Chapter 8

Environmental Sample Collection and Processing

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8.1 SOILS AND SEDIMENTS

Soils are discontinuous, heterogeneous environments that contain large numbers of diverse organisms. As described in Chapter 4, soil microbial communities vary with depth and soil type, with surface soil horizons generally having more organisms than subsurface horizons. Communities also vary from site to site, and even within sites because of natural microsite variations that can allow very different microorganisms to coexist side by side. Because of the great variability within communities, it is often necessary to take more than one sample to obtain a representative microbial sample at a particular site. Therefore, the overall sampling strategy will depend on many factors, including the goal of the analyses, the resources available and the site characteristics. The most accurate approach is to take many samples within a given site and perform a separate analysis of each sample. However, in many instances time and effort can be conserved by combining the samples taken to form a composite sample that is analyzed, thereby limiting the number of analyses that need to be performed. Another approach often used is to sample a site sequentially over time from a small defined location to determine temporal effects on microbes. Because so many choices are available, it is important to delineate a sampling strategy to ensure that quality assurance is addressed. This is done by developing a quality assurance project plan (QAPP) according to the guidelines shown in Information Box 8.1.

8.1.1 Sampling Strategies and Methods for Surface Soils

Bulk soil samples are easily obtained with a shovel or, better yet, a soil auger (Figure 8.1). Soil augers are more precise than simple shovels because they ensure that samples are taken to exactly the same depth on each occasion. This is important, as several soil factors can vary considerably with depth, such as oxygen, moisture content, organic carbon content and soil temperature. A simple hand auger is useful for taking shallow soil samples from areas that are unsaturated. Given the right conditions, a hand auger can be used to take samples to depths of 180 cm in 30 cm increments. However, some soils are

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Information Box 8.1 Collection and Storage Specifications for a Quality Assurance Project Plan (QAPP)

The QAPP involves delineating the details of the sampling strategy, the sampling methods, and the subsequent storage of all samples. The QAPP normally also includes details of the proposed microbial analysis to be conducted on the soil samples.

- Sampling strategies: Number and type of samples, locations, depths, times, intervals
- Sampling methods: Specific techniques and equipment to be used
- Sample storage: Types of containers, preservation methods, maximum holding times

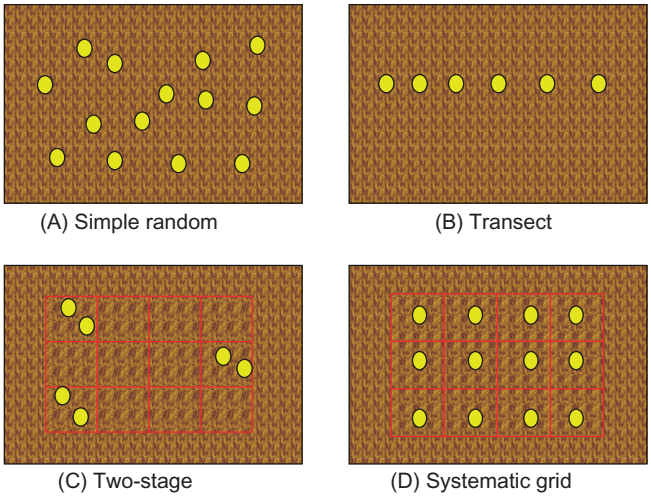

FIGURE 8.2 Alternative spatial sampling patterns.

FIGURE 8.1 Hand auger.

simply too compacted or contain too many rocks to allow sampling to this depth. When taking samples for microbial analysis, consideration should be given to contamination that can occur as the auger is pushed into the soil. In this case, microbes that stick to the sides of the auger as it is inserted into the soil and pushed downward may contaminate subsequent cores that are taken. To minimize such contamination, one can use a sterile spatula to scrape away the outer layer of the core and use the inner part of the core for analysis. Contamination can also occur between samples, but this can be avoided by cleaning the auger after each sample is taken. The cleaning procedure involves washing the auger with water, then rinsing it with 75% ethanol or 10% bleach, and finally rinsing with sterile water.

Composite samples can be obtained by collecting equal amounts of soil from samples taken over a wide area and placing them in a bucket or plastic bag. The whole soil mass is then mixed and becomes the composite sample. To reduce the volume of samples to be stored, a portion of the composite sample can be removed, and this becomes the sample for analysis. In all cases, samples should be stored on ice until processed and analyzed.

In some instances, a series of experimental plots or fields need to be sampled to test the effect of a soil amendment, such as fertilizer, pesticide or sewage sludge, on microbial communities. In this case, a soil sample must be taken from each of several plots or fields to compare control nontreated plots with plots that have received an amendment. For example, a researcher might be interested in the influence of inorganic nitrogen fertilizers on soil nitrifying populations. The investigator would then sample an unamended plot (the control) for comparison with a plot that had been treated with inorganic fertilizer. Another example would be the case in which soil amended with sewage sludge is sampled for subsequent viral pathogen analysis. In either example, multiple samples or replicates always give a more refined estimate of the parameters of interest. However, fieldwork can be costly and the number of samples taken must be weighed against the cost of analysis and the funds available. In the examples given, two-dimensional sampling plans can be used to determine the number and location of samples taken. In two-dimensional sampling, each plot is assigned spatial coordinates and set sampling points are chosen according to an established plan. Some typical two-dimensional sampling patterns, including random, transect, two-stage and grid sampling, are illustrated in Figure 8.2.

