Chapter 10

Cultural Methods

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10.1 INTRODUCTION

Isolation and enumeration of microorganisms by cultural methods is widely used to evaluate the diversity of microbial communities or quantitate a specific organism of interest. However, unlike the assay of pure culture samples, culture techniques involving the diverse microbial communities found in the environment are complex, and the numbers obtained depend on the specific culture techniques.

To determine the appropriate culture method, it is necessary to define which specific microorganism or group of microorganisms is to be enumerated. The methods for enumerating bacteria, fungi, algae and viruses are very different. In addition, within each group, special enumeration techniques may be needed, as in the case of anaerobic or autotrophic bacteria. Precise, standard methods are necessary for assaying indicator organisms such as fecal coliforms, or pathogens such as *Salmonella* spp.

The type of environmental sample under analysis must also be considered when enumerating microorganisms. Different techniques may be necessary for the extraction of the organisms from the sample, and, depending on the number of organisms of interest present in the particular sample, dilution or concentration of the sample may be necessary (see Chapter 8). Selective or differential growth media (see Section 10.4.1.2) may also be necessary to observe a small population of a specific organism when an abundance of other organisms is also present in the sample. For example, the enumeration of *Salmonella* in sewage samples is complicated by the presence of the large numbers of enteric bacteria.

10.2 EXTRACTION AND ISOLATION TECHNIQUES

10.2.1 Extraction of Cells from Soil

To obtain accurate bacterial numbers from soil it is necessary to have efficient recovery of the microorganisms that are attached to soil particles or are present in the pores of soil aggregates. Separation of bacteria from soil particles by physical and chemical dispersion methods is discussed by Lindahl and Bakken (1995). These include hand or mechanical shaking with or without glass beads, mechanical blending and sonication. Different extracting solutions may be used depending on the pH and texture of the soil. A surfactant such as Tween 80 (Difco) may be used, often with the use of a dispersing agent such as sodium pyrophosphate (see also Chapter 8).

Since most soils contain millions of bacteria, the extraction step is followed by serial dilution of the sample

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to separate the microorganisms into individual reproductive units. Frequently, the first step in the dilution process is the addition of 10 g of soil to 95 ml of the extracting solution, which results in a 10^{-1} weight by volume dilution. Sterile water, physiological saline and buffered peptone or phosphate solutions are a few of the solutions commonly used for this step. Although water is convenient and commonly used, it is not preferred because it does not prevent osmotic shock during the dilution process. Additional 10-fold dilutions are normally conducted to allow for individual colonies to form following plating.

10.2.2 Extraction of Cells from Water Samples

Some samples, such as marine water or drinking water, contain low bacterial numbers and require concentration rather than dilution before enumeration (see Section 8.2.1). In this method, a specified volume of water is filtered through a membrane using a vacuum. The bacteria are trapped on the membrane, which is placed on the agar medium or a cellulose pad soaked in medium to allow growth of individual colonies. This is the basis of the membrane filtration technique (see Section 10.4.1.3). Different volumes of water may need to be filtered to obtain the correct concentration of bacteria on the membrane for isolation and counting purposes. In this situation, it is critical to select a type and size of membrane appropriate to the bacteria to be collected. Often a nitrocellulose filter of pore size 0.45 μm is used. Care must be taken during processing of the sample to cause minimal stress to the organism with respect to such factors as processing time, vacuum pressure and desiccation.

10.3 PLATING METHODS

After dilution or concentration, the sample is added to Petri dishes containing a growth medium consisting of agar mixed with selected nutrients. Two different methods are used for application of the diluted sample to the growth medium. In the spread plate method, a 0.1-ml aliquot of selected dilutions of the sample is uniformly spread on top of the solid agar with the aid of a sterile glass rod (Figure 10.1). Alternatively, in the pour plate method, 1-ml aliquots of appropriate sample dilutions are mixed with molten agar (45°C) in a Petri dish and allowed to solidify (Figure 10.2). The spread plate technique is advantageous since it allows colonies to develop on the surface of the agar, making it easier to distinguish different microorganisms on the basis of morphology. It also facilitates further isolation of the colonies. The spread plate method generally gives bacterial counts that are higher than with the pour plate method (for the same size of inoculant), perhaps because of improved aeration and desegregation of clumps of bacteria that occur with this method.

After plating, the samples are incubated under specified conditions, allowing the bacteria to multiply into macroscopic, isolated colonies known as colony-forming units (CFU). Because it is assumed that each colony-forming unit originates from a single bacterial cell, it is critical for the organisms to be separated into discrete reproductive units in the dilution step prior to plating. In reality, however, colonies may arise from chains or clusters of bacteria, resulting in an underestimation of the true bacterial number. The total number of bacteria of interest is calculated from the number of colonies found on a specific dilution. The range of colonies acceptable for counting is 30−300 on a standard 150-mm-diameter agar plate. Below 30, accuracy is reduced; above 300, accuracy increases only slightly and, in fact, numbers may be reduced by overcrowding and competition between organisms growing on the plate. An example of a typical dilution and plating calculation is shown in Information Box 10.1.

The process of isolating microorganisms from environmental samples often necessitates further isolation of individual colonies arising from the spread or pour plate method, to allow for additional characterization or confirmation. In this technique, a sterile inoculating loop is used to pick colonies from the original agar plate and the loop of bacteria is streaked to dilution on a new agar plate. This process is shown in Figure 10.3.

10.3.1 Unculturable Bacteria

The standard plate count technique for the enumeration of microorganisms is one of the oldest and most widely used techniques in microbiology. Despite its popularity, the dilution and plating technique has been subject to much scrutiny and criticism almost since its inception (Information Box 10.2). One of the main criticisms is that only a small fraction of the total population, which can be observed microscopically, can be cultured on laboratory media. It is well documented that only 1−10% of the number of cells observed with direct microscopic counts can be recovered as viable bacteria using cultural plating techniques (see also Chapter 2).

There are many potential reasons for the "unculturability" of bacteria. For example, strict anaerobic organisms will not be able to be cultured in the presence of oxygen. Another simple reason for the apparent inability of an organism to be cultured is a slow growth rate such that visible colonies do not appear within the normal incubation period of a few days. Other more subtle factors are responsible as shown in Information Box 10.3.

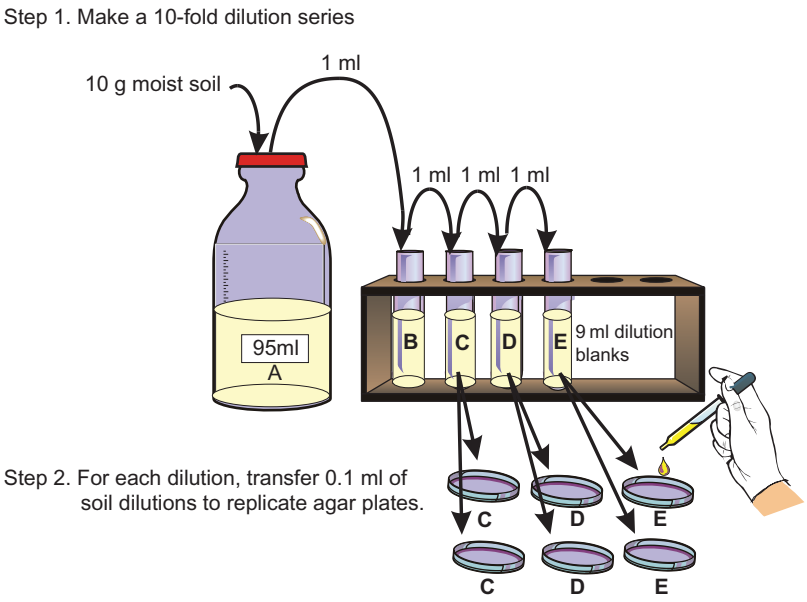
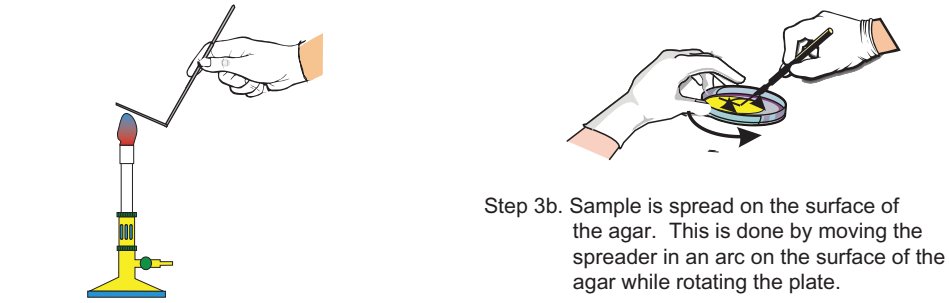
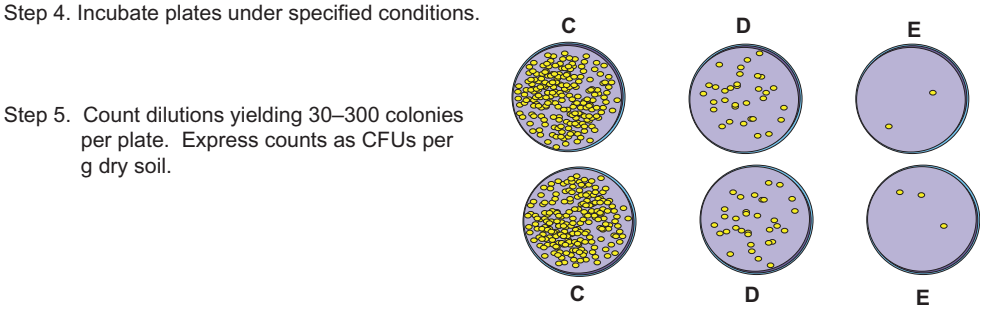


FIGURE 10.1 Dilution and spread plating technique. Here, soil that initially contains billions of microbes is diluted prior to being spread plated to enable discrete colonies to be seen on each plate. Numbers of colonies on each plate can be related to the original soil microbial population.





