrane) over time (Qian and Schoenau 1997). When choosing resin membranes, it is important that the resin is part of the membrane material, i.e., cannot be abraded by the soil, and that they are resistant to the chemicals used in P extractions, such as dilute HCl or chloroform (if microbial P is to be measured). It also helps if they are stiff enough to be easily handled.

The pool measured by resin extraction is very similar to that assessed with isotopic dilution (Amer et al. 1955). The sorption by a resin is usually complete within 20 h, and only minor
changes are observed thereafter. Isotopic exchange also reaches a relatively constant state within a few hours, and the disappearance of isotope from the solution is used to estimate the size of the labile pool into which the isotope has been diluted. In a variation of the isotopic dilution method, carrier-free $^{32}$P is added to a soil suspension, and the initial rapid removal of label is measured. This is followed by a determination of the continuing slow changes (Fardeau and Jappe 1980). These continuing changes represent the activity of less soluble or kinetically slower pools of soil P, which replenish available P at rates varying from days to years. On some soils with low or moderate P sorption, the continuing reduction in radioactivity in the liquid phase of the suspension has been extrapolated to times corresponding to seasons or longer with some success in estimating plant-available P. However, errors of extrapolation over long times can be large (Bühler et al. 2003; Chen et al. 2003). Phosphorus taking part in longer term transformations can be examined with sequential extractions, which first remove labile P, and then the more stable forms.

The sequential extraction method proposed by Chang and Jackson (1957) and modified by Williams et al. (1967) employs, sequentially, NH$_4$Cl to extract “labile” P$_1$, NH$_4$F to dissolve Al-associated P$_1$, NaOH to extract Fe-bound P$_1$, and dithionite–citrate to dissolve “occluded” P$_1$ forms. A subsequent extraction with HCl dissolves Ca-bound P$_1$ and the residue is analyzed by Na$_2$CO$_3$ fusion for residual total P. Alternatively, the residue can be analyzed for P$_o$ by ignition plus acid extraction before the Na$_2$CO$_3$ fusion (Williams et al. 1967). As in all other methods of P$_o$ determination, the amount of P$_o$ is not measured directly but calculated by difference: acid-extractable P$_1$ is subtracted from the greater amount of P$_1$ rendered acid extractable after ignition of the soil organic matter (Saunders and Williams 1955) (see Chapter 24).

An alternative P fractionation scheme was developed by Hedley et al. (1982a) building on the experience with previous extractions. This sequential extraction aims at quantifying labile (plant-available) P$_1$, Ca-associated P$_1$, Fe + Al-associated P$_1$, as well as labile and more stable forms of P$_o$. Labile P$_1$, i.e., P$_1$ adsorbed on surfaces of sesquioxides or carbonates (Mattingly 1975), is extracted with resin and bicarbonate. Hydroxide-extractable P$_1$ is less plant available (Marks 1977) and is thought to consist of amorphous and some crystalline Al and Fe phosphates. A more precise characterization of these P$_1$ forms is unlikely to be possible since mixed compounds containing Ca, Al, Fe, P, and other ions predominate in soils (Sawhney 1973). Organic P extracted with bicarbonate is easily mineralizable and contributes to plant-available P (Bowman and Cole 1978). More stable forms of P$_o$ are extracted with hydroxide (Batsula and Krivonosova 1973).

Each of the extracts obtained can be assigned some role in the P transformations occurring in soil under incubation (Hedley et al. 1982a) or cultivation (Tiessen et al. 1983), in the rhizosphere (Hedley et al. 1982b), or in soil development (Tiessen et al. 1984; Roberts et al. 1985; Schlesinger et al. 1998; Miller et al. 2001). These empirical assignments can then be used to characterize P status of the soil relative to a conceptual model of P pools and their transformations. Little has changed in the functional assignment or characteristics attributed
to those P extracts since the papers published in the 1980s, although some authors group fractions in ways that reveal interesting concepts on the function of the soil P cycle. Cross and Schlesinger (2001) group $P_i$ and $P_o$ fractions of the different extracts together reporting each extract’s total P, and implying that the mode of stabilization is the most important characteristic, not necessarily the distinction between organic and inorganic forms. Soil mineralogy clearly affects the interpretation of P fractions. In semiarid, calcareous soils, Cross and Schlesinger (2001) identified acid-extractable P not only as true calcium phosphates but also as various associations of P with carbonates. To distinguish such fractions more clearly, Samadi and Gilkes (1998) added (among other modifications) an ammonium acetate extract before the acid extraction.

This fractionation approach is currently the only one that can be used with moderate success for the evaluation of available $P_o$. Cross and Schlesinger (1995) used the ratio of bicarbonate $P_o$ to resin plus bicarbonate total P as an index for the bioavailability of P. This is probably only valid in temperate soils. Due to the reactivity of mineralized P with the soil’s mineral phase, determination of a potentially mineralizable $P_o$ pool, analogous to the mineralizable N or S pools measured with incubation and leaching techniques (Ellert and Bettany 1988), is not feasible. The nature of different extractable $P_i$ pools is even less well defined than that of the $P_i$ pools (Stewart and Tiessen 1987). Their turnover and availability frequently depend on the mineralization of C during which P is released as a side product, although solubilized $P_o$ will be rapidly mineralized by soil enzymes. Most progress on understanding soil $P_o$ has come from organic matter studies (Tiessen et al. 1983; Stewart and Tiessen 1987). It is often more appropriate to determine P in physical soil organic matter fractions, than to try and relate a chemically extracted $P_o$ to biological function. Unless one has good reasons to believe that an extracted organic fraction can be biologically defined, it is probably best to group the organic fractions and use the sequential fractionation as a multiple extractant to obtain as much as possible of the soil’s $P_o$.

The original fractionation (Hedley et al. 1982a) left between 20% and 60% of the soil’s P unextracted. This residue often contained significant amounts of $P_o$ that sometimes participated in relatively short-term transformations. On relatively young Ca-dominated soils, this residual $P_o$ can be extracted by NaOH after the acid extraction, while on more weathered soils, hot HCl (Mehta et al. 1954) extracts most of the organic and inorganic residual P. The hot HCl method appears to work satisfactorily on most soils, and is presented below as part of an extensive soil P fractionation.

### 25.4 P FRACTIONATION PROCEDURE

#### 25.4.1 Equipment and Materials

1. 50 mL centrifuge tubes with screw caps and refrigerated high-speed centrifuge
2. Shaker, preferably overhead type so that soils do not clump together in the round bottom of the centrifuge tubes
3. 0.45 µm membrane filter and filtration apparatus
4. Water bath
5. Block digester with 75 or 100 mL digestion tubes
25.4.2 EXTRACTING SOLUTIONS

1. 0.5 M HCl: dilute 88.5 mL conc. HCl to 2 L with deionized H₂O.

2. 0.5 M NaHCO₃ (pH 8.5): dissolve 84 g NaHCO₃ + 1 g NaOH in deionized H₂O and make to 2 L.

3. 0.1 M NaOH: dissolve 4 g NaOH in deionized H₂O and bring final volume to 1 L.

4. 1 M HCl: add 177 mL conc. HCl (11.3 M) to about 500 mL of deionized H₂O and bring to final volume of 2 L.

5. H₂O₂: 30% hydrogen peroxide.


7. Resin strips: use anion-exchange membrane cut into strips (9 x 62 mm) and convert to bicarbonate form. To regenerate after the adsorbed P has been extracted with HCl, wash resin strips for 3 days with 6 batches of 0.5 M HCl, followed by washing a further 3 days with 6 batches of 0.5 M NaHCO₃ (pH 8.5). Then rinse well with deionized/distilled water.

25.4.3 EXTRACTION PROCEDURE

Day 1: Weigh 0.5 g soil into a 50 mL centrifuge tube, add 2 resin strips + 30 mL deionized water, and shake overnight (16 h, and 30 rpm if using overhead shaker). See comments below of fineness of grinding of soil samples.

Day 2: Remove resin strips and wash soil back into tube using deionized water. Place resin strip in a clean 50 mL tube, add 20 mL 0.5 M HCl. Set aside for 1 h to allow gas to escape, cap and shake overnight. Determine P using Murphy and Riley method (see section at top of p. 301). Centrifuge soil suspension at 25,000 g for 10 min at 0°C. Decant water through a 0.45 μm membrane filter. Discard water and wash any soil off filter back into the tube with a little 0.5 M NaHCO₃ (pH 8.5) solution. Add more NaHCO₃ solution to bring solution volume to 30 mL (by weighing) and shake suspension overnight (16 h). Cap the tubes and resuspend soil by handshaking before putting on mechanical shaker.

Day 3: Centrifuge soil suspension at 25,000 g for 10 min at 0°C. Decant NaHCO₃ extract through a membrane filter into a clean vial. Determine inorganic and total P on bicarbonate extract. Wash any soil off filter back into the tube using a little 0.1 M NaOH. Make volume of NaOH solution to 30 mL and shake suspension overnight (16 h).
Day 4: Centrifuge suspension at 25,000 g for 10 min at 0°C. Decant NaOH extract through a membrane filter into a clean vial. Determine inorganic and total P on NaOH extract. Wash any soil off filter back into the tube using a little 1 M HCl. Make volume of HCl to 30 mL and shake suspension overnight.

Day 5:

1. Centrifuge soil suspension at 25,000 g for 10 min at 0°C. Decant HCl extract through a membrane filter into a clean vial. Determine P in extract. (In this step, any residue that right be on the filter paper is not washed back into the tube; decant gently so as to not lose any soil.)

2. Soil residue heated with 10 mL conc. HCl in a waterbath at 80°C for 10 min. (Vortex to mix soil and HCl well and loosen caps before putting into the hot bath. The mixture will take about 10 min to come to temperature—check with a thermometer in a tube containing HCl only—i.e., the tubes will be in the hot water for a total of 20 min.) Remove and add a further 5 mL conc. HCl, vortex and allow to stand at room temperature for 1 h (vortex every 15 min). Tighten caps, centrifuge at 25,000 g for 10 min at 0°C, and decant supernatant into a 50 mL volumetric flask. Wash soil twice with 10 mL H2O, centrifuge, and add supernatant solution to contents in the flask. Make to volume, and if necessary filter through a Whatman No. 40 paper (or equivalent), and determine inorganic and total P in HCl solution.

3. Add 10 mL deionized water to soil residue and disperse soil. Transfer suspension into 75 mL digestion tubes using the minimum amount of water possible to transfer all soil residues, add 5 mL conc. H2SO4 + one boiling chip (Hengar Granules, Hengar Co., Philadelphia, Cat. No. 136C), vortex and put on a cold digestion block. Raise the temperature very slowly to evaporate water and when 360°C is reached start treating with H2O2 in the following way: remove tubes from heat and let cool to hand-warm; add 0.5 mL of H2O2; reheat for 30 min, during which H2O2 is used up. Repeat H2O2 addition until liquid is clear (usually about 10 times). Make sure there is adequate heating after the final H2O2 addition, since residual H2O2 interferes with the P determination. Cool, make to volume, shake, and transfer to vials (either filter or allow residue to settle out overnight). Determine P in solution (see Section at top of p. 300). (This digestion is based on Thomas et al. 1967.)

25.4.4 Comments

1. The intensity of soil grinding greatly affects the amount of P extractable, particularly for the resin extraction which removes P from accessible surfaces. Interlaboratory testing has shown differences of an order of magnitude attributable to grinding between 2 mm screened and 60 mesh ground samples. The decision on how fine to grind should be based on a trade-off between sample homogeneity (important in a sample size of only 0.5 g) and preservation of the “natural” extractability of resin P. We have generally opted for moderate crushing of samples to 20 mesh.

2. Sequential extraction is a lengthy procedure. A batch of samples will take a week (including the weekend) to process. It is therefore important to reconcile the aim of the study with what this fractionation can produce. If geological transformations are the target, one can probably do without the resin and bicarbonate extracts; if labile pools are the target, the more resistant fractions may not be...
important, and it would be more useful to include microbial P or organic matter separations. In many highly weathered soils, cold acid-soluble P is so little that it is probably a “contaminant” from the previous extract.

25.4.5 ANALYSIS OF P IN EXTRACTS

Reagents for P Determination

1. Ammonium molybdate: dissolve 40.0 g ammonium molybdate in deionized H₂O and bring to a final volume of 1 L.

2. Ascorbic acid: dissolve 26.4 g L-ascorbic acid in deionized H₂O and bring to a final volume of 0.5 L.

3. Antimony potassium tartrate: dissolve 1.454 g antimony potassium tartrate in deionized H₂O and bring to a final volume of 0.5 L.

4. 2.5 M H₂SO₄: slowly add 278 mL conc. H₂SO₄ to 1 L of deionized H₂O and bring to a final volume of 2 L.

5. Color developing reagent: to 250 mL 2.5 M H₂SO₄, add 75 mL ammonium molybdate solution, then 50 mL ascorbate solution and finally 25 mL of antimony potassium tartrate solution. Swirl contents of flask after each addition. Dilute to a total volume of 500 mL with deionized H₂O and mix.

6. For organic matter precipitation and pH adjustment make up:
   - 0.9 M H₂SO₄: bring 100 mL conc. H₂SO₄ to 2 L with H₂O.
   - 0.25 M H₂SO₄: bring 100 mL 2.5 M H₂SO₄ to 1 L with H₂O.
   - 4 M NaOH: dissolve 160 g NaOH and dilute to 1 L with H₂O.

7. p-nitrophenol, 10% (w/v), aqueous solution.

8. Ammonium persulfate, (NH₄)₂S₂O₈.

Determination of P Recovered from the Resin Strip and of Pᵣ in HCl Extracts

This method (Murphy and Riley 1962) is used directly for the P recovered from the resin strip and for Pᵣ determination in the two HCl extracts:

1. Pipette a suitable aliquot into a 50 mL volumetric flask. The calibration curve is linear for up to a concentration of about 1 mg of P L⁻¹. Use two drops of p-nitrophenol as an indicator. If the extract is acid, first adjust pH with 4 M NaOH to yellow and then with ~0.25 M H₂SO₄ until the indicator turns clear. For alkaline extracts, just acidify until solution is clear. Note that most analytical problems are related to the solution being adjusted too acid.

2. Add 8 mL of color developing solution, make to volume, shake and read on spectrophotometer at 712 nm after 10 min (color is stable for several hours).
Determination of Inorganic P in 0.5 \(M\) NaHCO₃ and 0.1 \(M\) NaOH Extracts

1. Pipette 10 mL solution into a 50 mL centrifuge tube.
2. Acidify to pH 1.5 and set in fridge for 30 min:
   - (a) to acidify 0.5 \(M\) NaHCO₃ extract use: 6 mL of 0.9 \(M\) H₂SO₄;
   - (b) to acidify 0.1 \(M\) NaOH extract use: 1.6 mL of 0.9 \(M\) H₂SO₄.
3. Centrifuge at 25,000 g for 10 min at 0°C.
4. Decant supernatant into a 50 mL volumetric flask.
5. Rinse tube carefully so as not to disturb the organic matter with a little acidified water and add to the solution in the flask (2 or 3 times).
6. Adjust pH and measure P by the Murphy and Riley method (see section at bottom of p. 300).

Determination of Total P in 0.5 \(M\) NaHCO₃, 0.1 \(M\) NaOH, and Conc. HCl Extracts (EPA 1971)

Dissolved organic matter is oxidized with ammonium persulfate before P analysis:

1. Pipette 5 mL solution into a 50 mL volumetric flask.
2. To 0.5 \(M\) NaHCO₃ extract: add \(\sim\)0.5 g ammonium persulfate + 10 mL 0.9 \(M\) H₂SO₄.
   - (a) To 0.1 \(M\) NaOH extract: add \(\sim\)0.6 g ammonium persulfate + 10 mL 0.9 \(M\) H₂SO₄.
   - (b) To conc. HCl extract: add \(\sim\)0.4 g ammonium persulfate + 10 mL deionized water.
   The persulfate may be added by volume using a spatula with a spoon at one end rather than weighing every time.
3. Cover with tinfoil (double layer for conc. HCl) and autoclave:
   - NaHCO₃ and HCl extracts for 60 min, NaOH extract for 90 min. (Instead of an autoclave, a household pressure cooker can also be used.)
4. Adjust pH and measure P by the Murphy and Riley method (see section at top of p. 300).

25.4.6 Comments

1. The aliquot size of extract for the Murphy and Riley procedure may vary from 1 mL for high P concentration acid extracts up to 40 mL in the case of very low P resin extracts.
Most times when things go wrong, it is due to interferences in the Murphy and Riley colorimetry. Insufficient pH control before color development is the most common problem with color development. Residual oxidant from one of the digestion steps will of course interfere with the reduction step of the color development. In some soils, we have seen interference from soluble silica in the reacidified NaOH extract, resulting in a positive drift (i.e., increase) in absorbance. This interference is difficult to manage if it occurs. Very consistent absorbance reading at exactly 10 min helps but results will remain doubtful.

25.5 INTERPRETATION AND LIMITATIONS

The interpretation of data obtained from this sequential fractionation is based on an understanding of the action of the individual extractants, their sequence (Figure 25.1), and their relationship to the chemical and biological properties of the soil. It must be remembered that, while the fractionation is an attempt to separate P pools according to their lability, any chemical fractionation can at best only approximate biological functions. Resin P is reasonably well defined as freely exchangeable $P_i$, since the resin extract does not chemically modify the soil solution. Bicarbonate extracts a $P_i$ fraction, which is likely to be plant available, since the chemical changes introduced are minor and somewhat representative of root action (respiration). This fraction is not comparable to the widely used fertility test.

![FIGURE 25.1. Flow chart of the sequential P extraction.](image-url)
(Olsen et al. 1954) because the resin-extractable pool has already been removed at this point and because Olsen P is extracted over only 30 min.

Bicarbonate-P\textsubscript{1} and OH-P\textsubscript{1} are not really completely separate pools, particularly in acid soils, but represent a continuum of Fe- and Al-associated P extractable with increasing pH (the soils original pH to 8.5 to 13). The P\textsubscript{0} extracted with these two extractants is also likely to represent similar pools. Since P\textsubscript{0} is determined by difference between total P (P\textsubscript{t}) and P\textsubscript{1} in each extract, there is a source of error. The P\textsubscript{1} determination is quite reliable, but P\textsubscript{0} is determined in the supernatant after precipitation of organic matter with acid. Any nonprecipitated P\textsubscript{0} (fulvic acid P) will not significantly react with the Murphy and Riley reagent, so that P\textsubscript{1} is rarely overestimated. Any P\textsubscript{1}, though, that precipitates along with the organic matter upon acidification would be erroneously determined as P\textsubscript{0} (P\textsubscript{t} – P\textsubscript{1}). This may happen with P\textsubscript{1} associated with Fe or Al hydroxides, which are soluble at high pH but insoluble at low pH. It has so far been impossible to quantify the P\textsubscript{0} overestimation. In soils with low-extractable organic matter contents (low enough not to cause precipitation in the acid Murphy and Riley reagent), it is possible to determine P\textsubscript{1} in the extract without prior acid precipitation using a blank correction for the extracts’ color.