Random sampling involves choosing random points within the plot of interest, which are then sampled to a defined depth. Transect sampling involves collection of samples in a single direction. For example, transect sampling might be useful in a riparian area, where transects could be chosen adjacent to a streambed and at right angles to the streambed. In this way, the influence of the stream on the microbial community could be evaluated. In two-stage sampling, an area is broken into regular subunits called primary units. Within each primary unit,

subsamples can be taken randomly or systematically. This approach might be useful when a site consists of a hillside slope and a level plain, and there is likely to be variability between the primary units. The final example of a sampling pattern is grid sampling, in which samples are taken systematically at regular intervals at a fixed spacing. This type of sampling is useful for mapping an area when little is known about the variability within the soil.

Two-dimensional sampling does not give any information about changes in microbial communities with depth. Therefore, three-dimensional sampling is used when information concerning depth is required. Such depth information is critical when evaluating sites that have been contaminated by improper disposal, or spills of contaminants. Three-dimensional sampling can be as simple as taking samples at 50 cm depth increments to a depth of 200 cm, or can involve drilling several hundred meters into the subsurface vadose zone. For subsurface sampling, specialized equipment is needed, and it is essential to ensure that subsurface samples are not contaminated by surface soil.

Finally, note that there is a specialized zone of soil that is under the influence of plant roots. This is known as the rhizosphere, which is of special interest to soil microbiologists and plant pathologists because of enhanced microbial activity and specific plant−microbe interactions (see Chapter 16). Rhizosphere soil exists as a continuum from the root surface (the rhizoplane) to a point where the root has no influence on microbial properties (generally 2−10 mm). Thus, rhizosphere soil volumes are variable and are difficult to sample. Normally, roots are carefully excavated and shaken gently to remove bulk or nonrhizosphere soil. Soil adhering to the plant roots is then considered to be rhizosphere soil. Although this is a crude sampling mechanism, it remains intact to this day. As a result, the sampling of rhizosphere remains a major experimental limitation, regardless of the sophistication of the microbial analyses that are subsequently performed.

8.1.2 Sampling Strategies and Methods for the Subsurface

Mechanical approaches using drill rigs are necessary for sampling the subsurface environment. This significantly increases the cost of sampling, especially for the deep subsurface. As a result, few cores have been taken in the deep subsurface, and these coring efforts have involved large teams of researchers (see Case Study 4.4). The approach used for sampling either deep or shallow subsurface environments depends on whether the subsurface is saturated or unsaturated. For unsaturated systems, air rotary drilling can be used to obtain samples from depths up to several hundred meters (Chapelle, 1992). In air rotary drilling, a large compressor is used to force air down a drill pipe, out the drill bit, and up outside the borehole (Figure 8.3). As the core barrel cuts downward, the air serves to blow the borehole cuttings out of the hole and also to cool the core barrel. This is important, because if the core barrel overheats, microbes within the sample may be effectively sterilized, posing difficulty for subsequent microbial analysis. In normal air drilling, small amounts of water containing a surfactant are injected into the airstream to control dust and help cool the drill bit. However, this increases the possibility of contamination, so cores such as the one drilled in Idaho's Snake River Plain (see Section 4.6.2.1) have been drilled with air alone (Colwell, 1989). To help keep the core barrel cool, the coring was simply done very slowly to avoid overheating. To help maintain sterile conditions and prevent contamination from surface air, all air used in the coring process was prefiltered through a 0.3-μm high-efficiency particulate air (HEPA) filter (see Section 5.8.2). Immediately after the core was collected the surface layer was scraped away with a sterile spatula, and then a subcore was taken using a 60-ml sterile plastic syringe with the end removed. The samples were immediately frozen and shipped to a laboratory, where microbial analyses were initiated within 18 hours of collection.

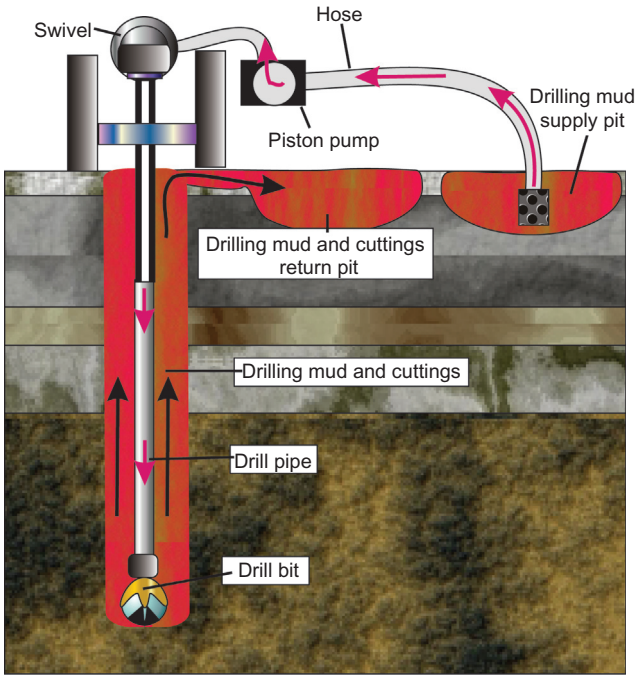

FIGURE 8.3 With rotary drilling the mechanical rotation of a drilling tool is used to create a borehole. Either air (air rotary drilling), or a fluid often called a drilling mud (mud rotary drilling), is forced down the drill stem to displace the borehole cuttings to the outside of the drill and upward to the surface. This figure illustrates mud rotary drilling.

