Chapter 36

Microbial Biomass

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The soil microbial biomass is an important component of the soil organic matter that regulates the transformation and storage of nutrients. It is a labile component of the soil organic fraction containing 1 to 3% of the total soil C and up to 5% of the total soil N (Smith & Paul, 1990). Microbially mediated processes affect ecosystem functions associated with nutrient cycling, soil fertility, global C change, and soil organic matter turnover. The size and activity of the soil microbial biomass must be assessed to fully understand nutrient fluxes in managed and natural ecosystems.

Soil microbial biomass estimations are useful in investigations that compare temporal nutrient fluctuations along natural and perturbed gradients. The effects of tillage, crop rotations, and soil type on organic C and nutrient turnover can be assessed by following nutrient pools and activity associated with the soil microbial biomass. Microbial biomass has been shown to be a sensitive indicator of differences in sustainable cropping systems (Anderson & Domsch, 1989). The toxicity of pollutants and the degradation of organic compounds (pesticides and industrial chemicals) can be monitored by following changes in the soil microbial biomass.

This chapter describes methods that estimate the size of the soil microbial biomass and associated nutrient pools and metabolites. The methods include the chloroform fumigation incubation method (CFI), chloroform fumigation extraction method (CFE), substrate induced respiration (SIR), and adenosine triphosphate (ATP) analysis. The CFI and CFE methods are also useful to recover tracers (e.g., ^{14}C and ^{15}N) from the microbial biomass. ATP and SIR cannot be used to measure tracer incorporation into the biomass. All the methods are sensitive to minor differences in technique and must be standardized for specific soil types and operating conditions.

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36–1 SOIL SAMPLING, PREPARATION, AND STORAGE

Representative soil samples that can be treated statistically will have the most significance in soil microbial biomass determinations. A knowledge of the site should be used to separate areas uncharacteristic or unrepresentative of the general landscape (see chapter 1 by Wollum in this book). Examples are low, poorly drained areas; these can be sampled separately. A minimum of four (preferably more) replicates each made from at least two separate composite samples are required to reduce the error to about 10% of the measurement (see chapter 2 by Parkin and Robinson in this book). The use of tracers to study nutrient cycling and flux rates in conjunction with biomass measurements is best accomplished in microcosms that can be completely sampled, mixed, and subsampled.

The soil is sampled by coring or removing a known dimensional quantity from the soil profile (see chapter 1). The soil should be removed from direct sunlight or placed in an ice chest. The soil can be stored overnight at 15 °C when microbial biomass determinations are to be done the following day. Soil can be stored at 4 °C for periods of a week, but the possibility of changes occurring during this storage period must be considered. Freezing of soil samples is not recommended due to the adverse biocidal effects on the soil microbial biomass. If samples must be frozen, they should be pre-incubated for 7 to 10 d before soil microbial biomass determinations are done. The drying of soil samples should be strictly avoided.

Soil samples are prepared by sieving through a 4 to 6 mm screen. This size mesh has been determined not to affect the soil microbial biomass size or activity (Jenkinson & Powlson, 1980; Ross et al., 1985). When soils are too moist for sieving they have to be dried to an adequate moisture content. The soil moisture content is determined after the sieving process. With the exception of ATP determinations, the described microbial biomass determinations do not work well for water-saturated samples.

36–2 PHYSIOLOGICAL METHODS

36–2.1 Chloroform Fumigation Incubation Method

The effect of fumigants on soil metabolism was established early during this century (Jenkinson, 1966). The respiration rate of a fumigated soil is initially less than an unfumigated sample, but as time proceeds, the respiration rate of the fumigated soil exceeds that of the unfumigated sample and eventually subsides to a lower level. The temporary flush of CO_2 from the fumigated soil is primarily due to the decomposition of microbial components from lysed microorganisms (Jenkinson, 1966). In addition, an increase in the NH_4^+ pool occurs as a result of the mineralization of nitrogenous substrates from the lysed microorganisms. The increase in CO_2 evolution and extractable NH_4^+ from fumigated samples has been used to estimate the size of the soil biomass (Jenkinson & Powlson, 1976a,b; Jenkinson, 1976; Anderson & Domsch, 1978a; Voroney & Paul, 1984).

36–2.1.1 Soil Samples

The amount of soil used will depend on its respiration rate or requirements for recovery of added tracers. Generally, 20 to 50 g (dry weight equivalent) of soil is placed in an appropriate sized container, this should allow for the addition of an extractant. Soil samples must be analyzed for initial inorganic N. Fumigated and control samples are analyzed for mineralized C and N. Analytical duplication of each soil sample is preferred. The samples to be fumigated are weighed into glass beakers and the beakers are marked with a chloroform-insensitive marker (e.g., pencil lead). The remaining treatments can be weighed into any suitable container, preferably with a closure to aid C or N extraction.

36–2.1.2 Fumigation of Soil Samples

Because of the carcinogenic-volatile properties of chloroform, all work must be done in an adequate fume hood. A beaker containing 50 mL of ethanol-free chloroform (Jenkinson & Powlson, 1976b) and antibumping granules is placed together with the soil samples into a vacuum desiccator. The desiccator is lined with moist paper towels to prevent the desiccation of soil samples during the fumigation. Commercially available ethanol-free chloroform preserved with heptachlor epoxide has been used to obtain similar results to that of purified $CHCl_3$ (Voroney et al., 1991). The desiccator is evacuated until the chloroform boils vigorously. This is repeated three times, letting air pass back into the desiccator to facilitate the distribution of the chloroform throughout the soil. The desiccator is then evacuated a fourth time until the chloroform boils vigorously for 2 min, the valve on the desiccator is closed, and the desiccator is placed in the dark at 25 °C for 18 to 24 h. Unfumigated samples are also kept in the dark in a desiccator or mason jars at 25 °C while the fumigation proceeds. Following this period, the chloroform and paper towels are removed, under the fume hood, and the desiccator evacuated 3 min for eight times letting air pass into the desiccator after each evacuation to remove residual chloroform. Never determine residual chloroform by sense of smell. Make sure the vacuum pump is periodically maintained to ensure proper operating condition.

