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The Determination of Microbial Biomass

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The soil biota represents 1–3% of the soil carbon and 3–5% of soil nitrogen. The microbial component, often ranging from 100 to >1000 μg C g^{-1}, is usually considered to contain those soil organisms not visible without magnification. It includes nematodes, protozoa, filamentous and yeast forms of the fungi, microalgae, and bacteria. The bacteria, in turn, contain great diversity, including the actinomycetes, archae, and chemo- and photolithotrophs plus many still uncultured forms. Often the effects of climate, landscape, soil type, and management on ecosystem functioning and nutrient dynamics in the environment are most easily interpreted through the highly responsive microbial component. Microbial biomass acts both as a nutrient reservoir and as a catalytic force in decomposition, and is crucial for understanding nutrient fluxes within and between ecosystems (Smith and Paul 1990). Microbial biomass can also be a sensitive indicator of environmental toxicity attributable to pesticides, metals, and other anthropogenic pollutants. Because microbial biomass is a sensitive indicator of many belowground components and interactions, it is an important measurement for cross-site comparisons in ecosystem studies.

Microbial biomass can be used to describe steady-state soil characteristics. It is more useful, however, when the dynamic nature of the microbiota is taken into account. Changes in size and composition represent the influence of above- and belowground inputs, climate, and disturbance. Although the MB is usually greatest near the soil surface, it occurs throughout the rooting zone and often at great depths in the vadose layer and in deeper subsurface sediments such as oil deposits.

The turnover rate of the biomass cannot be readily measured by changes with time because of concurrent growth. Tracers can be used to show the movement and turnover of nutrients in this active pool, especially when combined with mathe-

matical modeling. Growth studies also can be conducted by measuring the incorporation of specifically labeled substrates such as ^{3}H thymidine or ^{14}C leucine (Christensen and Christensen 1995; Harris and Paul 1994). A knowledge of the growth rate makes it possible to determine values for maintenance energy. The amount of substrate utilized for maintenance energy is of significance in many soils because of the slow turnover rate of the microbial biomass in these soils. Available methods include microscopy (Fry 1990) and the measurement of cell constituents released on fumigation (Horwath and Paul 1994; Joergensen 1995). Other methods include component analyses such as ATP (Inubushi et al. 1989), phospholipids (Tunlid and White 1992), ergosterol (Newell 1992), and ninhydrin-reactive materials (Amato and Ladd 1988). Substrate-induced respiration (SIR) is an activity-based method that has been applied to the measurement of biomass (Anderson and Domsch 1978). These techniques have not been conducted on a sufficient number of soils for us to be able to recommend them for routine measurements.

Microscopy facilitates the determination of size and shape, as well as the numbers of organisms such as bacteria, fungi, yeasts, protozoa, and diatoms. Fluorescent stains (Bloem et al. 1995b) make it possible to identify and measure the biota in the soil matrix. Analysis of digitized images by computer software can eliminate much of the operator error in cell identification and measurement. However, the high cost of the microscope and associated image analysis equipment and the lengthy time involved in analysis are deterrents to the use of this method. The relation of biovolume to biomass also is confounded by the fact that microorganisms can have variable C, N, S, and P contents.

Chloroform fumigation kills most soil organisms and destroys their membranes and cell walls. Fumigation can be combined with incubation (chloroform-fumigation-incubation, or CFI) to release the C as CO_2 and the N as NH_4. Alternatively, fumigation can be followed by extraction of the cell constituents (chloroform-fumigation-extraction, or CFE). Both approaches recover elemental cell constituents, making it possible to utilize tracers to determine pool sizes and turnover rates. A direct estimate of the pool size and turnover rate of the microbial biomass is a prerequisite for studies involving the role of the microbiota in organic matter dynamics and nutrient cycling. The tracers ^{14}C, ^{13}C, ^{15}N, and ^{32}P can be directly determined after lysis with $CHCl_3$ and incubation (CFI) or extraction (CFE).

Microscopy has been used to standardize fumigation methods. Although slow and tedious, it provides a number of advantages that include estimates of cell size and shape and bacterial-to-fungal ratios, as well as providing an overall estimate of biovolume. It is applicable to a wide variety of samples such as peats, sediments, litter, and contaminated sites where there can be problems using chemical approaches. Both fumigation methods can be set up to handle larger numbers of samples than microscopy within restricted time periods. The CFE method is faster than CFI and is now used on many sites where it gives reproducible results. The CFI method requires an incubation period but directly gives the products of decomposition as CO_2 and NH_4, which are readily measured. In some soils we have found CFI to give better reproducibility than CFE. Here we present methods for microscopy, CFI, and CFE. No one method is best suited for all purposes. All need to be calibrated and standardized before use. There is no simple approach to standardization of biomass measurements. Samples fixed on slides for microscopy are transportable between laboratories and should be used for comparison if possible. The interchange, between laboratories, of soil with a living biomass is not easy. Preincubating soil samples at constant moisture and temperature for 7 days results in a fairly stable biomass. Such samples could be sent to colleagues for cross-comparison. Sample standardization should be a prerequisite for funding and publication. When properly standardized, the different approaches give estimates that are reasonably well correlated with each other (Smith and Paul 1990; see also Wardle and Ghani 1995).

Soil Sampling and Handling

Sampling, in interdisciplinary studies, nearly always involves a number of analyses from one set of samples. As long as it is realized that one is working with living organisms, the sample coring and handling techniques used for other analyses are satisfactory for biomass determinations. Because biota are very site-specific and change during the season, compositing, replication, and recognition of site variability are most important and must be incorporated into the sampling design. The samples, stored in bags that retain moisture but allow respiration (thin polyethylene), should be placed in a temperature-controlled environment immediately upon sampling. A portable cooler has been found satisfactory for transportation. Immediate processing, although most desirable, is often not possible. Overnight storage at 5 °C or 15 °C depending on the original soil temperature is often used. The recognition that a delay in processing of field samples is often impossible to avoid has led Joergensen (1995) to recommend that all samples be preincubated for 5–7 days under laboratory conditions before analysis. This attenuates some of the disturbance associated with sampling but does not allow measurement of dynamic situations.

Sieving removes plant debris and large solids and provides mixing to decrease sample heterogeneity. Small soil samples (20 g) are attractive because of economy in containers, extractant volumes, and overall sample handling capacity, but they increase sample variance compared with larger samples. Fifty gram samples obtained from well-mixed composite subsamples are recommended as a compromise. Either 2 or 4 mm mesh sieve sizes are recommended, depending on the needs for other analyses also being conducted. Sieving removes much of the litter fraction and its associated microbiota. This should be analyzed separately.