Saturated subsurface environments are sampled somewhat differently because the sediments are much less

cohesive than those found in unsaturated regions. Therefore, the borehole must be held open so that an intact core can be taken and removed at each desired depth. For sampling of depths down to 30 m, hollow-stem auger drilling with push-tube sampling is widely used (Figure 8.4). The auger consists of a hollow tube with a rotating bit at the tip that drills the hole. The outside of the hollow auger casing is reverse threaded so that the cuttings are pushed upward and out of the hole as drilling proceeds. As the borehole is drilled, the casing of the auger is left in place to keep the borehole open. Thus, the casing acts as a sleeve into which a second tube, the core barrel, is inserted to collect the sample when the desired depth has been reached. The core barrel is basically a sterile tube that is placed at the tip of the hollow-stem auger, driven down to collect the sediment sample, and then retrieved. Drilling can then continue to the next desired depth and the coring process repeated. Each core collected is capped, frozen and sent to a laboratory for study. To avoid contamination of samples, the outside of the core is scraped away or the core may be subcored.

For cores that are deeper than 30 m, mud rotary coring is used (Chapelle, 1992). In this case, the hole is again bored using a rotating bit. However, drilling fluids are used to remove the borehole cuttings and to apply pressure to the walls of the borehole to keep it from collapsing. Mud rotary drilling has been used to obtain sediment samples to 1000 m beneath the soil surface. An example of such a core is one taken from the deep subsurface sediments of the Southeast Coastal Plain in South Carolina (see Case Study 4.4). During this coring, samples were retrieved from depths ranging from 400 to 500 m. In order to ensure the integrity of the cores obtained, the drilling fluids were spiked with two tracers, potassium bromide and rhodamine dye. The use of these two tracers allowed researchers to evaluate how far the drilling fluids had penetrated into the cores. Any areas of the cores that are contaminated with tracer must be discarded. The cores were retrieved in plastic liners, frozen, and sent for immediate analysis.

It is important to emphasize that coring either saturated or unsaturated environments is a difficult process

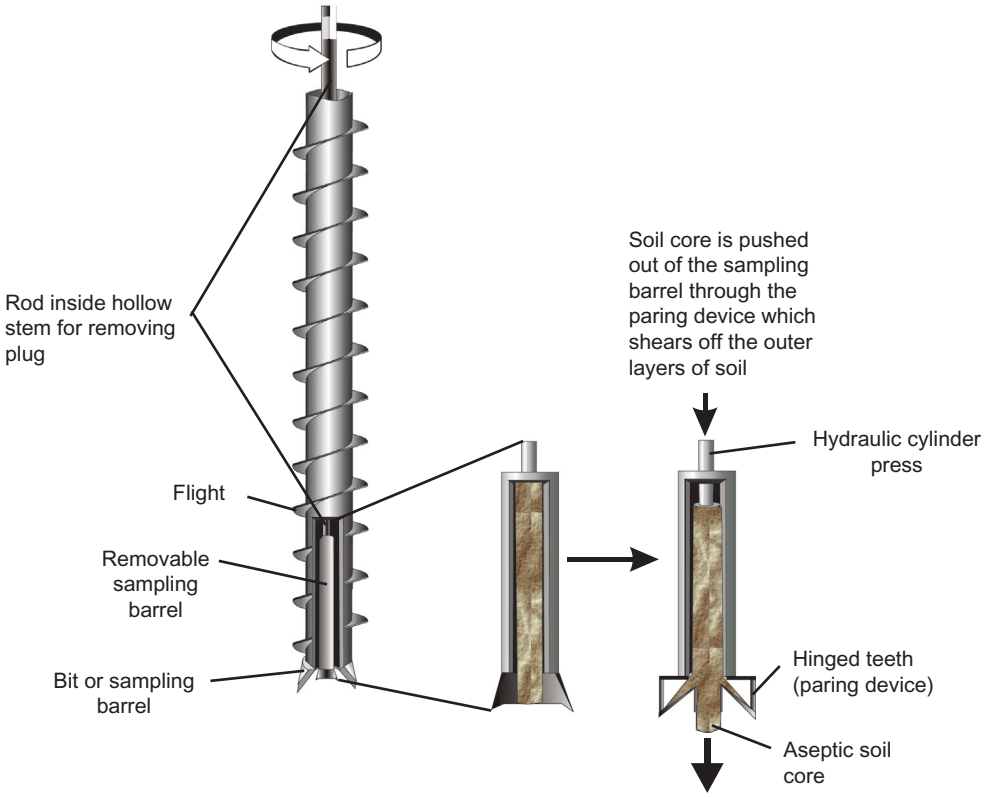

FIGURE 8.4 Diagram of a hollow-stem auger. Note the reverse threading on the outside of the auger. This is used to displace the borehole cuttings upward to the surface. This type of auger was used at Purdue University to collect core samples to a depth of 26 m for microbial and soil analysis as described by Konopka and Turco (1991). A subcore of each core collected is taken using a split spoon sampler or a push tube. In either case, the outside of the core must be regarded as contaminated. Therefore, the outside of the core is shaved off with a sterile spatula or a subcore can be taken using a sterile plastic syringe. Alternatively, as shown in this figure, intact cores are automatically pared to remove the outer contaminated material, leaving an inner sterile core.

