# 42

# Methods for Studying Terrestrial Fungal Ecology and Diversity

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## 42.1. INTRODUCTION

### 42.1.1. Importance of Terrestrial Fungi

Fungi are of fundamental importance in terrestrial ecosystems, and their roles and importance are usually overlooked or underestimated by ecologists who study plants or animals. Fungi drive terrestrial nutrient cycles through their abilities to decompose the complex carbohydrates—cellulose, hemicelluloses, and lignin—that make up the majority of plant biomass, which in turn accounts for approximately 90% of the total biomass in most terrestrial ecosystems (34, 37, 89). Other fungi, the mycorrhizal fungi, form symbiotic associations with the roots of living plants. These mutualistic symbioses are vital to the survival of most green plants in natural ecosystems. Mycorrhizal fungi help plants obtain nutrients and water in environments where these are chronically or periodically lacking, and they may also protect plant roots from attack by pathogens, including other fungi (4, 102). Fungi living within aboveground plant parts, the endophytic fungi, may protect these plants from herbivory or attack by pathogens, sometimes at a cost of flowering and sexual reproduction (33, 90, 92, 96). The filamentous growth form of many fungi and their ability to simultaneously or successively perform more than one nutritional role (decomposer, symbiont, or predator) allow them to act as bridges across time, space, and trophic levels in ecosystems (5, 76, 109, 111). In contrast, predominantly unicellular, nonfilamentous yeasts are adapted for rapid response to rich, moist environments and may convert sugar solutions such as nectar or plant sap into more nutritious—or alcoholic—food for associated insects or other animals, including humans (70, 115). Filamentous fungi with a habit of exuding adhesive extracellular polysaccharides or mucopolysaccharides are important in soil

stabilization through the formation of microaggregates and the binding of aggregates and particles (29, 122). In the human environment, fungi are important in many food and industrial fermentations, but they also cause food spoilage and grow unwanted in our living and working spaces, creating problems of environmental health (7, 41, 87, 100). This chapter can only touch on the fundamentals of discovering and identifying fungi in these diverse environments.

### 42.1.2. Groups of Fungi and Funguslike Organisms

#### 42.1.2.1. Nonfungi: *Actinomycetes, Oomycota* and *Hyphochytriomycota,* and Slime Molds

Several groups of organisms that are not true fungi have traditionally been studied by mycologists or thought of as fungi. *Actinomycetes* are prokaryotic and are members of the G+C-rich gram-positive *Bacteria* (85, 119). They resemble fungi because of their filamentous growth, reproduction by spores, and production of extracellular enzymes (46, 62, 88; http://www.nih.go.jp/saj/DigitalAtlas/index.htm). Although mycologists and soil biologists will encounter actinomycetes in their studies, they are not treated here. The *Oomycota* and *Hyphochytriomycota* are "water molds" with motile, flagellated spores. The anterior flagella in both groups are called tinsel flagella and have two rows of fine, tubular hairs resembling those of the golden-brown algae. The natural relationships of the water molds, as determined by DNA sequence analyses and other lines of evidence, are with the chrysophyte algae in the kingdom *Stramenopila* (113, 114) (also called *Chromista* or *Heterokonta* [13]). Slime molds (the *Mycetozoa* or "fungus animals") are relatives of amoebae and have an ameboid feeding stage and a spore-bearing reproductive stage (11–13, 56). None of these groups belongs in the kingdom *Fungi*.

#### 42.1.2.2. Kingdom *Fungi*: *Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota,* and *Basidiomycota*

Most current classifications of fungi recognize four phyla: *Chytridiomycota*, *Zygomycota*, *Ascomycota*, and *Basidiomycota* (3, 60). There is good evidence from molecular phylogenetic studies, fossil record, and ecology for a fifth fungal phylum, *Glomeromycota* (98). The Assembling the Fungal Tree of Life group (http://aftol.org/) has spearheaded recent phylogenetic analyses of the fungi based on sequences of ribosomal and other genes. These have led to substantial changes to classification and naming of fungal groups (52a) compared to the conservative approach taken here. In addition, sequencing of ribosomal DNAs (rDNAs) isolated from soils has led to the recent discovery of several unknown and uncultured lineages with very deep roots in the fungal kingdom (112). The truly innovative investigator may develop techniques to see, cultivate, and describe these fungi and discover their role in nature, but our discussion is limited to groups known as whole organisms.

Like the *Oomycota*, chytrids are aquatic fungi with flagellated zoospores that require free water for motility (Fig. 1c). Zoospores of most chytrids have one posterior flagellum, but members of one group found in the rumen of herbivorous mammals are multiflagellate. Soil-inhabiting chytrids include decomposers of cellulose, chitin, and keratin, as well as parasites of soil algae, invertebrates, and vascular plants (15). The chytrids in rumen contribute to the digestion of cellulosic plant cell walls in the animal's food (81). The four other phyla consist of predominantly filamentous forms, plus secondarily unicellular yeasts belonging to both *Ascomycota* and *Basidiomycota*.

The *Glomeromycota* (Fig. 1d) are the fungi that form mutualistic symbioses called arbuscular mycorrhizae with the roots of approximately 90% of the vascular plants in the world; these symbioses enable plants in natural environments to survive drought and nutrient stress (102). *Glomeromycota* are coenocytic, with tubular cells containing hundreds of nuclei. Although they form large spores, of 100 to 1,000 μm in diameter, and networks of hyphae that may extend a meter or more, they are otherwise only microscopically visible. Recognition of the monophyletic phylum *Glomeromycota* resolves the paraphyly of the formerly broad *Zygomycota* including *Glomales* with respect to the *Chytridiomycota* (98). The remaining *Zygomycota* (Fig. 1e) are a diverse group of fungi in terms of morphology and ecology and include soil saprobes, parasites of soil fungi or algae, and parasitoids of insects and soil invertebrates, but few significant plant pathogens (20).

The *Ascomycota* (Fig. 1a) are the largest group of fungi and include most of the asexual or "mitosporic" fungi that were formerly classified in the *Deuteromycetes* or "Fungi Imperfecti" (60, 93, 99). The *Ascomycota* include soil-borne pathogens of crop plants, endophytes that live within plant tissues, usually without causing disease symptoms, most fungi that cause human and animal diseases, plus groups that are mycorrhizal, parasitic on other soil fungi, or predatory or parasitic on insects or other invertebrates (16, 19, 30, 33, 45, 58, 86, 92, 94, 96). In addition, the majority of lichens are in the *Ascomycota* (60). Lichens are symbiotic associations between fungi and green algae or cyanobacteria. The fungal partner (mycobiont) forms the characteristic structure that we call a lichen, which encloses and protects the alga or cyanobacterium (photobiont) (2, 50). Finally, most yeasts (Fig. 1f), including the most economically important ones such as *Saccharomyces cerevisiae*, belong in the *Ascomycota* (65). Yeasts are fungi adapted to life in aqueous environments by growth as separate, usually elliptical cells that divide by budding or fission (14, 63) and are discussed further in section 42.2.3 below.

The *Basidiomycota* (Fig. 1b) are the second largest group of true fungi and may be divided into four major groups, the *Ustilaginomycetes* (smuts), *Urediniomycetes* (rusts), *Heterobasidiomycetes* (jelly fungi), and *Homobasidiomycetes* (mushrooms and relatives) (60). The thousands of species of *Homobasidiomycetes* whose mycelial phase occurs in soil have mostly been overlooked in surveys of soil fungi (55, 110). The *Homobasidiomycetes* include important crop pathogens, including *Rhizoctonia* (sexual state *Thanatephorus* [6, 103, 105]) and timber pathogens *Armillaria*, *Phellinus*, and *Ganoderma* (108), saprotrophic leaf- and wood-decomposing fungi (53, 121), and the majority of fungi that form ectomycorrhizal symbioses with woody vascular plants in 30 families (47, 54, 102).

The fundamentally different biologies of different groups of fungi mean that no single method will work to discover or isolate all fungi in any material or area. For this reason, some general methods are presented here, which users may need to modify to study their fungi of particular interest. *Biodiversity of Fungi* (81a) presents an extensive review of this topic.

## 42.2. METHODS

### 42.2.1. Terrestrial Filamentous Fungi

#### 42.2.1.1. Collecting and Culturing Macrofungi

Macrofungi are those fungi with fruiting bodies large enough to see with the naked eye, usually defined as being

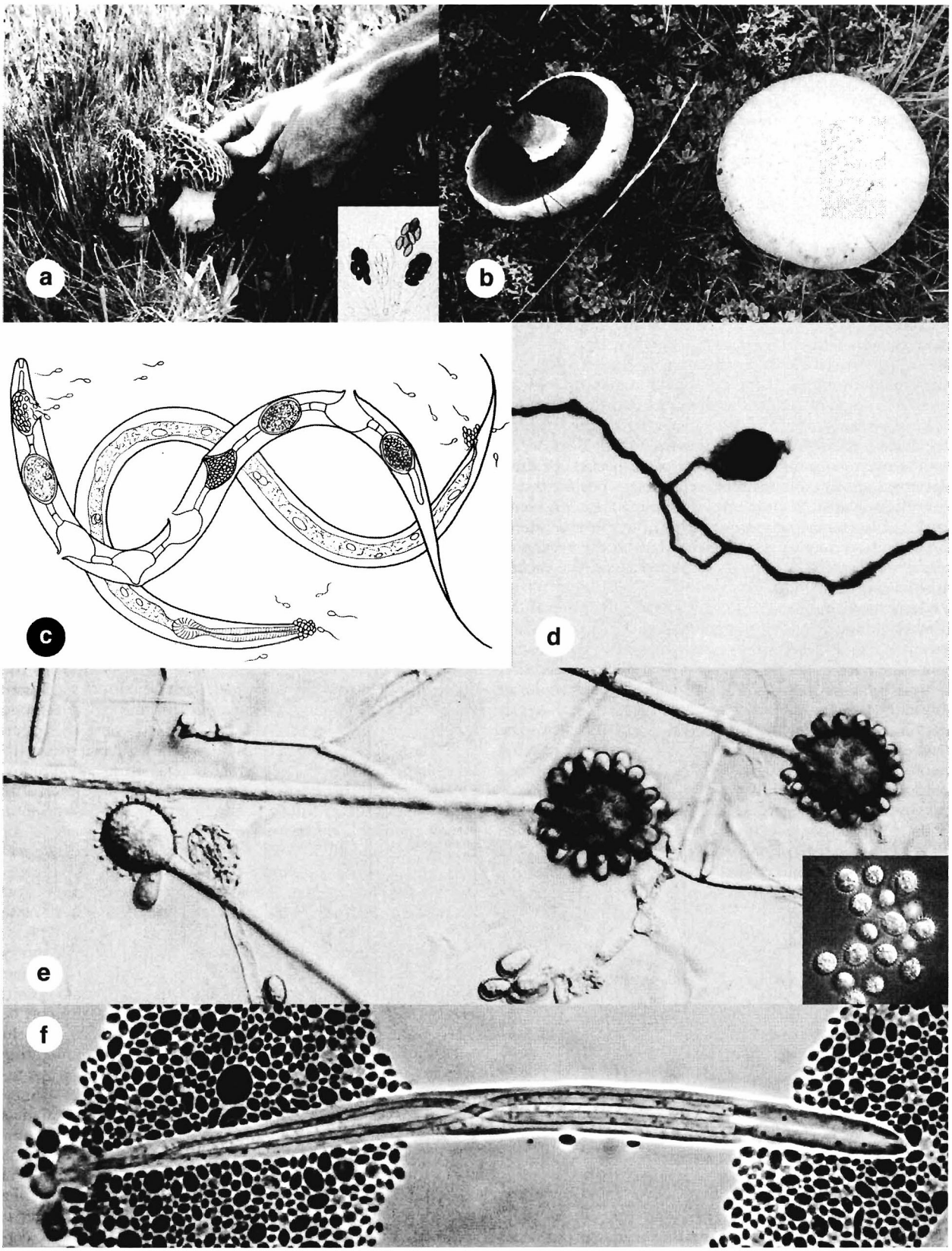


**FIGURE 1** The major types of fungi. (a) *Ascomyota*: *Morchella esculenta*, showing the correct method of collecting macrofungi; inset, eight ascospores are produced in each sac-like ascus in this *Saccobolus* (photo by G. L. Barron, University of Guelph). (b) *Basidiomycota*: *Agaricus*, associated with a fairy ring in Wyoming. (c) *Chytridiomycota*: *Catenaria anguillulae* attacking nematodes (drawing by G. L. Barron, University of Guelph, from reference 18). (d) *Glomeromycota*: a spore and hyphae of an endomyorrhizal fungus washed from soil. (e) *Zygomycota*: *Cunninghamella echinulata*, a striking and common fungus from soil. The inset shows a close-up of the sporangiospores (both photos by G. L. Barron, University of Guelph). (f) Vegetative cells and ascus of *Metschnikowia hawaiiensis*, an ascomycetous yeast.

at least 1 cm in one dimension. These fungi are relatively easy to collect, but a systematic approach and sampling design are required for studies intended to yield results that are comparable between areas or years. Sampling plots should be based on a stratified random or regular transect design for comparability between studies (Fig. 2), but for discovery of maximum diversity they should be combined with opportunistic sampling of known or suspected "good habitats" (91). Plots should be repeatedly sampled during the fruiting season or seasons, once a week being desirable but once per month probably more practical in terms of collection time and effort.