Overall, when isolated on a culture plate, microbial interactions and signals may be absent resulting in a lack of growth in monoculture. Additionally, when faced with this new environment devoid of necessary growth factors, some bacteria may enter into a dormant state of low activity which prevents growth and colony formation. Finally, note that numbers of viable bacteria may appear to be lower due to low efficiencies of extraction from soil, or poor separation of the bacteria during the dilution step. This could result in clumps of bacteria giving rise to a single colony. As a rule, the 1- to 2-log difference between direct and viable counts is referred to as viable but unculturable cells as it is assumed that dead cells would be rapidly degraded (Roszak and Colwell, 1987).

10.3.1.1 New Approaches for Enhanced Cultivation of Soil Bacteria

Scientists have known for decades that the total number of bacteria found in soil samples is far greater than the number that are found using culturable dilution and plating techniques (Joseph *et al.*, 2003). In fact, typically less

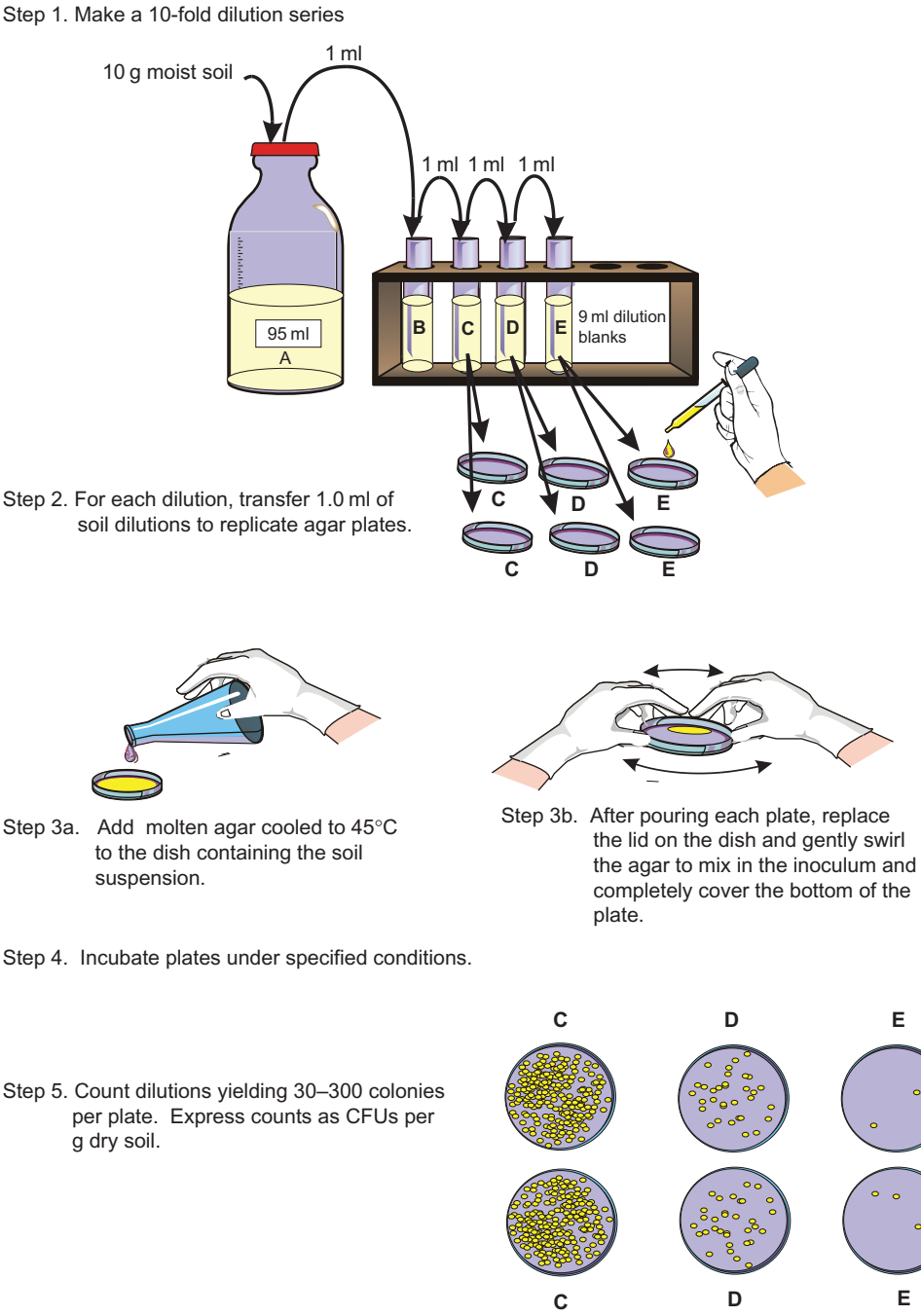


FIGURE 10.2 Dilution and pour plating technique. Here, the diluted soil suspension is incorporated directly in the agar medium rather than being surface applied as in the case of spread plating.

than 1% of the total bacterial population is culturable. The fact that such a small percentage is culturable means that the vast majority of soil organisms have not been obtained in pure culture to allow their characterization. In addition, cultural methods may actually select for less abundant rare microbes (see Section 10.3.1.2). Because of this, there have been several new approaches utilized to enhance the culturability of previously uncultured soil bacteria (Information Box 10.4).

These new approaches can be as simple as using longer incubation periods. Several investigators have seen enhanced culturability utilizing enhanced periods of incubation for colonies to evolve (30 days to 3 months) (Joseph *et al.*, 2003; Stevenson *et al.*, 2004). Other investigators have utilized dilute media with low nutrient content (Janssen *et al.*, 2002), or used hybrid methodologies combining cultural and molecular technologies (Stevenson *et al.*, 2004). Low nutrient media for dilution and plating can involve diluting nutrient broth to 1/100 of its normal concentration, or the use of more traditional soil plating media including soil extract agar derived from the soil under investigation.

Information Box 10.1 Dilution and Plating Calculations

A 10-g sample of soil with a moisture content of 20% on a dry weight basis is analyzed for viable culturable bacteria via dilution and plating techniques. The dilutions were made as follows:

Step	Dilution
10 g soil → 95 ml saline (solution A)	10^{-1} (weight/volume)
1 ml solution A → 9 ml saline (solution B)	10^{-2} (volume/volume)
1 ml solution B → 9 ml saline (solution C)	10^{-3} (volume/volume)
1 ml solution C → 9 ml saline (solution D)	10^{-4} (volume/volume)
1 ml solution D → 9 ml saline (solution E)	10^{-5} (volume/volume)

In plating, 1 ml of solution E is pour plated into an appropriate medium and results in 200 bacterial colonies.

$$\textbf{Number of CFUs} = \frac{1}{\text{dilution factor}} \times \text{number of colonies}$$

$$= \frac{1}{10^{-5}} \times 200 \text{ CFUs/10g moist soil}$$

$$= 2.00 \times 10^{6} \text{ CFUs/}g \text{ moist soil}$$

But, for 1 g of moist soil,

$$\text{Moisture content} = \frac{\text{moist weight} - \text{dry weight } (D)}{\text{dry weight } (D)}$$

Therefore,

$$0.20 = \frac{1 - D}{D}$$

$$D = 0.833 \text{ g}$$

$$\textbf{Number of CFUs per g dry soil} = 2.00 \times 10^{6} \times \frac{1}{0.833}$$

$$= 2.4 \times 10^{6}$$

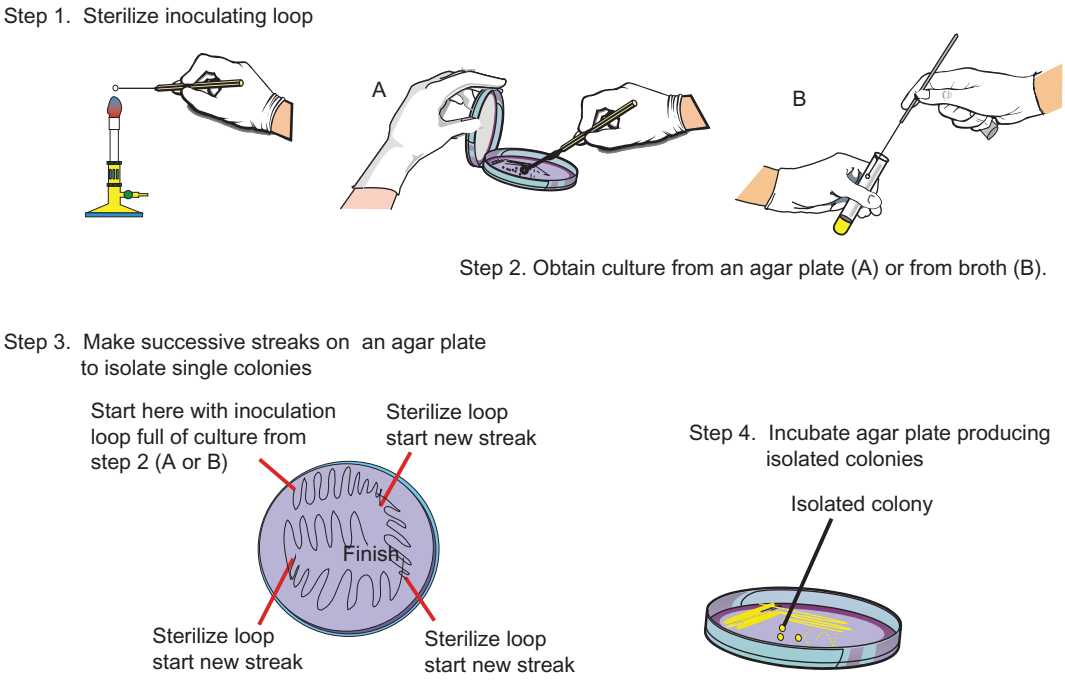


FIGURE 10.3 Isolation of a bacterial colony using the streak plate technique.