for several reasons. First, it may take years to plan and obtain funding to proceed with cores such as those described here for the Snake River Plain and the Southeast Coastal Plain. The actual drilling and recovery of such samples is an engineering problem whose sophistication has only been touched upon in this section. Also keep in mind that the cores obtained are not always truly representative of the sediments from which they are taken. For example, a 1-m core may be compressed considerably in the coring process so that it is difficult to identify exactly the depth from which it was taken. A second difficulty in obtaining representative samples is due to horizontal heterogeneities in the subsurface material. Such heterogeneities can mean that two samples taken a few meters apart may have very different physical, chemical and microbiological characteristics. Finally, for microbial analysis it is not enough merely to retrieve the sample; the logistics of sample storage and analysis must be considered as well.

8.1.3 Sample Processing and Storage

Microbial analyses should be performed as soon as possible after collection of a soil to minimize the effects of storage on microbial communities. Once removed from the field, microbial communities within a sample can and will change regardless of the method of storage. Reductions in microbial numbers and microbial activity have been reported even when soil samples were stored in a field moist condition at 4°C for only 3 months (Stotzky *et al*., 1962). Interestingly in this study, although the bacterial community changed, the actinomycete community remained unchanged.

The first step in microbial analysis of a surface soil sample usually involves sieving through a 2 mm mesh to remove large stones and debris. However, to do this, samples must often be air dried to facilitate the sieving. This is acceptable as long as the soil moisture content does not become too low, because this can also change the microbial community (Sparkling and Cheshire, 1979). Following sieving, short-term storage should be at 4°C prior to analysis. If samples are stored, care should be taken to ensure that samples do not dry out and that anaerobic conditions do not develop, because this too can alter the microbial community. Storage up to 21 days appears to leave most soil microbial properties unchanged (Wollum, 1994), but again time is of the essence with respect to microbial analysis. Note that routine sampling of surface soils does not require sterile procedure. These soils are continually exposed to the atmosphere, so it is assumed that such exposure during sampling and processing will not affect the results significantly.

More care must be taken with processing subsurface samples for three reasons. First, they have lower cultural counts, which means that an outside microbial contaminant may significantly affect the numbers counted. Second, subsurface sediments are not routinely exposed to the atmosphere, and microbial contaminants in the atmosphere might substantially contribute to microbial types found. Third, it is more expensive to obtain subsurface samples, and often there is no second chance at collection. Subsurface samples obtained by coring are either immediately frozen and sent back to the laboratory as an intact core or processed at the coring site. In either case, the outside of the core is normally scraped off using a sterile spatula or a subcore is taken using a smaller diameter plastic syringe. The sample is then placed in a sterile plastic bag and analyzed immediately or frozen for future analysis.

8.1.3.1 Processing Soil and Sediment Samples for Bacteria

Culture-Based Analysis

Traditional methods of analysis for microbial communities have usually involved either cultural assays utilizing dilution and plating methodology on selective and differential media or direct count assays (see Chapter 10). Direct counts offer information about the total number of bacteria present, but give no information about the number or diversity of populations present within the community. Plate counts allow enumeration of total cultural or selected cultural populations, and hence provide information on the different populations present. However, since less than 1% of soil bacteria is readily culturable (Amann *et al*., 1995), cultural information offers only a piece of the picture. The actual fraction of the community that can be cultured depends on the medium chosen for cultural counts. Any single medium will select for the populations that are best suited to that particular medium. Thus, the choice of medium is crucial in determining the results obtained. This is illustrated by the data in Table 8.1, which show that whereas direct counts from a series of sediment samples spanning a 5 m depth were similar, the culturable counts varied depending on the type of medium used. A nutritionally rich medium, PTYG, made from peptone, trypticase, yeast extract and glucose, consistently gave counts that were one to three orders of magnitude lower than counts from two different low-nutrient media that were tested. These were a 1:20 dilution of PTYG and a soil extract agar made from a 1:2 suspension of surface soil. These data reflect the fact that most soil microbes exist under nutrient-limited or oligotrophic conditions.