Care should be taken in collecting fruiting bodies of macrofungi to obtain the entire fruiting body (Fig. 1a). The base of the stem, if one is present, may be buried in the soil or other substratum. Collections should immediately be assigned a collection number and recorded in a field notebook. Collections should be packaged in the field to prevent damage during transit back to the lab, including drying or, in rainy areas, saturation. Waxed paper packets or Kraft paper bags are suitable for relatively robust specimens and dry collecting areas, aluminum foil for rainy areas, and hard plastic fishing tackle boxes for minute or fragile specimens. The collection number should be written on the packet, or a slip of paper with the collection number should be packed with the specimens.

Many macrofungi can only be reliably identified if detailed notes are made of characters detectable only when fresh (Fig. 3a). A good photograph, in situ or in the lab, can reduce the need for description of colors, textures, and size, but notes on taste, odor, staining reactions, and the color of a fresh and dried spore print are often vital for final identification. Too often a mycologist is asked to identify the voucher specimens from a detailed ecological study in some remote locale, but finds that the specimens are without notes and, because they were poorly dried, are useless for study of microscopic features as well. For details and terminology of macroscopic features of macrofungi, see reference 72. To obtain a spore print, lay a fresh, moist portion of the fruiting body, hymenium (spore-bearing surface) down on a piece of white paper or a microscope slide, and protect it from drying by covering with a cup or small collecting jar (Fig. 3c). Keep in a cool place overnight, and a pattern of thousands or millions of spores will be deposited (Fig. 3d), enabling the detection of spore color en masse and microscopic measurement of mature spores.

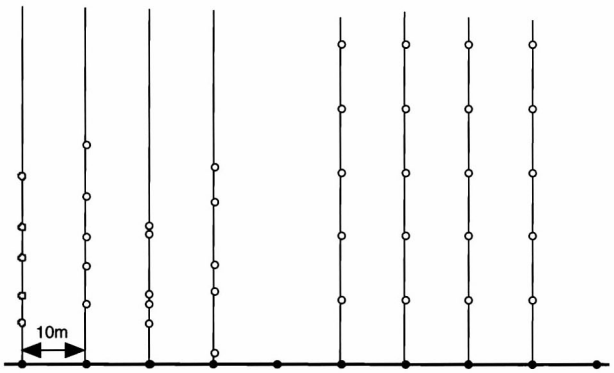


**FIGURE 2** Sampling designs: stratified random (left side) and regular grid (right). The heavy horizontal line represents the baseline, filled circles represent 10-m intervals from which sampling transects are struck at 90° to the baseline, and open circles represent sampling plots 4 $m^2$ in area, with a radius of 1.14 m (91). In stratified random sampling, sample plots are located on the transects at distances taken from a random numbers table, e.g., in the left line, with their centers at 6.33 m from the baseline, and then 2.17, 5.94, 4.67, and 8.07 m from the center of the previous plot.

Once a spore print is set up and notes are taken, including color annotations with reference to some color standard such as in the work of Kornerup and Wanscher (61), the decision is made whether to culture the specimen, preserve a small piece of tissue for future DNA studies, or dry it for the herbarium. A good field preservative for tissues intended for DNA studies is 2× CTAB buffer (see section 42.2.4.11 and reference 43). A piece of tissue approximately 3 by 3 by 3 mm, not including any contaminated external surface, can be cut out with a flamed and cooled scalpel and preserved in 0.5 to 1.0 ml of 2× CTAB in a 1.5- to 2.0-ml screw-cap vial and then kept at ambient temperatures until it is practical to refrigerate. Freezing is unnecessary and may not preserve the DNA as well as temperatures of 4 to 5°C. Specimens, and their field packets if Kraft paper bags were used, should be dried at 40 to 50°C with ample air flow. In many situations, a portable food dehydrator with an electric heating element and fan is ideal. Most specimens will dry overnight, and very large specimens may be cut into slices to speed drying. In humid areas, dried specimens should be packed immediately into airtight plastic bags such as Ziploc™ bags to prevent rehydration and attack by molds or insects.

Culturing from specimens of macrofungi is often very simple and adds a whole new biological dimension to the collection beyond the preserved, dead herbarium specimen. The spores of many saprotrophic macrofungi germinate readily on simple agar media, such as ME agar (see section 42.2.4.3). The isolation medium may be supplemented with antibacterial antibiotics and, for isolation of basidiomycetes, with benomyl. To obtain a polyspore culture, affix a small portion of fruiting body, approximately 2 by 2 mm, hymenium side down, to the lid of a petri dish using a dot of petroleum jelly. When spores on the agar surface are visible by eye or using a dissecting or compound microscope, the piece of tissue may be removed and a small piece of agar bearing spores may be transferred aseptically to a fresh plate of isolation medium. To obtain monospore, usually haploid, cultures for mating studies, make a dilution series of spores in sterile distilled water (sd$H_2O$). Spores deposited on agar should be resuspended and plated immediately after deposit, whereas spores collected on sterile microscope slides may be resuspended and plated up to several weeks after deposit. The correct dilution must be chosen so that germinating spores are well separated on the agar surface, and germinated spores should immediately be transferred to separate plates or tubes. For robust specimens, and for fungi such as ectomycorrhizal species whose spores do not readily germinate, it is easier or better to make a tissue culture, which will usually be dikaryotic. Break open the fruiting body and extract a small tissue piece from this freshly exposed surface using flamed and cooled forceps or scalpel, and transfer it directly to a plate of isolation medium (Fig. 3b). To be certain that you are growing what you intended, you should follow the germination and growth of spores or tissue pieces at frequent intervals using a dissecting and compound microscope, and immediately transfer the target fungus out of any plate that becomes contaminated with molds. Once cultures are growing cleanly, they should be transferred to plates (short-term working cultures) or slants (stock cultures) of medium without antibiotics. Slant cultures may be preserved by aseptically filling the vials with sterile, heavy

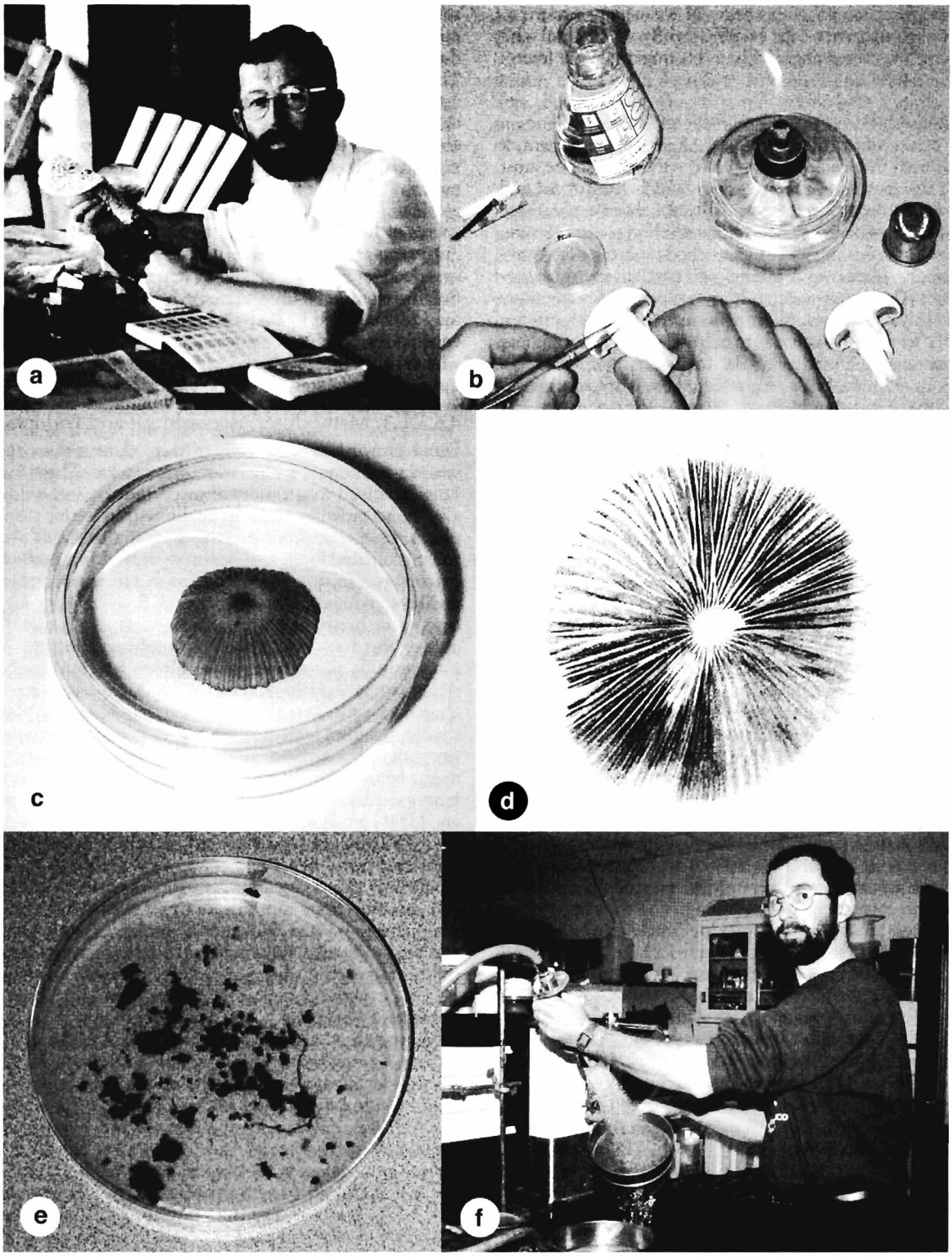


**FIGURE 3** Collecting and culturing macrofungi (a to d) and microfungi (e to f). (a) Taking notes on a fresh collection of *Amanita*, *Basidiomycota*, using a color guide (61); (b) making a tissue culture from a fruiting body of *Agaricus*, *Basidiomycota*; (c) the procedure for obtaining a spore print; (d) a spore print; (e) a soil sprinkle plate for isolation of nematophagous fungi; (f) particle washing to remove spores of abundantly sporulating molds to selectively recover more recalcitrant fungi from soil, including *Basidiomycota*. In this example, 5 g of soil was first dispersed by shaking for 1 h at 4°C in 0.1 M sodium pyrophosphate decahydrate and then poured through sieves of 250- and 53-μm mesh. Particles remaining on the 53-μm mesh sieve were washed with a stream of tap water. The remaining organic materials may be picked up in a 1-ml, broad-bore pipette and inoculated onto agar or in liquid medium or used in DNA extractions that are enriched for filamentous fungi.

mineral (paraffin) oil and storing at room temperature or with cryoprotectant such as 15% (vol/vol) glycerol and storing in the vapor phase of liquid nitrogen or in a freezer at −80°C (59).

### 42.2.1.2. Detection and Isolation of Microfungi

Many of the most interesting and important fungi cannot be seen with the naked eye and collected. Instead, one must collect the material on which or in which they occur and either examine this microscopically to observe the fungi or process it in some way to isolate the fungi into culture. What follows is a discussion of methods suitable for recovery of many filamentous microfungi, particularly those belonging to the *Ascomycota* and *Zygomycota*. Although many *Chytridiomycota* are members of terrestrial soil ecosystems, methods for their isolation into culture are essentially those for aquatic fungi and are not included here (23, 42). However, amplifications of genomic DNA from soil using fungus-specific primers yield diverse sequences in the *Chytridiomycota* and related basal lineages of fungi (112).

One of the best and easiest methods to detect microfungi is periodic observation of organic materials kept in moist chambers, using a dissecting microscope at ×5 to ×50 magnification. Fresh or moistened herbivore dung, leaf litter, tree bark, living or dead roots, and decorticated dead wood are all excellent materials for study by the moist-chamber method. A moist chamber may be as small as a petri dish or as large as a 10-liter plastic crisper. Depending on the material, a layer of moistened sand, perlite, or vermiculite covered with filter paper or paper towels may be required in the bottom of the chamber to maintain humidity. The soil sprinkle plate method for isolation of nematode-destroying fungi (18) is a variation on the moist-chamber technique, in which a small amount of soil or organic matter is sprinkled onto a plate of water agar (15 g of agar per liter of distilled water) and approximately 200 nematodes from a stock culture are added as bait to stimulate germination, trap formation, and development of nematode-destroying fungi (Fig. 3e). Astute fungal biologists may develop their own particular moist-chamber technique to detect or discover their fungi of interest. Observations are made periodically to note the appearance of novel fungi, and these may often be obtained in culture by picking single spores with a Pasteur pipette that has been drawn to a hair tip in a small flame and transferring these to plates of isolation medium.

In addition to the moist-chamber technique, two basic methods for culturing fungi from soil or organic materials are dispersion plating (also called dilution plating) and particle plating (with soil, also called soil washing). For dispersion plating of soil, a small amount such as 5 g (fresh weight) of soil is dispersed in a series of volumes of $sdH_2O$ to obtain a dispersion that yields 25 to 30 colonies on a petri plate of isolation medium. The series of 5 g of soil in 125 ml of $sdH_2O$, 1 ml dispersion 1 in 39 ml of $sdH_2O$, and 1 ml of dispersion 2 in 9 ml of $sdH_2O$ yields dispersion ratios of 1:25, 1:1,000, and 1:10,000. The last two are useful for many soils, with 0.5 to 1.0 ml spread evenly over the surface of a 100-mm petri dish. It is important that the soil be well dispersed and evenly suspended when taking the aliquot for the subsequent dispersion or for plating. Unused portions of soil samples are weighed, dried, and reweighed to back-calculate the dry weight of soil used in the dispersion. A low-nutrient medium supplemented with antibacterial antibiotics is generally used, and we recommend Martin's soil extract medium with rose bengal and chloramphenicol (see section 42.2.4.1). Dispersion plating yields a diverse subset of the soil fungal community, but it generally misses most basidiomycetes and nutritional specialists such as the nematode-destroying fungi, mycoparasites, and mycorrhizal fungi (32, 110). Comparability between studies requires a systematic approach to picking colonies to be identified: either all or the first 25 to 30 encountered randomly per plate should be chosen (32). Particle plating starts by dispersing the material, or grinding it to fine particles if necessary, and washing particles (Fig. 3f) through sieves of a chosen size range, such as 50 and 250 μm, with tap water or $sdH_2O$ to remove the spores of heavily sporulating molds such as *Penicillium* and *Aspergillus* (10, 22). Washed particles are plated on standard or selective isolation medium, depending on the group of fungi desired (21, 110). Although a few principal species may be recovered by such techniques, there may also be an enormously long "tail" to the species accumulation curve, with 8 to 15 previously unseen species added for every 100 incremental isolates past 1,100 (31, 32).