Adjustment of the water content of medium-textured soils to 50% of water-holding capacity (WHC) is recommended before proceeding with either analysis. Water-holding capacity (WHC) is defined as the gravimetric water content of sieved soil that has been saturated and allowed to drain for over 24 hours in a filter funnel at 100% humidity. Some sandy or high clay soils will require adjustment based on water potential (−5 to 10 kPa) rather than WHC (Voroney et al. 1993). The CFI method is not satisfactory for waterlogged soils due to methane production (Inubushi et al.

1984), but CFE can be used (Inubushi et al. 1991). Dryness affects CFE more than it does CFI. In CFE, moist soils are required for good fumigation and for the required proteolysis thereafter (Sparling and West 1989).

Microscopy

Fluorescence microscopes combined with a skilled observer or image analysis software can differentiate between the soil biota and other similar-sized particles such as clay minerals. Fluorescent stains that have been tested for soil microbial biomass measurement (Kepner and Pratt 1994; Bloem et al. 1995a) include protein stains such as fluorescein isothiocyanate (FITC) and DTAF (5-(4,6-dichlorotriazin-2-yl)aminofluorescein)). Acridine orange, europium chelate, and DAPI (4′,6-diamidino-2-phenylindole) stain nucleic acids. Research on fungal stains has concentrated on cell walls because many fungi have large sections free of cytoplasm. The stain now most often used for fungi is fluorescent brightener calcofluor white M2R (Bloem et al. 1995a).

Other available staining techniques include those that measure cell activity such as fluorescein diacetate (FDA) activated by esterase (Söderström 1979). The redox-sensitive dye INT (tetrazolium chloride), when reduced by microbial electron transfer, produces formazan visible within the cell (Dutton et al. 1983). The fluorescent redox probe, 5-cyano-2,3 ditolyl tetrazolium chloride, allows one to utilize the superior differential viewing of the fluorescence microscope to separate active from inactive cells (Rodriguez et al. 1992). Techniques that allow the use of molecular probes for direct analyses under the microscope open the field for phylogenetic analyses of that huge population of soil biota that can be microscopically seen but are not culturable. They are not yet available for routine analysis. Herein we report on the use of fluorescence microscopy using DTAF staining for bacteria and calcofluor M2R for fungi.

For microbial biomass measurements, the soil must be adequately dispersed and placed onto a microscopic slide or appropriate filter as a thin film. Smears produce flatter preparations with better contrast then membrane filters and are recommended for bacteria. Fungi in some soils do not adequately stick to smears; if so, filters instead of smears should be used for fungi. The individual fields must be selected and the biovolume in each field determined through measurement of the size, shape, and number of organisms.

Materials

1. Buffer: 0.05 mol/L Na_2HPO_4 (7.8 g/L) in 0.15 mol/L NaCl (8.8 g/L) adjusted to pH 9.0
2. DTAF stain: 2 mg (5-(4,6-dichlorotriazin-2-yl) aminofluorescein) in 10 mL buffer. Prepare fresh daily.
3. Calcofluor M2R (fluorescent brightener) stain: 2 g/L in water. Stain is stable at room temperature for 1 month.
4. Filter stains, buffer, and wash water filtered through a 0.2 μm filter

Procedure

Slide Preparation

1. Homogenize 10 g soil in 190 mL filtered water for 1 minute at full speed in a Waring blender.
2. Allow coarse particles to settle for 30 seconds. With a wide-bore pipette remove a bulk (5 mL) sample to a capped test tube.
3. Add 0.1 mL formalin (40% aqueous formaldehyde) as a preservative.
4. Vortex this bulk sample before subsampling to prevent further sedimentation.
5. Place 4 μL drops of soil suspension onto each 6 mm diameter "well" of printed slides (e.g., Bellco toxoplasmolysis slides). Spread the suspension uniformly across the well with the pipette tip without touching the tip to the slide surface.
6. Allow the smears to air dry completely; this fixes the organisms to the slide. Prepare two slides for each sample; one will be stained for bacteria, the other for fungi.

Staining Bacteria

1. Flood each smear (well) with 8 μL DTAF stain.
2. Store slides in a container with wet tissue (100% relative humidity) for 30 minutes.
3. Remove excess stain by placing slides in a series of staining jars containing buffer; make three changes of buffer (30 minutes each) and finally a change of water (30 minutes).
4. Air-dry slides flat.
5. Add a small drop of low-fluorescence immersion oil (Cargille type FF recommended) to each smear and cover the slide with a 50 × 25 mm coverslip.

Staining Fungi

1. Flood each smear with 10 μL Calcafluor M2R stain.
2. Stain in a covered container with wet tissue (100% relative humidity) for 2 hours.
3. Rinse by soaking slides in water in a staining jar for 30 minutes, three times.
4. Air-dry slides flat.
5. Add small drops of low-fluorescence immersion oil (Cargille type FF recommended) to each well and cover slide with a 50 × 25 mm coverslip.

Bacterial Counting and Biovolume Estimation

Observe the green bacteria using a fluorescence microscope with a 63× or 100× oil-immersion objective. Suitable filters are excitation 450–490 nm, dichroic 510 nm, and suppression <515 nm. The bacteria may be counted by image analysis or by a human observer. Counting by humans is not recommended because it is difficult to achieve consistency between individuals and by the same individual over time. It is also extremely difficult to estimate the dimensions of individual cells by

direct observation of fluorescent images because of their small size and unresolved edges. Important advantages of image analysis over human counting are consistent application of rules so that the same features in the image are counted on any occasion. It is possible to estimate the dimensions and volume of each cell counted. Collection of images typically is much faster than human counting, so photobleaching of the fluorochrome is minimized. Analysis of the images can be automated and run as a batch process separate from image acquisition. Comparisons of automatic image analysis counts with those made by skilled human observers show that average numbers of bacteria counted are similar but that the variance of counts on replicate slides by the image analysis system is greater (Bloem et al. 1995b; Harris, unpublished data). This effect is probably the result of the unconscious effort of the human counter to find more bacteria in slides where there are few and to miss bacteria where there are many.

Image analysis procedures are presented in the Appendix. Because relatively few laboratories will be suitably equipped, we describe a manual counting method that can be used in the absence of image analysis equipment. An ocular grid (10×10 squares) that encloses approximately 50% of the field of view is used to define a counting area in each field of view. The dimensions of the grid can be determined with a stage micrometer. A mechanical counter or simple computer program (Bloem et al. 1992) is used to tally the counted bacteria in each field. The observer should work methodically through the array of 100 squares within the grid, counting each cell recognized as a bacteria. Any cells crossed by the upper and left limits of the grid should be excluded, while those crossed by the lower and right lines are counted. Fields should be prearranged on equatorial transects of the smear such that at least 300 cells per smear are counted. Four to six replicate smears are recommended. Assuming a Poisson distribution, the 95% confidence interval is approximately twice the square root of the count. If $4 \times 300 = 1200$ cells are counted, then the 95% confidence interval is about $\pm$ 5%, equivalent to 60 cells.