Following the removal of chloroform, the fumigated soil samples are placed in mason jars (Fig. 36–1). Fumigated soil can be inoculated before the incubation by adding and thoroughly mixing 0.2 g of unfumigated soil to 50 g of fumigated soil (Jenkinson & Powlson, 1976b). Inoculation is often nonessential in soils with pH > 5 and high microbial populations since the fumigation procedure does not kill the entire population of the soil microbial biomass (Vance et al., 1987b). Inoculation of subsurface soils is often necessary. Soil samples are adjusted to an optimum soil moisture

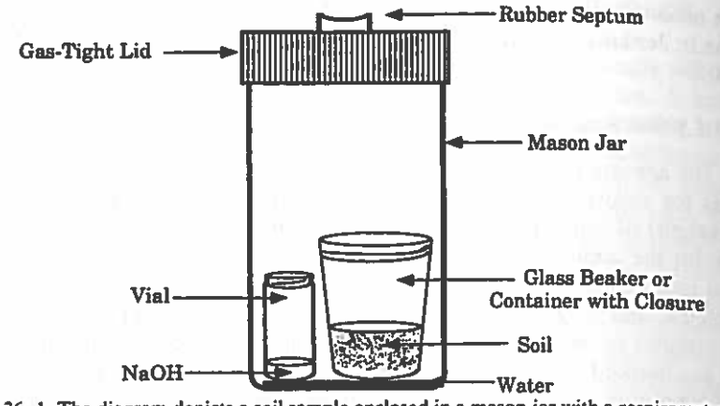

Fig. 36–1. The diagram depicts a soil sample enclosed in a mason jar with a respirometer and water. The respirometer is not used when sampling mineralized CO_2 with the gas chromatography (GC) method. When using the GC method a septum must be inserted into the mason jar lid to facilitate the sampling of the jar's headspace for CO_2 gas analysis.

content (55% of water-holding capacity). Soils prone to denitrification can be adjusted to lower water contents to reduce the gaseous loss of N (Jenkinson, 1988). Approximately 1.0 mL of water is added to the bottom of each mason jar to prevent soil desiccation (Fig. 36–1). The soils are then incubated in closed, gas tight mason jars under standard conditions (at 25 °C in the dark) for a period of 10 d.

36–2.1.3 Carbon Dioxide Mineralization Determination

A vial containing 1.0 mL of 2.0 *M* of NaOH is placed into each mason jar, exercising care not to partially neutralize the alkali by breathing into it. The volume or strength of the alkali can be adjusted to accommodate varying respiration rates of soils or recovery of tracers. Blanks consisting of jars without soil must be similarly maintained during the incubation period.

After the incubation period, the vials (respirometers) are titrated to determine the total C respired from the microbial biomass. An amount of $BaCl_2$ equivalent to the initial quantity of NaOH is added to each respirometer. The contents of the respirometer are then titrated to pH 7 or to a phenolphthalein endpoint using 0.1 *M* of HCl. The amount of CO_2-C evolved during the incubation is calculated from the volume of acid needed to attain pH 7 from the blank minus that required for the samples (1.0 mL of 2.0 *M* of NaOH can consume 12 mg of CO_2-C). When CO_2 levels are low, as is the case in subsurface soils, titration should be done using a double endpoint titration for bicarbonate (Jenkinson & Powlson, 1976b).

The double endpoint titration requires that the contents of the respirometer first be titrated to a pH of 9 to 10 with 1.0 *M* of HCl using thymolphthalein indicator solution (Sigma Chemical Co., St. Louis, MO). Add 50 µL of 0.1% carbonic anhydrase (Sigma Chemical Co., St. Louis, MO) and titrate to pH 8.3 with 0.05 *M* of HCl using a pH electrode. Titrate the respirometer contents to pH 3.7 with 0.05 *M* of HCl and record the volume of acid. The amount of CO_2 evolved during the incubation period is calculated from the volume of acid needed to decrease the pH of the respirometer from 8.3 to 3.7 subtracted from the blank respirometer (1.0 mL of 0.05 *M* of HCl being equivalent to 0.6 mg of CO_2-C in NaOH solution).

Alternatively, the CO_2 accumulated in the headspace of the mason jar may be measured by gas chromatography (GC) or with an infrared gas analyzer. The GC method gives a rapid and accurate measurement of CO_2 and can be used in acidic soils (see chapter 38 by Zibiliske in this book). However, this technique is prone to error in neutral and alkaline soils (Martens, 1987) as accumulation of carbonate species in the soil solution can lead to lowered CO_2 determinations. When working with neutral and alkaline soils the CO_2 absorption method described above should be used.

36–2.1.4 Nitrogen Mineralization Determination

Fumigated, unfumigated, and samples for initial inorganic N are extracted with 1 or 2 *M* of KCl at a ratio of 5:1 (extractant/soil). Clay soils require a ratio of 10:1. The soil and extractant are shaken on a reciprocal shaker at 180 strokes per minute for 0.5 h. The filtered extract is then analyzed for NO_3^- and NH_4^+ (see chapter 41 by Bundy and Meisinger in this book) to determine the flush of N from the microbial biomass (Quikchem Systems, 1987). The extract can be distilled or diffused for the analysis of ^{15}N in the biomass (Brooks et al., 1989; (see chapters 40 and 42 by Hauck et al. and Hart et al., respectively in this book).