### 42.2.1.3. Methods for Mycorrhizae and Endophytes

Fungi growing symbiotically within plant tissues require specific techniques for isolation into culture. These include fungi involved in a variety of mycorrhizal associations and also those growing endophytically within aerial tissues of plants, usually without causing disease symptoms. Among the mycorrhizal fungi are *Glomeromycota* forming arbuscular mycorrhizae (also called vesicular-arbuscular mycorrhizae or endomycorrhizae), *Ascomycota* and *Basidiomycota* forming ectomycorrhizae, and several other forms. As mentioned above, the basidiospores of most ectomycorrhizal basidiomycetes do not germinate readily on standard media, although they may be stimulated to do so using activated charcoal or filter-sterilized root extracts (25, 84). Many of these fungi form large fruiting bodies from which tissue cultures may be derived, using more complex media than might be used for saprotrophic fungi. Two agar media commonly used for isolation of ectomycorrhizal basidiomycetes are MMN medium and BAF agar (sections 42.2.4.4 and 42.2.4.5), usually supplemented with antibacterial antibiotics and with benomyl to suppress molds (57). These media may also be used for isolation of ectomycorrhizal basidiomycetes from mycorrhizal root tips that have been scrupulously washed and surface sterilized, although many attempts will still yield nonmycorrhizal ascomycetous molds that were on or in the root tip (107). MMN medium and BAF agar without benomyl may be used to isolate ascomycetous mycorrhizal fungi from root tips or fruiting bodies. Ectomycorrhizal root tips may be characterized and identified to some extent using morphological features (1, 49), and the fungal associate may be identified by selective amplification followed by sequencing of fungal ribosomal genes (27, 43, 44). Glomalean mycorrhizal fungi (GMF) have generally resisted attempts at axenic culture but can be cultivated from infected root pieces or spores recovered from soil in coculture with a host plant such as leeks (*Allium porrum* [25]). Selective primers are also available to amplify rDNA of GMF from roots or soil (101). Sequence-based identification of GMF promises to open the field of research into their ecology, previously inhibited by the difficult and imprecise identification using spore shape and wall structure (20). One difficulty with molecular identification of GMF, however, is that they are coenocytic. Their spores may each contain over 1,000 genetically distinct nuclei, with several different alleles for rDNA (95), and frequently also contain sequences of unrelated contaminant or endosymbiotic fungi as well (97).

Fungi in plant parts may be observed by direct microscopy following clearing of the plant tissues and staining of fungal structures. For nonpigmented roots and leaves, clear for 1 h in 1 M KOH at 60 to 80°C or 15 min at 121°C, neutralize in one to several rinses of 0.1 M HCl and then distilled water, and stain in one of the following: 0.05% (wt/vol) trypan blue in lactic acid, 0.1% (wt/vol) acid fuchsin in lactic acid, or 0.01% (wt/vol) chlorazol black E in water. For highly pigmented, tannin-rich roots, clear in 3% (vol/vol) hydrogen peroxide prior to KOH clearing (24–26).

Culturable endophytes may be isolated onto appropriate media containing antibiotics following surface sterilization and serial washings of the plant tissues to remove superficial contaminants. Surface sterilization using 75% ethanol and 3% bleach (sodium hypochlorite) may work for thick plant parts, but it may kill endophytic fungi in thinner tissues. For these samples, repeated washings using sd$H_2O$ containing 0.1% (wt/vol) detergent such as Tween-20™ are recommended. Portions of the plant tissue may then be aseptically chopped or macerated, and these fine particles, 1 $mm^2$ or less, may be spread on media containing antibiotics to permit the endophytic fungi to grow out (91).

## 42.2.2. Sampling and Analysis of Indoor Environmental Fungi

There is increasing recognition that exposures to indoor environmental fungi and their emissions are associated with a range of human illnesses. As a result, the detection, identification, and quantification of indoor fungi, particularly molds, has become an important part of the testing mandate of many environmental testing laboratories, particularly commercial laboratories. In contrast to bacteriology and food microbiology, the commercialization of analytical environmental mycology has posed a number of challenges, primarily due to the lack of standard testing procedures and evaluation criteria. This section discusses several sampling methods that are used commonly in commercial mycology laboratories for the assessment of populations of fungi in home and indoor work environments.

### 42.2.2.1. Sampling Methods

A wide range of sampling procedures exist for surveying environmental fungi. Primarily these methods are used (i) to evaluate hazards and (ii) to assess control and cleanup measures. Because of the highly episodic nature of spore release, short-term air sampling methods cannot be used to evaluate human exposures to fungi. In addition, the health hazards posed by fungal exposures are complex and may vary in terms of allergenic, toxic, and pathogenic effects, depending upon the species present, growth conditions, and individual human susceptibility. Therefore, the establishment of "safe exposure limits" is widely agreed to be untenable. However, the presence of indoor fungal growth has been strongly correlated to elevated levels of indoor airborne spores and increased symptom reporting by occupants. Thus, there has been widespread consensus that the elimination of indoor fungal growth sources is a prudent measure. Based on this guiding principle, most air sample interpretation guidelines have been developed in order to query the likelihood of indoor fungal growth rather than to recommend safe limits for human exposure.

#### 42.2.2.1.1. Bulk Samples

Bulk samples refer to (i) solid materials such as wood; (ii) fibrous materials, including broadloom and dust; and (iii) friable materials like gypsum wallboard. The assessment of bulk samples for fungal colonization is considered central because the presence of fungal growth on normally barren materials indicates a deficiency and is not acceptable. Furthermore, the examination of bulk samples is of fundamental value in permitting conclusive identification of the source of contamination.

**42.2.2.1.1.1. Direct microscopic examination.** Direct microscopy is considered the "gold standard" in environmental mycology for establishing the presence of fungal growth. Together, the observations of fungal spores, spore-bearing structures, and vegetative hyphae provide an unequivocal indication of past or current growth. All methods involving direct microscopy should first examine the bulk specimen in reflected light at a magnification of ×10 to ×40 for gross evidence of colonization. Subsequently, preparations from one or more areas of the specimen should be made in a suitable mounting fluid (discussed below) for microscopic evaluation in transmitted light. Two principal methods exist for the examination of bulk specimens by transmitted light microscopy: (i) adhesive tape lift and (ii) scraping/shaving.

42.2.2.1.1.1.1. Adhesive Tape Lifts. Methods for the collection of fungal growth from surfaces using shellac peels to preserve the arrangement of spores and spore-bearing structures have long been used to study fungi in situ (for example, see reference 106). Currently, the simplest method to do this is by using clear adhesive tape (28, 38, 39). Tape-based methods are more practical than liquid shellacs because they do not require setting time and they can be used easily on highly porous materials. Adhesive tape methods are rapid and practical for both field collection and laboratory analysis.

A number of genera of important indoor fungal contaminants may be recognized directly from spores alone (e.g., *Chaetomium* and *Stachybotrys*). Many more genera may be identified if both the spores and spore-bearing structures are preserved intact. Tape lift sampling allows for the identification and semiquantification of fungal colonists on a surface.

#### Indications

This sampling method is useful for (i) field collection of surface samples suspected of fungal colonization, (ii) laboratory examination of bulk specimens, and (iii) microscopic examination of delicate structures from cultures where minimal disruption is desirable.

#### Procedure

1. Press the adhesive side of a 2- to 5-cm segment of optically clear tape (e.g., 3M Scotch "Red Tartan" brand tape) to the sample to be tested. The collection of superficial debris from rough or irregular surfaces can be improved by gently rubbing the back of the tape.
2. For transport, adhere the sampled tape strip to a glass microscope slide or the interior surface of a sturdy closable plastic bag such as a zippered freezer bag.
3. Cut a piece of sampled tape small enough to fit beneath a glass coverslip.
4. Using a clean coverslip, gently scrape away any large surface debris (e.g., greater than 0.2 to 0.5 mm in diameter) from the tape adhesive such as soil particles, glass fibers, etc., as these may cause air bubbles to form in the preparation.

5. Place a small drop of 85% clear lactic acid (~10 μl) on a glass microscope slide, and mount the tape segment adhesive side up. Capillary action will help to draw the lactic acid throughout the space between the tape backing and the glass slide. Any air bubbles that form can be eliminated by gently lifting and repositioning the tape.

6. Place a small drop of mounting fluid such as lactofuchsin or lactophenol cotton blue (60) (~10 μl) in the center of a 22-mm no. 1 glass coverslip. Invert the coverslip to hang the droplet of mounting fluid.

7. Place the coverslip and hanging drop of mounting fluid on the adhesive side of the tape. Slide mounts should be prepared 5 to 10 min prior to examination in order to allow adequate time for staining.

8. Scan two or three microscopic transects of the tape specimen at ×400 magnification to examine a minimum of 20 to 50 $mm^2$ of tape area. Results should be collected on fungus type and category of structural element observed (i.e., spores versus vegetative hyphae/mycelia/ spore-bearing organs).

### Interpretation

Fungal spores produced on outdoor plant debris are common in indoor settled dusts. Therefore, the observation of fungal spores alone on a tape lift sample is insufficient to characterize the sample site as a fungal growth site. A minimum standard of evidence for the interpretation of active fungal growth requires the microscopic observation of fungal filaments (vegetative hyphae/mycelia/spore-bearing structures) together with spores.

### Notes

Tape used for sampling should be stored in a clean zippered plastic bag and not be used for any other purpose. Prior to sampling, the leading 2 to 5 cm of tape should be stripped off and discarded to guard against cross-contamination from earlier samples. Furthermore, to control against cross-contamination, one field blank (unsampled tape lift handled similarly to actual field samples) should be taken and analyzed for every 10 field samples.

Tape mounts prepared in the manner described above cannot be retained as a permanent record because lactic acid causes the tape adhesive to deteriorate within 12 to 24 h of preparation. If a longer-term specimen record is required, a sufficient portion of unmounted specimen should be retained in original packaging in a cool, dark, dry area.

Standard recipes for lactofuchsin and lactophenol cotton blue contain more stain than is necessary for this procedure. For tape lift mounts, these reagents are better prepared at 1/4- to 1/8-strength stain.

Confusion of small starch granules and fungal spores can be avoided by exposing the tape lift (adhesive side up) for 30 to 60 s in a chamber saturated with iodine vapor, which causes any starch granules to appear uniformly bluish, allowing them to be differentiated from fungal spores of similar size and shape. This method is similar to a standard procedure for visualizing chemicals separated by thin-layer chromatography. A suitable chamber can be made by placing several iodine crystals inside a closable wide-mouth amber glass jar set on its side. Once closed, the air inside the container will become saturated with iodine vapor after several hours. The chamber can be maintained by periodically replacing iodine crystals that have been lost to sublimation.

42.2.2.1.1.1.2. SCRAPINGS AND SHAVINGS. The simplest way to examine a bulk specimen for fungal colonization is by direct visualization of small particles of the specimen by transmitted light microscopy. This method has the advantage of allowing the analyst to observe both the sample surface and the subsurface aspect of the bulk material. Thus, this method may facilitate the detection of residual fungal contamination in specimens from which loose surface debris may have been cleaned or wiped. A second advantage of scrapings and shavings is that permanent microscopic mounts can be prepared from these sample formats as necessary. Thirdly, cultures can be prepared readily from scraping material should this be necessary. However, the scraping or shaving of substrates in the field may be time-consuming and requires the availability of appropriate sampling tools (e.g., disposable scalpels) as well as a means of safe disposal of sharps.

### Indications

Surface scraping and shaving is an appropriate sampling method in the field for samples with little evident surface irregularity. It can also be used as a fallback sampling method for those materials from which tape lift sampling fails to remove appreciable surface debris.

### Procedure

1. Scrape or shave the surface to be sampled, using a clean, sharp scalpel or other suitable tool.

2. Collect the scrapings in a clean tube, closable plastic bag, or paper packet. Plastic tubes are undesirable for the collection of scrapings due to their potential for dispersion of sample by static electric charge. For paper packets it is best to collect scrapings or shavings on gloss-coated heavy black paper because fine specimen fragments are easily seen and unlikely to adhere.

3. Place a small amount of scrapings or shavings on a clean glass microscope slide. Remove or further grind any large fragments, e.g., >200 μm in diameter, because particles of this size will cause uneven distribution of staining fluid. If scrapings or shavings are prepared in the laboratory directly from a bulk sample, they may be collected directly on a glass microscope slide.

4. Place a small drop of mounting fluid such as lactofuchsin or lactophenol cotton blue (60) (~10 μl) in the center of a coverslip, invert the coverslip to hang the droplet of mounting fluid, and place on the scraped material. Woody specimens or those containing alkaline minerals (e.g., wallboard samples containing gypsum dust) should be mounted instead in a solution of 10 to 15% potassium or sodium hydroxide with 10% glycerol to promote softening and avoid foaming. Hydroxide preparations incubated at 30 to 40°C for 30 to 60 min may be useful for wood shavings or other tough organic materials. The addition of a drop of 0.025% phloxine to hydroxide mounts may help differentiate hyaline fungal structures, especially those of basidiomycetes (74). Slide mounts should be prepared 5 to 10 min prior to examination in order to facilitate staining. An optional prewetting step may be useful for hydrophobic materials; this involves adding 1 or 2 drops of 95% ethanol to the specimen and allowing the ethanol to evaporate almost completely prior to the addition of mounting fluid.