Community DNA Analysis

In recent years, the advantages of studying community DNA extracted from soil samples have become apparent

TABLE 8.1 Total and Viable Cell Counts of Bacteria in Sediment Samples

Depth (m)	Saturation Status	AODC[a] (Cells/g Dry Weight)	Culturable Counts (CFU/g Dry Weight)		
			PTYG[b]	Dilute PTYG[c]	SSA[d]
1.2	Unsaturated	$6.8 \pm 4.9 \times 10^6$	$3.4 \pm 0.9 \times 10^4$	$1.9 \pm 0.4 \times 10^5$	$1.3 \pm 0.2 \times 10^5$
3.1	Interface[e]	$3.4 \pm 2.6 \times 10^6$	$2.0 \pm 0.5 \times 10^4$	$2.6 \pm 0.2 \times 10^6$	$2.9 \pm 0.6 \times 10^6$
4.9	Saturated	$6.8 \pm 4.3 \times 10^6$	$2.6 \pm 0.7 \times 10^3$	$3.5 \pm 0.1 \times 10^6$	$4.1 \pm 0.2 \times 10^6$

Adapted from Balkwill and Ghiorse (1985).
[a]*AODC, acridine orange direct counts. Reproduced by permission of the American Society for Microbiology Journals Department.*
[b]*PTYG, a nutritionally rich medium composed of peptone, trypticase, yeast extract, and glucose.*
[c]*Dilute PTYG, a 1:20 dilution of PTYG medium.*
[d]*SSA, soil extract agar. This medium was made by autoclaving a 1:2 suspension of surface soil in distilled water and then centrifuging and filtering the extract to clarify it.*
[e]*This sample was taken at the interface between the unsaturated and the saturated zone.*

Information Box 8.2 Comparison of Bacterial Fractionation and *In Situ* Lysis Methodologies for the Recovery of DNA from Soil

Issue	Bacterial Fractionation	*In Situ* Lysis
Yield of DNA	1–5 μg/g	1–20 μg/g
Representative of community	Less representative because of cell sorption	More representative, unaffected cell sorption
Source of DNA recovered	Only bacteria	Mostly bacteria but also fungi and protozoa
Degree of DNA shearing	Less shearing	More shearing
Average size of DNA fragments	50 kb	25 kb
Degree of humic contamination	Less contaminated	More contaminated
Ease of methodology	Slow, laborious	Faster, less labor-intensive

(see Chapter 13). This nonculture-based approach is thought to be more representative of the actual community present than culture-based approaches. In addition to providing information about the types of populations present, this approach can also provide information about their genetic potential. As with any technique, there are limitations to the data that can be obtained with DNA extraction. Therefore, many researchers now use DNA extraction in conjunction with direct and cultural counts to maximize the data obtained from an environmental sample.

Initially, two approaches were developed for isolation of bacterial DNA from soil samples. The first was based on fractionation of bacteria from soil followed by cell lysis and DNA extraction (Holben, 1994). The second method involved *in situ* lysis of bacteria within the soil matrix with subsequent extraction of the DNA released from cells (Information Box 8.2). Subsequent to the development of these two approaches, *in situ* lysis has become the commonly used extraction procedure primarily because it is easier and faster, because it yields more representative DNA, and because commercial kits have made it easier to purify the DNA.

The *in situ* lysis method involves lysing the bacterial cells within the soil and releasing their DNA prior to extraction of DNA from the sample. Lysis methodology has usually involved a combination of physical and chemical treatments. For bacteria, physical treatments have involved freeze–thaw cycles and/or sonication or bead beating, and chemical treatments have often utilized a detergent such as sodium dodecyl sulfate (SDS) and/or an enzyme such as lysozyme or proteinase (Moré *et al.*, 1994). Following lysis, cell debris and soil particles are removed by precipitation and centrifugation, and the DNA in the supernatant is precipitated with ethanol. The DNA can be further purified by sorption onto homemade or commercial columns packed with ion-exchange resins or gels that can subsequently be rinsed for removal of humic materials that can inhibit DNA analysis. Further purification can be achieved with phenol–chloroform/isoamyl alcohol extractions, followed once more by ethanol precipitations (Xia *et al.*, 1995). Pure samples of DNA are necessary to allow subsequent molecular analyses such as with the polymerase chain reaction (PCR) (see Chapter 13). However, regardless of what purification methodology is employed, each step in the purification process causes loss of DNA. Thus, purified DNA is obtained only at the expense of DNA yield.

Commercial kits, which have optimized the procedures described above, are now available for processing

FIGURE 8.5 UltraClean™ Soil DNA Isolation Kit.

soils for community DNA. Examples of such kits are Ultraclean™ Soil DNA Isolation Kit (MoBio) and Fast DNA Spin for Soil (MP Biomedicals) (Figure 8.5). Typically, these kits utilize physical bead beating technology followed by chemical lysis of microbes and subsequent DNA extraction and purification. These kits can even be used to extract DNA from high organic matter environmental samples including compost, sediments, manures and biosolids. But in this case, additional purification of the extracted DNA may be necessary in conjunction with the kit. One common approach is to repeatedly rinse the DNA with guanidine thiocyanate while it is sorbed to the extraction column provided by the kit. Overall these commercial kits have dramatically increased the ease and rapidity of community DNA extractions from soil. Note that there are also kits available to extract community DNA from water samples, e.g., UltraClean™ Water DNA Kit.

Although direct lysis using commercial kits has many advantages, it also has some problems. Sorption of DNA from lysed cells by clay or humic colloids can reduce the yield of extracted DNA (Ogram *et al.*, 1987). Another problem associated with direct lysis is distinguishing free from cellular DNA. Free DNA released from microbes that lysed naturally some time before the DNA extraction can sometimes be protected from degradation by sorption to soil particles (Lorentz and Wackernagel, 1987). This DNA may be extracted along with DNA from the viable cells. In addition, DNA isolated by direct lysis tends to be randomly sheared due to the bead beating procedure associated with most extraction kits. Finally, most kits will lyse all soil microorganisms including fungi and protozoa. Thus, the DNA extracted is not limited to bacterial DNA. Fortunately, fungi ($\approx 10^5$ per gram) and protozoa ($\approx 10^4$ per gram) are present at significantly lower numbers in most soils, and thus will not contribute significantly to DNA obtained from 10^8–10^9 bacteria per gram, even allowing for the larger genome size of protozoa.