R2A (Difco, Detroit, MI) has also long been utilized for soil dilution and plating. Other new approaches have utilized additions of humic acid or humic-like compounds (anthraquinone disulfonate) to media, or the addition of quorum sensing signaling compounds including acyl homoserine lactones (Stevenson *et al.*, 2004). Recently, a new soil diffusion system was utilized to enrich the growth of novel bacterial taxa (Information Box 10.5). Researchers have also increased the numbers of culturable bacteria from soil by protecting cells from exogenous peroxides. This can be done by adding pyruvate or catalase to growth media, two compounds known to eliminate H_2O_2 (Misunoe *et al.*, 1999). Other researchers have hypothesized that incubation in CO_2-enriched atmospheres with reduced oxygen content may enhance culturability since these are the atmospheric conditions that soil-borne organisms encounter (Stevenson *et al.*, 2004).

Information Box 10.2 Errors and Assumptions Associated with Dilution and Plating Assays

1. Clumping of cells may result in an underestimation of the true count. The assumption of one organism per colony is rarely satisfied as several cells associated with a soil particle may give rise to one colony (sample sonication may alleviate this problem). Microscopic direct counts do not make the assumption.
2. Errors in diluting the soil can arise from either particles not dispersing entirely (less dilution occurs) or from particles settling out of solution prior to the next dilution (more dilution occurs).
3. Only a small fraction of organisms will grow on a given medium. Microscopic direct counts do not make this assumption. Therefore direct counts are often referred to as total counts, which will typically be one to two orders of magnitude greater than the culturable count (see Section 9.4.1).
4. As soil is a heterogeneous medium, biological variability may be high even between adjacent areas of soil.
5. Heavily sporulating organisms are often overemphasized.
6. Slow growing bacteria may not give rise to visible colonies within reasonable time frames (2 weeks).
7. Organisms may become nonviable due to stress imposed during soil extraction.
8. Only aerobic organisms are cultured (unless anaerobic conditions are imposed).

Information Box 10.3 Reasons for the "Unculturability" of Bacteria

- Slow growing bacteria that do not form visible colonies within several days of incubation
- Fastidious growth requirements including specific nutrients, pH regime, incubation temperature or redox conditions (Kopke *et al.*, 2005)
- Competition for nutrients
- Growth inhibition due to bacteriocins or antibacterial substances released by other soil bacteria (Tamaki *et al.*, 2005)
- Need for cross-feeding or metabolic cooperation between species for the provisions of nutrients (Belenguer *et al.*, 2006)
- Requirement for community communication through a network of signals found only in natural environments (Nichols *et al.*, 2008)

Information Box 10.4 New Techniques for Culturing the Unculturable

- Use of low nutrient media (Connon and Giovannoni, 2002)
- Longer incubation periods (Davis *et al.*, 2005)
- Physically reducing the number and diversity of bacteria within mixed samples before cultivation (Song *et al.*, 2009)
- Co-cultivation with helper strains to enhance beneficial interactions (Nichols *et al.*, 2008)
- Addition of spent culture media to fresh media to provide growth stimulants (Kim *et al.*, 2008)
- Addition of signaling molecules (Bruns *et al.*, 2002)
- Encapsulation of individual cells in gel microdroplets (Ben Dov *et al.*, 2009)
- Mimicking natural conditions using microcolony cultivation on polycarbonate membranes with soil extract (Ferrari and Gillings, 2009)
- Use of flow cytometry and cell sorting to isolate individual bacterial cells and cell encapsulation within gel microdroplets (Zengler *et al.*, 2002)
- Use of optical tweezers (infrared laser or Raman tweezers) for micromanipulation of single cells and transfer to growth media (Huang *et al.*, 2009)

Information Box 10.5 Use of a Soil Diffusion System to Enrich the Growth of Novel Bacterial Taxa

The Soil Diffusion System (SDS) has been developed to coax previously uncultivated bacteria into growth. The SDS allows microbes to be cultivated in an environment that more closely mimics the natural soil environment. Specifically, the technology allows bacteria to grow in close association with their native soil, but isolated from the soil by a polycarbonate nano-membrane (0.003 μm). On top of the nano-membrane, a regenerated cellulose filter is placed to support the growing microbial community that arises following inoculation of the cellulose filter with a dilute soil inoculum (10^{-3} dilution). The filter is thought to allow for diffusion of soil-derived organics, signaling molecules and nutrients. This technology has been used to culture previously unculturable bacteria. Thus, it appears that proximity to a living microbial community appears to enhance community growth on the cellulose filter.

Source: Kakumanu and Williams, 2012

More sophisticated approaches to soil dilution and plating have included the use of cell encapsulation wherein individual cells are enclosed within micro gel capsules (Zengler *et al.*, 2002). Researchers are now starting to use molecular characterization of microbial communities to select custom media for previously uncultured bacterial groups (Joseph *et al.*, 2003). The molecular analyses use 16S rRNA gene sequences to identify the major divisions of bacteria present in an environmental sample. This information can aid in the selection of a suite of media to culture the diverse populations present

in a given sample. Other recent innovations involve the use of flow cytometry and cell sorting followed by cell encapsulation with gel microdroplets. Finally, optical tweezers for micromanipulation of single cells and subsequent transfer to growth media can be used.

10.3.1.2 Culturing Members of the Soil Rare Biosphere

Microorganisms that are found in low abundance have been referred to as "the rare biosphere" (Sogin *et al.*, 2006). However, little is known of the ecological significance of rare microorganisms within complex microbial communities. In a recent study, agricultural soil samples were analyzed by both conventional cultural techniques and culture-independent pyrosequencing technology (Shade *et al.*, 2012). Specifically, soil samples were diluted and plated on a rhizosphere isolation medium (culture dependent). In addition, the same soil samples were extracted for community DNA (culture independent). Both the community DNA and DNA from the cultured bacteria were subjected to pyrosequencing of the 16 SrRNA genes. Interestingly, the analyses showed that soil bacteria captured by culturing were in very low abundance or absent in the culture-independent community. Thus, molecular culture-independent assays tend to identify the most abundant microbes in an environmental matrix, whereas culture-dependent assays actually give greater access to "the rare biosphere." This raises the question: "Are cultured bacteria actually atypical members of the community?"

10.3.2 Most Probable Number Technique

The most probable number (MPN) technique is sometimes used in place of the standard plate count method to estimate microbial counts in the environment. In this method, the sample to be assayed is dispersed in an extracting solution and successively diluted, as in the plate count. This method relies on the dilution of the population to extinction, followed by inoculation of five to 10 replicate tubes containing a specific liquid medium with each dilution. After incubation, the tubes are scored as plus or minus for growth on the basis of such factors as turbidity, gas production and appearance or disappearance of a substrate (Figure 10.4). Scoring a tube positive for growth means that at least one culturable organism was present in the dilution used for its inoculation. The number of positive and negative tubes at each dilution is used to calculate the number present in the original sample through the use of published statistical MPN tables or computer programs designed to simplify the analysis. MPN tables can be found in American Public Health Association (2012).

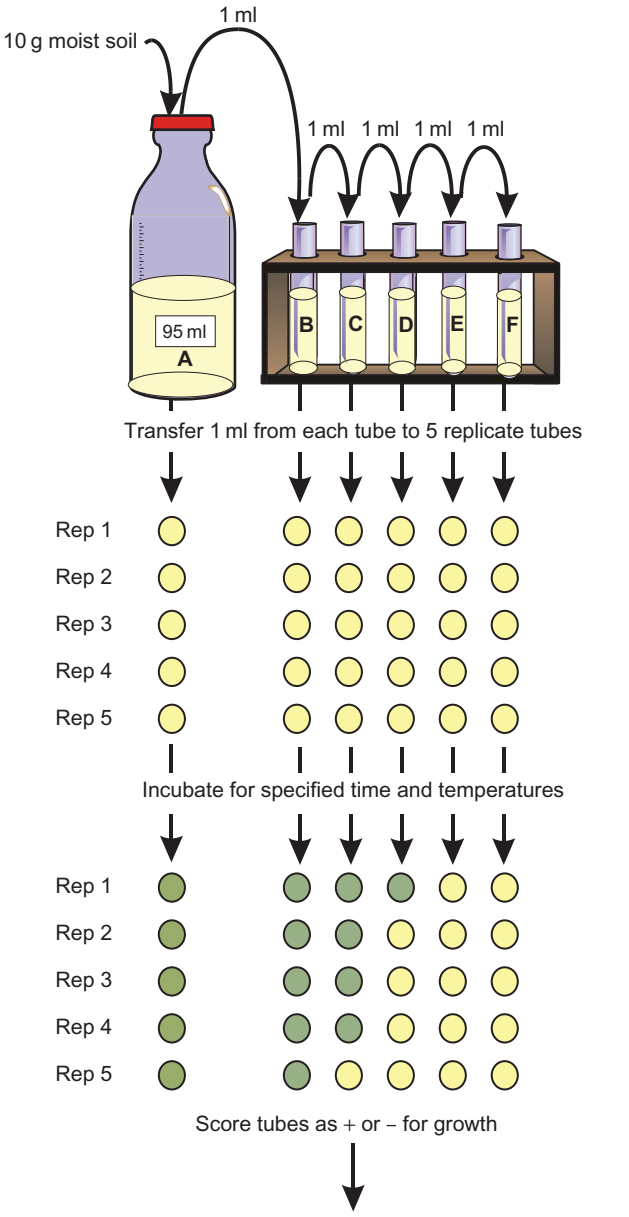


FIGURE 10.4 Most probable number technique. Here, an environmental sample is diluted to extinction. Diluted samples are used to inoculate replicate tubes at each dilution. The presence or absence of the microorganism of interest at a given dilution can be analyzed statistically to estimate the original population in the environment.

MPN methodology is useful because it allows estimation of a functional population of bacteria based on a process-related attribute such as nitrification, nitrogen fixation or sulfur oxidation. It is mandatory to use MPN analysis when enumerating a microorganism requiring broth enrichment prior to culturing. It is also essential when assaying many foods, sediments and soils. However, the MPN technique is very labor intensive, and

results are usually less precise than those obtained with direct plating methods.