5. Scan 20 to 50 microscopic fields at ×400 magnification. Record the fungus type and category of structural element observed, e.g., spores versus vegetative hyphae/ mycelia/spore-bearing organs).

### Interpretation

As with adhesive tape lift sampling, the visualization of fungal filaments en masse with or without the observation of fungal spores provides the best indication of fungal growth on

a substrate. Scrapings or shavings are not readily quantifiable, and thus this technique is best suited to qualitative analysis.

### Notes

Materials containing significant amounts of friable alkaline mineral salts (e.g., gypsum wallboard) for which microscopic analysis is required are usually best sampled on-site (by either adhesive tape lift or scraping) since transport of the entire bulk specimen to the laboratory for testing tends to cause powdering of the surface with gypsum dust, impeding microscopy.

**42.2.2.1.1.2. Culture.** Assay of bulk samples by direct microscopic examination is not always feasible, particularly for samples that are fibrous (e.g., dust, broadloom, and insulation) or granular (e.g., soil, plaster, and concrete). For these matrices, culture-based methods are preferred in conjunction with direct microscopy. A number of methods exist for culture-based analysis of bulk materials, largely based on techniques developed for soil fungi (17). The most commonly used methods are (i) elution, (ii) particle plating, and, occasionally, (iii) swabs and wipes (however, swabs and wipes offer little valuable information in environmental fungal sampling and should not be used as a routine sampling method). The suitability of elution or particle plating depends on the composition of the sample, as discussed below.

The validity of culture results depends on accurate identification of fungi to as detailed a taxonomic level as practical—in most cases to species level. Certain groups of fungi (notably *Fusarium* and *Penicillium*) require considerable expertise and extensive testing on an isolate-by-isolate basis to provide accurate identification to the species level. For these fungi, species identification is beyond the scope of routine practice, although identification to subgenus or morphological group may provide a practical alternative. If multiple cultures derived from a single material or site yield high numbers of what appears to be a single species of a taxonomically difficult genus, referral of one or more isolates to a relevant expert for accurate species identification may be useful.

42.2.2.1.1.2.1. Elution Methods. Elution methods are best used for samples that are soluble or insoluble but readily dispersible in liquid. Typically, elution methods measure an amount of sample (mass, volume, or area) and disperse the sample in a known volume of liquid eluent, usually a sterile isotonic broth. However, $sdH_2O$ is also satisfactory because fungal cell walls provide considerable protection against plasmolysis. Aliquots of eluent may be either plated directly or diluted serially prior to plating. Following incubation, growing colonies are enumerated and the culturable fungal burden of the specimen may be determined in CFU per unit of sample.

### Indications

Unlike other culture-based analytical methods, elution methods yield quantitative results. This permits evaluation of the test results against qualitative as well as quantitative interpretation criteria. One disadvantage of elution-type methods over other culture-based techniques is that they tend to overrepresent heavily sporulating fungi, producing results that do not necessarily reflect the true or active fungal populations of the specimen in terms of relative biomass. Even with this limitation, these methods remain widely used because they permit the comparison of specimens of similar composition using numerical data that are well suited to statistical analysis. Usually the elution results are expressed according to mass units (e.g., CFU per gram or CFU per milligram). However, area-based measures (e.g., CFU · 100 $cm^{-2}$ or CFU · $cm^{-2}$) may be better suited for specific applications such as duct interiors. Two procedural variations are commonly used for the plating of eluted samples: (i) serial dilution and (ii) direct plating. Serial dilution is a more cumbersome method than direct plating due to the need for dispersion of aliquots of eluent in molten agar. Also, there is potential for reduction of fungal viability due to heat shock. Compared to direct plating, however, serial dilution provides more even dispersion of sample and improved quantitation.

### Procedure

1. Disperse a known quantity of specimen in a volume of eluent such as $sdH_2O$ or 2% peptone broth. The addition of 10 to 200 ppm of a wetting agent such as Tween 20 is helpful for wetting hydrophobic specimens. The ratio of specimen to eluent is arbitrary, but a good starting point is in the range of 20 to 100 mg of specimen per ml of eluent. Higher elution factors may be needed if the test material contains fungicidal additives.

OPTION A—*Serial dilution*

2a. Transfer 1 ml of well-mixed eluent to another tube containing 9 ml of sterile eluent and mix. Transfer 1 ml from this tube to another containing 9 ml of eluent broth and mix; repeat two or three times.

3a. Transfer 1 ml from each of the dilution tubes separately to an empty sterile petri plate.

4a. Disperse the elution aliquot by gentle swirling in 20 to 30 ml of sterile molten agar cooled to 45°C. Let plates cool to room temperature overnight to allow excess moisture to evaporate from the agar surface.

OPTION B—*Direct plating*

2b. Using a sterile disposable micropipette or calibrated loop, dispense aliquots of 100, 10, and 1 μl separately on the surfaces of agar plates.

3b. Disperse the aliquot on the medium surface by first spreading the fluid in a central streak across the diameter of the plate and then rotating the plate 90° and streaking back and forth across the entire length of the initial central streak. For small aliquot volumes (e.g., 1 μl) it may be useful to dispense the eluent in a larger volume of sterile fluid previously transferred to the plate.

4b. Allow inoculated plates to sit overnight to let excess moisture evaporate.

5. Incubate the plates inverted, under appropriate conditions, for 7 to 21 days. The inclusion of a diurnal light/dark cycle during incubation may promote sporulation in some fungi and facilitate identification. Alternatively, exposure to blue-black (near-UV) light may be helpful in inducing sporulation in some fungi, especially dematiaceous phylloplane molds.

6. Select one plate from the prepared concentration series to identify and enumerate colonies. Ideally, the number of colonies on the enumerated plate should be between 30 and 60. Analyzing plates with higher colony densities may lead to underrepresentation of slow-growing fungi (e.g., *Stachybotrys*) due to competitive inhibition or overgrowth by more rapidly growing taxa (73 [section 5.2.3.1]).

### Interpretation

Fungi and their propagules are ubiquitous and are likely to be recovered during any investigation employing culture-based methods, even in the absence of indoor fungal growth.

Without direct microscopic evidence of the presence of fungal growth, the simple isolation of a fungus from a bulk sample does not indicate colonization. The interpretation of elution culture results is one of several areas in environmental mycology where objective interpretive criteria are almost entirely lacking and subjectivity dominates.

There has been long-standing recognition that these methods do not provide reliable measures of the biological activity of environmental samples. Excellent discussion of the limitations of these methods is given by Barron (17) and Miller (78). For the purposes of indoor mycology, the quantitative burden of culturable fungi does not correlate well with the presence of fungal growth. This is especially true of indoor dust. For example, in strict quantitative terms a concentration of 10 to 100 CFU of culturable fungi $mg^{-1}$ in quiescent surface dust is at most an ambiguous finding and arguably an expected finding in the absence of a moisture or mold problem. In contrast, the qualitative composition of the fungal community of environmental specimens is often relevant to the evaluation of a problem.

The following general guidelines are proposed for culture-based results.

*The following apply if fungal growth is confirmed by direct microscopy of a specimen.*

- The recovery of the same fungus from culture of a specimen is confirmatory. This procedure is indicated only where species level identification or any other information not available by means of direct microscopy is relevant to remedial, protective, or control measures.
- The failure to recover fungi from a specimen may be related to reduced viability of propagules or vegetative hyphae or unsuitability of the culture conditions for cultivating the taxa concerned. Nevertheless, the result of direct microscopy is definitive in establishing the presence of fungal colonization, and this conclusion is not weakened by the lack of a positive culture result.

*The following apply if fungal growth is* not *confirmed by direct microscopy of a specimen.*

- Failure to recover viable fungi from a specimen supports but does not confirm the absence of contamination or colonization.
- Recovery of viable fungi from a microscopically "clean" specimen presents one of the greatest interpretive challenges in environmental mycology. The extent to which a positive culture result indicates fungal growth on a specimen or surface contamination from a local source usually requires establishing that the recovered flora differs from the expected "normal flora" of the material. Normal flora in this case refers to the expected surface load of viable fungi on the material. Because fungal propagules are ubiquitous, the recovery of low levels of environmentally common species from otherwise clean surfaces is an unremarkable finding. Several examples of expected microfungal flora of various materials under normal conditions compared to fungal contamination follow. These examples are not intended as safe limits.
  - *Wood and wood products*—A wide range of fungi are known to inhabit wood, ranging from basidiomycetes to molds. Recovery of basidiomycetes from wood by dilution methods is poor since many of the common wood rot basidiomycetes that are likely to be encountered as indoor contaminants bear their spores on a highly differentiated fruiting body and do not typically produce asexual spores on the surface of their vegetative mycelium. Thus, the isolation of low levels of wood rot basidiomycetes by dilution methods is strongly suggestive of colonization. In contrast, most molds are copious sporulators. For example, a microcolony of *Cladosporium* or *Penicillium* on a wood surface shaving may yield very high levels per unit mass in a culture-based assay (e.g., >100 or 1,000 CFU · $mg^{-1}$) but have little relevance to the condition of the material.
  - *Fibrous insulation*—Fibrous insulation products are used for thermal and sound insulation. These materials consist of fibrous batts or panels (usually fiberglass) containing a resin binder sometimes affixed to a backing membrane. Inorganic insulation materials are fairly resistant to fungal attack. Under conditions of persistent moisture, however, these materials are prone to mold growth (often yielding high numbers of a single or a few species of *Aspergillus* or *Penicillium*, e.g., >100 CFU · $mg^{-1}$). Fibrous insulation on the interior of ducts or wall cavities can act as a filter to trap spores and other airborne debris. In such cases, insulation becomes discolored or dirty, yielding moderate to high levels of culturable fungi (e.g., up to 100 CFU of mostly phylloplane molds $mg^{-1}$) even though the materials themselves may not exhibit fungal growth.
  - *Broadloom and broadloom dust*—As with insulation, the fibrous nature of broadloom and broadloom dust imparts a high capacity to trap and retain ambient airborne fungal spores. In addition, the common North American habit of wearing street footwear indoors further contributes to the content of fungi in these materials. Thus, by culture methods alone it is extraordinarily difficult to distinguish conclusively a carpet that is actively growing fungi from one that is simply dirty. In both cases, levels of total culturable fungi in vacuum-collected broadloom dust are typically up to 500 CFU · $mg^{-1}$. In dirty carpets, phylloplane fungi (e.g., *Alternaria*, *Cladosporium*, *Epicoccum*, and *Ulocladium*) and soil fungi (e.g., *Mucor*, *Rhizopus*, and *Trichoderma*) predominate. In contrast, the fungal flora of actively moldy carpets usually includes at least several of the following taxa in a dominant rank proportion: *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Eurotium*, *Paecilomyces*, *Penicillium* subgenus *Penicillium*, *Scopulariopsis*, and *Wallemia*.
  - *Wallboard*—Paper-faced gypsum wallboard is the most commonly used wallboard product in North America today. This product category has been in widespread use for nearly 40 years as a rigid-sheet finishing panel for interior walls. More recently, gypsum wallboard has gained notoriety for the propensity of its paper facing layers to serve as substrates for hazardous molds, notably *Stachybotrys chartarum*. In the absence of moisture under normal conditions of use, gypsum wallboard should be devoid of fungal growth. The interior-facing surfaces of gypsum wallboard are painted or finished, greatly reducing their porosity and surface irregularities that might otherwise trap moisture or airborne particles. Gypsum wallboard paper with more than 10 CFU · $mg^{-1}$ suggests growth on the material itself, particularly if the taxa recovered comprise only one or a few nonphylloplane molds. Levels below 1 to 10 CFU · $mg^{-1}$ may represent passive deposition of spores or hyphal fragments on the

paper surface (especially if the taxa recovered are phylloplane molds). However, low levels of nonphylloplane molds (e.g., *Acremonium*, *Aspergillus*, *Chaetomium*, *Paecilomyces*, *Penicillium*, *Scopulariopsis*, or *Stachybotrys*) may indicate a nearby mold growth site.

- *Quiescent settled dust (swab or wipe collected, not vacuum collected)*—Like broadloom dust, settled dust is a complex mix of inorganic and organic particles and fibers, most of which at one time were airborne. Because quiescent dusts remain dry under normal circumstances, their microfungal content tends to remain intact and ungerminated. For this reason, sampling passively settled dusts has been suggested as a good way to measure the long-term indoor airborne spore load. Thus, the common analytical goal in testing settled dust is the determination of a fungal growth source in the area where the dust was taken rather than investigating the dust itself for growth. Negative results should be interpreted cautiously because the presence of fungal growth in a building interior does not always cause an increase in the fungal content of settled dust.

### Notes

Most laboratories express the results of quantitative culture of bulk specimens as a function of the wet mass of the material. Although the expression of viable fungal content by wet mass is an underestimate of the same calculation by dry mass, the difference is likely to be insignificant with most bulk materials. Thus, adjustment for dry weight of sample is not indicated unless there is considerable variation in the moisture content of a sample set or there is a need to compare results with existing data expressed by dry mass. For quality control, one sterility control should be performed on the elution buffer for each batch of 10 samples processed.

For nonporous or very dense materials such as linoleum flooring or solid wood, quantitative culture expressed by unit mass may be less informative than an expression based on surface area since the interior of the specimen is likely to be barren yet may account for the majority of the sample's mass.