36–2.1.5 Calculation of Biomass Carbon

The amount of CO_2-C respired from fumigated and unfumigated samples is used to calculate soil microbial biomass C. Soil microbial biomass C, calculated using a control, is shown by the following equation:

$$\text{Biomass C} = (F_c - UF_c)/K_c$$

where

F_c = CO_2 flush from the fumigated sample

UF_c = CO_2 produced by the control.

The value of K_c is defined as the fraction of biomass C mineralized to CO_2.

Biomass C can also be calculated without the subtraction of a control as shown in the following equation (Voroney & Paul, 1984).

$$\text{Biomass C} = F_c/K_c$$

This method of biomass C calculation is useful in soil with high basal respiration rates. The value of K_c is of considerable importance since it relates the size of the biomass to the fumigated flush of CO_2. The value of K_c can be obtained by adding a known quantity of ^{14}C-labeled microorganisms to soil and determining the proportion of the added ^{14}C that is mineralized (Anderson & Domsch, 1978a).

The culture of indigenous labeled (^{14}C and ^{15}N) microorganisms is necessary to determine realistic K values for the accurate determination of microbial biomass in soils. Chapters 7 to 9 describe techniques and provide references for isolating microorganisms from soil. The liquid medium described by Anderson and Domsch (1978a) and modified by Wardle and Parkinson (1990) is used to culture labeled organisms. One liter of medium contains 10 g of U-^{14}C-labeled D-glucose (specific activity 370 Bq mg^{-1} of glucose), 1 g of NH_4NO_3, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 50 mg of $CaCl_2$ and 20 mg of $FeCl_3$. In addition, yeast extract (DIFCO) is added to the media, 1 g for fungi and 3 g for bacteria. Labeled NH_4NO_3 (4–10% atom % excess) may be used to enrich the microbial cultures with ^{15}N. The medium is autoclaved prior to the addition of labeled glucose. Bacteria and fungi are cultured in 50 mL of media in a 125-mL Erlenmeyer flask, on a culture shaker at 22 °C. Bacteria are harvested in late logarithmic phase and actinomycetes and fungi in the late linear phase. Radioactive cells are harvested by centrifugation (2000 *g*, 4 °C) for 20 min and washed free of adhering media with purified water by repeated centrifugation and resuspension. Fungi can be harvested by filtration and rinsed. The pellets are resuspended in water or dried and ground (0.5 mm) for introduction into soil samples.

An alternative technique to determine K factors is to label soil organisms in situ. Carbon-14-glucose (specific activity of 925 Bq mg^{-1} of C) and $^{15}NH_4^+$ (Atom % excess of 4–10%) are added directly to the soil (Voroney & Paul, 1984). The substrates can be added in dry form using a talc carrier (Anderson & Domsch, 1978b), misted and mixed into the soil or added in solution to bring the soil moisture to 55% of water-holding capacity. The C/N ratio of the added substrates should be approximately 10:1. The amount of ^{14}C-glucose and inorganic ^{15}N added to soil will depend on the amount of glucose and N that can be immobilized by the soil biomass in a 24-h period without a major change in the microbial biomass size. The short incubation time ensures minimal new biomass production. Standard glucose (e.g., Sigma Diagnostic Kit no. 510-A) and inorganic N assays can be done on 2 *M* of KCl extracts of soil to determine the glucose and N amendment.

It is assumed that added organisms grown in vitro give results applicable to the native soil population. Common values for K_c, subtracting a control, range from 0.45 (Jenkinson & Ladd, 1981) to 0.41 (Anderson & Domsch, 1978a). Voroney and Paul (1984) labeled the soil biomass in situ using ^{14}C-glucose and ^{15}N-NH_4^+ and developed a K_c of 0.41 without subtracting a control. It is difficult for every researcher to develop a K_c for each soil and literature values are often used. The value of K_c used and whether a control was subtracted should be reported when reporting biomass size. We always recommend reporting of the data for fumigated and control soils irregardless of the method of calculation. This allows other workers to reinterpret the data if they wish.

The use of an appropriate control has been problematic since the conception of the CFI method. Not all of the CO_2 evolved from a fumigated soil is derived from lysed microorganisms. The basal respiration of the fumigated and control samples can also vary depending on the moisture content and amount of root fragments contained in the soil. It is, therefore, difficult to ascertain the amount of the control to subtract from the fumigated sample, especially when basal respiration is high.

Preincubation of soil samples for 7 to 10 d prior to biomass determination has been recommended to eliminate interference from sieving, wetting, and root fragments (Sparling et al., 1985). However, if time zero soil microbial biomass determinations are desired or the experiment involves tracers, preincubation of soil is not an option. Voroney and Paul (1984) suggest an alternative method excluding the use of a control since they found that in situ labeling of the soil biomass produced a K_c of 0.41 without subtracting a control.

In soils of low microbial activity the control is low. Soils with low biomass but high background activity result in negative biomass calculations when a control is subtracted. In many soils, when a control is not subtracted the biomass estimates can be too high. Jenkinson and Powlson (1976b) suggest subtracting the CO_2 produced from a 10 to 20 d incubation to reduce the amount of the control subtracted from the fumigated sample in soils described above. The possibility of subtracting a partial control based on the size of the CO_2 flush from the 0 to 10 d control using internal standards also exists. This can be calculated by adding labeled substrate to both fumigated and unfumigated samples and calculating the difference of substrate utilization between the treatments (Voroney & Paul, 1984).