Information Box 8.3 Spectroscopic Analysis of DNA

The amount of DNA is estimated from the 260 nm reading. An absorbance reading of 1.0 is equivalent to 50 μg of DNA per ml of solution.

The purity of DNA is estimated from the ratio of the reading at 260 nm to that at 280 nm. A value > 1.7 indicates relatively pure DNA. The maximum theoretical value is 2.0.

Once a sample of purified DNA is obtained from the soil sample, it can be quantified by ultraviolet (UV) spectroscopy or fluorometry. Normally, UV readings are made at wavelengths of 260 and 280 nm, from which the purity and quantity of DNA can be estimated (Information Box 8.3). One limitation of quantification by UV spectroscopy is that readings will be affected by any compound that absorbs at 260 nm. Quantification by fluorometry is more sensitive and more specific, but does not allow the evaluation of extract purity obtained by comparing readings at 260 and 280 nm. DNA concentrations as low as 1 picogram per μl can be measured with a fluorometer using picogreen dye.

Once the amount of DNA per mass of soil is known, estimates can be made of the microbial community. For bacteria such as *Escherichia coli*, a typical chromosome contains 4–5 million base pairs, equivalent to about 9 fg (9×10^{-15} g) of DNA. However, the amount of DNA per cell varies and other estimates are lower, approximately 4 fg per cell. The amount of DNA per cell can also vary because of chromosome replication occurring faster than cell division, resulting in two or three chromosomes per cell (Krawiec and Riley, 1990). These theoretical DNA estimates can be used to relate total extracted DNA to the number of microbes within a sample. Table 8.2 shows the total DNA extracted from four soils amended with glucose. The amounts of DNA obtained increased with the amount of soil organic matter (silt loam and loam), presumably because of larger sustainable bacterial communities. Extracted DNA also decreased in soils high in clay (clay loam), most likely because of sorption of DNA by soil colloids (Ogram *et al.*, 1987). Overall, the influence of the amendments can be seen over time as microbial communities get larger through growth, resulting in more extractable DNA. The theoretical number of bacterial cells that the extracted DNA represents can be calculated as illustrated below.

For example, at time zero for the clay loam soil:

$$\text{Extracted DNA} = 0.12\ \mu\text{g/g soil}$$

TABLE 8.2 Total Community DNA Extracted from Four Soils Amended with 1% Glucose and 0.1% Potassium Nitrate[a]

Soil	Extracted DNA (μg/g Soil) with Time (Days)				
	0	2	4	6	8
Clay loam	0.12	0.04	0.21	1.3	0.52
Silt loam	17.80	17.6	16.6	18.4	19.9
Sandy loam	0.63	0.60	1.90	5.50	1.90
Loam	1.30	0.90	1.30	4.20	7.70

[a]*Amendments were added at time zero and DNA was obtained via direct lysis.*

Therefore, if each cell has 4 fg of DNA:

$$\text{Number of cells} = \frac{0.12 \times 10\ \text{g}^{-1}\ \text{DNA}/g\ \text{soil}}{4 \times 10\ \text{g}^{-15}\ \text{DNA/cell}} = 3.0 \times 10^7\ \text{cells/g soil}$$

Similar values at time zero for the other soils are 1.6×10^8 cells/g soil (sandy loam), 3.3×10^8 cells/g soil (loam), and 4.5×10^9 cells/g soil (silt loam).

8.1.3.2 *Processing Soil Samples for Fungal Hyphae and Spores*

As with bacteria, it is also impossible to culture all species of viable fungi from a soil or sediment sample. Cultural methods for fungi are described in Section 10.5. However, several approaches have been developed for direct isolation of fungal hyphae or spores from soil. The first is a soil washing methodology. This involves saturating small volumes of soil with sterile water. Aggregates of soil are gently teased open with a fine jet of water, allowing heavier soil particles to sediment and finer particles to be decanted off. The procedure is repeated several times until only the heavier particles remain. These are then spread in a film of sterile water and examined under a dissecting microscope. Sterile needles or very fine forceps can then be used to obtain any observed fungal hyphae.

For spores, a different approach can be used. Soil samples are placed in separate sterile boxes each of which contains a number of sieves of graded size. The soil samples are washed vigorously in each box, and soil of defined size is retained on each sieve. Spores are determined empirically by plating successive washings (two minutes per wash) from each sieve. Because hyphae are retained by the sieves, any fungal colonies that arise must be due to the presence of spores. Information on fungi present as hyphae can be obtained by plating the washed particles retained by the sieves. However, these washing methods are labor intensive, and rely on trial and error in terms of which size fractions are most relevant with respect to individual spore size.