42.2.2.1.1.2.2. PARTICLE PLATING. Particle plating, also called the "soil crumb" method, involves the direct transfer of small fragments of material to a suitable fungal growth medium (78, 117). This method can provide a more reliable qualitative determination of active fungal flora than elution-based methods, which tend to overrepresent heavily sporulating fungi and underrepresent mycelial taxa. The plating of multiple particles from a single test material can provide a semiquantitative assessment of fungal colonization by expressing the recovered fungi in terms of the proportion of particles colonized.

### Indications

Particle plating favors the isolation of fungi that are present as actively growing hyphae as opposed to dormant spores. Thus, it is a useful method where mycelial fungi are the suspected agents of biocontamination, such as wood rot basidiomycetes.

### Procedure

1. Remove a fine particle of material from the test specimen using sterilized fine forceps or a fine needle. Ideally, the particle should be smaller than 1 mm in diameter. It may be helpful to use a stereomicroscope for the selection the particle. Rolling the tip of the transfer tool in sterile mineral oil may assist in particle recovery.

2. Transfer the particle to the surface of a petri plate containing a suitable growth medium. If the particle is very fine, it is a good idea to circle the area on the underside of the plate where the particle was placed in order that resulting fungal colonies are properly attributed. Up to 10 or 12 particles can be inoculated on a single petri plate as long as they are from the same specimen.

3. Incubate the plates upright under appropriate conditions and examine them under a stereo microscope for signs of growth at 24 and 48 h. Subculture emergent hyphal tips individually for identification.

### Interpretation

The first fungal filaments to emerge from a plated particle may represent the active fungal flora of the particle. This is based on two assumptions: (i) actively growing fungi exist in a state of log-phase growth, and (ii) most fungal spores possess a brief lag phase between exposure to suitable growth conditions and germination. Comparing the results of particle plating to those of other culture-based methods or direct microscopy may help to establish the fraction of fungal flora that is biologically active.

### Notes

This procedure complements direct microscopic examination by assisting in the determination of active fungal colonists. However, not all fungi present and actively growing can be recovered by culture methods, particularly if complex physiological requirements are not met by the isolation conditions.

Based on the notion that actively growing fungi are more likely than dormant spores to adhere to particles, Warcup (116) suggested that particle plating be preceded by gentle washing of the particles in water to remove loose spores and enhance the recovery of mycelial fungi. Similarly, surface sterilization may be used on larger solid particles to eliminate exterior contaminants.

## 42.2.2.1.2. Volumetric Air Sampling

Air sampling for fungi was first employed as a deliberate sampling method by Pasteur in the 19th century. Although the principle remains the same, a large number of sophisticated modern fungal air sampling methods exist that are used widely. These techniques can be divided into two categories according to detection method: (i) spore trap sampling and (ii) viable sampling. Spore trap sampling involves the collection of airborne particles by filtration or impaction. The analysis and quantitation of fungal content are done by light microscopy. In contrast, viable sampling collects airborne particles by impaction or centrifugation on the surface of a suitable growth medium. Following incubation, the resulting fungal colonies are counted and identified. A third technique, liquid impinging, is suitable for culture or direct microscopy, but is cumbersome and seldom used.

It is important to note that for several reasons air sampling data derived by either technique cannot be used to assess human exposure. (i) Airborne spore loads vary greatly over time, changing by orders of magnitude during the course of a single day. Brief "grab sample" techniques characteristic of most air sampling methods cannot account for these fluctuations. (ii) Results of air sampling methods cannot readily be converted to standard units of exposure assessment (milligrams of contaminant per cubic meter of

air). (iii) The airborne fungal spores are a complex, ever-changing mixture where each taxon may contribute a unique hazard set. Despite these limitations, air sampling methods remain widely used primarily for the detection of indoor fungal growth sites.

**42.2.2.1.2.1. Spore traps.** The spore trap method involves collecting total airborne particulate on a filter membrane or adhesive-coated slide. Analysis is by direct examination by light microscopy. A number of collection devices are used for this method. The most commonly used devices are (i) mixed cellulose ester membrane filters (MCEMs) and (ii) slit impactors such as the Allergenco MK-3 (Allergenco, Charleston, SC) and the convenient, disposable Air-O-Cell® cassette (Zefon International, St. Petersburg, FL).

The high variability in airborne spore content greatly reduces the importance of variation in counting method as a significant source of error. The most critical aspect of spore trap analysis is accuracy in identification. Spore trap analysis is the most technically challenging task in environmental mycology, requiring considerable skill and experience of the analyst for accurate identification of spores and other particles. It literally requires years of experience, not just analyzing spore trap samples but also examining whole specimens from field collections, in order to develop the depth of knowledge required to reliably and accurately analyze these kinds of samples. This level of experience cannot be trained in a short time, nor can it be acquired easily by individuals lacking advanced training in mycology or botany. Therefore, laboratories engaging in the analysis of spore trap samples are strongly encouraged to ensure that spore trap analysts possess a minimum of a graduate level university degree in mycology with an emphasis on fungal taxonomy and identification as well as field experience. Analyst certification programs will undoubtedly help to promote uniformity in these analytical procedures (e.g., Pan-American Aerobiology Certification Board [www.paacb.org]).

## Indications

Spore trap methods have an advantage over culture-based techniques in that they enumerate fungal particles regardless of viability. Because of this, spore trap methods are sometimes incorrectly termed "nonviable" techniques when they might more appropriately be called "total" sampling methods since both viable and nonviable propagules are counted. The major limitation of these techniques is their inability to provide low-level identifications of spores because many species level and even genus level fungal identifications require the examination of spores as well as spore-producing structures. The identification of certain fungi additionally requires knowledge of physiological characteristics. All of this information can be derived from a growing culture but not from spore trap specimens. As a result, spores in spore trap samples are only identified presumptively based on size, shape, and color.

## Procedures

### MCEM

MCEM spore traps typically use longer-term sample periods than slit impactor methods (e.g., 1 to 8 h versus 0.5 to 30 min). However, the fact that particles tend to be collected in an even distribution on the membrane in a single focal plane makes this sampling method appealing from an analytical standpoint. In addition, breakthrough (re-entrainment of sampled particles) is not a problem with this technique because the size of fungal cells greatly exceeds the pore size of the membrane filters used for collection.

Generally, a 25-mm MCEM is used with a 0.8-μm pore size supported by a backup pad housed in a three-piece electrically conductive cassette with a 50-mm extension cowl to reduce the dispersion of collected particles due to static electric effects. This is a standard sampling device used for airborne asbestos sampling as set out by National Institute for Occupational Safety and Health Analytical Method No. 7400. The laboratory analysis of MCEM samples requires the use of a "hot block" vaporizer to generate acetone vapor for clearing the filter. Preassembled cassettes and acetone vapor generators are available from health and safety supply houses.

#### Sample acquisition

1. Calibrate a high-volume air sampling pump using an equivalent calibration cassette in-line to draw between 2 and 15 liters of air $min^{-1}$.
2. Connect the pump hose to the outlet nipple of the test cassette and remove the end cap from the cowl. Samples should be collected with the cassette facing downward to reduce oversampling due to particle deposition by gravity. A total of 1.0 to 1.2 $m^3$ of air should be collected. In dusty areas a lower sampling volume may be used.
3. One blank MCEM should be analyzed for every 10 field samples. Blank MCEMs should be handled exactly as test samples with the exception that air should not drawn through them.
4. Completed samples and blanks should be re-capped, labeled, and placed in a clean box or bag away from light and moisture until analysis.

#### Laboratory analysis

1. Remove the MCEM from the cassette and backup pad and place it, sampled surface upward, on a clean glass microscope slide.
2. Insert the slide and filter into the vapor chamber of a hot block set at 70 ± 2°C.
3. Inject 100 to 200 μl of acetone into the injection port. Wait 3 to 5 s before removing the slide and filter. If the filter did not clear completely, repeat the procedure. A minimum volume of acetone should be used to avoid dislodging sampled particles.
4. Place a small drop of mounting fluid in the center of a square 22-mm no. 1 glass coverslip (e.g., 5 to 10 μl). Suitable mounting fluids include lactophenol cotton blue and lactofuchsin.
5. Place the coverslip on the cleared filter and allow to stain for 5 to 10 min prior to examination. Using a darkly colored fine-point permanent marker, trace a line corresponding to the sampled area of the filter on the reverse of the microscope slide.
6. Using a microscope with a calibrated stage micrometer, examine sufficient microscopic fields to evaluate a minimum of 3.5% of the sample area of the filter. For densely sampled filters, it may be sufficient to stop counting once 100 fungal elements have been counted. Warner and Emberlin (118) suggested that counting a minimum of 1.4% of the area of a Hirst spore trap provides a satisfactory representation of the total sample in terms of taxonomic diversity; however, it is unclear if this threshold can be applied to other sample formats. In any case, microscopic fields should be enumerated in tandem along three or four adjacent transects of the full diameter of the filter. Each transect should be separated from its neighbor by at least

one field diameter. Care must be taken not to count beyond the sampled area of the filter. Identify and count all fungal elements that lie within the field of view, including floating elements present initially. As a general rule, elements lying partially outside of the field of view should be counted on only one half of the field (e.g., right side) and ignored on the other half of the field (e.g., left side).

7. Extrapolated airborne concentration in counts per cubic metre of air (C) is given by

$$C = \frac{A_T}{n \times A_f} \times \frac{C_R}{Q \times t} \times 10^3 \text{ liters} \cdot \text{m}^{-3} \quad (1)$$

where C is extrapolated airborne counts (per cubic meter), $A_f$ is area of one microscopic field (in square millimeters), $A_T$ is total sampled area of the MCEM (in square millimeters), $n$ is number of microscopic fields evaluated, $C_R$ is raw count, $Q$ is flow rate of sampler (in liters per minute), and $t$ is duration of sample (in minutes).

### Air-O-Cell®

Air-O-Cell® is a sticky slide packaged in a single-use slit impactor cassette. Airborne particles are collected on a glass wafer coated with an optically clear gel. The cassettes are individually serial numbered and stamped with an expiry date (Fig. 4).

#### Sample acquisition

1. Calibrate a high-volume air sampling pump using an equivalent calibration cassette in-line to draw 15 to

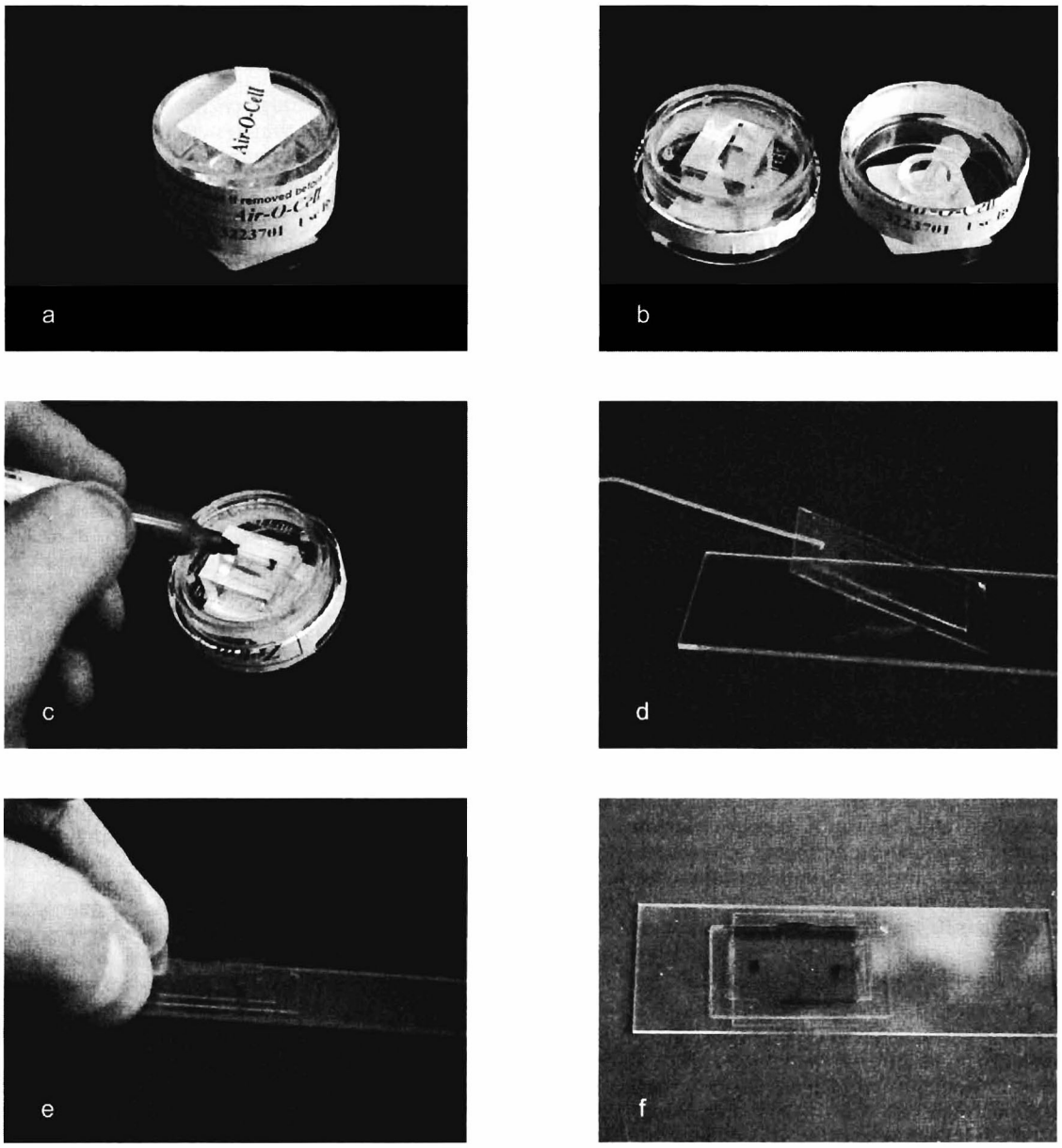


**FIGURE 4** (a) Preparation of the Air-O-Cell® spore trap. (b) Separate the cassette by breaking the shrink seal. (c) Mark the ends of the inlet slit. (d) Remove the gel-coated glass wafer and gently lower it, gel side up, onto a drop of clear lactic acid. Using the placement marks, adjust the wafer so that the sampled area lies parallel to the sides of the microscope slide. (e) Hang a drop of mounting fluid on a coverslip and gently lower it onto the gel surface. (f) Completed mount.