36–2.1.6 Calculation of Biomass Nitrogen

Microbial biomass N (B_n) is calculated similarly to biomass C as shown:

$$B_n = (F_n - UF_n)/K_n$$

where

F_n = The flush of NH_4^+ due to fumigation

UF_n = The NH_4^+ mineralized during 0 to 10 d from a control.

The value of K_n is the proportion of microbial N mineralized to NH_4^+ during the 10-d incubation period. The value of B_n also has been calculated without the use of a control. The equation is:

$$B_n = F_n/K_n$$

Values of B_n calculated by either of the above equations are generally in agreement if the background NH_4^+ levels of soils taken from the field are low (Voroney & Paul, 1984).

The establishment of K_n is difficult since the N content of the microbial biomass is variable depending on substrate availability, fungal/bacterial ratio, litter C/N ratio or environmental conditions (moisture and temperature). The immobilization of mineralized N after fumigation can also complicate the calculation of K_n. Voroney and Paul (1984) related the flush of C and N from the fumigation response and developed a floating K_n based on the expression:

$$K_n = -0.014*(C_f/N_f) + 0.39$$

The inclusion of C_f/N_f ratio accounts for the reimmobilization of N during the N flush.

The values of K_n developed by adding ^{15}N-labeled microorganisms to soil range from 0.54 to 0.62 and are reviewed by Jenkinson (1988). Shen et al. (1984), obtained a value 0.68 by determining the immobilization of ^{15}N-NH_4^+ in fumigated and unfumigated soil. Jenkinson (1988) suggests a weighted mean for K_n of 0.54, for samples with C_f/N_f ratio of < 6.7. The value of K_n should be included with all reported microbial biomass N data.

36–2.2 Substrate-Induced Respiration Method

The substrate-induced respiration (SIR) method was introduced by Anderson and Domsch (1978b) to rapidly estimate the amount of C held in living, non-resting microorganisms in soil samples. The initial respiratory response to glucose as an added C and energy source was taken as an index of existing soil microorganisms before new synthesis of microorganisms occurred.

For incubations at 22 °C, a substrate-induced maximal respiration rate of 1 mL of CO_2 h^{-1} corresponds to about 40 mg of microbial C. The SIR method has been correlated against microbial biovolume measurements (West & Sparling, 1986; Beare et al., 1990) and ATP determinations (West & Sparling, 1986; Kieft & Rosacker, 1991). The SIR method as applied to replicate soil samples should be done according to the following steps.

36–2.2.1 Optimal Glucose Amendment

It is essential to standardize each soil relative to the respiratory response to glucose. The lowest glucose concentration that will give the maximum initial respiratory response (mL of CO_2 h^{-1}) is determined as measured by CO_2 evolution. The glucose amendment will range from 5 to 400 μM g^{-1} soil solution. Glucose concentrations should be reported in relation to the total soil solution as well as per gram dry weight of soil to avoid misinterpreting glucose concentrations as a result of varying soil water contents.

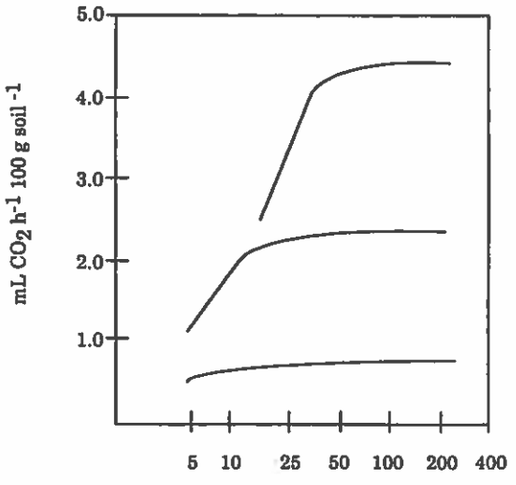

Fig. 36–2. The figure exemplifies the respiratory response from different levels of glucose amendment. The asymptote of each curve represents the minimum concentration of glucose that produces the maximal respiration rate.

Anderson and Domsch (1978b) suggest adding the glucose in dry form (0.5 g of talc plus glucose ground to a fine powder with mortar and pestle) to facilitate thorough mixing of the amendment. Our laboratory routinely adds amendments in liquid form to bring slightly dried soil to 55% of water-holding capacity with good results. West and Sparling (1986) suggest using a water-soil slurry (2:1) to minimize substrate dispersion problems and water limitation during the incubation period.

The soil is placed in a gas-tight container suitable for CO_2 headspace analysis. For soils low in organic matter, as much as 100 g of dry weight is necessary, whereas for organic layers of forest soils 10 g may be sufficient. A concentration series of glucose amendments are added to replicate soil samples (see above) to determine the lowest glucose level that yields maximum respiratory response. Figure 36–2 exemplifies a series of responses obtainable from varied soil samples (Anderson & Domsch, 1978b). When the respiratory response approaches an asymptote, the corresponding glucose level is defined as the minimum concentration of glucose invoking maximal respiratory response. The CO_2 is analyzed using a CO_2 analyzer, gas chromatography, or infrared gas analyzer to measure the amount of CO_2 respired and is expressed as mL CO_2 h^{-1} g^{-1} of dry weight soil.