10.4 CULTURE MEDIA FOR BACTERIA

10.4.1 General Media Used for Culturing Bacteria

Liquid and solid media for the cultivation of microorganisms must contain substances that will support their growth, and the media available are as diverse as the microorganisms themselves. Many media are available from commercial manufacturers such as Difco (Detroit, MI) or BBL (Sparks, MD), and variations and new formulations are published by researchers available through journals and volumes such as the *Handbook of Microbiological Media* (Atlas, 2010). The major nutrient components of microbiological media include: (1) a source of carbon for incorporation in biomass, such as glucose for heterotrophic bacteria or CO_2 for autotrophic bacteria; (2) nitrogen, which is needed for growth and commonly supplied as ammonia, proteins, amino acids, peptones or extracts from plants or meat; (3) buffers to maintain a suitable pH; and (4) growth factors such as defined trace minerals or metals, or undefined factors such as those found in extracts made from the environmental samples themselves. Many media also contain selective components that favor the growth of specific organisms while inhibiting the growth of nontarget organisms (see Section 10.4.1.2).

For solid media used in plating, agar is the most commonly used solidifying agent. It is a polysaccharide of an extract from marine algae. Agar exists as a liquid at high temperatures but solidifies on cooling to 38°C. Although agar ideally does not supply nutritional value to the medium, variations in growth may be observed with different types of agar.

While cultural media are generally used to enumerate or isolate specific microorganisms, they also can be used in metabolic fingerprinting analysis to allow identification of microbial isolates. Such systems include API strips (BioMérieux, Durham, NC) and Enterotube (BBL, Sparks, MD), which are routinely used for clinical identification of Gram-negative microorganisms. For environmental microbiology, the most commonly used of the metabolic fingerprinting systems is Biolog. Biolog is used for identification of single isolates as well as for analysis of community composition. The Biolog Company (Hayward, CA) makes an EcoPlate which contains 31 carbon sources chosen for soil community analysis. The basis for the Biolog system is a 96-well microtiter plate, whereas for the EcoPlate, the 31 carbon sources are each in triplicate wells. Each well is inoculated with the same isolate or community sample. If the substrate is utilized, the well turns purple. The plates can be read either manually or automatically using a plate reader. The EcoPlate can be used to provide a comparison of the metabolic fingerprint of the community before and after a perturbation, or simply to monitor the community for a period of time.

10.4.1.1 Heterotrophic Plate Counts

Heterotrophic bacteria are "consumers" that obtain energy and carbon from organic substances. Heterotrophic plate counts give an indication of the general "health" of the soil as well as an indication of the availability of organic nutrients within the soil. Two basic types of media can be used for this analysis: nutrient-rich and nutrient-poor media. Examples of nutrient-rich media are nutrient agar (Difco), peptone-yeast agar (Atlas, 2010), and soil extract agar amended with glucose and peptone (Atlas, 2010). These media contain high concentrations of peptone, yeast and/or extracts from beef or soil. Nutrient-poor media are often called minimal media, and contain as much as 75% less of these ingredients, often with substitutions such as casein, glucose, glycerol or gelatin. Examples of minimal media are R2A agar (BBL), m-HPC agar (Difco) and soil extract agar with no amendments. In many cases, higher colony counts are obtained with a minimal medium because a large number of oligotrophic organisms in the environment cannot be cultured on a rich medium. This is true for most water and subsurface porous medium samples, for which the nutrient-poor medium, R2A, is often used (Figure 10.5). Growth on a nutrient-poor medium may take longer (5–7 days), and the colony sizes are often smaller than for a rich medium. In addition, the

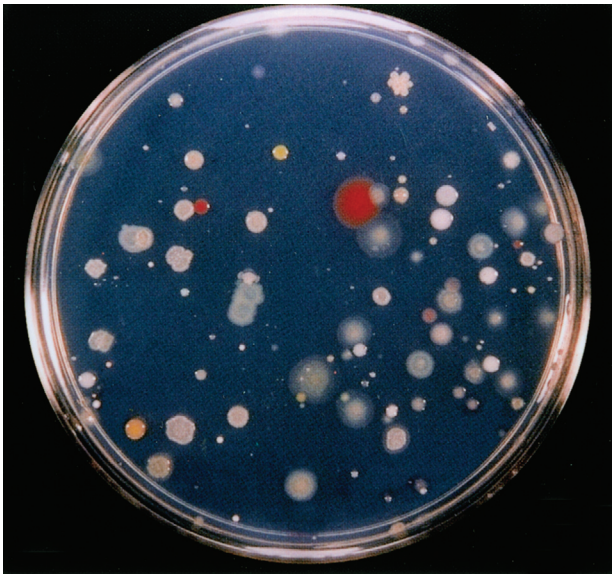


FIGURE 10.5 Heterotrophic colonies on an R2A agar plate. A number of discrete colonies with diverse morphology arise after dilution and plating from soil.

TABLE 10.1 Common Antibiotics Often Used in Selective Media

Name	Spectrum	Mode of Action
Chloramphenicol	Broad spectrum	Inhibits protein synthesis by binding to 50S ribosomal subunit
Erythromycin	Mostly Gram-positive	Inhibits protein synthesis by binding to 50S ribosomal subunit
Tetracycline	Broad spectrum	Inhibits protein synthesis by binding to 30S ribosomal subunit
Streptomycin	Broad spectrum	Inhibits protein synthesis by binding to 30S ribosomal subunit
Polymyxin	Gram-negative bacteria, especially *Pseudomonas*	Disrupts cell membrane
Nalidixic acid	Gram-negative bacteria	Inhibits DNA synthesis
Novobiocin	Gram-negative bacteria	Inhibits DNA synthesis
Trimethoprim	Broad spectrum	Inhibits purine synthesis
Rifampicin	Gram-positive bacteria	Inhibits RNA synthesis
Penicillin	Mostly Gram-positive bacteria	Inhibits cell wall peptidoglycan synthesis

community of isolates from a single sample may be entirely different when plated on the two media types.

10.4.1.2 Culturing Specific Microbial Populations

It is often necessary to detect or enumerate a specific population of microorganisms, or even a very specific bacterial isolate, from the total population of bacteria found in an environmental sample. This may necessitate culturing with one or more specialized media, and often requires that a specified sequence of steps be performed to ensure maximum culturability of the target organism. We will now define the media involved in these steps and explain why they are important.

Pre-enrichment or resuscitation medium is a liquid medium that allows the microorganisms in a sample to begin to actively metabolize and increase in number. There are two reasons for a pre-enrichment medium. First, damaged cells are given time and the necessary nutrients to repair and grow. This step is important because direct inoculation of a sample into a selective enrichment medium (discussed next) may result in the death of some bacteria. This is especially critical for sublethally injured organisms, which may not be recoverable under selective conditions. The second purpose of pre-enrichment is to increase the number of cells. This step is crucial when trying to enumerate low numbers of a target organism in environmental samples.

Enrichment medium is a liquid medium that promotes the growth of a particular physiological type of microorganism present in a mixture of organisms, while suppressing the growth of competitive background flora. It aids in the detection of the desired environmental isolate when the specimen contains a high population of normal flora. Enrichment media may be elective or selective. Elective enrichment medium allows growth of a single or limited type of bacteria based on a unique combination of nutritional or physiological attributes. Selective enrichment medium involves the use of inhibitory substances or conditions to suppress or inhibit the growth of most organisms while allowing the growth of the desired organism.

A selective plating medium is a modification of an agar medium to suppress or prevent the growth of one group of organisms while fostering the growth of the desired organism. Antibiotics are among the most widely used and effective selection agents. Cycloheximide is often used in heterotrophic media to inhibit the growth of fungi from soil. Table 10.1 lists some of the more common antibiotics, with their spectrum and mode of action. Other examples of selective agents often added to growth media are metals such as mercury or lead, which are usually used in the presence of a minimal salts medium. Selection may also be accomplished by the use of toxic chemicals such as high salt concentrations or dyes. For example, the dye crystal violet inhibits most Gram-positive bacteria while allowing the Gram-negative bacteria to grow. Adjustments in pH or osmotic conditions are also used for selection. Finally, selectivity may be based on incubation conditions of the inoculated growth medium. These may include temperature or oxygen levels.

A specialized isolation medium contains formulations that meet the nutritional needs of specific groups of organisms, such as *Staphylococcus* or *Corynebacterium*, thereby allowing differentiation and identification.

A differential medium contains ingredients to allow distinction of different microbes growing on the same medium. It includes an indicator, usually for pH, to distinguish certain groups of organisms on the basis of variations in nutritional requirements, and the production of

acid or alkali from various carbon sources. This results in a distinguishable morphological characteristic of the colony, usually color. In addition, it may support the growth of a selected group of bacteria while inhibiting the growth of others. Thus, a medium can be selective as well as differential.

Although culturing procedures for pure laboratory strains or clinical samples are fairly straightforward, the same techniques may not be successful for culturing the same organisms found in soil or water. Often it is necessary to follow a series of steps or modifications in procedures along with a combination of media to obtain optimal culturing of the target environmental organism. Furthermore, once colonies are isolated by cultural methods, they may be characterized by physiological (see Chapter 11), immunological (see Chapter 12) or molecular (see Chapter 13) techniques. In the next sections we present five examples of the application of these methods to culture specific microbes from soil and water samples. These examples were chosen to illustrate a wide range of bacteria and problems encountered in the environment. These examples are far from exhaustive, but the selection represents a variety of cultural methodologies, and their application to environmental samples.

10.4.1.3 Fecal Coliforms and the Membrane Filtration Technique

Coliform bacteria are nonpathogenic bacteria that occur in the feces of warm-blooded animals. Their presence in a water sample indicates that harmful pathogenic bacteria may also be present. Coliforms are found in numbers corresponding to the degree of fecal pollution, are relatively easy to detect and are overall hardier than pathogenic bacteria. For these reasons, coliforms are important "indicator organisms" in an environmental sample to assess water quality prior to or in place of culturing other organisms (see also Chapter 23).