20 liters · $min^{-1}$. Lower flow rates reduce the impaction efficiency of the device and broaden the particle trace, whereas higher flow rates may produce "bounce off" and breakthrough of sampled particles.

2. Note that the shelf life of the cassette is current, and connect the outlet of the cassette to the sampling pump. Suspend the cassette so that the sample slit faces downward. Retract the vinyl slit cover.

3. The manufacturer-recommended sampling volumes are 150 liters for generally clean indoor environments and 75 liters for dusty environments or outdoors. Lower sample volumes (e.g., 10 to 25 liters) may be appropriate for very dusty environments such as remediation enclosures or industrial processes.

4. A minimum of one field blank should be collected for every 10 field samples. Field blanks should be handled exactly as test samples with the exception that air is not drawn through them.

5. Completed samples and blanks should be resealed, labeled, and placed in a clean box or bag away from light and moisture until analysis.

### Laboratory analysis

1. Separate the upper and lower halves of the sample cassette using a coin or a fork.

2. Mark the location of the sample trace on the underside of the glass wafer using a fine-point indelible marker.

3. Place a small drop (e.g., 5 μl) of clear lactic acid on a microscope slide. Remove the glass sample wafer from the plastic cassette housing and place it, gel side up, in the lactic acid. Using the pen marks for reference, adjust the sample so that the trace is parallel to the long axis of the microscope slide.

4. Place a drop of mounting fluid (e.g., lactophenol cotton blue or lactofuchsin) in the center of a 22-mm square coverglass. Invert to hang the drop of mounting fluid and carefully lower onto the sticky surface of the Air-O-Cell® sample.

5. Allow to stain for 5 to 10 min prior to examination.

6. The field diameter of the microscope lens used to analyze spore trap samples must be calibrated using a stage micrometer. Identify and enumerate the fungal elements present in a minimum of one-third of the trace by counting along transects perpendicular to the trace. The microscopic transects should be enumerated in the central portion of the trace and separated from each other by at least one microscopic field diameter. In samples showing an elevated density of fungal elements the number of transects evaluated may be reduced to the number of complete transects required to count a total of 100 or more fungal elements. Note that all of the transects counted must be counted in their entirety (i.e., from one side of the slide to the other).

7. Extrapolated airborne concentration in counts per cubic metre of air (C) is given by

$$C = \frac{L}{n \times \Phi} \times \frac{C_R}{Q \times t} \times 10^3 \text{ liters} \cdot \text{m}^{-3} \qquad (2)$$

where C is extrapolated airborne counts (per cubic meter), $L$ is 14.4 mm (length of Air-O-Cell® sampling slit), $n$ is number of transects evaluated, $\Phi$ is diameter of microscopic field (in millimeters), $C_R$ is raw count, $Q$ is flow rate of sampler (in liters per minute), and $t$ is duration of sample (in minutes).

## Interpretation

In the absence of indoor fungal growth, outdoor air can be expected to be the largest contributor to the indoor airborne spore load. Thus, an indoor fungal growth source should be suspected if the taxonomic profile of fungi in the indoor air varies significantly from that of the outdoor air. One of the major difficulties in interpreting the results of any air sampling method is the problem of accounting for the enormous quantitative fluctuations in airborne spore load that occur over time. There has been general acknowledgment that the numerical data generated by these methods are unreliable and should not be used as a primary determinant in interpretation (78). A more meaningful index of interior condition as reflected by the results of air sampling can be determined by a comparison of the organisms recovered indoors relative to outdoors. When the predominant organisms indoors are not present outdoors or vice versa, the interpretation of spore trap results is straightforward. However, where an objective interpretation is required, the comparative assessment of the biota of indoor versus outdoor air can be accomplished statistically using Spearman's rho or rank correlation (36, 104).

Taxa observed in all samples are assigned a rank according to airborne concentration from highest to lowest. For each taxon observed, the absolute value of the difference between indoor and outdoor rank is summed and divided by the total number of taxa seen. Spearman's coefficient ($r_s$) is given by

$$r_s = 1 - \frac{6\sum(d_i)^2}{n(n^2-1)} \qquad (3)$$

where $d_i$ = difference between indoor and outdoor rank for the same taxon, and $n$ = number of taxa observed.

An example dataset is shown in Table 1. The average value of tied ranks is substituted when ties occur. In the case where a taxon is absent from one site yet present in the other, that taxon is assigned the lowest rank where it is absent. In the example data set shown in Table 1, the sum of the squared rank differences is

$$(0)^2 + (-4)^2 + (+2)^2 + (-2)^2 + (+3.5)^2 + (-2)^2 + (+2.5)^2 = 46.5$$

and Spearman's coefficient for this data set is

$$r_s = 1 - \frac{6 \times 46.5}{7(7^2-1)} = 0.170$$

The null hypothesis of Spearman's test is that there is no correlation between the sample sets (i.e., the biodiversities in the two compared are different). Degrees of freedom (*df*) for Spearman's coefficient is calculated as $n - 2$, or 5 for this data set, and the corresponding critical value, $r_s$*, at $\alpha = 0.05$ with 5 degrees of freedom is 0.900. Critical values for Spearman's coefficient are given in many general statistics textbooks (e.g., reference 83). In our example, the computed value $r_s$ is well below the critical value, $r_s$*, and the null hypothesis cannot be rejected. In other words, the taxonomic profiles of indoor and outdoor air are statistically different, implying the existence of an indoor growth source.

This test is useful but does not account for nominal variation between indoor and outdoor airborne taxa that might directly suggest an indoor growth site. For example, "indicator fungi" such as *Chaetomium* and *Stachybotrys* are sufficiently rare in outdoor air samples that their presence indoors individually or collectively even at detectable levels is decisively symptomatic of indoor mold growth. A good discussion on limitations of Spearman's rank correlation in the interpretation of air sample results is given by Miller (78).

**TABLE 1** Ranking of indoor versus outdoor air spora

| Taxon | Indoor | | Outdoor | | $d_i$ |
|---|---|---|---|---|---|
| | cts[a] · $m^{-3}$ | Rank[b] | cts · $m^{-3}$ | Rank | |
| Basidiospores | 660 | 1 | 1,980 | 1 | 0 |
| *Aspergillus/Penicillium* | 506 | 2 | 22 | 6 | −4 |
| Ascospores NOC | 220 | 4 | 990 | 2 | +2 |
| *Fusarium* | 440 | 3 | 44 | 5 | −2 |
| *Alternaria* | <LOD[c] | 6.5 | 264 | 3 | +3.5 |
| *Scopulariopsis* | 110 | 5 | <LOD | 7 | −2 |
| *Cladosporium* | <LOD | 6.5 | 154 | 4 | +2.5 |

[a]cts, counts.
[b]1 = most abundant.
[c]LOD, limit of detection.

## Notes

Spore trap specimens should be examined at a minimum of ×600 magnification in bright-field microscopy or ×500 magnification if an oil immersion objective is used. Other light microscopy formats such as phase contrast or, still better, Nomarski differential interference contrast microscopy offer improvements over basic bright-field microscopy for the analysis of spore trap samples. Regardless of the illumination method, planachromat or planapochromat objectives should be used in the analysis of spore trap samples to ensure an optically flat field of view free of chromatic aberration. This will greatly help to reduce the possibility of misidentifying or miscounting peripheral particles. The use of a graticule may improve counting by permitting more accurate measurement of area assessed and help to reduce eye fatigue.

Standard recipes for microscopic mounting media often include much more dye than is desirable for the preparation of spore trap samples. Preparation of mounting fluids with 10 to 20% of the recommended dye content is usually adequate for staining and makes the preparations easier to examine. Iodine vapor staining (discussed above) may help to distinguish small hyaline spores from starch granules. As well, many fungal spores have diagnostically relevant surface features that can be revealed by iodine staining.

While a number of resources exist as aids to spore identification, there has been little literature discussion of the standardization of criteria for counting particles on spore traps. Although variability is normally quite high as a function of the nonhomogeneous dispersion and episodic nature of spore aerosols, several common procedural variations in spore counting may increase imprecision and thus warrant discussion.

- *Single spores, spore chains, and clustered elements*—In general, most analysts count the total number of spores or other elements observed with little regard to grouping or distribution. In the context of a naturally produced bioaerosol, the propagules of many aerially dispersed fungi exist commonly as aggregates rather than as single spores. This is certainly true of many plant-pathogenic conidial fungi and ascomycetes for which spore aggregation is a beneficial property, increasing the terminal velocity of the infective particle and enhancing its penetration of the boundary layer of air surrounding plant leaf surfaces. In contrast, a culturable, multispored particle will yield a single colony if deposited on a growth medium and thus will be counted only once by viable sampling methods (viable sampling is discussed in the next section). This methodological difference contributes to the unjustified notion that a vast majority of airborne fungal spores are nonviable or at least nonculturable. Given that (i) single and aggregated airborne spores both behave as single particles and (ii) measurement units used for air sampling are not relevant to exposure measures (e.g., counts per cubic meter versus milligrams per cubic meter), it is recommended for comparative purposes that airborne fungal particles on spore trap samples be tallied by impaction event rather than spore number.
- *Elements floating freely in the mount*—The use of viscous mounting media and removal of excess mounting fluid by gently blotting will help to reduce the movement of spores in microscopic examination of a spore trap preparation. Nevertheless, with all spore trap sampling methods there are occasional "floaters." One way to deal with drifting particles is to count only those particles present in the field initially and ignore those that enter the field during counting.
- *Elements within but extending beyond the field of view*—An approach based on that of Lacey and Venette (66) and adopted by many analysts is to count such elements on only one half of the field of view and to ignore them on the other half.
- *Taxa observed during setup but not encountered during analysis proper*—The low-level presence of certain problem indicator taxa (e.g., *Chaetomium* and *Stachybotrys*) should be reported if observed both formally during counting and informally during setup. If these taxa are only observed informally, then they should be reported but at a concentration below the limit of detection (LOD). The inclusion of a deliberate final raster scan of the complete slide may help to formalize the reporting of rare taxa below the LOD.

**42.2.2.1.2.2. Viable sampling.** The mainstay of fungal air sampling is viable sampling. This method involves the collection of airborne particulate matter on the surface of a culture medium by impaction or centrifugation. Airborne fungal content is extrapolated from the colony counts obtained following an appropriate incubation period. The results of viable air sampling are usually expressed in CFU per cubic meter of air. As is the case with spore trap air sampling, viable sampling methods cannot provide measurements relevant to the assessment of exposure. Even so,

these techniques are useful in detecting the existence of indoor fungal growth. Viable air sampling offers the practical benefit of basing identifications on actively growing colonies of fungus rather than solitary spores, permitting a low level of taxonomic discrimination (e.g., species level identifications may be determined directly for many genera of microfungi). Viable sampling methods have several limitations.

- Short-duration sampling is necessary to prevent excessive drying of growth media. The use of short sampling times increases the extent to which sample results are influenced by transient airborne spore bursts.
- Standard growth media do not support the growth of all fungi present at a given time in the air. A great number of commonly occurring environmental fungi have specific nutritional requirements that collectively cannot be met by the use of a single growth medium. Sometimes these fungi are referred to as nonculturable. Thus, the media that are typically recommended for viable sampling procedures are intended to optimize recovery of the most relevant subset of the problem indoor aeromycota.
- An incalculable proportion of the fungal component of air is nonviable and thus cannot be detected by viable sampling methods.
- Lengthy incubation times are often required for sufficient growth and sporulation to perform accurate identifications (e.g., 7 to 21 days). Reducing incubation time or increasing the incubation temperature subselects fast-growing or thermotolerant taxa, respectively, biasing results.

## Indications

Viable sampling is useful as a frontline investigative technique where indoor fungal biocontamination is suspected but not directly observed during a walk-through inspection. Viable sampling is also the preferred air sampling method when low-level identifications (e.g., species level) are required. This method is indicated where excessive nonfungal background particulate present in the air may interfere with spore trap analysis.

A number of commercially available devices exist for viable air sampling such as centrifugal samplers (standard and high-flow RCS; Biotest, Dreieich, Germany), Anderson N6 and two-stage samplers (Thermo Anderson, Smyrna, GA), and the Surface Air Sampler (Bioscience International, Rockville, MD).

## Procedure

1. The sampling device should be wiped with a disinfectant solution such as 70% isopropyl alcohol and allowed to dry. This will help to prevent carryover contamination from previous work. As well, a sampling plan should be clearly laid out prior to beginning.

2. Insert the sampling medium into the air sampler by following the manufacturer's recommendations. Wear latex or nitrile gloves during this process to prevent handling contamination.