The dilute HCl P\textsubscript{1} is clearly defined as Ca-associated P, since Fe- or Al-associated P that might remain unextracted after the NaOH extraction is insoluble in acid. There is rarely any P\textsubscript{0} in this extract. Dilute acid is well known to be inefficient in extracting organic carbon from soils, and therefore, does not extract much P\textsubscript{0}.

The hot concentrated HCl extract does not present the same problems as the other P\textsubscript{0} extracts, since P\textsubscript{1} is determined directly. This extract is useful for distinguishing P\textsubscript{1} and P\textsubscript{0} in very stable residual pools. However, at the same time, P\textsubscript{0} extracted at this step may simply come from particulate organic matter that is not alkali extractable but may be easily bioavailable. Any P protected by cellulosic structure would be biologically available as a byproduct of cellulose breakdown, but would only become extractable in the hot concentrated acid step. The residue left after the hot concentrated HCl extraction is unlikely to contain anything but highly recalcitrant P\textsubscript{1}.

It is important to remember that this sequential extraction does not provide direct measures of biologically or geochemically important P pools. It provides circumstantial evidence that is more valuable if it can be corroborated by other methods such as isotope or organic matter studies. Particularly, for a reliable interpretation of P\textsubscript{0} transformations, it is advisable to supplement the fractionation with a suitable characterization of soil organic matter, so that characteristics can be inferred from the combined results of different techniques.

**REFERENCES**


26.1 INTRODUCTION

The dissolution methods for extracting Al, Fe, Mn, and Si are valuable tools to help determine the chemical forms of these elements in soils. The results are useful in studies of soil classification, soil genesis, soil reactivity, and metal mobility or bioavailability in soils. For example, the nature and amounts of extractable organic and inorganic Al and Fe may reflect the pathway of soil genesis. Also, extractable soil constituents are generally fine grained with large specific surface area and therefore, have a marked effect on physical and chemical soil properties and behavior. For these reasons, extraction data, notably for Al and Fe, are commonly used as chemical criteria for soil classification. Moreover, extractions are often performed to establish the mechanisms of metal retention and fractionation in contaminated soils. A variety of chemical extractants are used to approximate the amounts and forms of Al, Fe, Mn, and Si in soils. Five of the most commonly used extractions are discussed here and four methods are presented.

Dithionite–citrate removes organically complexed Al, Fe, and Mn, amorphous inorganic Al, Fe, and Mn compounds, noncrystalline aluminosilicates as well as finely divided hematite, goethite, lepidocrocite, and ferrihydrite (Mehra and Jackson 1960; Guest et al. 2002). It is much less effective in removing crystalline oxides and hydroxides of Al. The method extracts virtually no Al, Fe, Mn, or Si from most crystalline silicate minerals or opal, and thus, provides an estimate of “free” (nonsilicate) Fe in soils. The procedure may have to be repeated to dissolve silt- and sand-size goethite and hematite completely (Kodama and Ross 1991). Magnetite is not dissolved. Ross and Wang (1993) indicated that coefficients of variation at Fe levels of 1.4% and Al levels of 0.45% are 6.3% and 7.8%, respectively.

Acid ammonium oxalate removes organically complexed and amorphous inorganic forms of Al, Fe and, to a lesser extent, Mn and noncrystalline aluminosilicates from soils (McKeague 1967). It also dissolves poorly ordered phases like allophane and imogolite to some extent and their amount in soils can be estimated from oxalate-extractable Al and Si concentrations, taking into account that oxalate also extracts organically complexed Al (Parfitt and Henmi 1982). Oxalate only slightly attacks crystalline Al and Fe oxides, most crystalline silicate minerals, opal, goethite, hematite, and lepidocrocite, but it dissolves considerable amounts of...
magnetite (Baril and Bitton 1967) and of finely divided, easily weathered silicates, such as olivine. Ross and Wang (1993) indicated that coefficients of variation at Fe levels of 0.67% and Al levels of 0.67% are 7.2% and 4.1%, respectively.

Hydroxylamine is closely similar to oxalate in its extraction capacity (Chao and Zhou 1983). It is also commonly used to extract soil Mn. Unlike ammonium oxalate, however, hydroxylamine does not dissolve magnetite and can therefore be used as an alternative to ammonium oxalate for soils containing magnetite (Ross et al. 1985). Ross and Wang (1993) indicated that the coefficients of variation at Fe levels of 0.63% and Al levels of 0.62% are 4.5% and 3.0%, respectively.

Tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid (disodium salt), does not dissolve magnetite either and its use has been suggested instead of oxalate (Kodama and Ross 1991). Furthermore, Tiron dissolves pedogenic opaline silica (Kendrick and Graham 2004), whereas, neither oxalate nor hydroxylamine dissolves this soil component effectively. Tiron is currently used mainly to remove coatings on clays (Ross and Wang 1993). However, it should also be suitable for soils ground to pass a 0.15 mm (100-mesh) sieve.

Sodium pyrophosphate extracts organically complexed Al and Fe from soils. Manganese compounds are also dissolved. It only slightly dissolves noncrystalline inorganic forms, and it does not significantly attack silicate minerals and crystalline Al and Fe oxides or hydroxides (McKeague et al. 1971). The pyrophosphate solution does not dissolve opal and is a poor extractant for allophane or imogolite (Wada 1989). Ross and Wang (1993) indicated that the coefficients of variation at Fe levels of 0.64% and Al levels of 0.69% are 5.9% and 6.0%, respectively. The specificity of the method for organic complexes of Al and Fe has been challenged because amorphous and poorly ordered inorganic Al and Fe solid phases were found to be significantly removed by the pyrophosphate extract (Kaiser and Zech 1996).

From the results of these methods, the following quantities can be estimated:

A. Finely divided crystalline Fe solid phases like goethite, hematite, and lepidocrocite: dithionite Fe–oxalate Fe or dithionite Fe–hydroxylamine Fe or dithionite Fe–Tiron Fe

B. Noncrystalline inorganic forms of Fe including ferrihydrite: oxalate Fe–pyrophosphate Fe or hydroxylamine Fe–pyrophosphate Fe or Tiron Fe–pyrophosphate Fe

C. Organic complexed Fe: pyrophosphate Fe

Relationships B and C also hold approximately for Al; this is not the case for relationship A. In the case of Mn, both dithionite and oxalate attack crystalline oxide forms to some extent and differences between extracts are not easy to interpret. The noncrystalline forms of Si, such as opaline silica, are completely extracted only by Tiron (Kodama and Ross 1991). They are not extracted by oxalate and only partly by dithionite and hydroxylamine. Poorly crystalline and noncrystalline aluminosilicates, including allophane and imogolite, are extracted by oxalate, hydroxylamine, and Tiron. Dithionite and pyrophosphate are much less effective in extracting these compounds.

A survey of the literature on the extraction of Al, Fe, Mn, and Si from soils clearly shows that the laboratory procedures employed vary considerably among extractions and between
studies. Yet, results from different extractions are frequently compared in studies on soil genesis and metal fractionation. Moreover, the effects of the grinding of soil samples and of the filtration of extracts on the amounts of Al, Fe, Mn, or Si extracted are documented (Loveland and Digby 1984; Neary and Barnes 1993). The reduction of particle size by grinding is nonetheless necessary when weighing out small subsamples because it increases the homogeneity between subsamples and, thus, the repeatability of the extraction. In this context, and because of the operational character of the extraction schemes, investigators are strongly encouraged to report the procedures they used, notably, with respect to the preparation of soil samples (sieving, grinding) and to the centrifugation (g force) or the filtration of extracts (type of membrane, pore size). In the methods proposed here, all the samples are ground to 0.15 mm.


The dithionite–citrate method consists of shaking soils overnight in the presence of a reducing and complexing solution. Dithionite creates a reducing environment and dissolves metallic oxides whereas the Na-citrate chelates the dissolved metals and buffers the pH to near 7 to avoid the precipitation of FeS compounds. This treatment is particularly useful for dissolving the "free" Fe in soils. Caution must however be exercised when interpreting extracted Al. The overnight shaking procedure is simpler than the dithionite–citrate–bicarbonate method of Mehra and Jackson (1960), and it gives closely similar results (Sheldrick and McKeague 1975). This extractant is used in the Canadian System of Soil Classification (Soil Classification Working Group 1998) for describing Fe accumulation in Gleysols.

**26.2.1 REAGENTS**

1. Sodium hydrosulfite (dithionite), Na$_2$S$_2$O$_4$
2. Sodium citrate (Na$_3$C$_6$H$_5$O$_7$ · 2H$_2$O), 0.68 M (200 g L$^{-1}$)
3. Certified atomic absorption standards, ±1%

**26.2.2 PROCEDURE**

1. Weigh 0.500 g of <2 mm air-dry soil, ground to pass a 0.15 mm (100 mesh) sieve, into a 50 mL screw-cap plastic centrifuge tube (use 0.2 g for clays and 1 g for coarse soils).
2. Add 25 mL of the sodium citrate solution.
3. Add about 0.4 g of dithionite (a calibrated scoop may be used).
4. Stopper tightly and shake overnight (16 h) in an end-over-end shaker. A horizontal shaker can also be used although interparticle abrasion can be increased.
5. Remove stoppers and centrifuge for 20 min at 510 g (centrifuge at higher speed for samples rich in clay particles). Filter extracts containing suspended materials.
For determining Al, Fe, Mn, and Si by atomic absorption spectroscopy (AAS), prepare standard solutions of these elements containing the same concentration of extracting solution, here Na-citrate with dithionite, as the extracting solution. Gently heat the solution to dissolve dithionite. Note that at high concentration, the precipitation of dithionite can rapidly block the AAS burner. The amount of dithionite added to standard solutions can be lowered to reduce this effect.

An air–acetylene flame is suitable for the determination of Fe and Mn, and a nitrous oxide–acetylene flame is used for Al and Si.

If it is necessary to dilute the extracts, either dilute them with the extracting solution or prepare standards containing the same concentration of extracting solution as the diluted extracts.

26.2.3 Calculations

1 \% Fe, Al, Mn, Si = \frac{\mu g mL^{-1} \text{ in final solution} \times \text{extractant (mL)} \times \text{dilution}}{\text{sample weight (g)} \times 10,000} \quad (26.1)

For example, for 0.500 g of sample, 25 mL of extractant, 5 times dilution, and a 48 \mu g Fe mL^{-1} concentration:

% Fe in sample = \frac{48 \times 25 \times 5}{0.500 \times 10,000} = 1.2 \quad (26.2)

26.3 Acid Ammonium Oxalate Method (in the Dark)
(McKeague and Day 1966)

The acid NH₄-oxalate method was developed in 1922 by Tamm to remove the sesquioxide weathering products from soils. It was revised by Schwertmann (1959), who showed that it could estimate noncrystalline and poorly ordered Al and Fe forms in soils. It extracts the amorphous Al and Fe accumulated in podzolic B horizons (McKeague and Day 1966) and is thus useful to identify podzolic B horizons. Oxalate also dissolves allophane and imogolite (Wada 1989). In the soil taxonomy, amounts of Al and Fe extracted with oxalate are criteria for andic soil properties (Soil Survey Staff 1990). The extraction must be conducted in the dark to prevent photodecomposition of the oxalate solution.

26.3.1 Reagents

1 Solution A: Ammonium oxalate solution (NH₄)₂C₂O₄ · H₂O, 0.2 M (28.3 g L⁻¹).
2 Solution B: Oxalic acid solution H₂C₂O₄ · 2H₂O, 0.2 M (25.2 g L⁻¹).
3 Mix 700 mL of A and 535 mL of B, check pH, and adjust to 3.0 by adding A or B.
4 Certified atomic absorption standards, ±1%.
26.3.2 Procedure

1. Weigh 0.250 g of <2 mm air-dry soil, ground to pass a 0.15 mm (100 mesh) sieve, into a 15 mL screw-cap plastic centrifuge tube (weigh 0.125 g for samples with >2% extractable Fe or Al).

2. Add 10 mL of the acid ammonium oxalate solution and stopper the tube tightly.

3. Place the tubes in an end-over-end shaker and shake for 4 h in the dark. A horizontal shaker can also be used although interparticle abrasion can be increased.

4. Centrifuge the tubes for 20 min at 510 g (centrifuge at higher speed for samples rich in clay particles), decant the clear supernatant into a suitable container, and analyze within a few days. Extracts should be stored in the dark to avoid the photoinduced degradation of oxalate and the subsequent precipitation of dissolved metals.

5. For determining Al, Fe, Mn, and Si by atomic absorption, follow standard atomic absorption procedures. Consider the points mentioned in Section 26.2.2.

26.3.3 Calculations

\[
\% \text{ Fe, Al, Mn, Si} = \frac{\mu g \text{ mL}^{-1} \text{ in final solution} \times \text{extractant (mL)} \times \text{dilution}}{\text{sample weight (g)} \times 10,000} \quad (26.3)
\]

2. For example, for 0.250 g of sample, 10 mL of extractant, 5 times dilution, and a 12 \( \mu g \text{ Fe mL}^{-1} \) concentration:

\[
\% \text{ Fe in sample} = \frac{12 \times 10 \times 5}{0.250 \times 10,000} = 0.24 \quad (26.4)
\]

26.4 Acid Hydroxylamine Method (Ross et al. 1985; Wang et al. 1987)

The acid hydroxylamine extraction is used in geochemical studies for removing noncrystalline material, notably hydrous Mn oxides, from crystalline Fe oxides with minimal dissolution of associated Fe oxides like magnetite (Chao and Zhou 1983). Ross et al. (1985) and Wang et al. (1987) modified this procedure and tested it on soil samples. For Al and Fe, the results were similar to those obtained by oxalate extraction with the advantage that hydroxylamine did not dissolve magnetite. There was less agreement between the Si results obtained by the two methods. The suitability of hydroxylamine as an extractant for Mn in soils has not been fully tested yet. Hydroxylamine solutions are also more easily analyzed than oxalate solutions by AAS because the latter tend to clog the burner.
26.4.1 REAGENTS

1. Prepare a hydroxylamine hydrochloride–hydrochloric acid (0.25 M \( \text{NH}_2 \text{OH} \cdot \text{HCl} \), 0.25 M HCl) solution by adding 21.5 mL of concentrated HCl and 17.37 g of \( \text{NH}_2 \text{OH} \cdot \text{HCl} \) to a 1 L volumetric flask and making to volume with deionized water.

2. Certified atomic absorption standards, ±1%.

26.4.2 PROCEDURE

1. Weigh 0.100 g of <2 mm air-dry soil, ground to pass a 0.15 mm (100 mesh) sieve, into a 50 mL screw-cap plastic centrifuge tube.

2. Add 25 mL of the hydroxylamine solution and stopper the tube tightly.

3. Place the tubes in an end-over-end shaker and shake overnight (16 h). A horizontal shaker can also be used although interparticle abrasion can be increased.

4. Centrifuge the tubes for 20 min at 510 g (centrifuge at higher speed for samples rich in clay particles), decant the clear supernatant into a suitable container, and analyze within a few days.

5. For determining Al, Fe, Mn, and Si by atomic absorption, follow standard atomic absorption procedures. Consider the points mentioned in Section 26.2.2.

26.4.3 CALCULATIONS

1. % Fe, Al, Mn, Si = \( \frac{\mu g \text{mL}^{-1} \text{in final solution} \times \text{extractant (mL)} \times \text{dilution}}{\text{sample weight (g)} \times 10,000} \) \hspace{1cm} (26.5)

2. For example, for 0.100 g of sample, 25 mL of extractant, 5 times dilution, and a 6 \( \mu g \text{ Fe mL}^{-1} \) concentration:

   \[ \% \text{ Fe in sample} = \frac{6 \times 25 \times 5}{0.100 \times 10,000} = 0.75 \] \hspace{1cm} (26.6)

26.5 SODIUM PYROPHOSPHATE METHOD (MCKEAGUE 1967)

Sodium pyrophosphate is a common extractant for Al, Fe, and Mn associated with soil organic matter. It does not extract opal or crystalline silicates. The method is used in the Canadian System of Soil Classification as chemical criteria for identifying podzolic B horizons, in the soil taxonomy for spodic horizons and by the FAO for classifying podzolic soils (Soil Survey Staff 1990; FAO 1990; Soil Classification Working Group 1998). The pyrophosphate extraction is strongly dependent on the centrifugation and filtration procedures because, in some cases, finely divided colloidal silicates and oxides remain dispersed after low-speed centrifugation. High-speed centrifugation or ultrafiltration is then necessary to clear the extracts (McKeague and Schuppli 1982; Schuppli et al. 1983).
26.5.1 REAGENTS

1 Sodium pyrophosphate solution (Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7} \cdot 10H\textsubscript{2}O), 0.1 M (44.6 g L\textsuperscript{-1}).

2 Superfloc (N-100) 0.1% (1.0 g L\textsuperscript{-1}). Available from Cytec Canada Inc., 7900 Taschereau Bld, A-106 Suite, Brossard, Que., J4X 1C2.

3 Certified atomic absorption standards, ±1%.

26.5.2 PROCEDURE

1 Weigh 0.300 g of <2 mm air-dry soil, ground to pass a 0.15 mm (100 mesh) sieve, into a 50 mL screw-cap plastic centrifuge tube (use 1 g for samples low in extractable Fe and Al).

2 Add 30 mL of sodium pyrophosphate solution and stopper the tube tightly.

3 Shake overnight (16 h) in an end-over-end shaker. A horizontal shaker can also be used although interparticle abrasion can be increased.

4 Centrifuge at 20,000 g for 10 min or, alternatively, add 0.5 mL of 0.1% superfloc solution and centrifuge at 510 g for 10 min. Note the following points:
   a. Concentrations of Fe and Al in sodium pyrophosphate extracts of some samples may decrease progressively by centrifugation for longer times or at higher speeds.
   b. Ultrafiltration through a 0.025 μm Millipore filter is recommended for tropical soils and for samples giving questionable results by the centrifugation methods.