It is too slow to manually measure the dimensions of individual cells in fluorescent images because of photobleaching. A subsample of fields should be photographed on 35 mm transparency film (ISO 400 or faster), and the images should include a size marker or grid. The dimensions of bacteria can be measured more easily and accurately in projected images calibrated with the known scale. Two perpendicular diameters represent the maximum and minimum dimensions of the cell. The transparencies can also be used to train counters and to test interoperator calibration, and they may be archived for comparison between dates, experiments, and sites.

The bacterial biovolume is calculated from measurements of the large and small diameters of each cell by assuming that the cells are prolate spheroids (Sieracki et al. 1989):

$$bv = (\pi/6) \times d^2 \times l$$

where

bv = bivolume
d = small diameter
l = large diameter

The average bacterial volume from the measured sample can be multiplied by the count of bacteria per smear to give bacterial biovolume per smear.

Fungal Counting and Biovolume Estimation

Observe the Calcofluor M2R (blue)–stained fungi under epifluorescence. Suitable light filters are excitation 340–380 nm, dichroic 400 nm, and suppression <430 nm. Fluorescent dyes usually fail to stain heavily melanized hyphea, which may be common or prevalent in some soils. Low magnification ($10 \times$ objective), to include a relatively large field, can be used with imaging systems. A method for measurement of fungal hyphae by image analysis is given in the Appendix (see also Morgan et al. 1991; Daniel et al. 1995). Higher magnification objectives (25–40×) may be needed for manual measurements, especially for hyphal diameter estimation. Increased magnification decreases the frequency of hyphal fragments per field; use the lowest magnification that allows clear discrimination of hyphae.

Manual measurements of hyphal length can be made by the grid intercept method (Newman 1966) where intersections between hyphae and a set of lines of known length distributed in a fixed area are counted. Hyphal length (hl) is estimated by:

$$hl = (\pi \times n \times a)/(2 \times l)$$

where

hl = length of hyphae, in μm
n = the number of intersections
a = the defined area, in μm^2
l = the length of all the lines, in μm

The 10×10 ocular grid used for bacteria counting is suitable. Scan the grid and count each contact between hyphae and each of the lines of the grid. In soils with relatively low hyphal populations (<100 m hyphae/g soil), many microscope fields will contain no hyphae. For example, in a smear prepared as described earlier, from a soil with 100 m hyphae/g, a 1 mm^2 grid with a total line length of 22 mm would contain about 6 μg soil and an average of about 600 μm hyphae corresponding to an average of 8.4 grid intersections. In our experience 1 minute homogenization at full speed in a Waring blender yields hyphal fragments with a median length of about 40 μm, which is close to the optimum fragment size recommended by Sundman and Sivelä (1978). The error of the measured hyphal length is proportional to the number of hyphal fragments measured (Hanssen et al. 1974); therefore, preparations with larger hyphal fragments require a larger counting area (more fields) for equal precision.

Hyphal diameters can be estimated by visual comparison with the lines in the grid, by using an ocular scale, or, better, by measurement of projected photographic images. The volume (hv) of each hyphal fragment can be calculated as a cylinder:

$$hv = \pi \times (d/2)^2 \times l$$

where

hv = hyphal volume, in μm^3
d = diameter, in μm
l = length, in μm

The hyphal biovolume per image is the sum of the volumes of the fragments.

Many, perhaps most, hyphae in soil are apparently dead or empty. This poses the greatest problem in the estimation of fungal biomass by microscopic means. Vital staining (activity detecting) with FDA (Söderström 1979; Ingham and Klein 1984) has been tried but fails to penetrate many hyphae. Other vital stains such as sulphofluorescein diacetate (SFDA; Tsuji et al. 1995) or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Rodriguez et al. 1992) may prove useful in this regard. However, in preliminary work with CTC many regions of soil hyphae where dye reduction occurred were colonized by superficial or intralumenar bacteria; these appeared to be the active agents in dye reduction (D. Harris, unpublished data).

Calculations

Mass of Soil Per Image (field of view)

The initial suspension (200 mL) contains 10 g moist soil, and each smear contains 4 µL of the suspension. Thus each smear contains the following mass of soil:

$$\text{g soil per smear} = \{10\text{g soil}/[(\%H_2O/100) + 1]\} \times (1/200\text{ mL}) \times (4/1000\text{mL})$$

The mass of soil per field is given by

$$s = [(x \times y)/(\pi \times r^2)] \times \{10/[(\%H_2O/100) + 1]\} \times (2/10^5)$$

where

s = soil mass per field
x = length of the counting area, in mm
y = width of the counting area, in mm
r = radius of the smear, in mm

The sum of the volumes of bacteria (bv) or fungal hyphae (hv), divided by the appropriate mass of soil (s), represents biovolume per gram soil ($\mu m^3/g$). If filters are used, instead of smears, for fungi the calculations will need to be modified.

Calculation of Biomass

The biovolume is converted to biomass C using a conversion factor for the C content per unit volume. Conversion factors for C have been estimated by a variety of methods using different preparation techniques, organisms, and growth conditions (van Veen and Paul 1979; Bakken and Olsen 1983; Bratbak and Dundas 1984; Fry 1990; Bloem et al. 1995b). These factors have shown a range for the C content of bacteria of 150–310 $fg/\mu m^3$. Norland (1993) compared four models of variation in the C content-to-volume ratio with cell size in marine bacteria. In these models the

C content ($fg\ C/\mu m^3$) increases as biovolume decreases. The variation in C content of bacteria with cell size may help to explain some of the differences in reported conversion factors. Three of these models, applied to our measurements of soil bacteria, gave biomass C values that agreed well (±5%) with those obtained using the factor 200 $fg\ C/\mu m^3$ (Bloem et al. 1995b). Carbon contents of cultured fungi were estimated by van Veen and Paul (1979) and correspond to 150 $fg/\mu m^3$.