3. Switch on the sampler or sampling pump to take the air sample. The flow rates of most units are fixed according to the manufacturer in the range of 25 to 60 liters · $min^{-1}$. Air samples taken in the summer months should be taken with a reduced sample volume to avoid oversampling due to the normally elevated outdoor levels of plant leaf surface fungi. For example, a final sample volume of 80 to 100 liters is usually sufficient for an Anderson sampler or Biotest RCS during the summer months. Accordingly, the total sampling volume of samples taken during the wintertime may be doubled. The intent of varying the sample volume according to the expected level of culturable airborne fungi is to ensure reliability of counting. Specifically, the sample volume should be roughly calibrated to deliver a colony density on agar plate or strip samples in the range of 10 to 60 fungal colonies (e.g., roughly 1 colony · $cm^{-2}$) (73 [section 5.2.3.1]). It is not recommended to sample volumes larger than 200 liters with any viable sampling instrument, in order to avoid desiccation of the surface of the sampling medium, reducing spore viability by hyperosmolar action. Impaction samplers are especially susceptible to these effects (79).

4. Incubate samples inverted under appropriate conditions for 7 to 21 days. The inclusion of a diurnal light/dark cycle during incubation may promote sporulation in some fungi and facilitate identification.

5. Colonies should be counted and identified to as low a level as practical. Care should be taken to notice patterns of nonhomogeneous distribution of colonies on sampling medium. For instance, confluent or patchy distribution of a single species restricted to the margin is suggestive of handling contamination. Similarly, patchy distribution of small colonies around or in a plume extending from a single larger colony may indicate satellite colony formation, particularly where dry-spored molds such as *Aspergillus* and *Penicillium* are present. These artifacts result in spurious elevation of airborne counts and should be noted in the laboratory report whenever they are observed. Generation of satellite colonies can be avoided by incubating sampling media in a still environment and not handling samples until the time of analysis.

6. Level of airborne culturable fungi (C) can be determined from the raw colony count such that

$$C = \frac{C_R}{Q \times t} \times 10^3 \text{ liters} \cdot \text{m}^{-3} \quad (4)$$

where C is extrapolated airborne concentration (CFU per cubic meter), $C_R$ is raw count, $Q$ is flow rate of sampler (in liters per minute), and $t$ is duration of sample (in minutes).

## Interpretation

Several qualitative and semiquantitative guidelines have been widely used for interpreting the results of viable air samples (36, 51, 73). These guidelines use air samples to predict indoor fungal growth. It bears repeating that neither these nor any other currently available guidelines are intended to give safe limits for exposure. Many interpretive guidelines for viable air sampling use the rank order criteria set out by Health Canada (51) based on a set of more than 3,000 viable air samples taken from more than 100 Canadian government buildings in the decade between 1986 and 1996 with an RCS taking 160-liter samples on rose bengal agar. The nine-point Health Canada interpretive criteria are given below.

- Significant numbers of certain pathogenic fungi should not be present in indoor air (e.g., *Aspergillus fumigatus*, *Histoplasma*, and *Cryptococcus*). Bird or bat droppings near air intakes, in ducts, or in buildings should be assumed to contain these pathogens. Action should be taken accordingly. Some of these species cannot be measured by air sampling techniques.
- The persistent presence of significant numbers of toxigenic fungi (e.g., *Stachybotrys chartarum*, toxigenic

*Aspergillus*, *Penicillium*, and *Fusarium* species) indicates that further investigation and action should be taken accordingly.

- The confirmed presence of one or more fungal species occurring as a significant percentage of a sample in indoor air samples and not similarly present in concurrent outdoor samples is evidence of a fungal amplifier. Appropriate action should be taken.
- The "normal" air mycoflora is qualitatively similar to and quantitatively lower than that of outdoor air. In federal government buildings, the 3-year average has been approximately 40 CFU · $m^{-3}$ for *Cladosporium*, *Alternaria*, and nonsporulating basidiomycetes.
- More than 50 CFU of a single species present per cubic meter (other than *Cladosporium* or *Alternaria*) may be reason for concern. Further investigation is necessary.
- Up to 150 CFU · $m^{-3}$ is acceptable if there is a mixture of species reflective of the outdoor air spores. Higher counts suggest dirty or low-efficiency air filters or other problems.
- Up to 500 CFU · $m^{-3}$ is acceptable in summer if the species present are primarily *Cladosporium* or other tree and leaf fungi. Values higher than this may indicate failure of the filters or contamination in the building.
- The visible presence of fungi in humidifiers and on ducts, moldy ceiling tiles, and other surfaces requires investigation and remedial action regardless of the airborne spore load.
- There are certain kinds of fungal contamination not readily detected by the methods discussed in this chapter. If unexplained (sick building syndrome) symptoms persist, consideration should be given to collecting dust samples with a vacuum cleaner and having them analyzed for fungal species.

While very useful, it is important to note that air spora vary considerably by geography. Thus, the numerical and to some extent the species composition data upon which the above guidelines are based are likely to be less relevant at southern latitudes. Dillon et al. (36) suggested the use of Spearman's rho coefficient for detecting differences between the indoor and outdoor air spora for a particular location (as discussed previously in the interpretation of spore trap samples). Other authors have also supported this approach (78), acknowledging that the presence of certain indicator taxa (e.g., *Cladosporium sphaerospermum*, *Aspergillus sydowii*, *Aspergillus versicolor*, *Paecilomyces variotii*, *Scopulariopsis* spp., *Stachybotrys* spp., *Chaetomium* spp., and *Wallemia sebi*) in indoor air samples is strongly suggestive of indoor fungal growth.

### Notes

The validity of viable sample results is dependent on the accurate identification of the taxa present. Certain groups of fungi (notably *Fusarium* and *Penicillium*) require considerable expertise and extensive testing on an isolate-by-isolate basis to provide accurate identification to the species level. For these fungi, species identification is beyond the scope of routine practice, although identification to subgenus or morphological group may provide a practical alternative. However, if multiple cultures derived from a given material or site yield high numbers of what appears to be a single species of a taxonomically difficult genus, referral of one or more isolates to a relevant expert for accurate species identification may be warranted.

Rapidly growing fungi pose a particular problem on cultured air samples by overgrowing less competitive fungi and biasing sample counts. Often it is possible to observe overgrown colonies by examining the colony reverse. Overgrown samples should be retested at a lower sample volume. Additionally, the use of restrictive growth media (e.g., rose bengal agar and DG18) may help to prevent sample overgrowth.

## 42.2.3. Principles and Methods of Yeast Community Analysis

### 42.2.3.1. Introduction

Information on the ecology of yeast communities is scattered across a profuse taxonomic literature and otherwise overshadowed by studies of industrially or medically important species. The following is therefore based largely on our own experience and practice. For a recent review of the subject, see reference 70.

The position of yeasts in nature is founded on the intrinsic properties of the yeasts themselves (fundamental niche) and the environmental factors (realized niche) that ultimately determine where and how yeasts grow in nature. Yeasts are a highly polyphyletic collection of ascomycetous and basidiomycetous fungi (40, 64). Because they share a predominantly unicellular growth cycle, specifically lacking aerial hyphae, yeasts behave very much like heterotrophic bacteria in the laboratory. Consequently, the methods used in the study of yeasts are typical of those used in bacteriology and quite different from those used with the filamentous fungi. The unicellular habit defines both the methodology used in exploring yeast communities and the habitats that should be explored for the occurrence of yeasts.

#### 42.2.3.1.1. The Fundamental Niche

Nutritionally, yeasts share a strictly organotrophic habit (for a comprehensive discussion of yeast physiology, see reference 115). Suitable carbon sources may include many carbohydrates and their polyol derivatives, simple alcohols, organic acids, ketones, esters, alkanes, or more complex compounds, but only a small number of sugars, including D-glucose, are universally assimilated by all known species. Likewise, the nitrogen requirement of yeasts may be satisfied by a variety of inorganic and organic molecules, but ammonia is a quasiuniversal nitrogen source. Most other nutrients may be supplied in the inorganic form, except for vitamins, which are frequently required. A few species require the presence of some amino acids. Various yeasts differ in their growth temperature responses, but most grow well at 25°C. A few species are psychrophilic and only grow at temperatures below 18 to 25°C, and in rare cases, growth is limited to a narrow range from ca. 30 to 40°C. No truly thermophilic yeast has yet been identified. Only a handful of species grow at 45°C or slightly above. The vast majority of yeasts tolerate a broad range of pH values between ca. 3.5 and 8.5. Most are moderately osmotolerant.

Among the yeasts, considerable variation exists with respect to nutritional breadth and metabolic versatility within the limits set above. As a very broad generalization, and with many exceptions, basidiomycetous species tend to be polyphagous with respect to carbon sources and strictly oxidative in the utilization of carbohydrates. The growth of ascomycetous yeasts is usually limited to a narrower range of substrates, and carbohydrates often can be used both oxidatively and fermentatively. Most yeasts appear to be copiotrophic with respect to carbon, and many appear to be oligotrophic with respect to nitrogen.

#### 42.2.3.1.2. The Realized Niche

The characteristics of the fundamental niche not only serve as a basis for the design of culture media but also define the potential range of habitats where yeasts abound in nature (the realized niche). Unicellularity favors submerged growth in liquid substrates or surface growth on moist, solid substrates, although several species are dimorphic and produce invasive hyphae or pseudohyphae. Yeast communities thrive in habitats that are relatively rich in organic carbon, and where other biotic or abiotic factors may restrict the intensity of competition with bacteria and molds. Such selective conditions are most frequently met in the immediate vicinity of other living organisms or their early decay products. Yeasts are considered saprotrophic, although some are intimately associated with other living organisms. Some yeasts are found in association with vertebrates as opportunistic pathogens, but very few have an absolute dependency on their animal hosts and most act as epizootics. A small group of yeasts are able to produce specialized structures that allow them to penetrate other yeasts or even filamentous fungi, resulting in their death (invasive necrotrophy). This may result in a nutritional benefit to "predacious" yeasts, although all are capable of growth at the expense of inert material (68).

Communities consisting of large populations of ascomycetous yeasts commonly occur in tree sap flows, nectar, necrotic tissue of succulents, or damaged fruit. Insects and other invertebrates that utilize these substrates as feeding or breeding grounds are important vectors. In contrast, basidiomycetous yeasts are more often found in the phylloplane. Many are thought to benefit from the formation of carotenoid pigments as protective agents against toxic photoreaction products. Basidiomycetes appear to be less dependent on invertebrates for their movement. Many are ostensibly wind or water dispersed, and some even discharge their propagules actively. As living beings invariably die and find their way to soil and water, populations of yeasts also exist in soils and aquatic habitats. It is not clear, however, whether soil and seawater are habitats that harbor dynamic yeast communities.

#### 42.2.3.1.3. The Place of Yeasts in Nutrient Cycling and the Food Chain

Compared to bacteria, yeasts have rather modest physiological abilities, as all are heterotrophs that require fairly abundant carbon sources. In terms of carbon cycling, yeasts can be regarded as decomposers, although their role in that capacity is unquestionably minor in comparison with that of filamentous fungi. Only a fairly small proportion of yeasts produce extracellular enzymes capable of hydrolyzing polysaccharides, proteins, or fats, and the degradation of the most naturally abundant polymers such as cellulose, lignin, or chitin seems to be confined to the filamentous fungi. Yeasts may effect a reversal of mineralization with respect to nitrogen, thereby enriching the diet of insects that feed on plant exudates, nectar, sap, or decaying tissue (71).

### 42.2.3.2. Collection Methods

Although in situ identification and enumeration of species with molecular probes may become practicable in the future, ecological studies of yeasts currently require that pure cultures be isolated on culture media and purified prior to characterization in the laboratory. The methods described below were developed for the study of natural communities and may differ from those used in clinical or industrial contexts.

#### 42.2.3.2.1. Media

Isolation is generally done on plates. Common media contain various mixtures of glucose and peptones. YM agar is adequate for most situations. Media that do not contain yeast extract should be avoided. As the nutritional requirements of yeasts also satisfy those of many bacteria, the addition of antibiotics (e.g., 50 to 100 ppm of chloramphenicol) is usually necessary for isolation purposes. Acidification of the medium to pH 3.5 (after autoclaving) has been used also, but it is less effective. Molds such as *Mucor* or *Penicillium* also grow well on yeast media, and even a single colony can rapidly invade the surface of a plate. Molds cannot be suppressed selectively without harming some yeasts. Young mold colonies may be excised from agar plates with a sterile instrument, but in cases where they are abundant, a liquid enrichment step may be necessary prior to plating. A small amount of sample is inoculated into a tube of liquid medium (YM broth with antibiotics) and incubated on a Rollordrum™ (New Brunswick Scientific). The rotational mixing delays the formation of conidia and causes the mold to grow as a mycelial ball that is easily removed from the tube with a bent inoculation needle. A loopful of the remaining yeast suspension is then streak inoculated onto agar. Because of the potential for enrichment of antibiotic-resistant bacteria, it is prudent not to use the same antibiotic in both the broth and the agar medium. Kanamycin, streptomycin, and doxicycline have been used.

#### 42.2.3.2.2. Substrate Type

Liquids containing relatively low cell numbers (e.g., seawater) should be passed through a filter membrane (0.45-μm pore size) that is then plated. If cell numbers are high, a liquid substrate may be streak inoculated directly. The streak pattern should ensure that a broad dilution gradient is formed on the plate so that single colonies will arise regardless of the cell concentration in the sample. Colony separation may be facilitated by diluting the liquid substrate with water. Solid materials are mixed vigorously with a suitable amount of sterile water, followed by streak-inoculation of a loopful of suspension. Addition of a small amount (ca. 10 mg/liter) of Tween 80 to the water improves the dispersion of yeast cells stuck to solids. Small insects are conveniently sampled by allowing them to walk over the agar for a few minutes. Larger insects may require dissection or homogenization. Surfaces of solid substrates may be swabbed or scraped with an inoculation loop and sterile water. Ballistosporogenous yeasts can be sampled by affixing the substrate, usually a leaf, to the lid of the petri dish to allow for spore discharge.