The minimum concentration of glucose giving maximal respiratory response is added to replicate subsamples of soil. Enough replicate subsamples to reduce measurement error and express statistical variation are analyzed. The response of soil to the amendment is variable and, therefore,

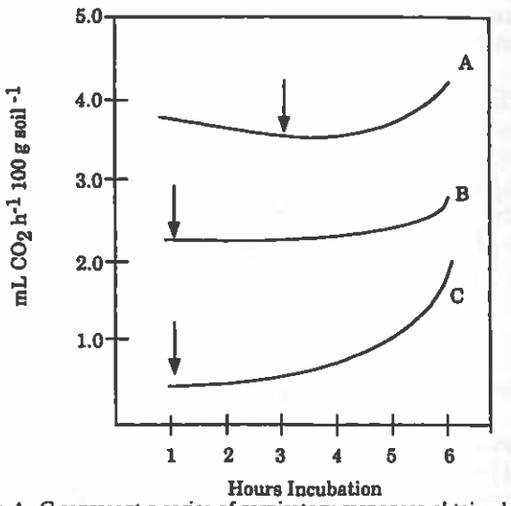

Fig. 36–3. Curves A–C represent a series of respiratory responses obtained from the predetermined glucose amendment. The arrows indicate CO_2 (mL CO_2) values used to calculate microbial biomass C. Measurements are taken before CO_2 increases which indicates the synthesis of new microbial biomass C.

hourly measurements are done to encompass increases, decreases or lags in CO_2 efflux that is then followed by a normal increasing rate. The minimum hourly rate of CO_2 production is recorded to determine biomass size (Fig. 36–3). Curves A–C of Fig. 36–3 represent possible respiratory responses as a result of the predetermined glucose amendment. The CO_2 mineralization rates show negative, zero, and positive slopes and associated rate maximum for each response. A positive tendency or increase in CO_2 production is interpreted as new biomass synthesis indicating that measurements must precede this event (Anderson & Domsch, 1975).

36–2.2.2 Calculation of Biomass Carbon

Anderson and Domsch (1978b) using 12 soils ranging from 0.778 to 39.2% C correlated the SIR method to CFI method to estimate total microbial biomass C and developed the expression:

$$x = 40.04y + 0.37 \quad (r^2 = 0.96)$$

where

$$x = \text{total microbial biomass C}$$

$$y = \text{maximum initial rate of } CO_2 \text{ respiration}$$

$$(\text{mL of } CO_2\ g^{-1} \text{ dry weight soil}).$$

The above relationship is valid only for SIR incubations done at 22 °C.

The SIR method correlates well to other biomass methods and involves a short analysis period (1–3 h). The method has been modified through additions of mineral salts, nutrient broth, and yeast extract to produce maximal respiratory response from nutrient imbalanced soil or long-term incubations where nutrient status may change (Sparling, 1981; Smith et al., 1985). The SIR method has also been used to evaluate pesticide damage to the soil microorganisms (Anderson, 1981). Additional modifications include measuring bacterial and fungal contributions to soil and litter metabolism using selective inhibitors (Anderson & Domsch, 1973; Beare et al., 1990). A discussion of these modifications can be found in chapter 9 by Turco in this book. Under standard assay conditions (22 °C and 55% of soil water-holding capacity), this method accurately estimates biomass C by probing the respiration response of in situ soil microbial populations.

36–3 CHEMICAL METHODS

The extraction of unique compounds representative of the microbial community is an attractive method for determining the size of the microbial biomass. The extraction of ATP, nucleic acids, muramic acid, chitin, and other biomass components have been reviewed (Jenkinson & Ladd, 1981; Nannipieri et al., 1990). Methods for determining the lipid P and ergosterol content of microbial biomass are reviewed by Grant and West (1986). The determination of microbial C and N by direct extraction and the determination of ATP are covered in detail here.

36–3.1 Chloroform Fumigation Extraction Method

Microbial constituents released by fumigation and extracted directly can be used to determine the size of the soil biomass. The CFE is correlated to biomass C and N as determined by CFI (Brookes et al., 1985; Vance et al., 1987a; Gallardo & Schlesinger, 1990). This method has several potential advantages over CFI including:

1. No NH_4^+ immobilization or denitrification activity.
2. Low interference from nonmicrobial labile C and N substrates that can be used during the incubation.
3. Shorter analysis time.

However, ^{14}C specific activity of the microbial biomass C from the CFE does not correlate well with that obtained by CFI method in soil labeled with ^{14}C-labeled plant residues (Horwath, 1992, unpublished data). We have found that when extracting soils incubated (up to 1 yr) with ^{14}C substrates, the CFE specific activity is approximately one-half that of CFI indicting that different C pools are being sampled. Recent investigations (Merckx & Martin, 1987; Badalucco et al., 1990) suggest that additional anthrone-reactive and ninhydrin-reactive C of nonbiomass origin is released from fumigated soil. Even though the CFE and CFI methods release

similar amounts of microbial C, the prospect of extracting dissimilar soil organic C pools with these two methods is disturbing and requires further study.

Soils held under chloroform fumigation retain protease activity, but lose dehydrogenase and C and N immobilization activity (Amato & Ladd, 1988). As a result, soluble C, organic N, and NH_4^+ levels increase until extracellular enzyme activity ceases or substrate becomes limiting. In some soils, a chloroform treatment of 1 d releases all the potentially extractable microbial products, while other soils require an exposure of up to 5 d (Brookes et al., 1985; Davidson et al., 1989).

36–3.1.1 Fumigation and Extraction

Soil samples are prepared and weighed as outlined in the sample preparation section 36–2.1.1, 10 to 20 g of dry weight, are weighed in triplicate into containers suitable for fumigation and extraction. The fumigation of soil samples is done according to CFI outlined in section 36–2.1.2, but soil fumigation is extended to attain maximum levels of soluble C and N. Fumigation periods of 1 d can be done for rapid soil biomass determinations (Brookes et al., 1985; Voroney et al., 1991). However, since soils vary in microbial activity, we recommend a 5-d fumigation as a standard time for the analytical determination of the soil biomass unless analysis of the particular soil has shown that the longer fumigation time is not necessary.