Chloroform-Fumigation-Incubation

We describe both chloroform fumigation techniques: (1) the release of CO_2 and NH_4 by mineralization in a 10 day incubation following fumigation (CFI) and (2) the extraction of cell constituents by K_2SO_4 after fumigation (CFE). The CFI method has been calibrated for temperate soils against direct microscopy and by the addition of cells with known C and N contents (Jenkinson 1976; Voroney and Paul 1984). Calibration factors for soils of nontemperate regions have not yet been developed. The major problem with CFI is the question of what proportion of the C mineralization flush following fumigation is due to continued mineralization of soil organic matter rather than mineralization of the killed biomass; this is still controversial (Wu et al. 1996). Studies using ^{14}C-labeled straw additions to soil indicate that fumigation reduces but does not eliminate soil organic matter mineralization (Smith et al. 1995; Horwath et al. 1996). The respiration of the unfumigated control soil has been used to estimate this background mineralization rate. But many soils have respiration rates comparable to the CO_2 flush following fumigation. This, if subtracted from the mineralization in the fumigated sample, can result in a low or even negative microbial biomass C estimate. The opposite assumption, that all of the 10 day postfumigation CO_2 flush arises from mineralization of the killed biomass, can result in excessively high microbial biomass C estimates.

Development of a method for estimating the proportion of the unfumigated control mineralization to subtract for each sample (Horwath et al. 1996) has overcome the control problem in CFI. The proportion (p) of the control to subtract is calculated by using the ratio of CO_2 mineralized in the control (C_C) relative to that in the fumigated sample (C_F). Two parameters, k_1 and k_2, are used to relate C_F/C_C to p. These have been obtained by fitting the data obtained by fumigation in a series of soils to the biomass estimated by microscopy (Horwath et al. 1996).

The control problem is not as serious with biomass N determinations by CFI because the unfumigated control values for N mineralization are typically low relative to those in fumigated samples (Jenkinson 1988). However, the N mineralized in CFI is affected by the C:N ratio of the biomass before and after fumigation and by the growth yield of the biomass that develops after fumigation. This results in the mineralization of variable proportions of the biomass N and may lead to net immobilization of N, especially where fungi are prevalent. Harris et al. (1998) adjusted for this variation in biomass C:N by relating the mineralization of N and C after fumigation to the N:C ratios of organisms added to soil. The parameters of this relationship were found applicable to other soils; this approach allows calculation of biomass N even for soils where CFI results in net immobilization of N.

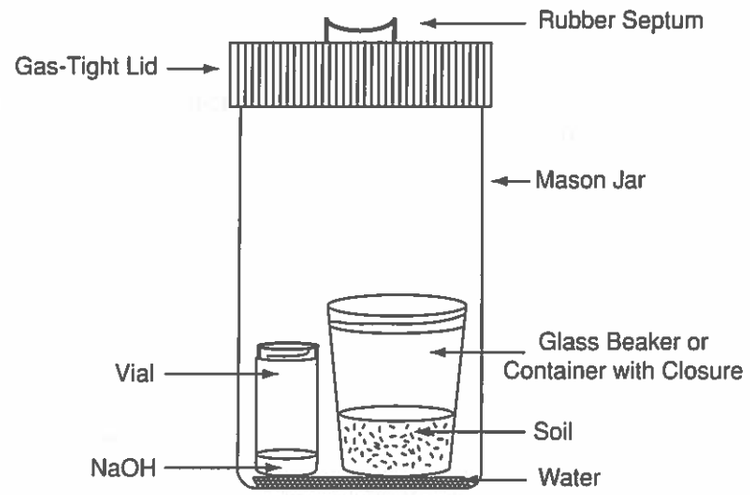

Figure 15.1. Soil incubation jar. The respirometer (vial + NaOH) is not used when mineralized CO_2 is sampled using the gas chromatography (GC) method. The rubber septum is not needed when using the respirometer method to assay CO_2.

Materials

1. Commercial chloroform stabilized with ethanol (0.75%)
2. Anhydrous K_2CO_3
3. 2 mol/L NaOH solution
4. 1 L separatory funnel
5. 100 mL glass beakers for fumigated samples. Containers for controls need not be glass.
6. Incubation containers of approximately 1 L (Fig. 15.1). Home canning jars, such as Mason jars, are readily available and work well if new lids are used.
7. 20 mL scintillation vials. (The dissolution of silica in base traps can be a problem with soda glass vials; high-density polyethylene vials have good base resistance but relatively high permeability to CO_2 and so should be protected from atmospheric CO_2 during any storage before titration.)
8. Large glass vacuum desiccators (20 L desiccators will hold about 20 soil samples in 100 mL beakers)
9. Aspirator or rotary vacuum pump with cold trap

Procedure

Soil Preparation

1. Mix and composite soil samples by sieving to 4 mm. Do this at field moisture (unless partial drying is necessary) to maintain microaggregate stability.

2. Determine the soil moisture content by oven drying samples of each composite.
3. Determine the WHC for each soil type (see Chapter 3, this volume).
4. Weigh 50 g (dry weight equivalent) samples into 100 mL beakers; three samples of each soil are required, one for initial mineral N determination plus one for control and one for fumigated samples. Label beakers to be fumigated with pencil (chloroform will dissolve most markers). Smaller samples (e.g., 20 g) have many advantages, such as the use of smaller containers and extractant volumes, but they may increase sample variability.
5. Add water to bring each sample to 50% of its WHC (or to −5 to −10 kPa).
6. Preincubate the samples for 5 days at 25 °C in closed incubation jars that have a few milliliters of water in the bottom to provide 100% humidity. This preincubation should be omitted in tracer experiments where nutrient dynamics are being measured.

Fumigation

In a fume hood:

1. Line the vacuum desiccator(s) with wet paper towels.
2. Put fumigation samples (approx. 20 in a 20 L desiccator) and a conical flask containing 75 mL ethanol-free chloroform and a few boiling chips in each desiccator.
3. Connect the desiccator(s) to a vacuum manifold that includes a vacuum gauge. If a rotary vacuum pump is used, it should be protected from excessive chloroform vapor with a cold trap (−20 °C or better) and should vent to a fume hood. Change the pump oil frequently. Alternatively, an aspirator pump may be used.
4. Evacuate the desiccators until the chloroform boils for 1 minute.
5. Incubate the samples in chloroform vapor in the dark at 25 °C for 24 hours.
6. After fumigation, open the desiccators in a fume hood and remove the paper towel and the flask of chloroform.
7. Remove the residual chloroform from the soil by evacuating the desiccator to 250 Pa and venting to atmosphere. Repeat this step six times. The meticulous removal of residual chloroform is essential.
8. During the fumigation the unfumigated control samples are incubated in closed canning jars.

Incubation

1. Check the sample weights and readjust the moisture content if necessary.
2. Place the fumigated and control samples in canning jars with an open 20 mL scintillation vial containing 2 mL of 2 mol/L NaOH (base trap) to trap CO_2 (for soils with very high organic matter content the volume or molarity of the trap can be adjusted to be equivalent to about twice the expected CO_2 flush). A few milliliters of water in the bottom of the jar will maintain 100% humidity. Open the canning jars of the unfumigated controls for at least 5 minutes

to equilibrate with atmospheric air. Breathing directly into the jars or preparing the samples in a small, poorly ventilated space (such as a temperature control room) can significantly increase the initial CO_2 concentration in the jars and should be avoided. The O_2 content of the canning jar should be adequate for the 10 day incubation (50 g soil producing 1 mg C/g as CO_2 would consume approximately 40 of the 200 mL of O_2 in the jar).
3. Include several blank jars that contain everything except soil.
4. Tightly close the jars and incubate for 10 days at 25 °C.
5. After 10 days remove the base traps and close with gastight screwcaps (Poly Seal).