Isolation of yeasts from natural substrates is most effective when the material is fresh. Once harvested, yeast-rich materials, especially moist solids, tend to be overcome by molds. As it is rarely convenient to plate in the field, samples can be carried to the laboratory in sterile Whirl-Packs or in small vials containing sterile water. Refrigeration may or may not be advantageous, but protection from excessive heat is essential.

#### 42.2.3.2.3. Isolation

Inoculated plates must be examined periodically. As yeast colonies develop, usually after 2 days, cells from representative colonies are picked with a sterile inoculation needle, suspended thinly in sterile water, and streak inoculated onto a plate of nonselective medium to ascertain the absence of contaminants. Direct plating of yeast cells without resuspension is less effective. The use of dilution blanks

and purification plates in the field, away from the laboratory, may be impractical. In that case, selected colonies may be transferred to small agar slants (1 ml in 2-ml vials). Purified cultures are stored on agar slants or in $sdH_2O$. Permanent preservation requires cryogenic storage or lyophilization. Liquid nitrogen storage in small vials containing porcelain beads (Microbank™ [ProLab Diagnostics]) is particularly convenient.

The details of strain characterization exceed the scope of this discussion, and the reader is referred to reference 123. However, it is useful to mention that standard growth tests are conducted with two chemically defined media available commercially. Yeast nitrogen base may be used in combination with individual carbon sources in searching for yeasts that have the ability to utilize specific carbon compounds. Similarly, yeast carbon base (1.17%) may be supplemented with a nitrogen source to look for yeasts with specific nitrogen assimilation abilities. Until recently, standard growth tests were essential for identification. They now serve a descriptive function. Accurate identification is based on DNA sequencing.

#### 42.2.3.3. Sampling and Data Management, Sample Size, and Numbers

Samples should be assigned simple consecutive reference numbers, to which is attached information about the locality, type of substrate, and voucher specimens. As individual yeast isolates are recovered, they should be assigned a unique number, preferably a decimal extension of the sample number, which serves to cross-reference information on colony characteristics, approximate abundance in the sample, microscopic features, photographs, physiological responses, and any other information. The use of identification codes or acronyms based on the isolation locality, the type of substrate, or any other descriptor inevitably leads to loss of data due to irretrievability in the long term. The information as a whole can be managed using database or spreadsheet software.

If required, the determination of cell numbers in natural substrates may follow any of the procedures used in bacteriology, such as the determination of CFU or most-probable-number approaches. However, in studies of natural yeast communities, establishing the magnitude of species diversity and determining which species are autochthonous members of each community are the most important objectives. Cell numbers vary enormously from one sample to the next and contribute little information relevant to the pursuit of those objectives. The strongest indication that a species is an important member of a community is its repeated occurrence in multiple, independent samples. Indeed, many common species diversity indices are based on presence or absence data and not on relative numbers of individuals (8). Such measures typically stabilize once ca. 15 samples have been screened (69). Accordingly, effort should be spent on examining a sufficient number of representatives of the community, and not on determining cell numbers.

The primary agar plate obtained from inoculation of a sample invariably contains replicates of each species. A decision must therefore be made about how many representatives of each colony type should be picked and purified for further study. When large numbers of samples from the same habitat are examined, the chances of overlooking distinct species because their colonies are similar are minimal, and one representative of each type is sufficient. The cost of identifying large numbers of replicates from a few samples is not justified if many independent samples are available.

#### 42.2.3.4. Identification

Correct identification of species is probably the most important step of an ecological study and once was the most difficult. The so-called conventional approach is based on morphological traits that may be difficult to interpret, even by experienced workers, plus a battery of onerous growth tests (63). As our knowledge of species diversity continues to expand, the discriminatory power of those tests is increasingly insufficient. Identification keys based on morphology and physiology are becoming less and less satisfactory and have a high probability of ambiguous results or error. This is not to say that the knowledge of the reproductive and nutritional features of yeast species is not essential to the characterization of yeast communities. Indeed, they provide important insights on the fundamental niche.

At present, rapid and correct identification is best achieved by determining the sequence of the D1 and D2 variable domains of the large rDNA subunit. The target sequence of an unknown is conveniently amplified by suspending a small amount of a pure yeast culture directly in a PCR mixture containing suitable primers (67). The purified amplification product is sequenced and the result is compared with known sequences. The D1/D2 region offers three important advantages. Most importantly, a comprehensive database for all known species is available in GenBank. Other popular targets, such as the internal transcribed spacer or the small subunit rDNA (SSU) regions, have not been determined systematically for all species. Secondly, for identification purposes, the complete D1/D2 sequence can be obtained in a single reaction, as opposed to three for the SSU. Last, the phylogenetic signal of D1/D2 sequences is normally strong at the species level and the sequences are sufficiently conserved to allow assignment of new species to a genus. In most cases a perfect match or the presence of fewer than two or three substitutions provides a conclusive species identification (64). Exceptions do exist, and some cases may not be resolved without the intervention of an experienced yeast systematist. Better alternatives are not available at this time, although they no doubt will be in the future. The cost of sequencing still precludes its indiscriminate application to very large collections of strains unless one has continuous access to a high-throughput sequencing facility. The present solution is to subject isolates to a preliminary screening procedure based on colony or cell morphology, selected growth tests, or simple molecular fingerprinting. The last should be used with the utmost caution (52).

#### 42.2.3.5. Ecological Interpretation

Yeast community ecology is still in its infancy. Considerable effort is still devoted to the physiological and biochemical characterization of yeasts, but our understanding of their interactions among themselves and with other species, microbial or otherwise, is limited. It would be presumptuous to propose analytical methods purported to lead to genuinely new insights. Great advances will no doubt be made when the methodology is such that identification can be performed in the field.

### 42.2.4. Common Media and Reagents

#### 42.2.4.1. Rose Bengal Agar

Rose bengal agar is a good, widely available medium for general-purpose isolations that permits the growth of most commonly occurring indoor fungal contaminants and rapidly sporulating soil fungi. Although there are a number of

variations, the basic formula uses a base of peptone (5 g/liter), glucose (10 g/liter), mineral salts ($KH_2PO_4$, 1 g/liter; $MgSO_4 \cdot 7H_2O$, 0.5 g/liter), and agar (15 g/liter) to which antibiotics are added to prevent the overgrowth of bacterial contaminants and rose bengal (25 mg/liter) is added to restrict and delimit the growth of fungal colonies as an aid to enumeration. For soil fungi, the nutrients are omitted and half of the water is replaced with 500 ml of boiling water extract of 75 to 200 g of soil. Dichloran may be added to reduce growth of zygomycetous molds, and prepared dichloran rose bengal chloramphenicol (DRBC) agar is available from Oxoid. Rose bengal is known to become somewhat toxic following exposure to light mainly due to the liberation of singlet oxygen species ($^1O_2$). For this reason, these plates should be incubated in the dark.

#### 42.2.4.2. DG18 Agar

Dichloran 18% glycerol (DG18) agar is a good medium for xerophiles such as *Aspergillus* and *Penicillium*, but it does not generally support the growth of *Stachybotrys*. The formula, per liter, includes the following: $KH_2PO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g, dichloran, 1.0 ml of a 0.2% solution in ethanol; peptone, 5 g; glucose, 10 g; and agar, 15 g. These ingredients are steamed to dissolve and melt the agar, and then 220 g of glycerol is added prior to autoclaving and 0.1 g of chloramphenicol in ethanol is added after autoclaving.

#### 42.2.4.3. ME Agar

Malt extract (ME) agar is an excellent medium for culturing wood-inhabiting and litter-decomposing basidiomycetes, but it is not recommended for sampling fungi of human or indoor environments. Several standard formulations exist in the literature, of which the best is that of Nobles (82): 12.5 g of ME and 15 g of agar per liter of distilled water. Antibacterial agents and antifungal agents may be added to improve isolations of basidiomycetes. Addition of sugars increases production of toxic metabolites by the growing cultures and is not recommended. A weak ME broth, with 2 to 5 g of ME per liter of $dH_2O$, is excellent for preparing mycelia of many saprotrophic fungi for DNA isolation.

#### 42.2.4.4. MMN Medium (9, 75)

Modified Melkin-Norkrans (MMN) medium and BAF agar are excellent for isolating cultures of fastidious ectomycorrhizal fungi. Benomyl and dichloran may be added to reduce growth of ascomycetous and zygomycetous molds, respectively, and antibacterial antibiotics are recommended to prevent the growth of bacterial contaminants. The formula, per liter, includes the following: $CaCl_2$, 0.05 g; NaCl, 0.025 g; $KH_2PO_4$, 0.5 g; $(NH_4)_2HPO_4$, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $FeCl_3$, 1.2 ml of a 1% aqueous solution; thiamine HCl, 100 μg; ME, 3 g; sucrose, 10 g; and agar (optional), 15 g.

#### 42.2.4.5. BAF Agar (9, 80)

BAF agar has threefold more sugar than MMN medium and contains a few additional micronutrients and growth factors; BAF agar and MMN medium seem to work equally well for isolating ectomycorrhizal fungi. The formula, per liter, includes the following: $CaCl_2 \cdot 2H_2O$, 0.1 g; NaCl, 0.025 g; $KH_2PO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 10 mg; $MnSO_4$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; thiamine HCl, 50 μg; folic acid, 100 μg; inositol, 50 μg; biotin, 1 μg; yeast extract, 0.2 g; peptone, 2 g; glucose, 30 g; and agar (optional), 15 g. As with MMN medium, benomyl and dichloran may be added to reduce growth of ascomycetous and zygomycetous molds, respectively, and antibacterial agents are recommended to prevent the growth of bacterial contaminants.

#### 42.2.4.6. YM Agar

Available commercially (Difco), "yeasts and molds" agar contains 1% glucose, 0.5% peptone, 0.3% ME 0.3% yeast extract, and 2.0% agar. It can be used for isolation and maintenance of most yeast species.

#### 42.2.4.7. Yeast Nitrogen Base

Yeast nitrogen base is a synthetic medium available from Difco in three formulations. All contain a complete array of defined nutrients, including several vitamins. The standard medium is supplemented with 0.5% ammonium sulfate and three amino acids, some of which are required by a few yeast species. One variant does not contain the amino acids and can be used to isolate prototrophs. Either medium is used at a concentration of 0.67%. Another variant contains neither supplement so that an alternative nitrogen source can be chosen. The normal concentration is 0.17%. All three formulations require the addition of an organic carbon source at the rate of 0.5 to 1.0%.

#### 42.2.4.8. Yeast Carbon Base

Similar to yeast nitrogen base, yeast carbon base contains glucose as a carbon source, but no nitrogen source. Trace amounts of the three amino acids are included to ensure the growth of yeasts that have such a requirement. The normal concentration is 1.17%. Nitrogen sources can be added at a concentration of 0.05% or less in the case of compounds such as sodium nitrite.

#### 42.2.4.9. Antibacterial Agents

Rose bengal, used at 25 mg/liter, is light sensitive and may be toxic to fungi as well as bacteria, but it may be added to media prior to autoclaving (see section 42.2.4.1). Streptomycin sulfate, tetracycline hydrochloride, penicillin G, or chloramphenicol may be added at 50 to 100 mg/liter to media after autoclaving. Addition of a 5-ml/liter concentration of a solution of 20-mg/ml chloramphenicol in 70 to 95% ethanol is convenient and effective for reducing bacterial contamination in isolations from soil or contaminated tissues.

#### 42.2.4.10. Antifungal Agents

Various fungicides may be used to make an isolation medium more selective for the fungi of interest. Fast-growing ascomycetous and zygomycetous molds are sometimes problematic when isolating basidiomycetes, and these may be combated with benomyl and dichloran, respectively (120). Add 1 ml per liter of medium of a solution containing 2 to 5 mg of active ingredient/ml of benomyl or dichloran in 1:1 acetone/ethanol. Cycloheximide (Actidione) has broad toxicity to fungi but may be used at 2 mg/liter to isolate certain fungi that have some tolerance, such as *Ophiostoma* (48, 77). Cycloheximide is added as 2 ml per liter of a 1-mg/ml aqueous solution, before or after autoclaving.

#### 42.2.4.11. 2× CTAB for Preserving Samples for DNA Studies

CTAB is a useful preservative composed of 2% (wt/vol) hexadecyl trimethylammonium bromide (also known as cetyl trimethylammonium bromide or CTAB), 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 1.4 M NaCl in

distilled water (43). The mixture may require heating to bring the CTAB into solution. For convenience, you may choose to buy ready-made 1 M Tris (pH 8) and 0.5 M EDTA (pH 8) from your chemical supplier.

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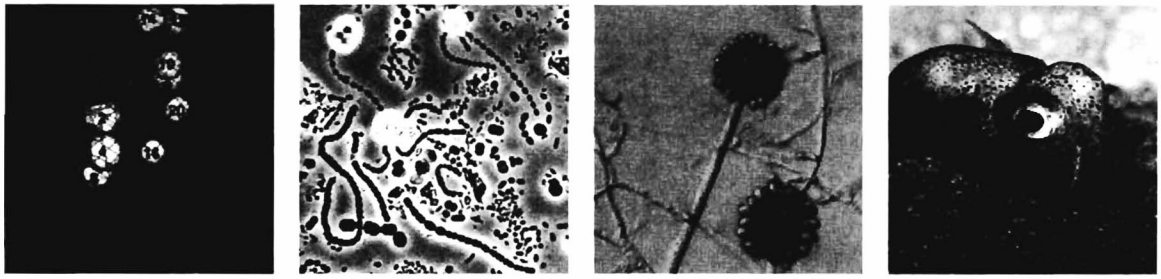

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