5 Decant a portion of the clear supernatant into a suitable container and analyze within a few days. Extracts containing suspended materials should be filtered.

6 For determining Al, Fe, and Mn by atomic absorption, follow standard atomic absorption procedures. Consider the points mentioned in Section 26.2.2.

26.5.3 CALCULATIONS

1 % Fe, Al, Mn = \frac{\mu g \text{ mL}^{-1} \text{ in final solution} \times \text{ extractant (mL)}}{\text{sample weight (g)} \times 10,000} \quad (26.7)

2 For example, for 0.300 g of sample, 30 mL of extractant, and a 75 μg Fe mL\textsuperscript{-1} concentration:

   % Fe in sample = \frac{75 \times 30}{0.300 \times 10,000} = 0.75 \quad (26.8)
REFERENCES


Nitrogen (N) is the major nutrient determining tree growth, and this has been demonstrated abundantly in the Boreal Shield with fertilization trials or net N mineralization studies (e.g., Attiwil and Adams 1993; Reich et al. 1997). However, Ingestad (1979a,b) also showed that any other nutrient (but particularly phosphorus (P) and potassium (K)) could be limiting if supplied at a rate lower than tree demand, even if N was in excess. For example, fertilization trials with N alone or in combination with P, K, or both stimulated the growth of black spruce (Picea mariana (Mill.) BSP) (e.g., Wells 1994; Paquin et al. 1998) and jack pine (Pinus banksiana Lamb.) (e.g., Morrison and Foster 1995; Weetman et al. 1995). A much lower number of studies have showed the benefits of increased calcium (Ca) and magnesium (Mg) availability on tree nutrition and yields in Canadian forests (Hamilton and Krause 1985; Bernier and Brazeau 1988; Thiffault et al. 2006). A review by Binkley and Högberg (1997) suggested that fertilization trials with Ca and Mg have only occasionally favored the growth of northern tree species. The benefits of Ca and Mg fertilization may actually be related to an indirect effect of liming on N availability (Nohrstedt 2001; Sikström 2002). The lack of scientific evidence about the role of soil nutrients (other than N) on improved tree nutrition and growth may be due to the fact that permanent site variables such as climate, drainage, and soil physical properties have a stronger influence on trees (Post and Curtis 1970).
The forest floor has been the focus of many nutrition studies because it has a large fraction of the fine roots (Steele et al. 1997), it can be used for linking N and P turnover to tree productivity and nutrition (e.g., Paré and Bernier 1989; Reich et al. 1997), and it generally represents a large fraction of the total soil nutrient pools (Bélanger et al. 2003). However, K, Ca, and Mg in trees are believed to be derived primarily from mineral weathering and recent studies suggest that parent material elemental composition and estimates of mineral weathering can be better indicators of their availability (van Breemen et al. 2000; Bailey et al. 2004; Thiffault et al. 2006). As for P, its availability is not only constrained by the decay process and biological sinks (plant and microbial uptake) but also by geochemical sinks. Forest soils share many characteristics with agricultural soils, but the way they are used and managed requires a different approach in many situations. The objective of this chapter is to suggest what we believe are the most acceptable analyses for determining N, K, Ca, and Mg availability in forest ecosystems and establishing a link with tree nutrition, growth, and mortality. We focus on (1) mineralizable N; (2) pH, effective cation exchange capacity (ECEC), and exchangeable cations; and (3) elemental Ca, Mg, and K composition and their release from mineral weathering. Indices of available P are mostly limited to the extraction of labile P and the reader should refer to Chapter 24 and Chapter 25 for more details.

27.2 MEASURING AVAILABLE NITROGEN IN FOREST SOILS

Several techniques have been used to estimate net N mineralization in the field. Each method has its own limitations and there is no consensus on a best method (see Binkley and Hart 1989). These methods could be divided into field incubation, laboratory incubation, chemical extraction, and measurements of gross N fluxes using \(^{15}\)N (to better understand the microbial dynamics of N transformations). Binkley and Hart (1989) provided a comprehensive review of the components of N availability assessments in forest soils. In recent years, the view of the N cycle in forested ecosystem has substantially changed. The following findings may have a large impact on the measurement techniques that are considered most appropriate for forest soils as well as on the interpretation of the results:

1. Ericoid and ectomycorrhizal fungi have the capacity to scavenge organic sources of N and P and to participate in the decomposition process (Read et al. 2004). Therefore, incubations that exclude active plant roots may underestimate fluxes, especially in boreal or coniferous forests.

2. Organic N is the dominant form of N in soil solutions (Qualls et al. 2000) and some plants and associated mycorrhizal fungi can absorb dissolved organic N (Näsholm et al. 1998).

3. Studies reporting gross N fluxes have indicated substantial rates of gross mineralization and nitrification even in systems where little mineral N accumulates during mineralization assays.
Given this information, net N mineralization measured with incubation techniques cannot be viewed as a direct measure of plant-available N but rather as an index of this process (see Schimel and Bennett 2004). We provide here the description of two incubation techniques that would likely be correlated with field N fluxes even though the soils are not in contact with living roots. The methodologies described consider periods of incubations that are long enough to avoid the immobilization phase typical of forest soils with high C:N ratios and therefore allow part of the more labile fractions of soil N to be mineralized and measured. The first technique is a long-term laboratory incubation that assesses the potentially mineralizable N fraction of the soil. The second method is a field incubation that is sensitive to field microclimatic conditions. These two techniques have been compared in Brais et al. (2002).

27.2.1 LONG-TERM LABORATORY INCUBATION

The fraction of potentially mineralizable N ($N_0$) and its mineralization constant ($k$) can be assessed with long-term laboratory incubations (Stanford and Smith 1972); some related methods for measuring mineralizable N in agricultural soils are given in Chapter 46. The long-term laboratory incubation technique given here can be used to measure production of dissolved organic N, C, and P (Smith et al. 1998), and CO$_2$ (Côté et al. 2000). The effect of temperature on mineralization rates can be assessed with this technique to give insight on the reactivity of soil organic matter to changes in temperature regime (Paré et al. 2006). Soil disturbance during sampling and sample preparation (e.g., drying, grinding, or sieving; and refrigerating or freezing) can have an impact on microbial activity and this is of importance for obtaining indices of N turnover and availability (e.g., Van Miegroet 1995; Ross and Hales 2003). The field logistics and the study’s objectives will determine the methodology used and the interpretation of the data must be done accordingly. For the sake of simplicity, however, these effects and the different methods used are not further considered in this chapter. Rather, we describe a technique using fresh moist samples that we believe yields reliable estimates of the potential of the soil for N and C release under standard conditions.

**Materials and Reagents**

1. Plastic filtration units are used (Falcon Filter units, Becton Dickinson, Model 7102) but the original nitrocellulose filter of the microlysimeter has to be replaced by a glass–fiber filter (Nadelhoffer 1990).

2. Glass wool.

3. 1 $M$ HCl.

4. 0.005 $M$ K$_2$SO$_4$.

5. Vacuum pump (60 mm Hg).


7. Whatman No. 42 filter paper.

8. Acid washed 1 mm (18 mesh) silica sand.
Procedure

1. Volumetric soil samples are collected; fresh, moist samples are sieved through 6 and 4 mm screens for organic humus layers (FH) and mineral horizons, respectively, to remove coarse fragments and roots. The samples are then weighed. To maintain the soil structure of the moist samples as much as possible, the large mesh sizes are used while homogenizing the material.

2. The soil material is weighed to obtain samples of 25 g of the fresh organic humus layer (about 9 g of dry FH material on average) and 100 g of fresh mineral soil (about 73 g of dry mineral soil on average), and inserted into the top part of the filtration units above a layer of prewashed (1.0 M HCl and deionized water) glass wool. The soil material is then packed slightly to obtain a total volume of soil of 70 and 100 cm$^3$ for the organic layer and the mineral layer, respectively. Fine-textured mineral soils can be mixed with acid-washed silica sand (50% soil volume). Silica sand is washed with 1 M HCl and rinsed until the conductivity falls to that of demineralized water.

3. Soil samples are incubated in growth chambers at 22°C. The relative humidity level is maintained around 85% to keep the soil moist. The microcosm remains open to air exchange inside the growth chamber unless a respiration measurement is taken over for short periods (24–48 h). Water content is verified by weighing and adjusted to 85% of field capacity weekly.

4. Soils are flushed monthly with 100 mL 0.005 M K$_2$SO$_4$. Solution is gently added to the soil with a burette (2 x 50 mL) to limit disturbance to the soil structure. Soils are allowed to drain freely; the excess solution is removed by applying vacuum. If the solution contains soil particles, it can be refiltered using a Buchner funnel and filter paper. Samples are transferred to the refrigerator at 4°C and should be analyzed for ammonium, nitrate, and total N within 2 weeks.

Calculations

Cumulative mineralized N through time ($N_t$) is fitted to the following simple negative exponential model (Stanford and Smith 1972):

$$N_t = N_0(1 - e^{-kt})$$

(27.1)

where $N_0$ is potential mineralizable N, and $k$ is mineralization rate and is unitless. $N_0$ can be expressed on a total N basis to estimate organic matter quality or on an area basis to give the reserve of potentially mineralizable N for a given soil depth. See Chapter 46 for more details.

Comments

Higher temperatures of incubation are often used (e.g., 35°C). These high temperatures often provide a better fit and convergence of first-order models. However, Equation 27.1 parameters ($N_0$ and $k$) are sensitive to temperature (MacDonald et al. 1995; Paré et al. 2006) and we recommend using a soil temperature that is high (e.g., 22°C), but not outside the range of temperatures observed in surface soils under a closed forest canopy.
27.2.2 Field Incubation

Field incubation techniques include incubating a soil sample in the field with the least possible disturbance and estimating the net amounts of ammonium and nitrate that accumulated in the sample. The incubation period varies from a week to a year. These techniques originate from in situ buried bags (Eno 1960) where samples are incubated in a polyethylene bag. The main drawback of this method is the disturbance to the soil sample, which can increase mineralization rates from 2- to 10-fold according to Raison et al. (1987). The latter authors have described the use of in situ incubations in closed-top tubes perforated on the sides and open at the bottom. This technique limits disturbance of the soil samples while allowing the use of the samples to a greater depth (40 cm). In addition, Di Stefano and Gholz (1986) have proposed the use of a resin core above and below the incubated core. The resin core above is discarded at the end of the measurement period. Its only use is to prevent contamination with atmospheric N inputs. Nitrogen mineralization is estimated as the net amount of N mineralized in the soil core in addition to the N captured by the bottom resin core. This technique may provide conditions that more closely mimic those in intact soils because it allows water movement in the soil core as well as the removal of the products of mineralization. A simpler alternative is the use of closed-top cores; since there is no water flux into the top of the cores, it is assumed that there is no leaching loss from the bottom. We describe here closed-top field incubations.

Materials and Reagents

1. ABS cores, 30 cm long, 4.5 cm in diameter, capped
2. 2 M KCl solution
3. Whatman No. 42 filter paper
4. Reciprocating shaker
5. Erlenmeyer flasks (250 mL)
6. Funnels

Procedure

1. Two tubes are brought to the field. The first one is used to collect a soil sample for initial determination of mineral N content. The second one is inserted in the soil to the required depth near the first tube and is left in the field for the incubation period (i.e., 1 week to 1 year; 6 weeks incubations gave reproducible results on a rich soil in the boreal mixedwood although it was too short to measure net mineralization in black spruce sites).

2. Tubes collected from the field are kept in a cooler and should be extracted within 48 h.

3. The soil samples are separated into forest floor and mineral soil samples. They are sieved through 6 and 4 mm screens for organic humus layers and mineral horizons, respectively. The total wet weight of the soil that is kept is weighed.
A subsample is dried at 65°C for organic horizons and at 105°C for mineral soil horizons to estimate water content, and these subsamples are also used for determination of total N and total C preferably on a CN analyzer (see Chapter 21 and Chapter 22).

An amount of fresh soil that corresponds to 10 g dry weight (about half for strongly organic samples) is placed into a 250 mL Erlenmeyer flask. Then add 100 mL of KCl solution. Flasks are capped and shaken for 1 h and then filtered through a Whatman No. 42 filter. Solutions are analyzed for NH$_4$-N and NO$_3$-N (see Chapter 6).

**Calculations**

The difference between final and initial concentrations is used to express net N mineralization, net nitrification, net ammonium production, or all the above. Production rates are expressed in N weight, on time, and on either soil dry weight, total N or C basis to express the quality of the soil organic matter, or on an area basis to get an estimate of nutrient fluxes.

**Comments**

1. We would advise the use of long incubation periods or the use of laboratory incubations in soil with high C:N, high organic matter content, low N turnover, little net nitrification, little nitrate in soil solution, or presence of ericoid and ectomycorrhizal fungi. On the other hand, short-term incubations would be suitable for forests with thin or nonexistent organic layers that undergo net nitrification (such as sugar maple [Acer saccharum Marsh.] forests).

2. We often found very low and negative rates of net mineralization (net immobilization) in boreal black spruce forests with thick organic layers (D. Paré, unpublished data). Such results are frequent and not often published. In all cases, it is advisable to compare results with the nutrient budget.

3. Estimates of N in litterfall and immobilization in biomass provide estimates of N mineralization that are totally independent of incubation estimations and that should match them. Although it is not always possible to obtain such an estimate, the comparison of incubation results with budget estimates should be done on a few plots within the forest and soil types investigated.

**27.3 SOIL pH, EFFECTIVE CATION EXCHANGE CAPACITY, AND EXCHANGEABLE CATIONS**

**27.3.1 Soil pH**

Because of the variations in ionic strength of agricultural soils, the most common method of measuring their pH in Canada is the 0.01 M CaCl$_2$ method. By measuring the pH in an electrolyte of known concentration, the effects of variable ionic strength of the soil solutions are largely eliminated. Forest soils, on the other hand, tend to have close to the same ionic strength throughout the year, except as influenced by variations in water content. For this reason, many researchers choose to use pH measured in water. Since the ionic strength of the measurement solution is lower, the pH obtained will be closer to that observed by plants.
growing in the field. Researchers should be aware that the disturbance caused by sampling and drying soils does have an effect on the measured pH (Courchesne et al. 1995). A discussion on the choice of pH methods can be found in Chapter 16 along with a detailed description of the methods themselves.

### 27.3.2 Effective Cation Exchange Capacity and Exchangeable Cations

The use of an unbuffered BaCl₂ solution is now generally preferred for determination of ECEC. The BaCl₂-compulsive exchange procedure (Gillman and Sumpter 1986) is recommended for determining CEC on all soils (except soils containing salts, carbonates, or zeolites) (Sumner and Miller 1996). Similarly, Chapter 18 suggests the use of a simplified BaCl₂ extraction. The BaCl₂ extraction has the ability to displace trivalent cations at lower ionic strength without being preferentially adsorbed compared to the NH₄Cl or KCl extractions. Although the extraction can modify pH because the ionic strength of 0.3 mol L⁻¹ (for 0.1 M BaCl₂) of the solution is still about three orders of magnitude higher than the soil solution of a sandy Podzol, it causes smaller changes in pH than the more concentrated solutions (e.g., 1 mol L⁻¹ for 1 M KCl or NH₄Cl). In this method, ECEC is calculated by summing exchangeable cations (Ca, Mg, K, Na, Al, Fe, and Mn).

The method of Chapter 18 is strongly recommended for acid forest soils such as Podzols as well as Sombric and Dystric Brunisols (or Dystric Cambisols according to the Food and Agriculture Organization of the United Nations (1974)). For boreal plain forests with higher soil pH values (above pH 5.5–6.0) and low levels of exchangeable aluminum and manganese (e.g., Melanic and Eutric Brunisols [or Eutric Cambisols], Gray Luvisols [or Albic Luvisols], and Chernozems), the unbuffered NH₄Cl extraction is also acceptable and is commonly used (Kalra and Maynard 1991). However, although most agronomists are interested in determining the amount of exchange sites for management or control of soil pH, usually by liming, forest soil scientists are also interested in knowing the cation species (base vs. acid) held on these exchange surfaces. The method proposed here is therefore a one-step extraction that uses an unbuffered NH₄Cl solution and that allows the measurement of ECEC as well as the individual contribution of Ca, Mg, K, and Na to ECEC (and Al, Fe, and Mn if needed).

### Materials and Reagents

1. Centrifuge tubes (50 mL) with screw caps.
2. Ultracentrifuge accepting 50 mL tubes.
3. End-over-end shaker.
4. Ammonium chloride, 1 M: dissolve 53.5 g of NH₄Cl with double deionized (d.d.) water and make to volume in a 1000 mL volumetric flask.
5. Standards of Ca, Mg, K, Na, Al, Fe, and Mn are prepared using atomic absorption reagent-grade standards of 1000 mg L⁻¹. The matrix in the standards must correspond to the NH₄Cl concentration of the analyzed sample (diluted or nondiluted matrix).
6. Lanthanum solution, 100 mg L⁻¹: dissolve 53.5 g of LaCl₃·7H₂O in a 200 mL volumetric flask and make to volume (for analysis by atomic absorption spectroscopy [AAS]).

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Cesium solution, 100 g L\(^{-1}\): dissolve 25.2 g CsCl in a 200 mL volumetric flask and make to volume (for analysis by AAS).

Whatman No. 41 filter paper.

**Procedure**

1. Weigh out about 2.5 g of dry organic soil (FH samples) or fine-textured mineral soil and about 12.5 g of dry (<2 mm) coarse-textured mineral soil into a 50 mL centrifuge tube. Record the exact weight of soil used to the nearest 0.001 g. Include blanks, duplicates, and quality control samples.

2. Add 25.0 mL of 1 M NH\(_4\)Cl to each tube and shake slowly on an end-over-end shaker (15 rpm) for 2 h.

3. Ultracentrifuge (15 min, 7000 g) and filter the supernatant with Whatman No. 41 filter paper.

4. Analyze Ca, Mg, K, Na, Al, Fe, and Mn in the supernatant solution with AAS or other suitable instrument. Dilution (10- or 100-fold) will likely be required for Ca, K, Mg, and Na. The addition of 0.1 mL of La solution and 0.1 mL of Cs solution to a 10 mL aliquot of diluted extract is required for the determination of Ca, Mg, and K by AAS (for detailed instructions on this and other aspects of analysis refer to the manual for your AAS). If needed, preservation of samples by acidifying to 0.2% HNO\(_3\) will prevent the loss of Fe and Al.