Measurement of CO_2 Mineralized—Double End-point Titration

When no stable isotopic analysis of the CO_2 is required, the double end-point titration is preferred because it is a direct analysis of HCO_3^-.

Materials

1. Dual end-point automatic titrator (or pH meter with semi-micro combination electrode, magnetic stirrer and burette)
2. Carbonic anhydrase solution (1 mg/mL)
3. 2 mol/L HCl, not standardized
4. 0.3 mol/L HCl, standardized
5. Thymolpthalein indicator solution (Sigma Chemicals)

Procedure

1. Add 1 drop of indicator and a small stir bar to the base trap.
2. While stirring add 2 mol/L HCl from a pipette (or burette) until the indicator is very pale blue (pH 9.0–9.5; use a white surface under the stirrer).
3. Add 50 µL carbonic anhydrase solution.
4. Transfer the vial to the titrator and add 0.3 mol/L HCl to the first end point of pH 8.35.
5. Titrate to the second end point of pH 3.5.
6. Record the volume of acid added between the two end points.
7. Denatured carbonic anhydrase and indicator will accumulate on the pH electrode. It can be removed, as necessary, with 0.5 mol/L NaOH.
8. The sample titre (T) minus the blank (B) is equivalent to $(T - B) \times M \times 12$ mg C where M is the molarity of the HCl in µmol/L.

Measurement of CO_2 Mineralized—Single End-point Titration

Back titration of excess NaOH after precipitation of CO_3 with $SrCl_2$ is the preferred method when ^{13}C analyses are required because it enables collection of the C as $SrCO_3$, that can be collected for ^{13}C analysis in automatic mass spectrometers (Harris et al. 1997).

Materials

1. Automatic titrator (or pH meter with semi-micro combination electrode, magnetic stirrer and burette)
2. Standardized 0.3 mol/L HCl
3. 2 mol/L $SrCl_2$

Procedure

1. Add 2 mL 2 mol/L $SrCl_2$ to the base trap.
2. Titrate to pH 7.0 with 0.3 M HCl.
3. The blank titre (B) minus the sample titre (T) is equivalent to the CO_2 in the trap ($[B - T] \times M \times 6$ mg C when M is the molarity of the HCl in mol/L).

N Mineralized during Incubation

Materials

1. 1 mol/L KCl (use 2 mol/L for soils with high CEC)
2. 500 mL flasks or bottles
3. Filter funnels and multiposition rack
4. 15 cm Whatman no. 1 filter paper (a finer grade [no. 5] may be necessary for clay soils) washed in 0.1 mol/L HCl and rinsed with water

Procedure

There are three extractions for each soil sample, an initial (day 0) extract (N_0), at the start of the fumigation, and control (N_C) and fumigated (N_F) samples after the 10 day incubation.

1. Measure 250 mL 1 mol/L KCl.
2. Transfer the soil from beakers to the extraction flasks, use some of the KCl to wash residual soil from the beakers, and add the remainder to the flask.
3. Shake vigorously (keep the soil suspended) on a reciprocating or orbital shaker for 30 minutes. It may be necessary to use ultrasound to disperse strongly aggregated clay soils.
4. Filter the extract, and collect the filtrate, which may be stored frozen prior to analysis.
5. Analyze the extract for NH_4^+ and NO_3^-. Automated colorimetric analyzers are preferred.

Calculation of Biomass C and N from CFI

Five measurements are required: the 10-day C mineralization in fumigated and unfumigated soils (C_F, C_C), initial soil mineral N (ammonium plus nitrate), and mineral N in the fumigated and unfumigated samples after 10 days incubation. Four of these measurements are required for biomass C and N determination by previous

methods (Jenkinson 1976, 1988). The fifth measurement, that of initial mineral N, is often routinely conducted when taking such samples.

The amounts of CO_2–C evolved in the fumigated (C_F) and unfumigated (C_C) samples are used in the following equation to calculate the biomass C when using a corrected control (Horwath et al. 1996):

$$MBC = [C_F - (p \times C_c)]/k_c$$

where

MBC = biomass C in μg C/g soil
C_F = C mineralized from the fumigated sample (μg C/g soil)
C_C = C mineralized from the unfumigated sample (μg C/g soil)

$$p = [k_1 \times (C_F/C_c)] + k_2$$

where

k_1 = 0.29 (see below)
k_2 = 0.23 (see below)
k_c = 0.41 (see below)

The value 0.41 for the decomposition factor k_c is the average value determined by a number of laboratories for temperate soils incubated at 25 °C. Values for the parameters k_1 and k_2 have been estimated by minimizing the sums of squares of the differences between MBC and microscopic biomass of a number of soils. This equation has given meaningful biomass estimates in a number of soils using parameter values of k_1 = 0.29 and k_2 = 0.23 (Horwath et al. 1996). Substituting these values, the above equation can be simplified to

$$MBC = 1.73C_F - 0.56C_c$$

The calculation of biomass N is based on the determination of biomass C and the N:C ratio. The addition of organisms to soil was used to determine the parameters involved (Harris et al. 1998). Calculation of biomass N:C ratio (MBN/MBC) can be made without correction for mineralization of nonbiomass material in the fumigated sample:

$$MBN/MBC = (0.56 \times N_F/C_F) + 0.095$$

where

MBN = biomass N in μg N/g soil
N_F = N mineralized from the fumigated sample (μg N/g soil)
N_C = N mineralized from the control sample (μg N/g soil)

The preceding uses only the measured C and N mineralization following fumigation-incubation and parameters determined from added organism experiments

(Harris et al. 1998). Biomass N is obtained by multiplying biomass N:C by biomass C:

$$MBN = MBC \times \{[0.56 \times (N_F/C_F)] + 0.095\}$$

The biomass N:C estimate best reflects soil conditions when it includes the contribution of nonbiomass materials to N mineralization in the fumigated soil by using the following relationships:

$$MBN/MBC = \{0.56 \times [(N_F - qN_C)/(C_F - pC_C)]\} + 0.095$$

where

qN_C and pC_C = N and C mineralization, respectively, in the fumigated soil from sources other than chloroform-killed biomass
N_C = mineral N after 10 day incubation of unfumigated soil minus soil mineral N at day 0
N_F = mineral N after 10 day incubation of fumigated soil minus soil mineral N at day 0 and
$q = p = [0.29 \times (C_F/C_C)] + 0.23$

This calculation includes measurements of C and N mineralization in the unfumigated sample and a fraction p (Horwath et al. 1996) determined by calibration of CFI biomass C using microscopy. We found that the calculation with partial control correction yields values 20% higher than the uncorrected version. The corrected values should be more representative of the populations in nature than uncorrected ones because differences in the N:C ratio of biomass and nonbiomass materials decomposed in the fumigated soil are considered.