The membrane filtration technique is used for the detection of fecal coliforms in water samples (see Figure 23.3). The volume of water required to enumerate fecal coliforms varies, but a volume of 100 ml is normally processed for drinking water, lakes and reservoirs. After filtration, the membrane containing the trapped bacteria is subjected to cultural methods. For fecal coliform analysis, the membrane is applied to a Petri dish containing m-FC agar (Difco) and incubated at 45°C for 24 hours. Fecal coliforms appear as blue colonies and nonfecal coliform colonies are gray to cream colored. Colonies are viewed and counted on a microscope under low magnification. The fecal coliform–membrane filtration procedure uses an enriched lactose medium with 1% rosalic acid to inhibit the growth of noncoliform colonies, and an elevated incubation temperature is a critical component in the selection for fecal coliforms.

This is a published standard method (*Standard Methods for the Examination of Water and Wastewater*, 2012), but there are also variations to help recover injured bacteria. These include an enrichment–temperature acclimation in which the membrane is incubated on nonselective agar at 35°C for 2 hours prior to incubation on the m-FC medium, and deletion of the rosalic acid suppressive agent from the m-FC medium. Individual colonies must also be confirmed to be fecal coliforms.

10.4.1.4 Salmonella and the MPN Technique

Salmonella is an enteric bacterium that is pathogenic to humans and causes a wide range of symptoms, primarily gastroenteritis. It can be transmitted by drinking improperly disinfected water or contaminated recreational water, but infection in the United States is primarily due to foodborne transmission because *Salmonella* infects both beef and poultry. Municipal sewage sludge (biosolids) also contains many microorganisms including *Salmonella*. Some cities now apply biosolids to agricultural soil to improve nutrient quality. Thus, it has become necessary to monitor the biosolids for indicator organisms and pathogens such as *Salmonella*, to ensure the safety of the product. Culture methods for *Salmonella* are varied, complex and time consuming and require final confirmatory tests (Figure 10.6).

The traditional method for detecting *Salmonella* spp. relies on enrichment and a plating technique with subsequent estimation using MPN tables. Selected volumes of biosolids, generally ranging from 10 to 0.01 ml, are added to a pre-enrichment broth and incubated for 18–24 hours at 37°C. Laboratory studies have found peptone and lactose broth to be most effective for the resuscitation of most *Salmonella* spp. Aliquots from the pre-enrichment phase are added to a selective enrichment broth. Numerous media are available for this step, all with different selection techniques. Included are the use of the inhibitory agent sodium selenite in broth and brilliant green and tetrathionate, which are contained in Mueller–Hinton tetrathionate broth (Difco, Detroit, MI). Rappaport–Vassiliadis (RV) broth (Difco) is also widely used; selection is based on resistance of *Salmonella* to malachite green, $MgCl_2$, and ability to grow at a pH of 5.0. Recently, RV broth was modified to include the antibiotic sodium novobiocin with incubation at an elevated temperature of 43°C. This medium, known as NR10 broth, has shown much promise for isolation of *Salmonella* from marine water and possible deletion of the pre-enrichment phase of analysis (Alonso *et al*., 1992).

Growth from the enrichment phase is plated onto a selective or differential medium such as Hektoen enteric agar (Difco), brilliant green bile agar (Difco), or xylose–lysine–deoxycholate agar (Difco). Characteristics used for differential purposes are production of H_2S, and the inability of *Salmonella* to ferment lactose.

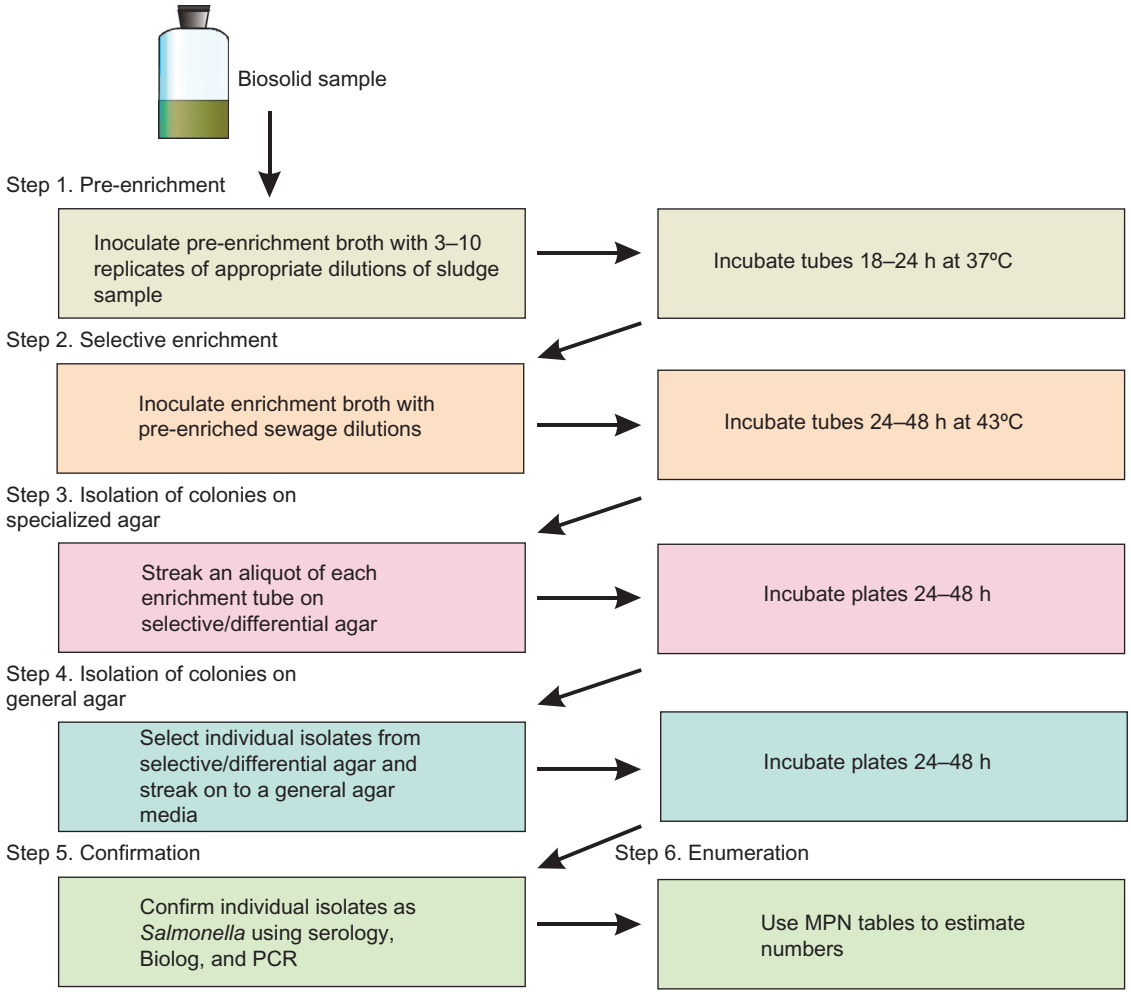


FIGURE 10.6 Protocol for the detection of *Salmonella* from biosolids.

Selective agents include triphenylmethane dyes, antibiotics such as sulfadiazine or sodium deoxycholate salts.

Individual colonies arising from this medium are streaked onto conventional growth media to allow confirmation using biochemical tests such as BIOLOG, serological identification (see Chapter 12), or confirmation using nucleic acid methods such as the polymerase chain reaction (PCR) (see Chapter 13).

10.4.1.5 Isolation of Fluorescent Pseudomonads from Soil

Pseudomonas are Gram-negative aerobic chemoheterotrophs that are commonly found in the environment. They exhibit diverse enzymatic systems and are capable of conducting many biochemical transformations. Fluorescent pseudomonads are characterized by the production of siderophores. Siderophores are compounds that are produced under low levels of iron. They have the ability to chelate iron and transport it into the microbial cell. These microbes also have a yellow–green pigment that diffuses through agar media during growth and fluoresces under ultraviolet light. Pigment production is enhanced by iron deprivation.

For enumeration of fluorescent pseudomonads, soil is added to an extracting solution and a dilution series is performed as outlined earlier (see Section 10.2). The spread plate technique is used to isolate individual colonies on selective media (see Section 10.4). One medium appropriate for isolation is called S1 medium (Gould *et al.*, 1985). Components of the media include sucrose, glucose, casamino acids, sodium lauroyl sarcosine (SLS), trimethoprim, various salts and agar. The SLS prevents the growth of Gram-positive organisms, and the trimethoprim is an effective inhibitor of facultative Gram-negative organisms. Sucrose and glycerol provide an osmotic stress that selects for the fluorescent pseudomonads. Fluorescent colonies are identified on the medium with the use of ultraviolet light.

10.4.1.6 Isolation of Nitrifying Organisms from Soil

Nitrosomonas and *Nitrobacter* are chemoautotrophic organisms found in soil and water, and are responsible for

the oxidation of ammonium to nitrite (*Nitrosomonas*) and nitrite to nitrate (*Nitrobacter*). This process, known as nitrification, is important because it can affect plant growth beneficially, but nitrate also contributes to potable water contamination. Direct plating techniques are difficult, because even with the use of strictly inorganic substrates in the medium, slight amounts of organic material introduced by inoculation with the environmental sample allow growth of faster growing heterotrophic organisms. One approach used to overcome this problem is a lengthy serial enrichment technique that includes soil enrichment, an initial culture enrichment step, a final enrichment step, isolation on agar and rigorous purity checks (Figure 10.7). To begin the isolation of *Nitrobacter* from soil, fresh field soil is leached with sodium nitrite on a daily basis for 2−3 weeks to enrich for *Nitrobacter*. The enriched soil is transferred to a liquid medium containing $NaNO_2$. Cultures are incubated with shaking at 25°C. As soon as turbidity is detected, the culture is transferred to fresh medium using a 1% inoculum. Prompt transfer reduces the development of heterotrophs in the sample. The initial enrichment goes through a series of six to eight passages to fresh medium using a 1% inoculum each time. After these steps, the culture is filtered through a membrane to trap the cells and washed to remove growth products. The membrane is transferred to fresh medium, and again undergoes five or six passages into fresh medium after filtration of cells onto a membrane. The final enrichment culture is streaked on *Nitrobacter* agar medium (Atlas, 2010) and tiny colonies appearing after 14 days are indicative of *Nitrobacter*. Purity of the test culture must be checked by using media with various carbon and nitrogen sources, and osmotic strengths for selection (Schmidt *et al*., 1973). Numbers of *Nitrobacter* spp. in a soil sample can also be estimated using an MPN technique. After extraction, the soil dilutions are added to replicate tubes of broth medium containing potassium buffers, magnesium salts, trace elements, iron, $NaNO_2$ as the inorganic substrate for oxidation and Bromthymol Blue as the indicator. A drop in pH monitored by a change in color of the tubes from blue−green to yellow over a period of 3−6 weeks indicates the oxidation of NH_4^+ to NO_2^-. Dilutions are scored as + or − for growth, indicated by the presence of NO_2, and the population is estimated using MPN tables.