8.1.3.3 *Processing Sludge, Soil and Sediment for Viruses*

To assess fully and understand the risks from pathogens in the environment, it is necessary to determine their occurrence in sewage sludges (biosolids), soils to which wastewater or sludge is applied, or marine sediments that may be affected by sewage outfalls or sludge disposal. In fact, the U.S. Environmental Protection Agency currently requires monitoring of sludge for enteroviruses for certain types of land application. To detect viruses on solids it is first necessary to extract them by processes that will cause their desorption from the solid. As with microporous filters (see Section 8.2.2.1), viruses are believed to be bound to these solids by a combination of electrostatic and hydrophobic forces (see Chapter 15). To recover viruses from solids, substances are added that will break down these attractive forces, allowing the virus to be recovered in the eluting fluid (Gerba and Goyal, 1982; Berg, 1987).

The most common procedure for sludges involves collecting 500−1000 ml of sludge and adding $AlCl_3$ and HCl to adjust the pH to 3.5. Under these conditions, the viruses bind to the sludge solids, which are removed by centrifugation, and then resuspended in a beef extract solution at neutral pH to elute the virus. The eluate is then reconcentrated by flocculation of the proteins in the beef extract at pH 3.5, resuspended in 20−50 ml, and neutralized. A major problem with sewage sludge concentrates prepared in this manner is that they often contain substances toxic to cell culture. A diagram of the details of this procedure is shown in Figure 8.6. Similar extraction techniques are used for the recovery of viruses from soils and aquatic sediments (Hurst *et al.*, 2007).

8.2 WATER

8.2.1 Sampling Strategies and Methods for Water

Sampling environmental waters for subsequent microbial analysis is somewhat easier than sampling soils, for a variety of reasons. First, because water tends to be more homogeneous than soils, there is less site-to-site variability between two samples collected within the same vicinity. Second, it is often physically easier to collect water samples because it can be done with pumps and hose lines. Thus, known volumes of water can be collected from known depths with relative ease. Amounts of water

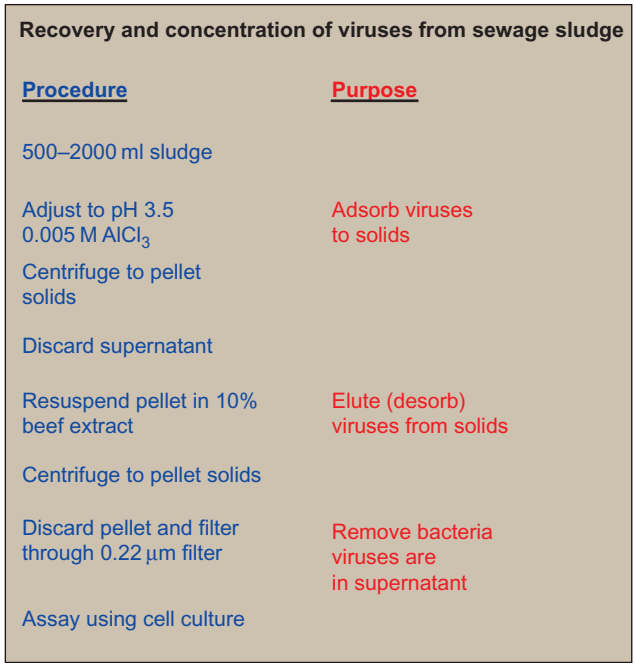

FIGURE 8.6 Procedure for recovery and concentration of viruses from sludge.

collected depend on the environmental sample being evaluated, but can vary from 1 ml to 1000 liters. Sampling strategy is also less complicated for water samples. In many cases, because water is mobile, a set number of bulk samples are simply collected from the same point over various time intervals. Such a strategy would be useful, for example, in sampling a river or a drinking water treatment plant. For marine waters, samples are often collected sequentially in time, within the defined area of interest.

Although the collection of the water sample is relatively easy, processing the sample prior to microbial analysis can be more difficult. The volume of the water sample required for detection of microbes can sometimes become unwieldy because the numbers of microbes tend to be lower in water samples than in soil samples (see Chapter 6). Therefore, strategies have been developed to allow concentration of the microbes within a water sample. For larger microbes including bacteria and protozoan parasites, samples are often filtered to trap and concentrate the organisms. For bacteria this often involves filtration using a 0.45-μm membrane filter (see Chapter 10). For protozoan parasites, coarse woven fibrous filters are used. For viruses, water samples are also filtered, but because viral particles are often too small to be physically trapped, collection of the viral particles depends on a combination of electrostatic and hydrophobic interactions of the virus with the filter. The different requirements for processing of water samples for analysis of viruses, bacteria and protozoa are outlined in the next two sections.

8.2.2 Processing Water Samples for Virus Analysis

The detection and analysis of viruses in water samples is often difficult because of the low numbers encountered and the different types that may be present. There are four basic steps in virus analysis: sample collection, elution, reconcentration and virus detection. For sample collection, it is often necessary to pass large volumes of water (100 to 1000 liters) through a filter because of the low numbers of viruses present. The viruses are concentrated from the water by adsorption onto the filter. Recovery of virus from the filter involves elution of the virus from the collection filter, as well as a reconcentration step to reduce the sample volume before assay. Virus detection can be done via cell culture or molecular methods such as PCR. However, both methods can be inhibited by the presence of toxic substances in the water that are concentrated along with the viral particles. Many strategies have been developed to overcome the difficulties associated with analysis of viruses, but they are often time consuming, labor intensive and costly. For example, the cost of enterovirus detection ranges from $600 to $1000 per sample for drinking water. Another problem with analysis of viruses is that the precision and accuracy of the methods used suffer from the large number of steps involved. In particular, the efficiency of viral recovery associated with each step is dependent on the type of virus that is being analyzed. For example, hepatitis A virus may not be concentrated as efficiently as rotavirus by the same process. Variability also results from the extreme sensitivity of these assays. Methods for the detection of viruses in water have been developed that can detect as little as one plaque-forming unit in 1000 liters of water. On a weight-to-weight basis with water, this is a sensitivity of detection of one part in 10^{18}. For comparison, the limit of sensitivity of most analytical methods available for organic compounds is about 1 μg/liter. This corresponds to one part in 10^9.