The fumigated and unfumigated soil are extracted with 0.5 *M* of K_2SO_4 at a ratio of 5:1 (weight of extractant to dry soil weight). The soil and extractant are usually shaken on a reciprocal shaker at 180 strokes per minute for 1.0 h. Dispersion such as in a Waring blender has been found necessary for well-aggregated soils in the determination of microbial plate counts and should be considered for CFE. After shaking, the soil suspension is filtered and the filtrate collected. The filtrate is stored at 4 °C (maximum of 1 wk) or frozen until analyzed. A blank filtrate, extractant alone, is run for each batch of samples analyzed to determine background levels of C and N in both the filter paper and extractant.

36–3.1.2 Determination of Biomass Carbon

Soluble organic C is determined on both the fumigated and unfumigated soil extracts. The soluble C is best analyzed on any suitable commercial soluble C analyzer. When such devices are not available, wet combustion techniques can be employed. Jenkinson and Powlson (1976a) describe a dichromate digestion in which an aliquot of soil extract is added to a mixture of potassium dichromate, sulfuric acid, phosphoric acid and mercury, and boiled under refluxing conditions for 30 min. The excess dichromate is titrated with ferrous ammonium sulfate using ferroin as an indicator.

Our laboratory routinely combines ^{14}C measurements with those of microbial biomass. We use a persulfate digest adapted from McDowell et al. (1987). The persulfate digest will be discussed in detail because of its ease and reduced safety requirements as well as its ability to capture the liberated $^{14}CO_2$. The method includes the following:

- 10 to 15 mL of soil extract
- 1 g of $K_2O_8S_3$ (persulfate)
- 1.0 mL of 0.025 *M* of H_2SO_4
- 1.0 mL of 0.1 *M* of NaOH placed in 15 × 45 mm vial

The digestion is done in 25 × 200 mm culture tubes equipped with a Poly Seal cap (Fisher Scientific, Fair Lawn, NJ) (Fig. 36–4). The persulfate, sulfuric acid, and filtrate (10–15 mL) are placed in the culture tube, the alkali trap is inserted, and the tube promptly capped producing a pressure-tight seal. The alkali is suspended above the digestion mixture by putting a restriction in the glass or by placing the alkali on a glass rod support. The samples are heated in a digestion block at 120 °C for 2 h. The digested samples are removed and allowed to stand overnight to complete the trapping of the liberated CO_2 into the alkali. A brown precipitate of oxidized Fe may form in the fumigated samples but does not affect the analysis. The base traps are then titrated according to section 36–2.1.3 to determine total sample C (1.0 mL of 0.1 *M* of NaOH can consume 600 μg CO_2-C). The base traps may be subsampled or the entire contents of the titrated traps used to determine ^{14}C activity. The blank filtrate is digested to determine background C for the control and fumigated sample. A set of glucose standards in 0.5 *M* of K_2SO_4 should be assayed with each sample run to verify the results of the assay.

36–3.1.3 Determination of Biomass Nitrogen

Total N in the extract (NO_3^-, NH_4^+ and organic N) is determined by Kjeldahl digestion (see chapters 40 and 42 in this book) (Brookes et al., 1985) or by ninhydrin-reactive N analysis (Amato & Ladd, 1988). Amato and Ladd (1988) used 2 *M* of KCl to extract the soil and determine organic N by the ninhydrin method, but this method has been modified using K_2SO_4 (Joergensen & Brookes, 1990) to allow for the analysis of both C and N from the same sample. When measuring ^{15}N-labeled biomass, the Kjeldahl procedure is used to convert organic N to NH_4^+, and distillation or diffusion is used prior to ^{15}N analysis (see chapters 40 and 42 in this book). Problems with NO_3-N if present in the extract are discussed elsewhere (chapter 42).

36–3.1.4 Calculation of Biomass Carbon

The amount of soluble C in the fumigated and unfumigated soil extract are used to determine biomass C expressed as:

$$\text{Biomass C} = (C_f - C_{uf})/K_{ec}$$

where

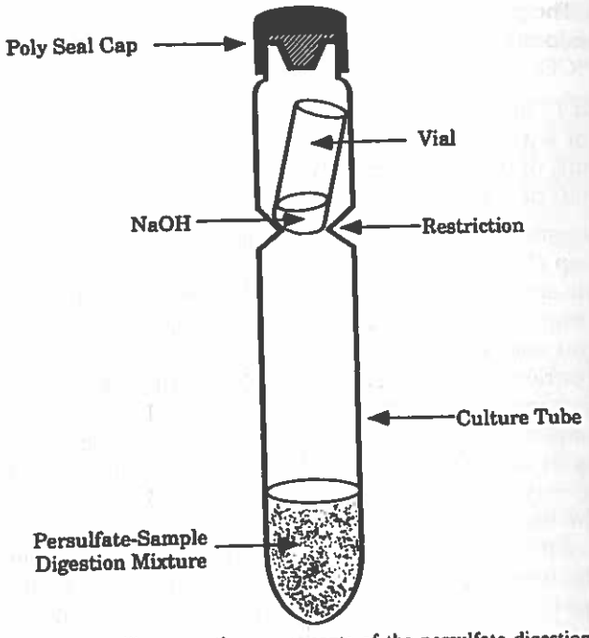

Fig. 36–4. The diagram illustrates the components of the persulfate digestion. The sample (extract of soil), digestion chemicals, and CO_2 trap are placed into the modified culture tube and sealed with a Poly Seal cap. Care must be exercised to avoid heating the cap excessively during the digestion that may distort the seal.