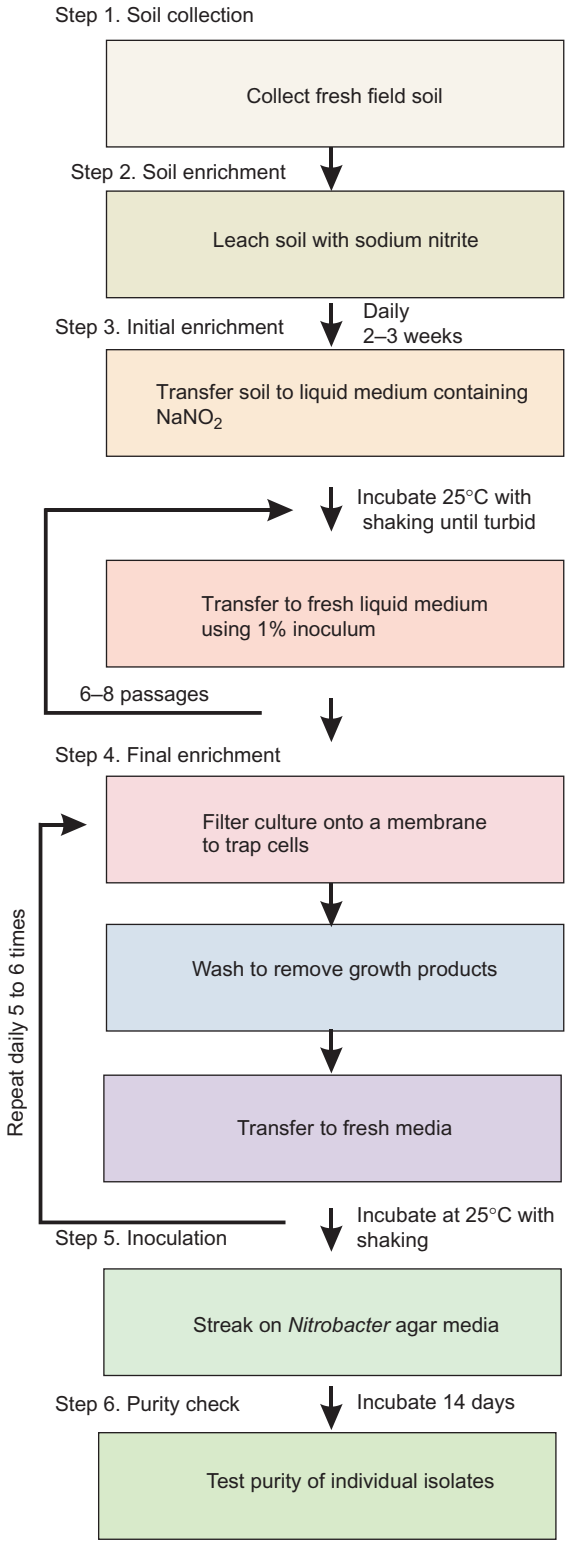


FIGURE 10.7 Protocol for the isolation of nitrifying organisms.

10.4.1.7 Isolation and Enumeration of 2,4-D Degrading Bacteria in Soil

Researchers often wish to determine whether bacteria that perform a known metabolic function exist in an environmental sample. As an example, the research community is very interested in the fate of pesticides that are introduced into the environment. The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) is one such pesticide that has been commonly used in fate and ecology studies (see Case Study 3.1). A cultural medium used for enumeration and isolation of viable 2,4-D degraders is a selective, differential enrichment medium called eosin−methylene

blue (EMB)-2,4-D agar (Neilson *et al.*, 1994). This agar contains minimal salts and 2,4-D as the carbon source. Indicators eosin B and methylene blue allow selection for Gram-negative bacteria and differentiation of 2,4-D-degrading colonies, which turn black. In essence, this is using 2,4-D as an elective carbon source, and the indicators as both selective agents and differential agents that distinguish on the basis of the black colonies that arise during 2,4-D degradation.

10.5 CULTURAL METHODS FOR FUNGI

Fungi are ubiquitous in nature and can be found in samples taken from soil, sediments and aquatic environments such as lakes, ponds, rivers, marine water, wastewater and well water. They are heterotrophic organisms, mostly aerobic or microaerophilic in nature. Fungi exist in a variety of morphological and physical states, which makes them difficult to quantitate and identify by cultural techniques. Cultural methods for fungi are similar to those for bacteria but must be modified to restrict bacterial growth. This is normally done by the addition of antibiotics or dyes, such as Rose Bengal, or by lowering the pH of the medium. Cultural methods for fungi are normally used to obtain pure fungal isolates, but these methods are not usually appropriate for quantitative dilution and plating analyses. This is because counts can be highly biased by the presence of spore-forming fungi, where colonies can arise from spores, and the number of culturable colonies obtained will not be a true reflection of the number of colonies in the environmental sample. For cultural assays of bacteria, it is assumed that each colony-forming unit originates from a single bacterial cell. However, in the same enumeration technique for fungi, fungal colonies may develop from a single spore, an aggregate of spores or a mycelial fragment containing more than one viable cell. Keeping in mind this limitation, plating techniques are still a valuable tool for the assessment of fungi in the environment. Techniques for culturing fungi include the pour plate technique (see Section 10.3), the spread plate technique (see Section 10.3) and the membrane filtration technique (see Section 10.4.1.3). These protocols as they relate specifically to the cultivation of fungi can be found in *Standard Methods for the Examination of Water and Wastewater* (2012). Modifications of the dilution and plating technique include directly picking fungal hyphae from samples with subsequent plating on nutrient agar, or washing the soil (or plant roots) and placing a small amount of the washed sample on the agar medium.

Fungi are often isolated on a nonselective agar medium, which allows the isolation of the maximum number of fungal taxa from the sample under study. As with bacteria, a wide selection of nutrient media is available. The most common include potato dextrose agar and malt extract agar (Figure 10.8). In some cases, a bacterial antibiotic such as streptomycin can be added to the agar, or alternatively, the agar can be acidified to pH 4.5 to inhibit bacterial growth.

Often, the scientist is interested in the isolation of a specific physiological group of fungi or a specific taxon such as Basidiomycetes or mycorrhizal fungi. A wide range of selective media have been developed for this purpose (Seifert, 1990). The MPN technique (see Section 10.3.2) is often used for the quantification of mycorrhizal fungi, which do not grow well on plating media. In this technique, soil dilutions are used to inoculate a host plant rather than a nutrient medium. After a specified incubation, plant roots are observed for colonization by the fungi, and calculations are performed using the MPN tables to estimate the numbers present in the original sample. After the initial isolation of fungi from the environmental sample, individual colonies must be selected for further purification and identification.

10.6 CULTURAL METHODS FOR ALGAE AND CYANOBACTERIA

Algae are unicellular or multicellular phototrophic eukaryotic microorganisms that occur in fresh and marine water and moist soil (see Section 2.3). In contrast, the so-called blue–green algae are not true algae—rather they belong to a group of bacteria known as cyanobacteria. The majority of soil algae are obligate photoautotrophs, using light to manufacture organic compounds from inorganic nutrients (phototrophic eukaryotes). Thus, their nutritional requirements include water, light, oxygen, carbon dioxide and inorganic nutrients. A small number of algae are photoheterotrophic, requiring organic compounds for growth. Algae and cyanobacteria can be enumerated by dilution and plating techniques or the MPN assay. Prior to dilution and culturing, because of the filamentous nature of some algal species, it is sometimes necessary to use more forceful methods for extraction than those used for bacteria. These include grinding soil samples with a mortar and pestle prior to extraction, as well as a longer and more vigorous homogenizing step using a blender or glass beads. When enumerating microalgal colony-forming units on solid media, the addition of antibiotics such as penicillin and streptomycin to the algal medium is recommended, to ensure their growth in the presence of the faster growing heterotrophic bacteria and fungi. Alternatively, low doses of UV radiation or exposure to nonspecific bactericides such as formaldehyde or sodium lauryl sulfate may be used to suppress growth of bacteria. An incubation period of 1–3 weeks or even longer may be necessary, requiring special care to ensure that desiccation of the medium does not occur. Recommended air temperatures are 20 to 25°C, with a