**Calculations**

1. Exchangeable cations:

\[
M^+ \text{ cmol}_c \text{ kg}^{-1} = C \text{ cmol}_c \text{ L}^{-1} \times (0.025 \text{ L/wt. soil g}) \times 1000 \text{ g kg}^{-1} \times \text{ DF}
\]  

(27.2)

where \(M^+\) is the concentration of an adsorbed cation (cmol\(_c\) kg\(^{-1}\)); \(C\) is the concentration of the same cation measured in the NH\(_4\)Cl extract (cmol\(_c\) L\(^{-1}\)); and DF is the dilution factor (if applicable).

2. Effective CEC:

\[
\text{Effective CEC cmol}_c \text{ kg}^{-1} = \Sigma \text{ cmol}_c \text{ Ca, Mg, K, Na, Fe, Al, Mn kg}^{-1}
\]  

(27.3)

See Section 18.2.4 in Chapter 18 for details on quality controls, standards, and the effects of different soil:solution ratios on results.

**27.3.3 Contribution of Exchangeable H\(^+\) to Effective Cation Exchange Capacity**

It is difficult to account for the amount of H\(^+\) coming from the exchange reaction and its contribution to ECEC from NH\(_4\)Cl or BaCl\(_2\) extractions because some H\(^+\) in the extract may come from sources other than exchangeable H\(^+\) (e.g., dissociation of organic acids) or be
produced or consumed in reactions involving Al–OH complexes or hydrolysis of free Al$^{3+}$ (Thomas and Hargrove 1984). The salt solution, ionic strength, and the soil: solution ratios have an influence on the amount of exchangeable H$^{+}$ displaced from exchange sites. Therefore, the contribution of exchangeable H$^{+}$ to ECEC or base saturation is operationally defined from titration of a 1 M KCl extract as suggested by Thomas (1982) (see Chapter 18 for details on methodology). Exchangeable H$^{+}$ is relatively abundant in acidic organic horizons (e.g., forest floor material), but acidic mineral soils such as Bhf and Bf horizons also have high enough amounts to draw our attention (Ross et al. 1996; Bélanger et al. 2006). Bélanger et al. (2006) noted that ‘‘fundamentally, any valid measure of ECEC must therefore include some estimate of exchangeable H$^{+}$ concentration or a demonstration that it is negligible’’. Unfortunately, the direct measurement of exchangeable H$^{+}$ is time-consuming and not practical for routine analysis. Therefore, Bélanger et al. (2006) have used soil pH in water (as proposed in Chapter 16) and ECEC (using BaCl$_2$ as described in Chapter 18) to estimate exchangeable H$^{+}$ concentrations in FH and podzolic (spodic) B samples of acidic forest soils developed from granitic bedrock or parent material. Although Equation 27.4 provides good estimates of the proportion of exchangeable H$^{+}$ on the exchange complex of organic and podzolic B horizons from all types of forests, we recommend the readers build specific relationships using their own samples if greater predicting power is required:

\[
\log(\text{exch. H}^+)/\text{ECEC} = 0.682 - (0.308 \times \text{soil pH in water}); \quad R^2 = 0.691 \quad (27.4)
\]

### 27.4 ELEMENTAL P, K, Ca, AND Mg COMPOSITION AND RELEASE BY MINERAL WEATHERING

It has long been recognized that Ca and Mg in trees are derived primarily from Ca and Mg released into the soil solution from mineral weathering (van Breemen et al. 2000; Blum et al. 2002), and additional studies suggest that parent material elemental composition can be a reliable indicator of tree Ca and Mg nutrition. For example, Thiffault et al. (2006) examined soil and foliar nutrient status of black spruce and balsam fir (Abies balsamea (L.) Mill.) stands in Quebec subject to whole-tree and stem-only harvesting and found that total mineral parent C elemental content was more indicative of nutrient limitations than surface soil-available nutrient concentrations: the signal of this low Ca and Mg availability was very weak in the upper soil layers, including the forest floor, probably because the chemistry of these layers is largely controlled by litter material with relatively well-balanced nutrient ratios (Knecht and Göransson 2004). Additional studies suggest that parent material elemental composition may be an important predictor of tree mortality as well as Ca and Mg nutrition. For example, van Breemen et al. (1997) showed that sugar maple mortality in the northeastern United States increased with decreasing elemental Ca in the parent C material. Sugar maple foliar Ca and Mg status and mortality were also more strongly linked to B horizons compared to forest floor Ca and Mg chemistry (Bailey et al. 2004).

Bailey et al. (2004) further suggested that a model that calculates release of Ca and Mg from soil mineral weathering of the parent C material would likely be successful in predicting stand nutrition and productivity. Many indices of soil mineral weathering have been developed in the past (Birkeland 1999), but one of the preferred approaches compares the concentration of elements in the various soil horizons to the concentration of elements on the presumably unaltered parent material in the C horizon (Kirkwood and Nesbitt 1991; Bain et al. 1994; Hodson 2002). Adjustments are made to consider additions of organic matter and the leaching of elements that are not of interest in the study because both will affect the concentration of the elements studied. Therefore, an equation using an element resistant to
weathering (most often zirconium and titanium) is used to normalize the data for mobile elements. Assuming the age of the soil is known, the release rate of a mobile element in a particular horizon can be calculated using the following equations (Hodson 2002):

\[
R = (E_{PM} - E_i^*) \times \rho \times Z / t
\]

and

\[
E_i^* = E_i \times C_{PM} / C_i
\]

where \(R\) is the element release rate (\(\mu g\ m^{-2}\ year^{-1}\)), \(E_{PM}\) is the concentration of element \(E\) in the parent material (\(\mu g\ g^{-1}\)), \(E_i^*\) is the adjusted concentration of element \(E\) in horizon \(i\) (\(\mu g\ g^{-1}\)), \(E_i\) is the concentration of element \(E\) in the horizon \(i\) (\(\mu g\ g^{-1}\)), \(r\) is the horizon density (\(g\ m^{-3}\)), \(Z\) is the horizon thickness (m), \(t\) is the soil age (years), \(C_{PM}\) is the concentration of immobile element \(C\) in the parent material (\(\mu g\ g^{-1}\)), and \(C_i\) is the concentration of immobile element \(C\) in horizon \(i\) (\(\mu g\ g^{-1}\)).

In forest soils, the concentration of immobile elements tends to decrease with depth; there is a concentration effect from bottom to top because of the loss of mobile elements and accumulation of organic matter in upper soil horizons (Melkerud et al. 2000; Courchesne et al. 2002; Hodson 2002). Research has shown that some of the elements that are resistant to weathering can nonetheless be eluviated and we recommend that anyone applying this technique study the results of authors such as those mentioned above.

Weathering rates in the >50 \(\mu m\) fraction are sometimes assumed to be negligible because of the relatively low surface area and lack of easily weatherable minerals in that fraction (Kolka et al. 1996). Therefore, the method is sometimes employed on the silt fraction (2–50 \(\mu m\)) alone after wet sieving to remove sand and by multiple centrifugations to remove clay. In this case, the expression \((E_{PM} - E_i^*)\) is multiplied by the silt mass in that horizon, which can be measured after determination of soil bulk density, particle size distribution, and horizon thickness.

Wavelength dispersive x-ray fluorescence spectroscopy on fused beads is generally the preferred approach to determine the elemental composition of soil samples (e.g., van Breemen et al. 1997; Melkerud et al. 2000), but this can also be determined by inductively coupled plasma (ICP) or AAS on samples digested using hydrofluoric acid.

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Chapter 28
Chemical Properties of Organic Soils

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28.1 INTRODUCTION

Organic soils are rich in fresh plant material or organic materials at various stages of decomposition, namely fibric, hemic, and sapric materials (Soil Survey Staff 2003). These soils usually form under conditions of water saturation. Organic soils include muck and peat soils or histosols (Canada and United States of America), the tundras, the Irish peat bogs, the moor peats (Australia), les sols hydromorphes organiques (France), and earthy peat soils in Great Britain (Okruszko and Ilnicki 2003). Common organic soil parent materials may include mosses (such as sphagnum), gyttja, dy, marl, volcanic ash, cattails, reeds, sedges, pondweed, grasses, and various “water-loving” deciduous and coniferous shrubs and trees. Organic soils can contain silicate minerals from trace to appreciable amounts. The characteristics of organic soils depend mainly on the nature of the vegetation that was deposited in the water and the degree of decomposition (Mokma 2005).

Although the chemical properties of organic soils are different from those of their mineral counterparts, the chemical methods given for mineral soils are also applicable to organic soils; therefore, these analytical procedures are not repeated here. The analyst may choose an appropriate method from other chapters.

28.2 SAMPLE PREPARATION (ASTM 1988)

28.2.1 INTRODUCTION

Soil testing is complicated by the larger range of volume percentage of the solid phase, computed as the ratio of bulk density to particle density, and water contents in samples of organic soils compared with mineral soils, and with a greater influence of organic soil drying on soil chemical properties (Parent and Khiari 2003). According to Watson and Isaac (1990), quantitative analysis of soil samples can be broken down into six steps: (i) handling and
preparation of the samples, (ii) weighing samples, (iii) dissolution of samples/extraction of elements, (iv) pretreatment or removal of interferences, if needed, (v) measuring a property of the sample, and (vi) calculating and reporting of concentration of analyte. Handling and preparation of organic soils before soil analysis are critical. Chemical analyses may be conducted on fresh samples (field moisture content) or on air-dried samples. It should be noted that drying organic soils increases dry bulk density, volume of the solid phase (Ilnicki and Zeitz 2003), and mineralization of organic P (Daughtrey et al. 1973), decreases pH measured in water, 0.01 M CaCl₂, and 1 M KCl compared with the field-moist condition (van Lierop and Mackenzie 1977), and may lead to higher soil test levels of certain nutrients, such as available P and K (Daughtrey et al. 1973; Anderson and Beverly 1985). These latter authors postulate that screened organic soils are more easily compacted upon drying, and conversely expand upon rehydration. Parent and Khiari (2003) noted that air-dried pristine peat contained more P in available form than fresh peat. Anderson and Beverly (1985) recommend that organic soils be sampled on a volume basis in order to ensure uniformity of results. Harrison (1979) suggested that where soils vary in bulk density, soil data should be expressed in terms of soil volume.

### 28.2.2 Materials

1. Analytical balance
2. Blender, high speed
3. Large flat pan or equivalent
4. Spoon or spatula

### 28.2.3 Procedure

1. Mix organic soil sample thoroughly and weigh a 100 to 300 g representative sample. Determine the mass of the sample and spread evenly on a large flat pan, square rubber sheet, or paper. Crush soft lumps with a spoon or spatula and let the sample come to moisture equilibrium with room air, not less than 24 h.

2. Stir occasionally to maintain maximum air exposure of the entire sample.

3. When the mass of the sample reaches a constant value, calculate the moisture removed during air drying as a percentage of the as-received mass.

4. Grind a representative portion of the air-dried sample 1 to 2 min in a high-speed blender. Determine the amount, in grams, of air-dried sample equivalent to 50 g of as-received sample as follows:

\[
\text{Equivalent sample mass, } g = 50.0 - \left[ \frac{(50 \times M)}{100} \right]
\]  

(28.1)

where \( M \) is the percent of moisture removed in air drying.

5. Place the sample in a moisture-proof container.
28.3 MOISTURE AND ASH CONTENT (ASTM 1988)

28.3.1 INTRODUCTION

The simplest method for the direct determination of moisture content is the gravimetric method, which involves the measurement of water lost by weighing a soil sample (as-received) before and after it is dried at 105°C–110°C in an oven. The moisture content is expressed either as a percent of the oven dry mass or of the as-received mass. This method may not be suitable when a dried soil sample is used to assess nitrogen, pH, cation exchange, and other soil chemical properties. An alternative method that removes the total moisture and provides a more stable sample, the air-dried sample, has been suggested by the ASTM Committee (ASTM 1997). This method includes two steps: (1) evaporation of moisture in air at room temperature (air-drying) and (2) the subsequent oven drying of the air-dried sample at 105°C.

There are basically two procedures involved in the determination of the ash (inorganic fraction) of a peat or organic sample: dry-ashing methods and wet-ashing methods.

The dry-ashing method involves the removal of organic matter by combustion of the sample at medium temperature (375°C to 800°C) in a temperature-regulated muffle furnace. The principal errors in dry-ashing arise through incomplete combustion when the temperature or time allowed for combustion is insufficient, and through losses resulting from the use of too high a temperature (Allen 1989).

If necessary, samples are dried (105°C–110°C) before ashing. The substance remaining after ignition is the ash and includes mineral impurities such as sand. The weight lost on ignition is calculated and considered as an approximate measure of the organic content of acid organic soils and noncalcareous peatlands. Vessels suggested for ashing are porcelain, quartz, or platinum dishes. Sample weights used vary from 0.25 to 2.00 g. The ash may be further dissolved in an acid solution for elemental analysis.

28.3.2 MATERIALS

1. Muffle furnace—controlled to ±5°C for ashing at 600°C
2. High-form porcelain, 30 mL crucible
3. Porcelain crucible cover or aluminum foil, heavy duty
4. Desiccator cabinet or nonvacuum, desiccator with desiccant
5. Analytical balance and spoons
6. Electric drying oven: regulated to a constant temperature of 105°C

28.3.3 PROCEDURE

1. Weigh a 2 g sample of 2 mm oven-dried soil (105°C) into a tared high-form porcelain, 30 mL crucible with 0.1 mg accuracy. Determine the mass of the covered high-form porcelain crucible. Remove the cover and place the crucible in a muffle furnace.
Gradually bring the temperature in the furnace to 370°C and maintain it for 1 h and then ash the sample either at 550°C for 16–20 h (Andrejko et al. 1983) or at 600°C for 6 h (Goldin 1987).

Remove the crucible from the furnace, cover, place it in a desiccator, allow to cool, and weigh with 0.1 mg accuracy. Save the crucible and its contents for metal ion determination.

28.3.4 **Calculation**

Calculate the ash content as follows:

\[
\text{Ash, g/100 g} = \frac{(a - c)}{(b - c)} \times 100
\]

where \(a\) is the final weight (g) of crucible and ash; \(b\) is the weight (g) of crucible and sample; and \(c\) is the weight (g) of empty crucible.

The procedure described above can be used to determine the amount of organic matter as follows:

\[
\% \text{ Organic matter} = 100 - \% \text{ mineral content (ash)}
\]

28.3.5 **Comments**

1. Using the above procedure for determining ash content it has been shown by Andrejko et al. (1983) that a temperature setting of 550°C is satisfactory for most purposes. The standard method approved by the ASTM Committee (ASTM 1997) proposes a temperature setting of 440°C and heating until the sample is completely ashed (no change of mass occurs after a further period of heating).

2. Dry ashing may overestimate the amount of organic matter in the soil. Positive errors are dependent on soil properties, such as the amount of carbonates and the amount and type of clay present in the mineral fraction of the soil (Goldin 1987).

3. The procedure outlined above measures the mass percentage of ash and organic matter in organic soil, including moss, humus, and reed–sedge types (Day et al. 1979).

4. Samples should be placed in the muffle furnace cold and the temperature allowed to rise slowly to avoid volatilization losses, which are aggravated by violent deflagration.

5. Use high-form porcelain crucibles with covers or equivalent if ashes are retained for elemental analysis. These crucibles eliminate possible contamination of the ash by boron, which may volatilize from the furnace walls (Williams and Vlamis 1961).

6. Values derived from loss-on-ignition results should only be considered as approximate (Allen 1989).
28.4 TOTAL ELEMENT ANALYSIS (ELEMENTS OTHER THAN NITROGEN, CARBON, OXYGEN, AND HYDROGEN)

28.4.1 INTRODUCTION

Most methods that have been developed for the determination of total elements in organic soils involve a two-step procedure, namely: (i) the complete destruction of both organic and inorganic fractions of the soil matrix by various digestion/oxidation procedures in order to liberate all elements in solution and (ii) the determination of soluble elements by various techniques. The chemical procedure involved in the destruction of organic materials (peat, plants, sediments, soils) falls basically into two groups: (a) dry-ashing methods and (b) wet-ashing (or digestion) methods.

In the dry-ashing procedure, the organic material is ignited in an electrically controlled temperature muffle furnace with fume disposal at low (400°C) or medium temperature (550°C–660°C) to oxidize organic matter and the ions are extracted from the ash with an acid solution: 1.5 M HCl (Ali et al. 1988), 6 M HCl (Kreshtapova et al. 2003), or 2 M HNO₃ (Day et al. 1979). Dry-ashed sample may be heated on a hot plate with dilute HCl to dissolve the residues and then with concentrated hydrofluoric (HF) acid to destroy any silicates present (Papp and Harms 1985).

Wet digestion involves complete dissolution of the organic material to convert elements to soluble forms by heating with concentrated acids in either open or closed vessels. This phase is then followed by determination of the liberated ions. Dissolution technique can be performed by hot plate, hot block digestion, or pressured digestion systems. Open or closed vessels can be used in microwave systems while open vessels are usually used in block digestion. Disadvantages of open vessel digestion systems include the greater risk of loss of volatile elements. Microwave digestion is a commonly used practice in many laboratories. Important variables in a microwave digestion procedure are the microwave energy power profiles (power, time, and pressure), the volume and combination of acids used, and the acid-to-sample ratio.

Recently, a new wet digestion procedure for the determination of As in biomasses, coal, and organic-rich sediment samples using hydride generation–atomic fluorescence spectrometry (HG–AFS) has been developed (Chen et al. 2005). This method involves digestion of 200 mg sample aliquots with 3 mL HNO₃ (65%) + 0.1 mL HBF₄ (~50%), heating in a microwave autoclave up to a temperature of 240°C. After digestion, no evaporation of HNO₃ to remove acid from the digests is needed before arsine generation can be carried out.

The following microwave acid digestion procedure is a modification and synthesis from methods proposed by Papp and Harms (1985), Weiss et al. (1999), and Morrell et al. (2003). It employs microwave heating of the sample, first in HNO₃ + H₂O₂ to oxidize organic matter and then in HNO₃ + HF to decompose any remaining organic material and complete dissolution of the inorganic fractions of the peat or organic soil. The first gentle phase of the digestion program, the organic, carbon-rich matrix components are slowly converted to CO₂ to avoid foaming (Krachler et al. 2002).