C_f = C in the fumigated extract

C_{uf} = C in the unfumigated extract.

The value of K_{ec} is the proportion of the microbial C that is extracted from the soil. The extraction of labile microbial C rendered soluble by fumigation may partition differentially between soil organic matter, clay, and extractant for different soils. Therefore, the value of K_{ec} will depend on the physical and chemical properties of the soil. Voroney et al. (1991) suggests a K_{ec} of 0.35 as a general value for microbial C-extraction efficiency.

36–3.1.5 Calculation of Biomass Nitrogen

Biomass N is calculated as the flush of N from a fumigated soil less that extracted from an unfumigated soil:

$$\text{Biomass N} = (N_f - N_{uf})/K_{en}$$

where

N_f = total N from the fumigated soil extract

N_{uf} = total N from the unfumigated extract

The value of K_{en} is the efficiency of extraction of organic microbial N and inorganic N from soil. The efficiency of extracting organic N has the same theoretical limitations associated with soluble organic C. The variable K_n equation (Voroney & Paul, 1984), discussed in section 36–2.1.6, has been used with some success to correlate to CFI (Davidson et al., 1989). Brookes et al. (1985) suggest an extraction efficiency of 0.68 across several soils for a 5-d fumigation period. A realistic value of K_{en} would have to be developed for each soil to determine exact values for biomass N. As in the case of CFI, the value of K_{en} should be included with all data sets to facilitate comparison with other microbial biomass values.

36–3.2 ATP Determinations

Much effort has been expended over the last 20 yr searching for efficient extractants of ATP from soils and sediments. Extractants such as H_2SO_4, DMSO, butanol, Tris buffer, $NaHCO_3$-$CHCl_3$, $NaHCO_3$, and $HClO_4$ have been tested (Lee et al., 1971; Conklin & MacGregor, 1972; Paul & Johnson, 1977). The ideal extractant should disrupt microbial cells rapidly, stabilize ATP by deactivating synthesizing and degradative enzymatic processes and quantitatively remove ATP from the soil matrix (Nannipieri et al., 1990).

Multi-component extractants hold promise for the quantitative extraction of cellular ATP from soil (Jenkinson & Oades, 1979; Webster et al., 1984; Martens, 1985). Jenkinson and Oades (1979) developed an extractant consisting of trichloracetic acid, Na_2HPO_4, and paraquat (TCAPP). A phosphoric acid mixture (PA) developed by Webster et al. (1984) was found to be more efficient than 12 other methods, including TCAPP. However, this work was done on oven-dried soil, and interpreting the results is difficult. Ciardi and Nannipieri (1990) found that the PA mixture recovered ATP 1.7 to 3 times more efficiently from two soils under different agronomic management than either the TCAPP or a $NaHCO_3$-$CHCl_3$ phosphate-adenosine mixture (Martens, 1985). Arnebrant and Bååth (1991) found no difference in ATP-extractant efficiencies in forest humus with TCAPP, PA or an extractant (500 m*M* of H_2SO_4 and 250 m*M* of Na_2HPO_4) proposed by Eiland (1983). In many cases, the light output of the luciferase reaction is influenced by the extracting agent and buffers and careful use of controls and standardized conditions are necessary so that extraction efficiencies of extractants can be thoroughly scrutinized.

Paraquat used in the TCAPP can be difficult to prepare and is listed as a hazardous substance at many research institutes. The PA method has been said to be an equally efficient extractant as TCAPP and has been employed on a variety of soils and sediments (Gregorich et al., 1990; Kieft & Rosacker, 1991; Arnebrant & Bååth, 1991). A variety of commercial extractants applicable to soil are also available (Fallon & Obrigawitch,

1990) but will not be discussed. We will describe the PA extractant in detail, but emphasize that no extraction methods have been thoroughly scrutinized over a broad range of soils.

36–3.2.1 ATP Extraction Method

The components of the PA mixture (Webster et al., 1984) are designed to quench metabolic processes by destabilizing cell compartmentalization and solubilizing ATP. The H_3PO_4 extracts ATP, inactivates ATPases and saturates phosphate group binding sites. EDTA chelates metal ions and prevents inhibition of the luciferin-luciferase reaction. The adenosine molecules saturate ATP-binding sites. Urea quenches metabolic reactions by denaturing enzymes that catalyze the metabolism of nucleotides. Dimethylsulfoxide (DMSO) and detergents such as polyoxeyethylene 10 laryl ether remove and lyse cells from soil surfaces. The procedure as adapted from Webster et al. (1984) modified by Vaden et al. (1987) and recently updated (F.R. Leach, 1992, personal communication) is outlined below.

36–3.2.1.1 Reagent Preparation. Dissolve 0.5 g of polyoxeyethylene 10 laryl ether (Sigma, St. Louis, MO) in 34 mL of warm water at 45 °C and maintain at 35 °C.

10 *N* of phosphoric acid, 228 mL (reagent grade) L^{-1} of water.

10 *M* of Urea (Fisher Scientific, Fair Lawn, NJ), 600 g L^{-1} of water, warm to dissolve, use at room temperature.

Dimethylsulfoxide (DMSO) (J.T. Baker Inc., Phillipsburg, NJ).

Adenosine (Sigma Chemical Co., St. Louis, MO) 2.5 g 500 mL^{-1} of water, warm to dissolve, use at room temperature.

1 *M* of EDTA (Fisher Scientific, Fair Lawn, NJ), 45.22 g 100 mL^{-1} of water.

Water used to make the above solutions should be purified, filtered through a 0.22-μm filter and autoclaved.