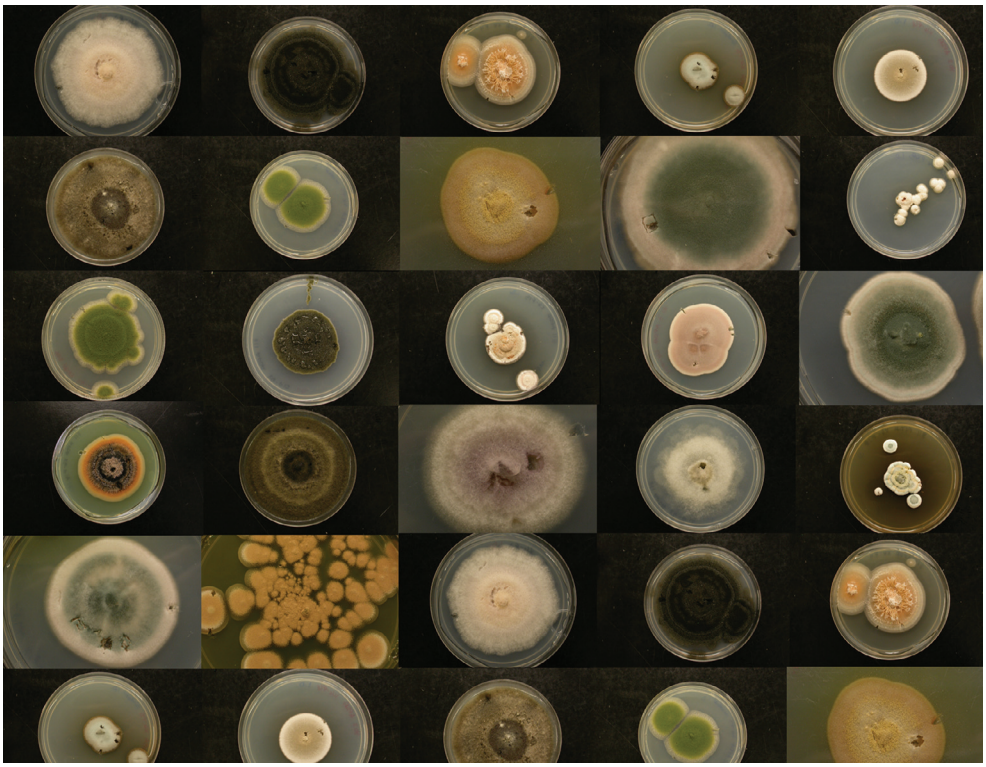


FIGURE 10.8 A collage of pure cultures of fungal isolates from a mine tailings site. Figure courtesy M.O. Mendez.

photoperiod of either 12 hours light/12 hours dark or 16 hours light/8 hours dark.

When isolating algae, the approach may be to select for a specific alga, or to obtain a quantitative index of the entire community. In the latter case, an MPN technique using a growth medium consisting of a soil−water mix is often used. Many media are available for specific enrichment and plating, including Bold's Basa medium for green and yellow−green microalgae (and cyanobacteria) (Figure 10.9).

A good review of culturable methods for algae methods was presented by Castenholz (1988), including several media suitable for freshwater cyanobacteria. The antibiotic cycloheximide is usually added to preclude growth of eukaryotic algae, diatoms and protozoa. The medium should be incubated at 24−26°C under fluorescent light of 2000−3000 lux (cool white light or daylight fluorescent light) (Figure 10.10). For agar plates, samples are usually diluted and applied as a spread plate.

10.7 CELL CULTURE-BASED DETECTION METHODS FOR VIRUSES

Living cells are necessary for virus replication, and originally human volunteers, laboratory animals or embryonated hens' eggs were the only techniques available to study and isolate human viruses. However, the advent of animal cell culture techniques after World War II revolutionized the study of animal viruses, where for the first time viruses could be grown and isolated without the need for animals. Two main types of cell culture are used in virology, the first of which is primary cell culture. In this procedure, cells are removed directly from an animal and can be used to subculture viral cells for a limited number of times (20−50 passages). Thus, a source of animals must be available on a continuous basis to supply the needed cells. Primary monkey kidney cell cultures (commonly from rhesus or green monkeys) are permissive, meaning that they allow the replication of many common human viruses. These are frequently used for the detection of enteric viruses found in the environment. A second type of cell culture is known as continuous cell culture, which is also derived from animals or humans, but may be subcultured indefinitely. Such cell lines can be derived from normal or cancerous tissue.

Cell cultures are initiated by dissociating small pieces of tissue into single cells by treatment with a proteolytic enzyme and a chelation agent (generally trypsin and EDTA). The dispersed cells are then suspended in cell culture medium composed of a balanced salt solution containing glucose, vitamins, amino acids, a buffer system, a pH indicator, serum (usually fetal calf) and antibiotics. The suspended cells are placed in flasks, tubes or Petri dishes, depending on the needs of the laboratory. Plastic

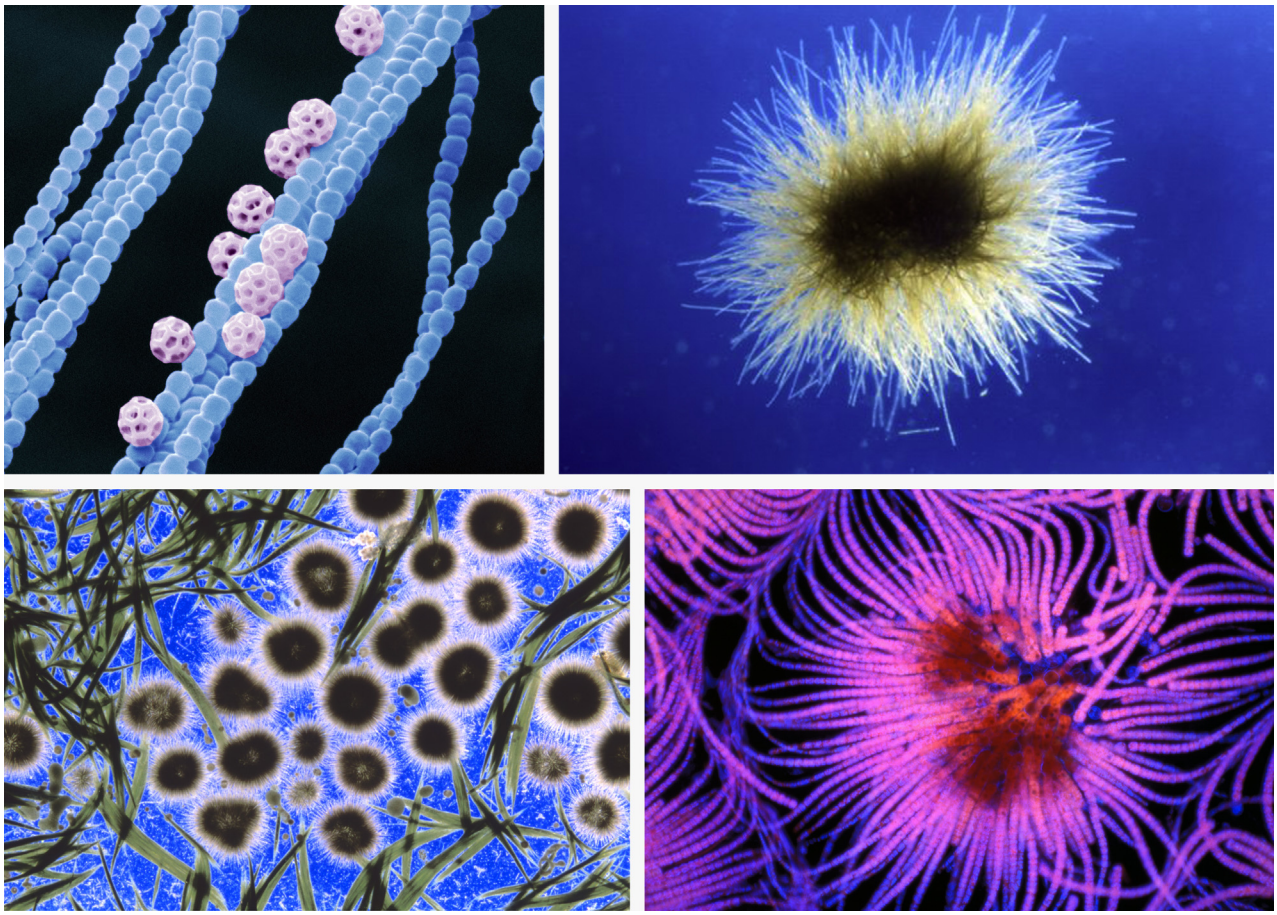


FIGURE 10.9 Various cyanobacteria (blue–green algae).

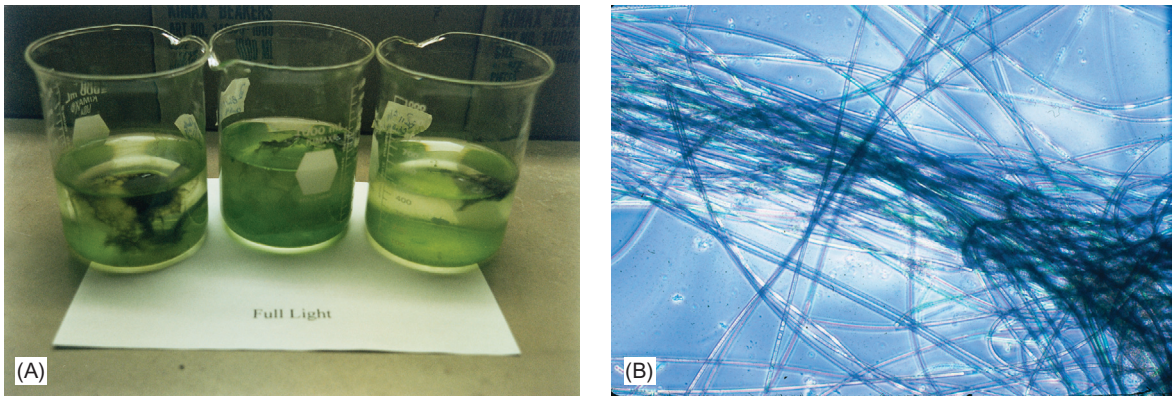


FIGURE 10.10 (A) Cyanobacteria (blue–green algae) grown in full fluorescent light in BG11 broth. (B) An example of a cyanobacterium *Lynabya* seen using phase contrast microscopy.

flasks and multiwell plates are especially prepared for cell culture use, and are currently the most common type of container used (Figure 10.11). The cells attach to the surface of the vessel and begin replicating. Replication ceases when a single layer or monolayer of cells occupies all the available surface of the vessel.