28.4.2 MATERIALS

1. Microwave oven-assisted sample digestion system, with closed vessels
2. Nitric acid (HNO₃), concentrated (trace metal grade), 65%
Hydrogen peroxide (H$_2$O$_2$), 30%

Hydrofluoric acid (HF), 40% or 48%, as recommended by the instrument manufacturer

Polyfluoroethylene (PTFE) Teflon vessels

Polypropylene volumetric flask, 50 mL

Screw-capped polypropylene bottles, 60 mL

### 28.4.3 Procedure

1. Weigh a 250 mg of air-dried and finely ground soil (100 mesh) of known moisture content in a 60 mL Teflon digestion tube with a cap.

2. Add 4 mL of trace metal-grade concentrated HNO$_3$ and 1 mL of H$_2$O$_2$.

3. Place the vessel in the microwave and digest the soil for 30 min at 296 W.

4. Seal the vessel with the cap and digest for 15 min at 296 W.

5. Cool for approximately 35 min well below the boiling point of the acid at atmospheric pressure, and then open the reaction chamber.

6. Add 4 mL concentrated HNO$_3$ + 1 mL concentrated HF. Seal the vessel with the cap and digest as follows: 4 min at 250 W, 8 min at 565 W, 4 min at 450 W, 4 min at 350 W, 5 min at 250 W, and vent for 35 min. Other operating power and temperature parameters can be set as specified by the instrument manufacturer.

7. After cooling, loosen the vessel cap in order to expel the interior gas into a fume hood. Remove the cap and allow the vessel to stand for ca. 2 min to remove any further gas.

8. Transfer the tube contents into a 50 mL polypropylene volumetric flask. Wash the inside of the tube and cap, and adjust the volume to 50 mL with distilled/deionized water. A colorless digestion solution is an indication of efficient destruction of the organic matter.

9. Store the sample solutions in 60 mL screw-capped polypropylene bottles before analysis for metals and other elements of interest.

10. Perform a blank containing all reagents used in the sample digestion.

### 28.4.4 Comments

This procedure does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this procedure to establish appropriate safety and health practices and determine the applicability of regulatory limitations before use. The analyst should read carefully all warnings.
and follow all hints and instructions provided with the instruction manual issued by the instrument manufacturer to ensure correct and safe operation of the instrument.

2 Peat samples containing mixtures of fine-grained matter and plant or fibrous material should be dried in an oven at 50°C and then mixed thoroughly in a low-speed blender to preserve all parts of the sample. To achieve the proper homogeneity with the use of small amounts of samples, dried soil or peat samples have to be ground to pass through 100 mesh sieve. The portion left on the sieve should be ground again for a short period, sieved, and so on until the complete sample could pass through the sieve.

3 All vessels have to be checked for metals and other elements contamination before use. Avoid using commercial detergents containing phosphate or other elements. The reagents and filter paper selected should be as free of metals and P as possible. It is essential to use reagents and distilled water of suitably low metal content, taking into consideration that the concentrated mineral acids are generally used in amounts several times that of the sample.

4 Low sample amounts may result in a good decomposition result, but may impair the analytical accuracy. Excessive sample amounts may lead to a poor decomposition result.

5 Add the nitric acid slowly, with swirling, to the sample. More HNO₃ may be needed to achieve the complete oxidation of organic matter. Nitric acid may react violently with some samples containing high organic material. Hydrogen peroxide has a high oxidization potential and can produce very strong reactions.

6 Addition of acids and sample digestion must be conducted in a fume hood with adequate ventilation.

7 Hydrofluoric acid is normally used in the acid mixture to dissolve silicates, which are present in the samples and more HF will be required for the decomposition of peat or organic soils high in silicate minerals. However, HF can give rise to problems in glassware and torch damage of some spectrometers, in particular inductively coupled plasma–mass spectrometry (ICP–MS) (Melaku et al. 2005). This problem can be avoided by using an HF-resistant nebulizing system and plasma torch (Swami et al. 2001). Special safety instructions must be observed when handling HF. Avoid the use of Pyrex glass materials or quartz vessels.

8 In the HNO₃/HF treatment, some elements such as Ca, Mg, Al, and rare earth elements may form insoluble fluorides that easily precipitate (Krachler et al. 2002; Wang et al. 2004); H₃BO₃ solution is often added to digestion mixtures to dissolve slightly soluble fluorides. In such cases, proceed as follows: add 10 mL of H₃BO₃ solution (5%, m/v) per 1 mL of HF (Swami et al. 2001) to the decomposed sample (step 7), seal the vessel again and subject it to a second decomposition run at high temperature or power rating for 10–15 min. After cooling, loosen the vessel cap in order to expel the interior gas into a fume hood. Remove the cap and allow the vessel to stand for ca. 2 min to remove any further gas. In cases of incomplete dissolution, continue to microwave until the sample is dissolved. Transfer the sample to a polypropylene volumetric flask and dilute with distilled/deionized
water to a fixed volume of 50 mL. This dissolution method is not suited for the determination of B in the soil digest.

Selection of the most suitable digestion method must be based on local requirements and facilities. Digestion mixture options include: HNO₃ + HClO₄ + HF (Papp and Harms 1985), HNO₃ + H₂O₂ + HF and HNO₃ + H₂O₂ + HClO₄ + HF (Weiss et al. 1999), HNO₃ + HBF₄ (Krachler et al. 2002), HNO₃ + HCl and HNO₃ + HCl + HF (Burt et al. 2003), HNO₃ + HClO₄; hot plate, microwave or block digestion, open or closed vessel. An HNO₃ + HClO₄ treatment of peat or soil samples is not always complete and a residue (siliceous materials) might remain. Filtration using Whatman No. 42 filter paper is desirable to keep the solution free of solid particles that cause clogging of the capillary tip of spectrometers. Acid digestion procedure using HClO₄ requires a HClO₄ fume hood. Perchloric acid is a very strong oxidizing agent that bears many risks and should not be used alone, but only in combination with other acids. As a safety precaution, it is recommended that organic samples be digested in HNO₃ before proceeding with HNO₃/HClO₄ digestion. Only use HClO₄ in microwave oven for processes that have been approved by the manufacturer. Perchloric acid can react with explosive force if the digestion mix approaches dryness. In general, perchlorates are easily soluble and the use of HClO₄ can considerably reduce the amount of HNO₃ required and complete the oxidation in a shorter time.

The analyst may use a suitable dilution factor depending on the detection limit of the instrument and the concentration of the element.

Metals in the digestion solution may be determined by atomic absorption spectroscopy (AAS) and La is added to the extracting solution (Ca and Mg determinations) as a suppressant. Sodium and potassium are commonly determined on a flame emission spectrophotometer. Low content at ppb level of some elements may be determined by using graphite furnace atomic absorption spectroscopy (GFAAS). The majority of elements may be determined by inductively coupled plasma–atomic emission spectrometry and –mass spectrometry (ICP–AES and ICP–MS). Selenium can be determined by using a hydride-generating system attached to an ICP emission spectrometer. If B is one of the elements of interest, it should be determined in H₃BO₃-free digestion solution. Phosphorus, sulfur, and boron may be determined by spectrophotometric methods.

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IV. SOIL BIOLOGICAL ANALYSES

Section Editors: E. Topp and C.A. Fox
Chapter 29
Cultural Methods for Soil and Root-Associated Microorganisms

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29.1 INTRODUCTION

Soil is an ecosystem that contains a variety of microbial populations whose members represent many physiological types. For example, some microorganisms, such as fungi, are aerobic chemoorganotrophs (heterotrophs) and use organic compounds as a source of carbon and energy. Others, such as nitrifying bacteria, are aerobic chemolithotrophs (autotrophs) using CO₂ as a carbon source and oxidizing reduced inorganic N compounds to obtain energy. Some microorganisms require special growth factors, a specific environmental pH, low O₂ levels, or the absence of O₂ (i.e., anaerobes) for optimum growth. The chemical, physical, and biological characteristics of a particular soil, as well as the presence of growing plants, will influence the numbers and activities of its various microbial components. Furthermore, because of the heterogeneous nature of soil, many different physiological types of organisms will be found in close proximity to one another. The microbial community in soil is important because of its relationship to soil fertility and the biogeochemical cycling of elements, and the potential use of specific members for industrial applications. Thus, there is a need to enumerate and isolate major and minor members of the microbial community in soils.

The nonselective enumeration and isolation of soil microorganisms is relatively straightforward, but the final result is not necessarily meaningful (Parkinson et al. 1971; Wollum 1994). On the other hand, selective enumeration and isolation of specific physiological types of microorganisms can provide useful and meaningful data (e.g., Lawrence and Germida 1988; Germida 1993; Koedam et al. 1994). Methods to enumerate and isolate soil microorganisms are constantly changing as our knowledge of the types of microorganisms present in soil expands (Vincent 1970; Woomer 1994; Pepper and Gerba 2005). This chapter provides basic principles and references on enumeration procedures and culture media for representative types of soil microorganisms.


29.2 PRINCIPLES

Enumeration of viable soil microorganisms may be accomplished by the plate count technique or most probable number (MPN) technique. The underlying principles are (i) dispersing of a sample in a suitable diluent, (ii) distributing an aliquot to an appropriate growth medium, (iii) incubating inoculated plates under suitable conditions, and (iv) counting the developed colonies or MPN tubes. These procedures are fairly standard and may be used to enumerate populations in bulk (Germida 1993) and rhizosphere (Goodfellow et al. 1968; Kucey 1983) soils along with root-associated and endophytic populations (Germida et al. 1998).

The composition of the growth medium used to enumerate microbial populations is important as it will affect the final result. Growth media may be selective or nonselective, although no medium is truly nonselective (James 1958). Selective media contain components, which allow or favor the growth of a desired group of organisms. Nonselective media should encourage growth of as many diverse groups of organisms as possible. To enumerate a specific physiological type of microorganism it is usually possible to design a growth medium which, when incubated under appropriate conditions of atmosphere and temperature, reduces interference from undesired populations. For example, chitin-degrading actinomycetes may be enumerated on medium containing chitin as the sole source of carbon and nitrogen (Lingappa and Lockwood 1962; Hsu and Lockwood 1975), and inclusion of specific antibiotics prevents growth of undesired organisms (Williams and Davies 1965). Similar selective media are available for many different soil microorganisms, e.g., phosphate-solubilizing bacteria (Kucey 1983), siderophore-producing microorganisms (Koedam et al. 1994), free-living nitrogen-fixing bacteria (Rennie 1981; Knowles and Barraquio 1994), nitrifying bacteria (Schmidt and Belser 1994), or sulfur-oxidizing organisms (Postgate 1966; Germida 1985; Lawrence and Germida 1988).

Although the plate count and MPN techniques are simple to perform, their usefulness will be limited by a number of key factors (Germida 1993; Wollum 1994; Woomer 1994). Many times choice of media, problems with dispersion, and even adsorption of microbes to pipette walls can interfere with standardization of these procedures. It should be pointed out that consistency and adequate use of replicate samples will help to minimize some of these problems. Investigators must realize that microorganisms are not uniformly dispersed within the soil environment, and that numbers of any particular microorganism are not synonymous with its importance.

29.3 SPREAD PLATE-COUNTING METHOD

Enumeration of microbial populations by the spread plate method is a simple and rapid method to count viable microbial cells in soil. However, counts obtained are generally 10- to 100-fold less than those determined by microscopic counts of soil smears (Skinner et al. 1952). Reasons for this discrepancy include measurement of viable and nonviable counts in soil smears, and the inability to provide adequate or appropriate nutrients in the growth media for spread plate counts (Germida 1993). Basically, this method consists of preparing a serial dilution (e.g., 1:10 dilutions) of a soil sample in an appropriate diluent, spreading an aliquot of a dilution on the surface of an agar medium, and incubating the agar plate under appropriate environmental conditions. These spread plates may be used not only for counting microbial populations but also as a starting source for isolation of organisms. In this latter case, an isolated colony is picked and repeatedly streaked on a suitable growth medium to check for purity. After several such transfers it may be cultured and preserved for future
study and identification. Selective or nonselective media may be used, depending on the nature of the desired microorganisms. Soil extract agar (James 1958) is commonly used as a nonselective medium for enumerating soil bacteria. Recently proposed alternatives to soil extract agar include a defined “soil solution equivalent medium” (Angle et al. 1991), “trypticase soy agar and/or R2A medium”—two commercially available complex media (Martin 1975; Reasoner and Geldreich 1985).

### 29.3.1 MATERIALS

1. Petri plates containing ca. 20 mL of an appropriate agar medium, e.g., soil extract agar
2. Dilution bottles (e.g., 50 × 160 mm; 200 mL capacity) and (or) test tubes (e.g., 18 × 150 mm) containing appropriate diluent such as sterile tap water
3. Sterile 1 and 10 mL pipettes
4. Glass spreader (i.e., glass rod shaped like a hockey stick)
5. Glass beaker containing 95% ethanol (ETOH)

### 29.3.2 PREPARATION OF AGAR PETRI PLATES

1. Agar media may be prepared from commercially available dehydrated components or from recipes found in the literature. The American Type Culture Collection (2005) and Atlas (1995) are an excellent source of media recipes and relevant references. Prepare media according to directions, sterilize in the autoclave at 1.05 kg cm\(^{-2}\) and 121°C for 20 min, cool to a pouring temperature of ca. 48°C. Some components of a medium may be heat labile and must be filter-sterilized and then added to the autoclaved agar medium (cooled to ca. 48°C–49°C) just before pouring plates.

2. Distribute ca. 20 mL of media into sterile glass or disposable, presterilized plastic Petri plates and allow the agar to solidify. The plates should be allowed to sit at room temperature for 24–48 h, allowing excess surface moisture to be absorbed into the agar; this helps prevent microbial colonies from spreading over the agar surface. Poured plates not used immediately may be stored under refrigeration (2°C–5°C) for up to 2 weeks in plastic bags. These stored plates should be removed and allowed to warm to room temperature before use.

### 29.3.3 PREPARATION OF SOIL DILUTIONS

1. Samples should be collected, handled, and stored with due consideration to their ultimate use, and the effects these steps will have on microbial populations.

2. Pass representative soil samples through a 2 mm mesh sieve and mix thoroughly.

3. Weigh out a 10.0 g portion of the soil into a dilution bottle containing 95 mL of a diluent. Glass beads (ca. 25 × 2 mm beads) may be added to this dilution blank to facilitate mixing. Cap the bottle, place on a mechanical shaker, and shake for
10 min. Alternatively, shake by hand moving the bottle through specified arc a number of times (e.g., a 45° arc at least 50–100 times). This first dilution represents a 1:10 or a $10^{-1}$ dilution.

After removing the bottle from the shaker, shake vigorously before removing aliquots. To prepare a serial 1:10 dilution series of the soil sample, transfer a 10 mL sample to a 90 mL dilution blank cap and shake the dilution bottle (alternatively it is possible to transfer 1 mL samples to 9 mL dilution blanks [prepared in 18 × 150 mm test tubes]). Continue this sequence until a dilution of $10^{-7}$ is reached. Subsequent spread plating of a 0.1 mL aliquot of this dilution will allow enumeration of up to $3 \times 10^{10}$ colony-forming units (cfu) per g soil. Experience will indicate the appropriate range of dilutions for samples being analyzed.

29.3.4 Preparation of Dilutions from Root-Associated Bacteria

For bacteria isolation from the root interior (endophytic), root material is collected from soil and maintained at ca. 5°C until processed in the laboratory (Foster and Rovira 1976). Approximately 10.0 g of fresh root material is shaken to eliminate adhering soil and then washed in sterile tap water. Subsequently, roots are placed in a 250 mL Erlenmeyer containing 100 mL of 1.05% sodium hypochlorite (NaClO) and shaken (200 rpm) for 10 min. Roots are washed (4×) in sterile tap water and aseptically blended in a sterile Waring blender containing 90 mL sterile phosphate buffered saline (PBS)—see recipe below. This will result in 1/10 dilution, i.e., 10.0 g roots and 90 mL sterile water. The root suspension is serially diluted in PBS and aliquots of appropriate dilutions spread plated onto selective and/or nonselective nutrient media depending on the nature of the study.

29.3.5 Preparation of Agar Spread Plates

1 Select a range of four dilutions that will adequately characterize the microorganisms in the sample. Transfer 0.1 mL aliquots to a separate plate from the highest dilution. Note that a 0.1 mL aliquot from a $10^{-7}$ dilution corresponds to an actual dilution of $10^{-8}$ on the plate. Repeat the process, transferring 0.1 mL aliquots from each of the next three successive and lower dilutions onto each of triplicate plates for each dilution.

2 Spread the suspension on the agar surface using a sterile glass spreader for each plate. The glass spreader is kept submerged in a beaker of ETOH and excess ETOH burned off before use. In the spreading step start with the highest dilution and progress to the next lower dilution, continuing the sequence until all the plates have been spread. Alcohol flame the glass spreader between each plate. Invert the plates and place in an incubator at an appropriate temperature.

3 Incubation conditions will depend on the facilities and the purpose of the study. When possible, one should try to mimic environmental conditions. Generally, spread plates samples are incubated in the dark, in an aerobic environment at a temperature between 24°C and 28°C. Incubation periods and conditions will vary depending on the nature of the organisms being enumerated.

4 After a suitable incubation interval, plates are removed from the incubator and those containing 30 to 300 colonies are counted. Plates with spreading or
swarming organisms should be excluded from the final count. The colonies can be counted manually or by an automated laser colony counter.

29.3.6 Calculations

Average the number of colonies per plate for the dilution giving between 30 and 300 colonies. Determine the number of cfu g\(^{-1}\) of dry soil (DW) as follows:

\[
\text{Number of cfu g}^{-1} \text{ soil}_{\text{DW}} = \frac{\text{mean plate count} \times \text{dilution factor}}{\text{dry weight soil, initial dilution}}
\]  

(29.1)

where

\[
\text{Dry weight soil} = \left( \frac{\text{weight moist soil, initial dilution}}{100} \right) \times \left( 1 - \frac{\% \text{ moisture, soil sample}}{100} \right)
\]  

(29.2)

29.3.7 Comments

Because bacteria may exist in soil as groups or clumps of cells, it is often desirable to disperse these cells so that colonies on spread plates arise from one cell. This may be accomplished by shaking on a mechanical shaker or by hand, through application of a high shearing force as with Waring blender, by sonic vibration (Stevenson 1959), mechanical vibration (Thornton 1922), and through the use of deflocculating agents.

29.3.8 Type of Dilutions

A number of diluents may be used. In most cases, tap or distilled water is adequate. Other diluents routinely used include:

1. Physiological saline: NaCl, 8.5 g; distilled water, 1 L.

2. PBS: NaCl, 8.0 g; KH\(_2\)PO\(_4\), 0.34 g; K\(_2\)HPO\(_4\), 1.21 g; distilled water, 1 L. Adjust pH to 7.3 with 0.1 M NaOH or HCl.