Combine the above in the following order:

34 mL of warm polyoxeyethylene 10 laryl ether
20 mL of 10 *N* phosphoric acid
20 mL 10 *M* of Urea
20 mL of DMSO
4 mL of adenosine solution
2 mL of 1 *M* of EDTA

36–3.2.1.2 Extraction Procedure. Soil samples (1–2 g wet wt.) are combined with 10 mL of the extractant in a sterile centrifuge tube and sonicated for 1 min (see Webster et al., 1984) or shaken vigorously for 30 min. The sample is then centrifuged (30 000 × *g*, 20 min, 20 °C). The supernatant is then diluted with 0.1 Tricine buffer (pH 11.2) yielding a desired pH of between 7 and 8.

36–3.2.1.3 ATP Measurement. ATP is measured in a reaction vessel containing 50 μL of sample; 100 μL of Analytical Luminescence Laboratory's fire fly luciferase (Firelight); and 50 μL of Tricine buffer pH 7.8 containing 25 m*M* of Tricine, 5 m*M* of $MgSO_4$, 1 m*M* of EDTA and 1 m*M* of dithiothreitol. Light production from the luciferin-luciferase reaction vessel is best measured on a ATP Photometer (Integrating Photometer). Since many models of photometers exist, it is left to the reader to develop a protocol for individual models. The luciferin-luciferase light reaction can also be analyzed in a Liquid Scintillation Spectrometer with gain set at 100%, narrow window setting and photomultiplier tubes switched out of coincidence (Jenkinson & Oades, 1979).

Variations of this procedure have been reported and applied to various soil and sediment samples and it is up to the reader to decide what modifications will be incorporated into their protocols (Ciardi & Nannipieri, 1990; Kieft & Rosacker, 1991; Arnebrant & Bååth, 1991). Internal standardization of this procedure can be done using either added *E. coli* cells (10^8 cells, see Webster et al., 1984) or by amending the extractant or soil with a known quantity of ATP (5 μ*M*). Additionally, the determination of adenylate energy charge can be done to measure metabolic energy stored in cells. Since this is not a measure of microbial biomass size, readers are referred to Vaden et al. (1987) for the analytical procedure and Nannipieri et al. (1990) for a review of this topic.

36–3.2.2 Calculation of Biomass

The relationship between C metabolized and biomass synthesized lead to the assumption that catabolic and anabolic reactions are synchronized (Tempest & Neijssel, 1987). However, studies have indicated that growth energetics and yield, especially ATP formation, are not coupled when growing conditions are adverse (Karl, 1980). Soil microorganisms are generally believed to be substrate limited, which complicates the relationship between ATP and cell biomass. Data obtained from ATP assays are difficult to relate to total microbial biomass determinations (biovolume or C).

Biomass C/ATP ratios ranging from 171 (Tate & Jenkinson, 1982) to 400 to 500 (Sparling, 1981; Martens, 1985), with many values in between, have been reported for a variety of soils. Long-term soil incubation experiments have suggested that little change in ATP concentrations occurs as the biomass is slowly starved of fresh substrate input (Brookes et al., 1987; Joergensen et al., 1990). However, results from amendment experiments show changing levels of ATP associated with substrate and time (Paul & Johnson, 1977; Nannipieri et al., 1978; Martens, 1985; Rosacker & Kieft, 1990). The discrepancy between reported results makes it difficult to compare data across a wide range of soils. It would be wise to supplement ATP determinations with one or more other biomass methods so that biomass data from different soils can be better compared.

36–4 COMPARISON OF METHODS

We have described several alternative and complementary methods since all are subject to different interpretations and require careful standardization for specific soil types. CFI, being one of the first methods developed, has been used as a baseline for correlations, but CFE is gaining acceptance because of its greater simplicity and lack of problems with the interpretation of a control. Both the CFI and CFE assay components of microbial biomass necessary to interpret nutrient-cycling processes, soil organic matter dynamics, cultural practices, and inputs associated with agronomic and natural systems.

The SIR method describes the soil microbial biomass by assaying the respiration dynamics of organisms as a result of added substrate and is useful to interpret temporal biomass changes and activity as affected by management and anthropogenic inputs. The respiratory quotient or CO_2 evolved by soil relative to the biomass as determined by SIR is particularly useful in cross comparing sustainable agriculture management techniques (Anderson & Domsch, 1989). The ATP method has been criticized because of the wide variance between ATP content and biomass C; it does, however, hold promise for studying microsites such as aggregates, rhizosphere, and deep sediment samples, since only a small amount of sample is required. It also is rapid and could be particularly useful for intra-site comparisons where a large number of replicates or treatments are involved.

We recommend careful use of internal standards and cross-referencing to other methods described in this chapter. If this is done, meaningful comparative values of microbial biomass can be determined by the method of choice. It is difficult to maintain soils at a known microbial biomass. If this were possible, the distribution of standard soil samples could eliminate problems in interpretation between laboratories.

The correlation of data from other methods to the results from CFI builds in the limits involved in this technique. Direct microscopy although slow and susceptible to differences in which person does the counting should be more often used to standardize techniques. This technique also gives estimates of sizes of organisms and fungal/bacterial ratios. Meaningful comparisons within sites on one study are fairly easy to achieve with any of the methods. Cross-site and cross-investigator comparisons, however, are also important. These should involve a thorough reporting of measurements made for $K_{c(ec)}$, $K_{n(en)}$, and control extractions. There is, unfortunately, too much information in the literature where internal standardization and cross comparison of techniques for a specific soil have not been carried out. We hope that results in the future will provide more meaningful cross-site data.

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