Chloroform-Fumigation-Extraction

Like the CFI procedure, the CFE procedure also has problems, although less severe, with controls. Here the considerations are the more difficult analysis of the low C and N contents of the extracts and the more involved preparations required when working with stable isotopic tracers. Some investigators have noted a greater inherent variability in the measurements (Sparling and Zhu 1993; Horwath and Paul 1994). Horwath and Paul (1994) also reported a lack of agreement in specific activity of ^{14}C obtained by CFE and CFI even though the overall correlation in total MBC between the two measurements was good. The radioactivity of the C extracted by CFE was one-half that of CFI. This verified findings by Merckx and Martin (1987) and Badalucco et al. (1990), who found anthrone and ninhydrin reactive material of nonbiomass origin in the CFE extract.

We give procedures for determination of C by persulfate digestion and N by Kjeldahl digestion. Other measurement methods are available. Dichromate digestion has been used for C (Jenkinson and Powlson 1976) and ninhydrin analyses for

N (Amato and Ladd 1988). Automated colorimetric methods for both C and N are also available (Voroney et al. 1993).

Materials

1. Ethanol-free chloroform prepared as in CFI procedure
2. Anhydrous K_2CO_3
3. 100 mL glass beakers for fumigated samples
4. Large glass vacuum desiccators (20L; each desiccator will hold about 20 soil samples in 100 mL beakers)
5. Aspirator or rotary vacuum pump with cold trap
6. 0.5 mol/L K_2SO_4
7. 500 mL flasks or bottles

Procedure

Fumigation

In a fume hood:

1. Line the vacuum desiccator(s) with wet paper towels.
2. Arrange fumigation samples (approx. 20) and a conical flask containing 75 mL ethanol-free chloroform and a few boiling chips in each desiccator.
3. Connect the desiccator(s) to a vacuum manifold that includes a vacuum gauge. If a rotary vacuum pump is used it should be protected from excessive chloroform vapor with a cold trap (−20 °C or better) and should vent to a fume hood. Change the pump oil frequently. Alternatively, a water (aspirator) pump may be used.
4. Evacuate the desiccators until the chloroform boils for 1 minute.
5. Seal the desiccator(s) and incubate at 25 °C for 24 hours in the dark. A 24 hour fumigation is widely used (Voroney et al. 1993), but a longer fumigation of 120 hour is recommended for full release of microbial C and N (Horwath and Paul 1994).
6. After fumigation, open the desiccators in a fume hood and remove the paper towel and the flask of chloroform.
7. Remove the residual chloroform from the soil by evacuating the desiccator to 250 Pa and venting to atmosphere six times.

Extraction of Microbial C and N

1. Add 250 mL 0.5 mol/L K_2SO_4 to the control samples; these should be extracted at the time the fumigation is initiated.
2. Measure 250 mL 0.5 mol/L K_2SO_4.
3. Transfer the fumigated soil from beakers to the extraction flasks; use some of the K_2SO_4 to wash residual soil from the beakers, and add the remainder to the flask.
4. Shake vigorously (keep the soil suspended) on a reciprocating or orbital shaker for 1 hour. It may be necessary to use ultrasound to disperse strongly aggregated, clay soils.

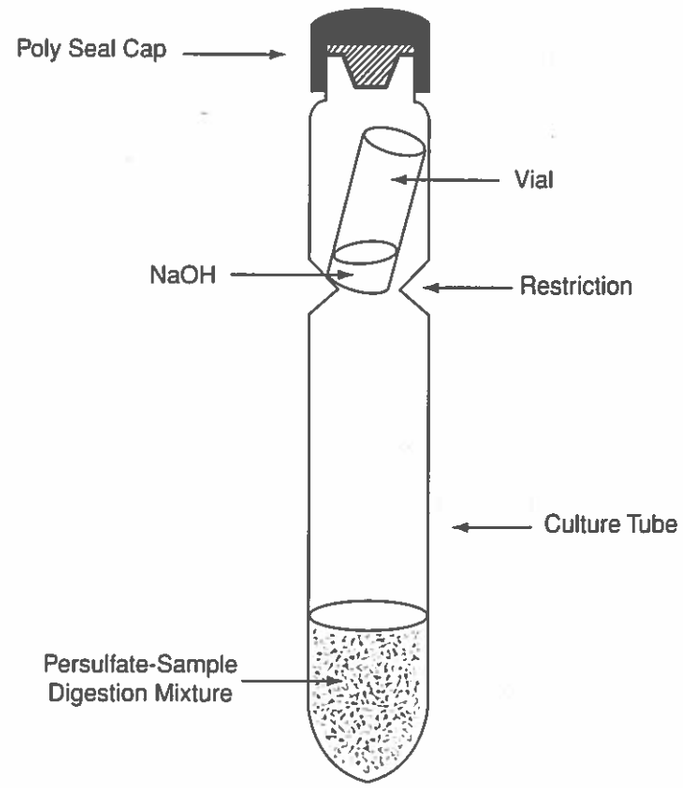

Figure 15.2. Components of the persulfate digestion. The sample, digestion chemicals, and CO_2 trap are placed into the modified culture tube and sealed with a Poly Seal cap. Avoid heating the cap excessively during digestion to avoid distorting the seal.

5. Filter the extracts through Whatman no. 5 filter paper and collect the filtrate; 5–30 mL is required depending on the method of analysis, although more may be needed for some isotopic analyses. The extracts should be stored frozen prior to analysis.

Analysis of Extract Persulfate Digestion (Total C)

Materials

1. 25 × 200 mm screw-cap culture tubes with Poly Seal caps. The tubes may be modified by forming a restriction at about 60 mm from the neck to support a vial containing alkali; otherwise the vial can be supported at about 150 mm on a bent glass rod (Fig. 15.2).
2. 15 × 45 mm glass vials
3. Heater block at 120 °C
4. 0.1 mol/L NaOH

5. Potassium persulfate ($K_2O_8S_2$)
6. 0.05 mol/L H_2SO_4
7. 0.05 mol/L HCl (standardized)

Procedure

1. Prepare an alkali trap containing 1 mL 0.1 mol/L NaOH.
2. Add 15 mL extract, 1 g $K_2O_8S_2$, and 1 mL 0.05 mol/L H_2SO_4 to the culture tube.
3. Insert the alkali trap, supported in the headspace of the tube by the restriction or by a glass rod.
4. Firmly cap the tube.
5. Heat the digests in the heater block at 120 °C for 2 hours. Do not allow the cap to get hot (it may leak or melt).
6. Remove the digests from the heater block and let stand overnight at room temperature to allow complete absorption of the CO_2.
7. Blanks should be prepared with 15 mL K_2SO_4.
8. Titrate the alkali traps by either the double or single end point (if ^{13}C is to be collected) methods (see earlier) except the titrator should be charged with 0.05 mol/L HCl.