3. Peptone water: Peptone, 1.0 g; distilled water, 1 L.

29.3.9 Types of Media

The choice of medium depends on the type of organism desired. Media may be made selective by omitting or altering a component, or by incubation conditions. For enumeration of total heterotrophic populations in soil, a general nonselective medium is usually employed. The following are examples of growth media commonly used to enumerate total soil bacteria, root-associated bacteria, actinomycetes, and fungi. Additional examples of specific media are described in Section 29.5.

Media for Total Heterotrophic Bacteria

1. Soil extract agar (James 1958): One kg of soil is autoclaved with 1 L of water for 20 min at 1.05 kg cm\(^{-2}\). The liquid is strained and restored to 1 L in volume. If it is
cloudy, a little CaSO$_4$ is added and after being allowed to stand, it is filtered through Whatman paper No. 5. The extract may be sterilized and solidified with agar (1.5%) as it is, or after the addition of other nutrients, e.g., 0.025% K$_2$HPO$_4$ or 0.1% glucose, 0.5% yeast extract, and 0.02% K$_2$HPO$_4$.

2 Tryptic soy agar (Martin 1975): Add 3.0 g of tryptic soy broth and 15.0 g of agar to 1 L distilled water. Sterilize the medium by autoclaving.

3 R2A (Reasoner and Geldreich 1985): Yeast extract, 0.5 g; Proteose Peptone No. 3, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; K$_2$HPO$_4$, 0.3 g; MgSO$_4$·7H$_2$O, 0.05 g; sodium pyruvate, 0.3 g; agar, 15.0 g; distilled water, 1 L. Adjust the pH to 7.2 with crystalline K$_2$HPO$_4$ or KH$_2$PO$_4$ and sterilize the medium by autoclaving.

### Media for Actinomycetes

Starch–casein agar (Küster and Williams 1966): Starch, 10.0 g; casein (vitamin free), 0.3 g; KNO$_3$, 2.0 g; NaCl, 2.0 g; K$_2$HPO$_4$, 2.0 g; MgSO$_4$·7H$_2$O, 0.05 g; CaCO$_3$, 0.02 g; FeSO$_4$·7H$_2$O, 0.01 g; agar, 15.0 g; distilled water, 1 L; pH, 7.2. Sterilize in autoclave as described above. Media can be improved by addition of fungistatic agents (Williams and Davies 1965).

### Media for Fungi

1 Czapek-Dox agar: Solution I: Sucrose, 30.0 g; NaNO$_3$, 2.0 g; K$_2$HPO$_4$, 0.1 g; KCl, 0.5 g; MgSO$_4$·7H$_2$O, 0.5 g; FeSO$_4$, trace; distilled water, 500 mL; pH, 3.5 using 10% lactic acid. Solution II: Agar, 15.0 g; distilled water, 500 mL. If desired, 0.5 g of yeast extract may be added (Gray and Parkinson 1968). Sterilize solutions I and II by autoclaving separately. Cool both solutions to pouring temperature, pour solution I aseptically into solution II and use immediately.

2 Streptomycin–rose bengal agar (Martin 1950): Glucose, 10.0 g; peptone, 5.0 g; KH$_2$PO$_4$, 1.0 g; MgSO$_4$·7H$_2$O, 0.5 g; rose bengal, 0.03 g; agar, 20.0 g; tap water, 1 L. Autoclave, cool medium to about 48°C and add 1 mL of a solution of streptomycin (0.3 g 10 mL$^{-1}$ sterile water). The final concentration of streptomycin in medium should be about 30 μg mL$^{-1}$.

### 29.4 MOST PROBABLE NUMBER METHOD

This method employs the concept of dilution to extinction in order to estimate the number of microorganisms in a given sample (Taylor 1962; Woomer 1994). It is based on the presence or absence of microorganisms in replicate samples in each of several consecutive dilutions of soil. For example, if a series of test tubes containing broth medium are inoculated with aliquots representing a dilution series from $10^{-4}$ through $10^{-7}$, and the highest dilution exhibiting growth is $10^{-5}$, then the number of cells present may be estimated to be between $10^4$ and $10^5$. The key is that the desired organism must possess a unique characteristic or metabolic trait, which can be detected. Thus, this technique can be used to count microorganisms based on growth (i.e., turbidity), metabolic activity such as substrate disappearance and product formation. Other uses for the MPN technique include enumeration of infective vesicular–arbuscular mycorrhizae (VAM) propagules in soil (see Chapter 30) or nodule-forming rhizobia in soil (see Chapter 31).
To estimate total heterotrophic counts of a soil sample the MPN procedure is similar to the spread plate count method, except that aliquots of dilutions are inoculated into test tubes of liquid medium. Alternatively, multiwell microtiter plates (e.g., 24 wells per plate) may be used in place of test tubes; this allows a savings of materials, reagents, incubator space, and allows for increased replication.

29.4.1 MATERIALS

1. Twenty five test tubes containing appropriate culture medium or 1 disposable sterile microtiter plates—24 or 96 wells per plate

2. Water dilution blanks (see Section 29.3.1)

3. Sterile 1 and 10 mL pipettes (see Section 29.3.1)

29.4.2 PROCEDURES

1. Prepare medium appropriate for the desired organism.

2. Dispense aliquots of medium in test tubes and sterilize or dispense aliquots of sterile medium into presterile microtiter plates.

3. Prepare a serial 1:10 dilution sequence of the soil sample (see Section 29.3.2).

4. Select a range of dilutions that will adequately characterize the organisms in the sample. Transfer 0.1 mL aliquots to each separate well in five replicate microtiter plate wells, starting with the highest dilution. Repeat the procedure, transferring 0.1 mL aliquots from each of the next successive and lower dilutions into each of the five replicate wells for each dilution.

5. Incubate MPN assay tubes and/or plates under appropriate conditions.

6. After suitable incubation, score wells positive for growth or physiological reaction.

29.4.3 CALCULATIONS

The MPN of organisms in the original sample is calculated by reference to an MPN table (e.g., Cochran 1950). Designate as \( p_1 \) the number of positive tubes in the least concentrated dilution in which all tubes are positive or in which the greatest number of tubes is positive. Let \( p_2 \) and \( p_3 \) represent the numbers of positive tubes in the next two higher dilutions. Refer to Table 29.1 and find the row of numbers in which \( p_1 \) and \( p_2 \) correspond to the values observed experimentally. Follow that row of numbers across the table to the column headed by the observed value of \( p_3 \). The figure at the point of intersection is the MPN of organisms in the quantity of the original sample represented in the inoculum added in the second dilution. This figure is multiplied by the appropriate dilution factor to obtain the MPN value for the original sample.

As an example, consider the instance in which a 10-fold dilution with 5 tubes at each dilution yielded the following numbers of positive tubes after incubation: 5 at \( 10^{-4} \), 5 at \( 10^{-5} \), 4 at \( 10^{-6} \), 2 at \( 10^{-7} \), and 0 at \( 10^{-8} \). In this series, \( p_1 = 5 \), \( p_2 = 4 \), and \( p_3 = 2 \). For this
A combination of \( p_1, p_2, \) and \( p_3 \), Table 29.1 gives 2.2 as the MPN of organisms in the quantity of inoculum applied in the \( 10^{-6} \) dilution. Multiplying this MPN by the dilution factor \( 10^6 \) gives 2.2 million as the MPN value for the original sample.

As a second example, consider the same situation as above except that the most concentrated dilution is \( 10^{-6} \). Under these circumstances, \( p_1 = 4, p_2 = 2, \) and \( p_3 = 0 \). For this combination of \( p_1, p_2, \) and \( p_3 \), Table 29.1 gives 0.22 as the MPN of organisms in the quantity of inoculum applied in the \( 10^{-7} \) dilution. Multiplying 0.22 by \( 10^7 \) yields 2.2 million organisms as the MPN value for the original sample, as before.

**TABLE 29.1 Table of Most Probable Numbers for Use with 10-Fold Dilutions and 5 Tubes per Dilution**

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The 95% confidence limits for MPN values can be calculated from prepared tables. A compilation of factors keyed to rate of dilution and to number of tubes per dilution is shown in Table 29.2. To find the upper confidence limit at the 95% level, multiply the MPN value by the appropriate factor from the table. To find the lower limit, divide the MPN value by the factor. In the first example above, the factor is 3.30, and the confidence limits are

\[(2.2)(3.30) = 7.26\]

and

\[(2.2)/(3.30) = 0.66.\]

**29.4.4 COMMENTS**

The MPN method is usually employed to enumerate and isolate organisms that will not readily grow on solid agar medium, or those that cannot be readily identified from the background community. For example, autotrophic-nitrifying bacteria (Schmidt and Belser 1994) and sulfur-oxidizing bacteria (Postgate 1966; Germida 1985; Lawrence and Germida 1988) are routinely enumerated using the MPN assay. The medium employed may be used to measure total growth, and hence optical density is satisfactory measurement. Alternatively, a physiological reaction may be monitored. For example, oxidation of sulfur to an acidic end product will alter pH and the difference may be recorded by using an appropriate pH indicator. The procedure may be used to provide a relative estimate of the numbers of many diverse physiological groups of organisms in soils. Choice of media and incubation conditions is limited only by our knowledge of specific physiological groups.

**29.5 MEDIA FOR THE ENUMERATION AND ISOLATION OF SOIL MICROORGANISMS (GRAY AND PARKINSON 1968)**

**29.5.1 MEDIA FOR ISOLATION HETEROTROPHIC BACTERIA**

1 Peptone yeast extract agar (Goodfellow et al. 1968): Peptone, 5.0 g; yeast extract, 1.0 g; FePO$_4$, 0.01 g; agar, 15.0 g; distilled water, 1 L; pH, 7.2.
Nutrient agar: Yeast extract, 1.0 g; beef extract, 3.0 g; peptone, 5.0 g; sodium chloride, 5.0 g; agar, 15.0 g; distilled water, 1 L; pH, 7.3.

Fluorescent pseudomonads (Sands and Rovira 1970; Simon et al. 1973): Proteose Peptone, 20.0 g; agar, 12.0 g; glycerol, 10.0 g; K2SO4, 1.5 g; MgSO4 · 7H2O, 1.5 g; distilled water, 940 mL. Adjust pH to 7.2 with 0.1 M NaOH before autoclaving. Sterilize by autoclaving. Add 150,000 units of penicillin G, 45 mg of novobiocin, 75 mg of cycloheximide, and 5 mg of chloramphenicol to 3 mL of 95% ethanol. Dilute to 60 mL with sterile distilled water, and add (filter-sterilized using a sterile 0.45 μm Millipore filter) to the cooled (48°C) medium before pouring. Prepared plates should be dried overnight before using and may be stored in the refrigerator for several weeks before use.

29.5.2 Media for Isolation of Specific Physiological Groups of Organisms

Microorganisms Involved in Carbon Transformations

1. Cellulose agar (Eggins and Pugh 1961): NaNO3, 0.5 g; K2HPO4, 1.0 g; MgSO4 · 7H2O, 0.5 g; FeSO4 · 7H2O, 0.01 g; cellulose (ball-milled), 12.0 g; agar, 15.0 g; distilled water, 1 L.

2. Chitin agar: Ball-milled, purified chitin, 10.0 g; MgSO4 · 7H2O, 1.0 g; K2HPO4, 1.0 g; agar, 15.0 g; distilled water, 1 L.

3. Starch agar: 0.2% soluble starch may be added to any suitable growth medium as an alternative or additional carbohydrate. Starch hydrolysis is shown by flooding incubated plates with an iodine solution and then noting clear zones.

Microorganisms Involved in Nitrogen Transformations

1. Combined carbon medium—Free-living putative nitrogen-fixing bacteria (Rennie 1981): Solution I: K2HPO4, 0.8 g; KH2PO4, 0.2 g; Na2FeEDTA, 28.0 mg; Na2MoO4 · 2H2O, 25.0 mg; NaCl, 0.1 g; yeast extract, 0.1 g; mannitol, 5.0 g; sucrose, 5.0 g; Na-lactate (60% v/v), 0.5 mL; distilled water, 900 mL; agar, 15.0 g. Solution II: MgSO4 · 7H2O, 0.2 g; CaCl2, 0.06 g; distilled water, 100 mL. Solution III: Biotin, 5.0 μg mL⁻¹; p-aminobenzoic acid (PABA), 10.0 μg mL⁻¹. Autoclave solutions I and II, cool to 48°C and mix thoroughly, then add (filter-sterilized using a sterile 0.45 μm filter) 1 mL L⁻¹ of solution III.

2. Azotobacter enrichment broth: MgSO4 · 7H2O, 0.2 g; K2HPO4, 1.0 g; FeSO4 · 7H2O, 0.02 g; CaCl2, 0.02 g; MnCl2 · 7H2O, 0.002 g; NaMoO4 · 2H2O, 0.001 g; distilled water, 1 L; pH, 7.0; ethyl alcohol (95%), 4.0 mL (add to autoclaved and cooled media).

3. Azotobacter chroococcum and A. agilis (Ashby’s medium): These two Azotobacter species utilize mannitol as their only carbon source. MgSO4 · 7H2O, 0.2 g; K2HPO4, 0.2 g; NaCl, 0.2 g; CaSO4 · 7H2O, 0.1 g; CaCO3, 3.0 g; Na2MoO4 · 2H2O, 25.0 mg; mannitol, 10.0 g; agar, 15.0 g; distilled water, 1 L. For isolation of A. indicus, substitute mannitol by glucose (5.0 g L⁻¹).
Yeast extract mannitol medium (Allen 1957): Mannitol, 10.0 g; K$_2$HPO$_4$, 0.5 g; NaCl, 0.1 g; MgSO$_4$·7H$_2$O, 0.2 g; CaCO$_3$, 3.0 g; yeast extract, 0.4 g; agar, 15.0 g; distilled water, 1 L.

Nitrifying bacteria (Lewis and Pramer 1958): Na$_2$HPO$_4$, 13.5 g; KH$_2$PO$_4$, 0.7 g; MgSO$_4$·7H$_2$O, 0.1 g; NaHCO$_3$, 0.5 g; (NH$_4$)$_2$SO$_4$, 2.5 g; FeCl$_3$·6H$_2$O, 14.4 mg; CaCl$_2$·7H$_2$O, 18.4 mg; distilled water, 1 L; pH, 8.0.

Microorganisms Involved in Sulfur Transformations

1. Thiobacillus thiooxidans or T. thioparus (Postgate 1966): (NH$_4$)$_2$SO$_4$, 0.4 g; KH$_2$PO$_4$, 4.0 g; MgSO$_4$·7H$_2$O, 0.5 g; CaCl$_2$, 0.25 g; FeSO$_4$, 0.01 g; powdered sulfur, 10.0 g or Na$_2$S$_2$O$_7$, 5.0 g; distilled water, 1 L; pH, 7.0. This medium can be made selective for T. thiooxidans-like bacteria by using S$^8$ as the sulfur source and adjusting the initial pH to <3.5.

2. T. denitrificans (Postgate 1966): KNO$_3$, 1.0 g; Na$_2$HPO$_4$, 0.1 g; Na$_2$S$_2$O$_7$, 2.0 g; NaHCO$_3$, 0.1 g; MgCl$_2$, 0.1 g; distilled water, 1 L; pH, 7.0. This medium may be used for agar plates, or dispensed into test tubes containing small Durham fermentation tubes to capture gas. Incubate under anaerobic conditions.

REFERENCES


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Chapter 30
Arbuscular Mycorrhizae

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30.1 INTRODUCTION

Most plant species live in a symbiotic association with mycorrhizal fungi whose establishment in roots increases the water supply and mineral nutrition. These soil-borne fungi colonize the root cortex and develop external filaments, making connecting bridges between roots and soil. They are recognized as improving plant fitness and soil quality. Widely distributed under all ecosystems, they are the most common type of symbionts involved in agricultural systems, influencing both plant production and plant protection.

The mycorrhizal fungi can be subdivided into three major categories: (1) the arbuscular mycorrhizal (AM) fungi, obligate symbionts, which belong to the Glomeromycota associated with the majority of herbaceous and cultivated plants and with some deciduous trees, (2) the ectomycorrhizal (EM) fungi taxonomically associated with Basidiomycetes and Ascomycetes found in symbiosis mostly with trees, and (3) the ericoid mycorrhizal (ERM) fungi, a symbiosis between mainly Ascomycetes and plants from the Ericaceae family (e.g., blueberry, rhododendron, and heather-type plants).

30.2 SAMPLING STRATEGY

Several procedures used in mycorrhizal research are time consuming. Thus, sampling plans will often have to be a compromise between a desire for precision and limited resources. More intensive or targeted sampling protocols are required in heterogeneous sites to allow for the accurate measurement of variables. Therefore, knowledge of the conditions causing variation in the distribution of AM structures in soil may help develop efficient sampling plans.

Sampling strategies must be planned carefully according to the experimental goal, the conditions of the study area, and knowledge on how these conditions may influence AM fungi distribution in soil. The AM fungi occurrence in soil is largely driven by the plant
distribution. Some plant species do not host AM fungi. These non-host species are found particularly in the families Polygonaceae, Juncaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Amaranthaceae, Cistaceae, Pinaceae, Fagaceae, Ericaceae, and Ranunculaceae. Furthermore, different plant species may selectively augment different AM species in their surroundings, and in some plant communities, AM fungi species distribution may follow plant species distribution (Gollotte et al. 2004).

AM spore abundance generally decreases with soil depth and spores are generally absent below the root zone. Approximately 75% of the AM spores and hyphae found in tilled and no-tilled fields in eastern Canada are found in the top 15 cm of the soil, but these structures are still present at 20–25 cm depth (Kabir et al. 1998b). Very few propagules are found below 40–50 cm (Jakobsen and Nielsen 1983). AM populations at depth are not only thinner, but their composition may also differ from that of the top soil populations. These populations may be more stable than those in the top soil layer, which is influenced by crop management (Oehl et al. 2005). AM fungi tend to follow plant root distribution and, in row crops, they are more abundant under the row than in between rows (Kabir et al. 1998a). AM root colonization development is a three-phase process including a lag phase, an exponential phase, and a plateau (McGonigle 2001). The length of the lag phase depends on the mycorrhizal potential of the soil and the mycorrhizal dependency of the host plant and may be increased by lower soil temperature. For example, root colonization peaks at flowering in maize and decreases thereafter up to host senescence (Kabir et al. 1998a).