Unfortunately, not all animal viruses will grow in the same cell line, and others have never been successfully grown in cell culture. Viruses require specific receptors for attachment and replication with the host cell, and if these are not present, no replication takes place. For example, rotavirus grows well in the MA-104 cell line,

but not the blue green monkey (BGM) cell line. The most commonly used cell line for enterovirus detection in water is the BGM cell line. In recent years, the cell line, $CaCO_2$, originating from a human colon carcinoma, has been found to grow more types of enteric viruses (hepatitis A, astroviruses, adenoviruses, enteroviruses, rotaviruses) than any other cell line, and is seeing increased use in environmental virology. Cell lines commonly used to grow human viruses are shown in Table 10.2.

The two most common methods for detecting and quantifying viruses in cell culture are the cytopathogenic effect (CPE) method and the plaque-forming unit (PFU) method. Cytopathic effects are observable changes that take place in the host cells as a result of virus replication. Such changes may be observed as changes in morphology, including rounding or formation of giant cells, or formation of a hole in the monolayer due to localized lysis of virus-infected cells (Figures 10.12 and 10.13). Different viruses may produce very individual and distinctive CPE. For example, adenovirus causes the formation of grapelike clusters, and enteroviruses cause rounding of the cells. The production of CPE may take as little as 1 day to 2−3 weeks, depending on the original concentration and type of virus. Not all viruses may produce CPE during replication in a given cell line. In addition, the virus may grow to high numbers, but no visible CPE is observed. In these instances, other techniques for virus detection must be used. Alternatives include the use of an immunoassay that detects viral antigens, or the use of PCR to detect viral nucleic acid.

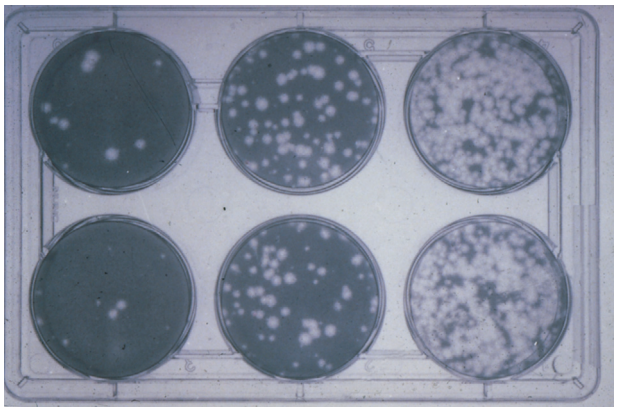


FIGURE 10.11 Multiwell plates for cell culture for virus detection. These wells show increasing dilutions (right to left) of virus on a monolayer. Each clear zone, or plaque, theoretically arises from a single infectious virus particle, i.e. a plaque-forming unit (PFU).

Generally, laboratory strains of viruses have been selected for their ability to grow in cell culture easily and rapidly with the production of CPE. In contrast, viruses isolated from environmental samples often come directly from infected animals or humans, and do not grow as rapidly in cell culture. Often, "blind passages" of cell culture showing no CPE are passed on to fresh monolayers of cells before a CPE is observed. Two procedures can be used to quantify viruses utilizing CPE. The first is the serial dilution endpoint or $TCID_{50}$ method, which involves adding serial dilutions of virus suspension to host cells, and subsequently observing the production of CPE over time. The titer or endpoint is the highest viral dilution capable of producing a CPE in 50% of the tissue culture vessels or wells, and is referred to as the medium tissue culture infective dose or $TCID_{50}$. The second method is the most probable number or MPN method, which is similar to that used to quantify bacteria. This can be used with viral cells by observation of CPE in monolayers inoculated with different dilutions of viral suspension, and subsequent use of MPN tables to determine viral numbers.

Some viruses, such as enteroviruses, produce plaques or zones of lysis in cell monolayers overlaid with solidified nutrient medium. These plaques originate from a single infectious virus particle, thus the titer can be quantified by counting the plaques or plaque-forming units (PFU). This can be thought of as analogous to enumeration of bacteria using CFU. There are variations of the basic plaque-forming unit assay, but usually a vital

TABLE 10.2 Commonly Used Continuous Cell Cultures for Isolation and Detection of Enteric Viruses

Cell Culture Line	Virus							
	Adeno	Astro	Coxsackie A	Coxsackie B	Echo	Polio	Rota	Hepatitis A
BGM	−[a]	−	−	+	+	+	−	−
BSC-1	−	*	−	*	*	+	*	*
$CaCO_2$	+	+	*	+	+	+	+	+
Hep-2 (HeLa)	+	*		+	*	*	*	*
RD	*	*	+	−	+	*	*	
RfhK	*	*	*	*	*	*	*	+

[a] *+, growth and/or production of cytopathogenic effect (CPE); −, no growth and/or production of CPE; *, no data.*

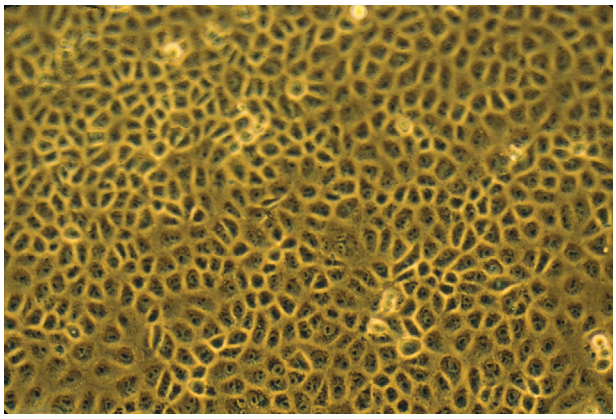


FIGURE 10.12 This figure shows a normal uninfected cell culture monolayer. Compare with Figure 10.13.

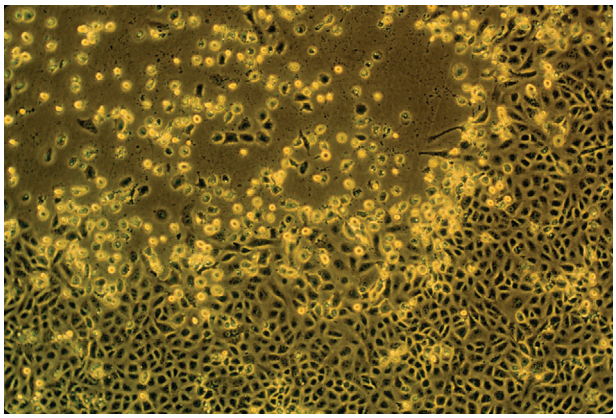


FIGURE 10.13 This figure shows an infected cell culture monolayer exhibiting CPE. Compare with Figure 10.12.

dye, which only stains living cells, is used. As the virus infection in the monolayer spreads and cells lyse, a clear plaque appears in a stained monolayer of living cells (Figure 10.11). A system commonly used for enteroviruses utilizes an agar overlay containing media components, serum and the dye neutral red. A virus kills and lyses infected cells, leaving a clear plaque in a red-stained cell monolayer, because the dye does not stain dead cells.

Cell culture is not 100% effective in detecting all of the viral particles that can be observed through use of an electron microscope. This is because not all the particles are infectious. A noninfectious viral particle may contain no nucleic acid in the capsid, or the nucleic acid may be incomplete. Other reasons for the inefficiency of cell culture may be that there is a lack of cell receptors, or the cell culture incubation period is insufficient for all of the viruses to attach to viral receptors on the host cells. Further, the plaquing efficiency or production of a CPE is influenced by the salts, additives or enzymes added to the medium. For example, good plaque formation for rotaviruses requires the addition of trypsin to the overlay medium. The susceptibility of cell lines can be enhanced by the addition of 5-iododeoxyuridine for enteric viruses (Benton and Ward, 1982). Generally, for laboratory-grown viruses, the ratio of PFU or infectious virus to total viral particles is 1:100, but it may be much greater for viruses from the host or environment (1:10,000) (Ward *et al.*, 1984). For this reason, techniques such as PCR, which can detect one to 10 copies of a viral genome, are more sensitive than conventional cell culture methods.

Use of cell culture for detection of viruses in environmental samples is also often made difficult by the presence of bacteria, molds and toxic substances. To overcome problems with other microorganisms, antibiotics are added, or the samples are filtered. Unfortunately, this sometimes results in loss of viruses that may be associated with solids. Problems with substances toxic to the cell culture are also difficult to overcome. The easiest solution is to dilute the sample until no more toxicity is observed. Unfortunately, this may require use of an excessive amount of cell culture. Treatment of the sample with Freon or chloroform is another technique that has often been employed.

QUESTIONS AND PROBLEMS

1. Student "A" performs a soil extraction with peptone as the extracting solution. A dilution and plating technique is performed using the spread plate method. The following results are obtained:
   a. 150 colonies are counted on an R2A plate at the 10^5 dilution
   b. 30 colonies are counted on a nutrient agar plate at 10^4 dilution

   Calculate the number of viable bacteria per gram of soil for R2A and nutrient agar. Why are the counts for nutrient agar lower than for the R2A media?
2. Student "B" performs a soil extraction with peptone as the extracting solution. A dilution and plating technique is performed using the pour plate method. The number of bacteria per gram of soil is calculated to be 8×10^8. What dilution plate contained 80 colonies?
3. Student "C" used direct microscopic methods to count bacteria in the same soil as student "A" above. How would student "C's" numbers compare to student "A's" numbers and why?
4. The last student also extracted the same soil in peptone, but let the extraction sit overnight at room temperature before diluting and plating. How would these numbers compare to student "A's" numbers and why?
5. Identify the major errors associated with dilution and plating techniques.
6. What is the difference between a primary cell line and a continuous cell line? Which cell line do you think is better for isolating viruses from the environment? Why?

7. What are the two most common methods used to assay viruses in cell culture?
8. Why are some virus particles unable to be detected by cell culture?
9. Discuss the advantages and disadvantages of cultural methodologies for the detection of microbes in the environment.

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