Analysis of Extract—Kjeldahl Digestion (Total N)

Materials

1. 25 × 250 mm, Pyrex, graduated (100 mL), constricted-neck digestion tubes
2. Block heater capable of achieving >375 °C
3. Concentrated H_2SO_4
4. $CuSO_4 \cdot 5H_2O$
5. Selenized antibumping granules (Hengar)

Procedure

In an acid-resistant fume hood:

1. Add 3 mL concentrated H_2SO_4 followed by 100 mg $CuSO_4$ and 25 mL extract and 2–3 antibumping granules to the digestion tubes.
2. Heat the tubes to 150 °C to evaporate most of the water, then increase the temperature until the acid refluxes.
3. Digest at acid reflux temperature (about 375 °C) for 1 hour.
4. Allow the tubes to cool, then carefully add water, mix, and adjust to the 100 mL graduation mark. If the digest solidifies on cooling it may be necessary to reheat to dissolve the salts.
5. Analyze the digest for NH_4^+, preferably by a colorimetric autoanalyzer method.

Special Considerations

See also the alkaline persulfate digest for total N described by Cabrera and Beare (1993), which could replace the TKN method, described earlier, and probably could be adapted for simultaneous measurement of C and N in K_2SO_4 extracts.

Calculation of Biomass C and N from CFE

1. Volume of solution in extracted soil:

$$V = FW - DW + EV$$

where

V = volume of solution in the extracted soil (mL)
FW = soil fresh weight, as g
DW = soil dry weight, as g
EV = extractant volume, as mL

2. Mass of extractable C and N in the fumigated and control samples:

$$C_F = EC_F \times V/DW$$
$$C_C = EC_C \times V/DW$$
$$N_F = EN_F \times V/DW$$
$$N_C = EN_C \times V/DW$$

where

C_F = extractable C in fumigated sample in μg/g soil
EC_F = extractable C in fumigated sample in μg/mL extractant
C_C = extractable C in control sample in μg/g soil
EC_C = extractable C in control sample in μg/mL extractant
N_F = extracted N in fumigated sample in μg/g soil
EN_F = extractable N in fumigated sample in μg/mL extractant
N_C = extractable N in control sample in μg/g soil
EN_C = extractable N in control sample in μg/mL extractant

3. Microbial biomass C and N in soil:

$$MBC = (C_F - C_C)/k_{EC}$$
$$MBN = (N_F - N_C)/k_{EN}$$

where

kEC = extraction coefficient for extractable carbon as described below
kEN = extraction coefficient for extractable nitrogen as described below

Voroney et al. (1993) proposed values of 0.25 for k_{EC} and 0.18 for k_{EN} based on in situ calibrations (Bremer and van Kessel 1990). These values are substantially lower than other estimates and will give correspondingly larger biomass estimates. Vance et al. (1987) obtained a k_{EC} of 0.38 by correlating the data for CFE with that obtained by CFI in 10 Rothamsted soils. A k_{EC} of 0.35 was obtained by Sparling and West (1988) using in situ labeling techniques. In later work Sparling and Zhu (1993) found a range of values for k_{EC} (0.30 $\pm$ 0.18) and k_{EN} (0.38 $\pm$ 0.14) for a number of Western Australia soils by comparing CFE to CFI. We recommend the use of factors derived directly from the soils under study either by in situ calibration or by comparison with other methodologies. The values used for all calculations should be reported, and reference soils from other laboratories should be included in the study.

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Appendix: Measurement of Bacterial and Fungal Biovolume by Fluorescence Microscopy and Image Analysis

General System Specifications

Hardware

1. Fluorescent images are very dim compared with those produced by most other forms of light microscopy. The camera should be sufficiently sensitive so that the bright features in the image have intensity values close to the maximum. This is commonly achieved by summing several frames in video-based systems or by variable shutter speeds in charge-coupled diode (CCD) digital cameras. A monochrome camera is usually preferred. Slow scan, cooled CCD cameras offer the best performance in terms of sensitivity and signal-to-noise ratio. Our system (Harris, Paul) is a Princeton Instruments cooled CCD camera with a Kodak 768 $\times$ 512 chip; each CCD is 9 $\times$ 9 μm.
2. The effective size of pixels in the image should be small so that each bacterial cell is represented by many pixels. Current systems use a pixel size of 0.1 μm or less. Pixel size is a function of the size of the detector elements and the magnification of the optical system. The detector elements should be small ($<$10 μm) to minimize the required magnification. High magnification, by reducing depth of field, increases the problem of analyzing those parts of the image that are out of focus. Confocal microscopy can be used to solve this problem (Bloem et al. 1995b) but is expensive and slow, since several images from different focal planes are required. The signal-to-noise ratio of photomultipliers typically used in confocal microscopes is also lower than that of cooled CCD detectors.

Software

Bacteria

Analysis of the images to detect bacteria will probably include these steps:

1. *Noise elimination:* Random noise can be removed by smoothing procedures, including averaging and median filters, and by morphological operations such as opening and closing (Russ 1990). Cooled CCD cameras produce less noisy images than other systems and may not require this step.
2. *Background subtraction:* Fluorescent images of soil smears contain a highly variable, nebulous background that must be removed before the bacteria can be detected. Morphological transforms called "top hat" are effective means to isolate small bright features (Bloem et al. 1995b; Bright and Steel 1987) and to effectively eliminate the background.
3. *Edge detection:* The intensity profile along a chord passing through an image of a bacterial cell approximates a Gaussian peak. The true edge is estimated either as the maximum gradient in the intensity profile (Sieracki et al. 1989) or as the half-height of the peak (Bloem et al. 1995b).
4. *Segmentation:* The gray-scale image is segmented at some threshold intensity; those parts of the image above the threshold are retained (presumed bacteria), and the rest are set to zero. Features outside a set range of area may also be rejected at this stage.
5. *Group decomposition:* Some bacteria occur either as dividing cells or as small groups. Image segmentation will frequently fail to separate these groups into individual cells. Groups can be found by searching for local maxima in the gray-scale image of the detected features. More than one local maximum suggests a group. This test is sensitive to noise and may require further image smoothing before use. Individual cells in groups can be reconstructed by isotropically expanding the local maxima until the segment boundary is reached or until further expansion would cause the "cells" again to merge (Bloem et al. 1995b). A related technique replaces pixel values with the distance to the nearest edge and then divides the feature along the watershed(s) of the distance values (Russ 1990); this technique has the advantage of preserving the size relationships of dissimilar structures like budding yeast cells. Another approach is to look for and connect inflections or turn points in the boundaries of groups (Dubuisson et al. 1994). Counts of dividing cells can be used as an estimate of the growth rate of the population (Bloem et al. 1995b).
6. *Cell measurement and biovolume estimation:* Most image analysis software readily calculates the area and perimeter of detected features and also the major and minor diameters of the minimum ellipse that will contain the cell. Cell volumes (*prolatev*) can be calculated from the major (l) and minor (d) diameters by assuming the bacterial cells to be prolate spheroids