Sampling strategy must be adapted to the requirements of the problems studied. The evaluation of AM biodiversity under mixed plant populations would require that samples be taken in the vicinity of all plant species. The evaluation of the abundance of AM fungi in a given area would require a more random sampling plan. For example, a stratified random sampling plan would be the most efficient strategy to generate a soil sample representative of an experimental monocultured plot at a given point in time. This procedure is given below.

30.2.1 MATERIALS AND REAGENTS

1. Labeled plastic bags
2. Graduated bulb planter or soil probe
3. Bucket

30.2.2 PROCEDURE

1. Take an equal number of soil cores at random in each of the following predetermined zones: (a) directly on the row and (b) in between rows. In crops with wide interrows, one may decide to sample (a) on the row, (b) between rows, and (c) midpoint between the row and midrow. The number of separate sampling sets will depend on plot size and homogeneity.

2. Place cores in a bucket as you go, up to completion of the plot sampling exercise.

3. Pour the content of the bucket into an appropriately labeled plastic bag. The soil cores can be conveniently pooled to produce a single representative sample for a plot, which can be sieved, mixed, and subsampled for different analyses.
Avoid leaving plastic bags with their contents under the sun as they may heat up quickly. The use of coolers is recommended.

30.2.3 Comments

Soils are commonly sampled to 15 cm depth, but the selection of a sampling depth may be based on specific considerations. For example, one may like to sample the plow layer, i.e., the top 20, 15, or 7.5 cm, depending on the tillage system used, or up to a known rooting depth. The number of cores required depends on the core size—the larger the core the smaller the number of samples—and site uniformity. Heterogeneous sites require more intensive sampling. In an apparently homogeneous field plot, it is advisable to take at least three core sets if a large core is used (e.g., a bulb planter). Using a soil probe, 10–12 cores can rapidly be taken from a plot and mixed in a bucket.

30.3 Determination of Root Colonization by Staining

Root clearing and staining reveal the intraradical phase of AM fungi and allow the evaluation of the extent of AM root colonization. Clearing is usually done by boiling root samples in a solution of KOH on a hot plate or in the autoclave, to remove alkali-soluble tannins. Tender roots like those of cucumber, corn, or onion may require only 5–10 min, whereas clearing some tree roots may require more than 1 h in a boiling 10% KOH solution. Roots can be cleared at room temperature as well. This method produces high quality root material, but requires several hours or days of soaking in the KOH solution. Further soaking in 30% H$_2$O$_2$ (Phillips and Hayman 1970) or in 3% NaOCl acidified with a few drops of 5 M HCl (Bevege 1968) solutions has also been used to remove some residual tannins in roots that are difficult to clear.

Staining solutions have evolved along a decreasing toxicity gradient from being lactophenol-based (Phillips and Hayman 1970) to lactoglycerol-based (Brundrett et al. 1994) and household vinegar-based (Vierheilig et al. 1998) or mild acids such as dilute hydrochloric acid (HCl) or even tonic water (Walker 2005). At last, ordinary permanent ink has been proposed as a replacement for possibly carcinogenic stains, such as chlorazol black E, trypan blue, and acid fuchsin (Vierheilig et al. 1998). The ink and vinegar staining technique is given here, as it is a safe and inexpensive method. Staining with trypan blue is given as an alternative. The performance of different stains varies with the quality of the AM root material under evaluation and with the plant species. Information on other commonly used stains can be found in an article by Brundrett et al. (1984) who compared the performance of chlorazol black E, trypan blue, acid fuchsin, and aniline blue.

30.3.1 Materials and Reagents

1. Aqueous solution of 10% (w/v) KOH.
2. Black Shaeffer ink solution: 5% black Shaeffer ink (Ft. Madison, Iowa) in household vinegar (5% acetic acid); or trypan blue solution: 0.5 g trypan blue in 500 mL glycerol, 450 mL H$_2$O$_2$, and 50 mL 1% HCl with a destaining solution: 500 mL glycerol, 450 mL H$_2$O$_2$, and 50 mL 1% HCl.
3. Water acidified with vinegar or 1% HCl.
Sample staining cassettes (Omnisette Embedding Cassettes, Fisher Scientific, Nepean, Ontario). In absence of cassettes, vials, beakers, or Erlenmeyer flasks can be used as recipients for roots.

Lead pencil.

1 L beaker.

Hot plate.

**30.3.2 Procedure**

1. Cut roots into 1 cm fragments and take a representative sample that is small enough to lay somewhat loosely in the sample staining cassettes in such a way as to permit the circulation of liquids around the roots being processed. A representative sample can be achieved by mixing the root pieces in a volume of water. Ensure that no soil adheres to the root pieces to be stained. For a representative evaluation of root colonization level, a minimum of 50 and an optimum of 100 mounted root segments are required.

2. Place the root samples in the sample staining cassettes labeled with a lead pencil (ink will vanish with KOH). In the absence of sample staining cassettes, the whole procedure can be performed using vials, beakers, or Erlenmeyer flasks. Root segments are introduced into the containers, soaked in the prescribed solutions, and recovered manually or by filtration to a 50 μm mesh nylon sheet.

3. Place sample staining cassettes in a 1 L beaker. The beaker should not be more than half full.

4. Cover with the KOH solution.

5. Place the beaker with its contents on a hot plate and boil for the time it takes to clear the roots. It usually takes 10–30 min, but it can also take hours, depending on the plant species and the quality of the root material.

6. Discard the KOH solution and rinse the cassettes several times with tap water.

7. It is then advisable to rinse in acidified water if Shaeffer ink is used for staining or in 1% HCl, in the case of trypan blue.

8. Boil gently in the ink–vinegar stain for 3 min or in the trypan blue solution for 15–60 min.

9. Discard the ink–vinegar solution and rinse the cassettes in tap water acidified with a few drops of vinegar. Trypan blue stained roots are placed in a destaining solution: 500 mL glycerol, 450 mL H₂O, and 50 mL 1% HCl over night. The trypan blue staining solution can be carefully filtered to remove root debris and reused for subsequent root staining. Store the stained root cassettes or the vials containing roots in acidified tap water or in destaining solutions at 4°C.
30.3.3 COMMENTS

Very small root fragments can be secured in between two pieces of nylon screen or filter paper before being placed in the sample staining cassette to prevent loss of material. In this case, care must be taken to ensure proper rinsing between the clearing and staining solutions. Toth et al. (1991) proposed a way to calculate AM fungal biomass from colonized root length and root radius.

30.4 DETERMINATION OF ROOT COLONIZATION USING GRID-LINE INTERSECT

Stained roots are most often scored for colonization using the grid-line intersect method (Giovannetti and Mosse 1980). McGonigle et al. (1990) modified this method into the “magnified intersection method” in which roots are examined at 200× magnification. This method involves the inspection of intersections between the microscope eyepiece crosshair and roots. Closer examination allows for accurate recognition and recording of arbuscular, hyphal, and vesicular colonization. Hyphal and vesicular colonization should be interpreted with caution as they can be produced by nonmycorrhizal fungi. This warning also holds for the grid-line intersect method. With the slide method (Giovannetti and Mosse 1980), 50 to 100 1 cm root sections are mounted on slides in polyvinyl lactoglycerol (PVLG) mounting media (Omar et al. 1979) (166 g polyvinylalcohol high viscosity—24–32 cP—dissolved in 10 mL H₂O is added to 10 mL lactic acid and 1 mL glycerol). The length of colonized root tissue is measured and compared to the total length of root observed. Results are expressed as a percent.

Biochemical methods have also been used to determine AM fungal colonization of roots. These sometimes present the advantage of discriminating between AM fungi and other root endophytes in addition to allowing the evaluation of lignified or large roots, which cannot be cleared. Chitin (Hepper 1977), 24-methyl/methylene sterols (Fontaine et al. 2004), and phospholipid fatty acid (PLFA) C16:1ω5 (van Aarle and Olsson 2003) were proposed as indicators of AM colonization. Biochemical methods of measurements of intraradical AM colonization have some limitations. Chitin is not specific to AM fungi; it is present in the cell wall of zygomycetous fungi. The relative abundance of sterols and fatty acid indicators is not consistent among AM fungal species. Thus, use of biochemical indicators of AM colonization is not recommended for the evaluation of field-grown plant roots as colonization, in this case, is likely the result of a mixed fungal population.

The grid-line intersect method is simple, relatively rapid, and appropriate for most routine determinations of the mycorrhizal colonization of roots. This method is described below.

30.4.1 MATERIALS AND REAGENTS

1. A plastic Petri dish on the underside of which lines were lightly etched with a sharp scalpel blade. Lines can be random or arranged in a grid pattern. Gridded dishes can also be directly purchased.

2. Wash bottle containing water acidified with a few drops of vinegar or HCl.

3. Tweezers and needle to disperse the roots.
Dissecting microscope.

Two-key desktop counter for keeping track of point counts.

Cleared and stained roots.

30.4.2 PROCEDURE

1. A stained root sample is placed in the etched Petri dish and dispersed using a jet of acidified water from the wash bottle, tweezers, and needle.

2. Scanning the grid-lines under the dissecting microscope, the total number of points where a root intersects a line is recorded using the key-counter. In parallel, the number of these intersects bearing AM colonization is also recorded. For example, if AM colonization is found in 42 out of a total of 100 intersects, AM root colonization would equal 42%.

30.4.3 COMMENTS

Root length can easily be assessed concurrently with root scoring, using the relationship of Newman (1966):

\[ R = An/2H \]  

(30.1)

where \( R \) is the root length, \( A \) the area of the Petri dish, \( n \) the total number of root × grid-line intersects, and \( H \) the sum of all etched lines’ lengths.

When root measurement is sought, care must be taken not to lose roots in the processes of staining, clearing, washing, and extracting roots from a known amount of soil. Colonized and total root length densities are best expressed as lengths of total or colonized roots per volume of soil.

30.5 DETERMINATION OF THE SOIL MYCORRHIZAL POTENTIAL

There are four methods for assessing the mycorrhizal potential of the soil:

1. The most probable number (MPN) method was applied to estimate AM fungal propagules in soil by Porter (1979). The method involves repeated serial dilutions with pasteurized volumes of the soil to be tested, and growth of a trap plant. Trap plant roots are examined for presence or absence of AM colonization, and values of MPN are derived from published statistical tables (Fisher and Yates 1963; Woomer 1994). The MPN method is laborious and yields only imprecise estimates of propagule numbers. Furthermore, the intense mixing of the soil under study dictated by the method disrupts AM hyphal networks that may also be involved in soil infectivity. Thus this method considers the MPN of infective spores and vesicles, underestimating soil infectivity.

2. An improved method for mycorrhizal soil infectivity (MSI) determination was proposed by Plenchette et al. (1989) as an alternative to the MPN method.
With the MSI, soil dilution series are made similarly to the MPN method, but population of 10 plants, rather than single plants, are grown. The relationship between the percentage of mycorrhizal plants and the minimum amount of nonsterile soil in a dilution allows for a calculation of the amount of soil required to induce mycorrhizal infection in 50% of the plants, which is one unit of MSI. This method is more precise than the MPN but involves growing a plant population rather than single plants as with the MPN, and thus requires the examination of hundreds and thousands of root systems.

Franson and Bethlenfalvay (1989) have proposed to count infection units formed on a trap plant root system directly extracted from the substrate as the expression of the mycorrhizal infectivity of a soil. This requires great skill and precision because infection points rapidly become sources of additional infections in addition to being hard to distinguish. It is unlikely that all AM fungal propagules in a soil sample will be synchronized in initiating root infection and, thus, this method will likely underestimate the number of propagules in a soil.

A simple infectivity assay can be more conveniently conducted. Trap plants are grown in the soil under examination for 2–4 weeks, a period of time long enough for colonization to occur but short enough to avoid mycorrhizal development to reach its full potential, a point at which plants may become uniformly colonized (see McGonigle 2001). Brundrett et al. (1994) proposed to grow trap plants in undisturbed cores to maintain an intact AM mycelium in the soil under evaluation. In contrast to the MPN and MSI methods, the intact core method accurately evaluates soil infectivity. The limitation of this method is that its end result is not a convenient number of propagules, but the percentage of colonization of a trap plant after a number of days. This method is appropriate to compare the mycorrhizal potential of soils or plots within the framework of an experiment. Furthermore, AM fungi are filamentous, a type of growth that makes it impossible to determine where a propagule starts and ends. Thus, rate of colonization expresses soil AM infectivity more realistically than a number of propagules. This method is reported below.

### 30.5.1 Materials and Reagents

1. Appropriately labeled steel cylinders with a tapered and sharpened lower end, which sends the pressure outward and preserves the soil core when it is pushed into the soil. These cylinders serve as growth containers. As many cylinders as there are plots multiplied by the number of desired subsamples per plot are needed.

2. Small wooden board to cover and push cylinders in the soil.

3. Trowel to lift the cylinders.

4. Knife to level off the lower end of the soil core.

5. Nylon mesh and high-gauge rubber bands to close the lower end of the cylinder while allowing drainage.
Growth chamber to control temperature, humidity, and lighting conditions to allow repetition of the assay at other times.

Perforated plastic trays with corrugated bottom that will allow cores to drain excess water while maintaining the soil in place upon watering.

Germinated seeds or seedlings of the desired species of trap plant. Note that more than one species can be grown simultaneously in each core. The number of plants of each species should be kept constant among growth cylinders. Clover seedlings inoculated with *Rhizobium* (Brundrett et al. 1994) have been used, but any mycotrophic plant species may be used.

Polyester wool may be used to cover the surface of cores, particularly in the case of soil rich in clay, in order to protect the soil surface structure from water damage during watering events.

### 30.5.2 Procedure

1. Cover the cylinders with the wooden board and push it in the soil until filled.
2. Carefully dig the cylinders out of the soil.
3. Invert the cylinders holding the surface with your palm or on the wooden board, level off the lower surface of the core, cover it with the nylon mesh, and secure this mesh with a high-gauge rubber band. Avoid exposing the cores to excessive heat during the collection process.
4. Carefully carry the cores to a clean greenhouse work bench.
5. Insert germinated seeds or plantlets in the center of the cores. Seeds can be placed in pairs and thinned to one per pair after a few days.
6. Cover the soil surface with polyester wool to protect the soil structure.
7. Place in corrugated trays in growth chamber at required preset settings.
8. Water very gently to field capacity when required, to avoid soil structure degradation.
9. Grow the trap plants for about 2–4 weeks (see Section 30.5.3).
10. Clear, stain, and assess the roots, as per the methods given in Section 30.3, for their percentage of mycorrhizal colonization using the grid-line intersect method (see Section 30.4), and determine simultaneously total root length.

### 30.5.3 Comment

It is worthwhile having extra cores available to assess periodically the status of root colonization before harvest and ensure that trap plants have adequate amount of mycorrhizal development.
30.6 EVALUATION OF THE EXTRARADICAL PHASE OF AM FUNGI

Several methods have been used to quantify the extraradical mycelium of AM fungi. Soil chitin measurement has been used to estimate AM fungal biomass in soil (Bethlenfalvay and Ames 1987). Chitin measurement does not give an estimate of the active AM extraradical hyphal biomass. Chitin is also abundant in invertebrates, exoskeletons, and zygomycetous fungi.

The spread of AM fungi extraradical hyphae in soil was studied using root exclusion chambers. Sequential sampling in compartmentalized growth containers allowed the comparison of the spread of AM fungal species from a root barrier into a hyphal compartment (Schuepp et al. 1987; Jakobsen et al. 1992). A rotating wire system was proposed to facilitate the task of extracting extraradical hyphae from mineral soil samples with low clay content (Vilarino et al. 1993; Boddington et al. 1999). The method of root exclusion chambers has been the most popular way to evaluate the size of the extraradical AM hyphae in soil, even though there are some limitations to direct measurement of hyphae extracted from soil. AM fungal hyphae are generally nonseptate and some researchers found morphological features typical of AM mycelia such as hyphal size (Ames et al. 1983), angle of branching, and wall characteristics. But considering the different morphologies found among AM genera, the range of possible AM extraradical hyphae size (from <1 μm in the fine endophytes to 18 μm in Glomus manihotis (Dodd et al. 2000)) and, on the other hand, the diversity of other soil fungi, one must conclude that the identity of coenocytic hyphae extracted from a soil sample is at best uncertain, especially in hyphal pieces bearing no branching. Also, much of the AM hyphae extracted from soil are neither viable nor functional and vital staining techniques must be applied if the amount of active hyphae is sought.

Inserted membrane techniques in which a membrane of a material resilient to decomposition is inserted in the soil to trap the hyphae that will cross it were proposed by Wright and Upadhyaya (1999) and Balaz and Vosatka (2001). These methods are simple and rapid. They give a measure of total hyphae cross-section plates. This method can be used with immunodetection of the AM fungi-specific protein glomalin (Wright and Upadhyaya 1999; Wright 2000) to assess the proportion of AM and saprophytic fungi. Inserted membrane techniques should be considered where data on hyphal density are not necessary.

Fatty acids are often specific to taxonomic groups. The phosphate group of phospholipids, the lipids making up membranes, is rapidly cleaved in soil, and PLFA measurement reflects the occurrence of living or recently dead organisms. The measurement of AM fungi PLFA indicator thus provides information on the functionality of the organisms. The PLFA 16:1ω5 is the preferred indicator of AM fungal biomass (Balser et al. 2005). The fatty acids 16:1ω5, 18:1ω7, 20:4, and 20:5 were proposed as indicative of AM fungi (Olsson et al. 1995; Olsson 1999). AM fungi do not have completely specific fatty acids; 20:4 and 20:5 are present in algae and protozoa but are rare in non-AM fungi and bacteria, and 16:1ω5 and 18:1ω7 occur in some bacterial genera but are not normally found in other fungi. Background level of the PLFA 16:1ω5 ranging from 30% to 60% was attributed to the presence of bacteria in soil. The fatty acid 16:1ω5 is dominant in many AM fungal species although it was absent from several Glomus species and from most Gigaspora species (Graham et al. 1995). Whole-cell fatty acid (WCFA) 16:1ω5, which is a more specific indicator of AM fungi than PLFA 16:1ω5, was correlated with hyphal length but the relationship varied seasonally (Gryndler et al. 2006). A nonmycorrhizal control can be used to correct for background levels of the fatty acid 16:1ω5. PLFA data on bacterial biomass, which can be generated simultaneously,
can be used to interpret changes in the abundance of the fatty acid 16:1ω5. Neutral lipid fatty acid (NLFA) 16:1ω5 should also be monitored because it highly dominates reserve fatty acids of all AM fungi tested, and because bacteria produce very little neutral lipids. The measurement of NLFA 16:1ω5 can be used to support observation on the variation in fatty acid 16:1ω5 from the PLFA fraction.

A method for lipid extraction from soil and measurement of PLFA and NLFA 16:1ω5 (J.M. Clapperton, personal communication, Agriculture & Agri-Food Canada, Lethbridge, Alberta) is given below.

### 30.6.1 Materials and Reagents

**Extraction**

1. Weighing boats
2. 35 mL glass centrifuge tubes
3. Dichloromethane (DMC)
4. Methanol (MeOH)
5. Citrate buffer
6. Saturated NaCl solution
7. 7 mL glass vials
8. Pipette
9. Nutating shaker
10. Centrifuge
11. N₂-gas flow drying manifold (we use a Reacti-Vap III)
12. Hot plate at 37°C (we use a Reacti-Therm III)

**Lipid-Class Separation**

1. Clamp-holder construction with 10 clamps (to hold columns)
2. Pasteur pipettes filled with silica gel up to 2 cm from the top (columns)
3. Pasteur pipettes fitted with pipetting bulb
4. 4 mL glass vials
5. DCM
6. Acetone
Transmethyl Esterization

1. N₂-gas flow drying manifold (we use a Reacti-Vap III)
2. Hot plate at 37°C (we use a Reacti-Therm III)
3. Pasteur pipettes fitted with pipetting bulb
4. Micropipette with tips
5. MeOH
6. H₂SO₄ (concentrated)
7. Water bath
8. Hexane
9. Vortex mixer
10. Ultrapure water
11. Methyl nonadecanoate (19:0; Sigma, Aldrich)
12. 200 μL glass syringe with needle
13. 100 μL tapered glass inserts and gas chromatograph (GC) vials

Gas Chromatography Measurement of Fatty Acids

1. 16:1ω5 standard fatty acid (from MJS Biolynx #MT1208).
2. Gas chromatograph with flame ionization detector (FID). We use a Varian 3900 GC equipped with a CP-8400 autosampler, helium as carrier gas (30 mL min⁻¹), and a 50 m Varian Capillary Select FAME #cp7420 column.

30.6.2 Procedure

Extraction

1. To extract total soil lipids, shake 4 g (dry weight equivalent) of frozen or fresh soil in 9.5 mL DMC:MeOH:citrate buffer (1:2:0.8 v/v) for 2 h in glass centrifugation tubes.
2. Add 2.5 mL of DMC and 10 mL of a saturated NaCl solution to each tube and shake for five more minutes.
Centrifuge tubes at 1500 g for 10 min.

Pipet the organic fraction into clean vials.

Add 5 mL of DCM:MeOH (1:1 v/v) to the tubes.

Shake for 15 min.

Centrifuge for 10 min at 1000 g.

Combine the organic fractions in the corresponding vials and dry under a flow of N₂ at 37°C in the fume hood.

Dissolve samples in 2 mL of DCM.

Samples can be stored at −20°C for a short time, if necessary.

**Lipid-Class Separation**

Lipid-class separation is conducted in silica gel columns made with Pasteur pipettes.

1. Using a pipette, load samples onto columns washing the vials twice with a small amount of DCM and adding the wash to the columns. Care must be taken to keep solvent level above the silica gel at all times.

2. Elute the neutral lipid fraction first by leaching columns with approximately 2 mL of DCM, collecting the eluent in 4 mL vials.

3. Elute the glycolipid fraction by leaching columns with approximately 2 mL of acetone, collecting the eluent in other 4 mL vials.

4. Elute the phospholipid fractions by leaching columns with approximately 2 mL of MeOH, collecting also the eluent in 4 mL vials.

5. Discard the glycolipid fraction.

6. Dry the neutral and phospholipid fractions under a flow of N₂ at 37°C in the fume hood.

7. Dissolve the dried fractions in a few mL of MeOH for PLFA or DCM for NLFA and store at −20°C.

**Transmethyl Esterization**

Fatty acid methyl esters are created through mild acid methanolysis as follows:

1. Dry neutral and phospholipids fractions under a flow of N₂ at 37°C in the fume hood.

2. Add half a Pasteur pipette full of MeOH/H₂SO₄ (25:1 v/v) to the vials.
3 Place vials in an 80°C water bath for 10 min.
4 Cool to room temperature.
5 Add 1 Pasteur pipette of hexane, vortex vials for 30 s, and leave to settle for 5 min.
6 Discard the lower fraction.
7 Add 1 mL of ultrapure water, vortex vials for 30 s, let stand for 5 min.
8 Discard the aqueous fraction entirely.
9 Add 10 µL of methyl nonadecanoate, the internal standard.
10 Dry samples under a flow of N2 at 37°C in the fume hood.
11 Wash vials with 50 µL of hexane using a glass syringe.
12 Transfer the samples into 100 µL tapered glass inserts, and place inside a GC vial.

Gas Chromatography Measurement of Fatty Acids

1 Sample (2 µL) injection is in 5:1 split mode.
2 In our program, for example, the injector is held at 250°C and the FID at 300°C. The initial oven temperature, 140°C, is held for 5 min, raised to 210°C at a rate of 2°C min⁻¹, then raised from 210°C to 250°C at a rate of 5°C min⁻¹, and finally held for 12 min.

Peak Identification

Identification of peaks is based on comparison of retention times to a known 16:1ω5 standard. Amounts are derived from the relative area under specific peaks, as compared to the 19:0 peak value, which is calibrated according to a standard curve made from a range of concentrations of the 19:0 FAME standard dissolved in hexane. The abundance of individual PLFAs is expressed as micrograms PLFA per gram dry soil.

The amount of fatty acid is calculated with the following formula:

\[ 16:1\omega5 = \frac{A_{16:1\omega5}}{A_{\text{istd}}} C_{\text{istd}} D \]  

(30.2)

where 16:1ω5 is the calculated concentration of the AM fungal indicator (moles or weight per unit volume), \( A_{16:1\omega5} \) is the GC area of the AM fungal indicator, \( A_{\text{istd}} \) is the GC area of the internal injection standard as determined by the GC data system (unitless), \( C_{\text{istd}} \) is the concentration of the internal injection standard given, and \( D \) is the appropriate dilution factor.

30.6.3 Comments

Solvents used throughout the procedure are HPLC grade, and tubes and vials are made of glass and their screw-top caps lined with Teflon. Organic solvents are toxic and must be
handled in the fume hood. The use of parafilm is prohibited. Care must be taken to avoid contamination with extraneous lipids. For example, the use of gloves is recommended. A bacterial and fungal saprobe fatty acid indicators mix (Supelco Bacterial Acid Methyl Esters #47080-U) can be used in conjunction with the AM fungal indicator 16:1ω5 to simultaneously obtain information on the whole soil microbial community.

30.7 METHOD TO EVALUATE AM EXTRARADICAL MYCELIUM

A number of methods have been used to study the extraradical mycelium of AM fungi. The cultivation of AM fungi on transformed root cultures has generated considerable knowledge on the physiology of AM fungi. This body of work was reviewed by Fortin et al. (2002). This system, in which the plant component of the AM symbiosis is reduced to a root often transformed by the Agrobacterium rhizogenes plasmid, is artificial. Giovannetti and her group successfully used a membrane sandwich method from which much knowledge on the extraradical phase of AM fungi was also gained (Giovannetti et al. 1993, 2001). This method is closer to reality as whole plants are used, although the environment of the symbioses formed is artificial and bidimensional. In this method clean AM spores are germinated in between two Millipore membranes (0.45 μm diameter pores) placed on moist sterile quartz grit in 14 cm diameter Petri dishes. Clean plantlets are added to the sandwich. Sandwiches are harvested at intervals to monitor mycorrhizal development. Rillig and Steinberg (2002) have used glass beads of different sizes to simulate different hyphal growing space conditions to show the large influence of the environment on hyphae length and glomalin production. Friese and Allen (1991) have used root observation chambers to describe runner hyphae, hyphal bridges, absorptive hyphal networks, germ tubes, and infection networks produced in soil by spores and root fragments. Although a root observation chamber allows the study of the morphology of arbuscular mycorrhizae formed in soil, this system may not be representative of the field situation. The film method in which a soil-molten agar suspension was poured into films was proposed by Jones et al. (1948). These agar films can be dried and stained to facilitate the enumeration of entrapped organisms under the microscope. A modification of this method was used to document AM hyphal links formed between the roots of different plant species. This method can be useful for examining interactions between roots, AM hyphae, and soil microorganisms in the field; it is described below.

30.7.1 MATERIALS AND REAGENTS

1 Warm 1.5% water agar
2 Microscope slides
3 Tray
4 Thin plastic film (Saran wrap)
5 Colored plastic flags to facilitate slides recovery in the field
6 A staining solution made of 15 mL phenol (5% aqueous), 1 mL of aniline blue W.S. (1% aqueous), and 4 mL of glacial acetic acid (use fume hood, gloves, and eye protection)
Euparal mounting medium (Bioquip, Gardena, California)
95% Ethanol

30.7.2 Procedure

1. Place the microscope slides side-by-side in a tray.
2. Pour the water agar on the slides to produce a thin agar coat on the slides.
3. When cold and solidified, free and remove the agar-coated slides from the tray using a scalpel. Store the fresh agar-coated slides wrapped in plastic film at 4°C. In the field, remove the plastic wrap from the slides. Bury the slides vertically in the rhizosphere at a chosen distance from the plant roots.
4. Mark the location of each buried slide with a plastic flag.
5. After a period of time, carefully dig out the buried slides cutting off the soil around the slide with a scalpel and any fine roots that might have grown in the agar coat. Agar-coated slides can remain buried for a few months.
6. In the laboratory, delicately wash the bulk of adhering soil off in a water bath.
7. Dry the agar films at 60°C for 15–20 min.
8. Under the fume hood, immerse the dried films for 1 h in the staining solution.
9. Wash and dehydrate in 95% ethanol and permanent mount in Euparal.

30.7.3 Comments

Phenol and phenol-containing solutions should be handled under the fume hood using gloves and eye protection. Good results are also obtained with fuchsin acid staining.

30.8 Extraction of AM fungi

Soil sampling strategies may generate the need to analyze in detail the composition of harvested soil samples for their AM spore population, spore abundance, and spore species diversity. Moreover, the extracted spores may provide starting inoculum in the form of isolated spores that may be used to obtain mixed or purified species inoculum.

According to the type of soil worked on, different approaches may be taken to facilitate the bulk isolation of spores from a soil substrate. The density gradient centrifugation method for spore extraction together with spore sieving and decanting is probably the most common method for AM spore extraction, especially for biodiversity and taxonomical studies (Khan 1999). However, it is very time consuming and less appropriate for most basic AM soil species and soil population investigations; the spore extraction method proposed below is the result of an adaptation of combined methodologies of soil sieving, decanting, sucrose...
centrifugation, and filtrating. Literature provides descriptions and evaluated methodologies that may help in refining the techniques when special requirements are needed. These requirements mainly concern diverse gradient methods (Ohms 1957; Allen et al. 1979; Furlan et al. 1980; Kucey and McCready 1982) and wet-sieving approaches (Gerdemann and Nicolson 1963; Daniels and Skipper 1982; Singh and Tiwari 2001). Book chapters and review articles have also been dedicated to the evaluation and description of procedures (Tommerup 1992; Brundrett et al. 1996; Clapp et al. 1996; Jarstfer and Sylvia 1997; Johnson et al. 1999; Khan 1999).

Once spores are extracted from soil material, they can be used directly as starting inoculum or classified by morphotypes as done for population studies (Smilauer 2001; Brundrett 2004). Spores may then be separated according to their size, their color, and their subtending hyphae morphology before being mounted on microscopic slides for AM fungi diversity assessment. When extracted spores are expected to serve as starting inoculum for the propagation of AM fungal strains, a preliminary spore vitality test is highly recommended. This can be done by a time-consuming evaluation of the germination potential of isolated spores. Moreover, dehydrogenase-activated stains such as tetrazolium bromide stain (3-(4,5-dimethylthiazol-yl) 2–5-diphenyl-2H-tetrazolium bromide (MTT)) and tetrazolium chloride stain (2-((p-iodophenyl)-3-((p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT)) have been frequently tested and used for the evaluation of AM spore vitality (An and Hendrix 1988; Meier and Charvat 1993; Walley and Germida 1995). Even though the interpretation of such staining approaches may be confusing, sometimes they remain the easiest and least time-consuming methods to estimate the spore vitality of a population. Procedures for spore extraction and viable staining of spores are given below.

Because of the laborious process associated with AM fungi spore extraction from soil samples, the limited species-specific spore morphological characters, and the incapacity to identify AM fungi from colonized roots, molecular-based techniques have been developed to study AM fungal communities. Several polymerase chain reaction (PCR)-based methods have been developed and applied to the detection of AM fungi and to the study of genetic diversity either directly from soil samples or from colonized root segments (Cläassen et al. 1996; Vandenkorochnuyse et al. 2002). The nested PCRs (two steps amplification) allow a rapid and efficient method for the study of soil and root AM fungal communities (van Tuinen et al. 1998; Jacquot et al. 2000; Jansa et al. 2003; de Souza et al. 2004). Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) can also be used for direct analysis of amplified DNA from soil and root samples. It allows the separation of DNA products of the same length and the distinction of single-base substitution between different nucleotide sequences, does not require a cloning process (Ma et al. 2005; Sato et al. 2005), and can discriminate between species (Kowalchuk et al. 2002; de Souza et al. 2004).

Finally, quantitative real-time PCR based on the detection of a fluorescent signal produced proportionally to amplification of a PCR product allows not only detection but also quantification of genomic DNA (Filion et al. 2003; Isayenko et al. 2004).

General discussion on the comparative value and applications of PCR-based method for the study of soil fungi communities and investigations with AM fungal diversity can be consulted respectively in Anderson and Cairney (2004) and Clapp et al. (2002).
30.8.1 EXTRACTION OF AM SPORES FROM SOIL BY SIEVING AND SUCROSE TECHNIQUES

Materials and Reagents

1. Scale
2. Flasks (1 L)
3. Sieves set (suggested mesh size: 1000 μm to intercept gravel, soil debris; 500, 150, and 50 μm to recover sporocarps and spores of different sizes)
4. Centrifuge tubes: round bottom 100 mL
5. Centrifuge
6. Vacuum filter apparatus (vacuum source, Buchner funnel and side-arm flask (2 L))
7. Filter paper (Whatman No. 1 (40 μm))
8. Plastic Petri dishes
9. Sucrose 50% (w/v)
10. Tween 80 (optional)

Procedure

1. Weigh 50 g of soil and pour in a 1 L flask with 200–300 mL of water.
2. Shake vigorously and then allow the soil to soak for 30 min to 1 h.
3. Pour the water and soil mix through the sieves piled in a decreasing order of mesh size (largest mesh on the top to retain debris), ensure recovery of the entire soil mix by carefully rinsing the flask. This allows the recovery of all soil material for spore extraction.
4. Wash the soil with running water, manually breaking soil aggregates if required, being careful not to clog the small mesh sieve. At this step, root pieces can also be recovered for either root colonization evaluation or inoculum material.
5. Recover the entire soil from each sieve, distribute the soil material in centrifuge tubes (max 10 mL of soil volume), fill the tube with a 50% (w/v) sucrose solution, thoroughly mix the tube content with a glass or metal rod, and centrifuge at 800 g for 4 min. (Optional step, see next section.)
6. Recover supernatants on a 40 μm paper filter and wash carefully to dilute sucrose concentration as it affects the spore wall morphology for the subsequent identification process.
Pour spores in water into a plastic Petri dish, or vacuum filter the spores on filter paper for examination under a dissecting microscope.

**Comments**

When an evaluation of the spore abundance is required, it is necessary to measure soil moisture content in order to express spore abundance as the number of spores recovered by weight in grams of dry soil weight. For steps 2–4, a drop of dispersant such as Tween 80 can be added to water to facilitate the separation of spores from soil debris. The dispersant does not seem to affect either spore morphology for further species identification, or spore germination potential. Depending on the soil texture, sucrose extraction and centrifugation (step 5) can be skipped especially when working with sandy soils because spores are easily separated from silica particles by a vigorous shaking of the soil–water–Tween mix. In this case, vacuum filtration is recommended and should be performed 2–3 times in order to recover a maximum number of spores. On the other hand, AM spore extraction from organic soil containing an abundance of partially decomposed plant debris requires the combination of sieving and sucrose extraction processes with, in some cases, the repetition of the extraction step 5 at least twice. The newly proposed use of low concentration of HCl or hydrofluoric acid for cleaning and separating spores from their surrounding organic material (Garampalli and Reddy 2002) yielded cleaner spores suitable for microscopic observations and *in vitro* culture propagation. However, extreme care should be taken in the use of such chemicals and the extraction should be performed under a fume hood to avoid inhalation. For hydrofluoric acid special fume hoods are needed.

### 30.8.2 EXTRACTION OF VESICLES THROUGH ENZYMATIC DIGESTION OF ROOTS

Vesicles are excellent AM fungal propagules. Strullu and Plenchette (1991) proposed their use in alginate beads as high quality root inoculum. Monoxenic culture of spores can also provide high quality inoculum, but only a few species can be grown *in vitro*. Vesicle extraction from root is another way to produce clean inoculum of the AM species that cannot be cultivated *in vitro*. Another application for extracted vesicles is the initiation of root organ cultures because intraradical vesicles are devoid of attached organic debris and cleaner than soil extracted spores.

**Materials and Reagents**

1. Scalpel
2. Enzyme solution made of 0.2 g L$^{-1}$ of macerozyme, 0.5 g L$^{-1}$ of driselase, and 1.0 g L$^{-1}$ of cellulase
3. 250 mL beaker
4. Blender
5. 50 μm sieve

**Procedure**

1. Cut 10 g of roots in 3–10 mm segments.
2. Place root pieces in 100 mL of the enzyme solution.
3 Incubate overnight at room temperature.
4 Wash digested tissues with demineralized water.
5 Homogenize in a blender.
6 Filter the homogenized sample on a 50 μm sieve and recover clusters of vesicles attached to hyphae under the dissecting microscope.

**Comments**

Jabaji-Hare et al. (1984) used mortar and pestle, a homogenizer, filtration, and density centrifugation to quantify and recover vesicles for biochemical analysis.

**REFERENCES**


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