$$prolatev = \pi/6 \times d^2 \times l$$

These assumptions work well for cocci and short rods but overestimate the volume of long rods and more complex shapes. Baldwin and Bankston (1988) de-

Table Appendix 1. Examples of Scripts Used to Count Bacteria and Fungi

Action	Comment
Bacteria	
Set variable	Width of "*Tophat brim*" 1
Set variable	Width of "*Tophat*" 15
Set variable	Height of "*Tophat*" 100
Start	Label to mark start of looping section
Open	Opens image file from supplied list
Show image	
Duplicate window	Copies original image; we work on the copy
Show image	Duplicate
Linear filter	Convolution with the 3 × 3 kernel 0,−1,0,4,−1,0,−1,0 (approx. Laplacian)
Linear filter	Convolution with the 3 × 3 kernel 3,5,3,5,8,5,3,5,3 (approx. Gaussian $s = 1$)
Tophat	"Horizontal" pass detects peaks in intensity profile, subtracts background; result in new window
Rename window	Renames result of "*Tophat*"—peaks "*h*"
Change window	Back to copy of original
Rotate and scale	Rotates copy of original 90°
Tophat	"Vertical" pass detects peaks in intensity profile, subtracts background; result in new window
Rename window	Renames results of "*Tophat*" peaks "*v*"
Rotate and scale	Rotates peaks $v - 90°$
Image arithmetic	Maximum value of peaks *h* and *v;* result to peaks *h,* which now contain the net result of peaks detected by both "horizontal" and "vertical" passes of *Tophat;* background is zero
Dispose window	Peaks *v*
Dispose window	Copy of original
Change window	Peaks *h*
Segmentation	Segments image at threshold intensity value 1
Change window	Original image
Transfer attributes	Copies overlay of segmented image (detected features) onto original image
Measurement options	Labels each measurement with image name, erases segments excluded from measurement, fills holes in segments
Set measurements	Collect area, perimeter, major and minor axes for each detected feature; excludes those not meeting set criteria" area > 5 < 200 pixels
Measure segments	Measure features matching set criteria
Dispose window	Clean up
Dispose window	Clean up again
Loop	Go to start and get another image from list
End	
Fungi	
Set variable	Width of "*Tophat brim*" 1
Set variable	Width of "*Tophat*" 10
Set variable	Height of "*Tophat*" 100
Start	Label to mark start of looping section
Open	Opens image file from supplied list
Show image	
Duplicate window	Copies original image; we work on the copy
Show image	Show copy
Tophat	"Horizontal" pass detects peaks in intensity profile, subtracts background; result in new window
MiniMax	"Horizontal" pass retains the value of pixels > ½ maximum peak height, sets others to zero
Rename window	MiniMax "*h*"
Dispose window	*Tophat h*
Change window	Copy of original
Rotate and scale	Rotates copy of original 90°
Tophat	"Vertical" pass detects peaks in intensity profile, subtracts background; result in new window
MiniMax	"vertical" pass retains the value of pixels > ½ maximum peak height, sets others to zero
Rename window	MiniMax *v*
Dispose window	*Tophat v*
Rotate and scale	Rotates MiniMax $v - 90°$
Image arithmetic	Maximum value of MiniMax *h* and MiniMax *v;* result to MiniMax *h,* which now contain the net result of peaks detected by both "horizontal" and "vertical" passes of *Tophat.* Background is zero
Dispose window	MiniMax *v*
Dispose window	Copy of original
Change window	MiniMax *h*
Median filter	Sets each pixel to the median value of the 9 pixels in its 3 3 3 neighborhood. Noise reduction
Segmentation	Segments image at threshold intensity value 1
Change window	Original image
Transfer attributes	Copies overlay of segmented image (detected features) onto original image
Measurement options	Labels each measurement with image name, erases segments excluded from measurement, fills holes in segments
Set measurements	Set criteria: area > 200 pixels, radial standard deviation > 35
Measure segments	Measures area of each detected feature matching criteria
Set measurements	No criteria
Modify segments	Erode each segment to a single connected line of pixels (skeletonize)
Measure segments	Area of skeleton estimates length
Dispose window	Original
Loop	Return to start
End	

scribe a stereological calculation of cell volume based on area and perimeter measurements. This avoids the difficult measurement of the diameter of the cell but is sensitive to overestimation of the perimeter due to "pixelation," resulting in underestimation of cell volumes. Sieracki et al. (1987) describe a method in which cell volume is estimated by rotating the cell outline about its long axis and summing the volumes of the disks swept by each edge pixel. This method is accurate for a wide range of cell shapes but is computationally complex.

Fungal Hyphae

Image-processing requirements are similar to those used for bacteria with some modification to detect filaments. The segmented image represents the plan area (*a*)

of the hyphae in the image. Length (l) can be estimated by eroding the image of each hypha to a single connected line of pixels (skeletonization). The diameter (d) of each hyphal fragment is estimated from the plan area (a) divided by length (l):

$$d = a/l$$

Alternatively, the logical term AND of the segmented image with an image of a grid can be used to estimate the total length of hyphae in the image by the grid intercept method. If this is used, an average hyphal diameter can be calculated for all the hyphae in the image by dividing the sum of the plan areas by the total length.

Specific Examples of Image-Processing Procedures

Image-processing steps to measure bacterial and fungal hyphal biovolume in soil smears using IPLab Spectrum v 3.0 image analysis software (Macintosh PPC) are shown later in the form of "scripts," which are executable lists of processing steps (see Tab. Appendix.1). The software has the capability to repeat the script for each of a list of image files and in this way can automatically analyze numerous images as a batch process. Two externally programmed functions are used; these are the filters "Tophat" and "MinMax," which were written in the computer programming language C and incorporated into the IPLab Spectrum software as external functions.

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