8.2.2.1 Sample Collection

Virus analysis is performed on a wide variety of water types. Types of water tested include potable water, ground and fresh surface waters, marine waters and sewage. These waters vary greatly in their physical–chemical composition, and contain substances either dissolved or suspended in solution, which may interfere with our ability to employ various concentration methods. The suitability of a virus concentration method depends on the probable virus density, the volume limitations of the concentration method for the type of water and the presence of interfering substances. A sample volume of less than 1 liter may suffice for recovery of viruses from raw and primary sewage. For drinking water and relatively

nonpolluted waters, the virus levels are likely to be so low that hundreds or perhaps thousands of liters must be sampled to increase the probability of virus detection. Various methods employed for virus concentration from water are shown in Table 8.3.

Most methods used for virus concentration depend on adsorption of the virus to a surface, such as a filter or mineral precipitate, although hydroextraction and ultrafiltration have been employed (Gerba, 1987). Field systems for virus concentration usually consist of the use of a pump for passing the water through the filter (at rates of 20 to 40 liters per minute), a filter housing and a flowmeter (Figure 8.7A). The entire system can usually be contained in a 20 liter capacity ice chest.

The filters most commonly used for virus collection from large volumes of water are adsorption–elution microporous filters, more commonly known as VIRADEL (for virus adsorption-elution). VIRADEL

TABLE 8.3 Methods Used for Concentrating Viruses from Water

Method	Initial Volume of Water	Applications	Remarks
Filter adsorption–elution			
Negatively charged filters	Large	All but the most turbid waters; best for seawater and sewage	Only system shown useful for concentrating viruses from large volumes of tap water, sewage, seawater, and other natural waters; cationic salt concentration and pH must be adjusted before processing
Positively charged filters	Large	Tap water; does not perform well with seawater because of positive charge	No preconditioning of water necessary at neutral or acidic pH levels
Adsorption to metal salt precipitate, aluminum hydroxide, ferric hydroxide	Small	Tap water, sewage	Has been useful in reconcentration
Charged filter aid	Small	Tap water, sewage	40-liter volumes tested, low cost; used as a sandwich between prefilters
Polyelectrolyte PE60	Large	Tap water, lake water, sewage	Because of its unstable nature and lot-to-lot variation in efficiency for concentration of viruses, the method has not been used in recent years
Bentonite	Small	Tap water, sewage	
Iron oxide	Small	Tap water, sewage	
Glass powder	Large	Tap water, sewage	Columns containing glass powder have been made that are capable of processing 40-liter volumes
Positively charged glass wool	Small to large	Tap water	Positively charged glass wool is inexpensive; used in pipes or columns
Protamine sulfate	Small	Sewage	Very efficient method for concentrating reoviruses and adenoviruses from small volumes of sewage
Hydroextraction	Small	Sewage	Often used as a method for reconcentrating viruses from primary eluates
Ultrafiltration			
Soluble filters	Small	Clean waters	Clogs rapidly even with low turbidity
Flat membranes	Small	Clean waters	Clogs rapidly even with low turbidity
Hollow fiber or capillary	Large	Tap water, lake water, seawater	Up to 100 to 1000 liters may be processed, but water must often be prefiltered; cannot easily be used in the field and requires longer processing time than filters
Reverse osmosis	Small	Clean waters	Also concentrates cytotoxic compounds that adversely affect assay methods; cannot easily be used in the field and requires longer filtering time than filters

FIGURE 8.7 (A) Field VIRADEL system for concentrating viruses from water. (B) Elution of virus from filter with beef extract.

involves passing the water through a filter to which the viruses adsorb. The pore size of the filters is much larger than the viruses, and adsorption takes place by a combination of electrostatic and hydrophobic interactions (Iker *et al*., 2012). Two general types of filters are available: electronegative (negative surface charge) and electropositive (positive surface charge). Electronegative filters are composed of either cellulose esters or fiberglass with organic resin binders. Because the filters are negatively charged, cationic salts ($MgCl_2$ or $AlCl_3$) must be added in addition to lowering the pH to 3.5. This reduces the net negative charge usually associated with viruses allowing adsorption to be maximized (see Chapter 2). Such pH adjustment can be cumbersome, as it requires modifying the water prior to filtering and the use of additional materials and equipment such as pH meters. The most commonly used electronegative filter is the Filterite. Generally, it is used as a 10-inch (25.4 cm) pleated cartridge with either a 0.22- or 0.45-μm nominal pore size rating. Electronegative filters are ideal when concentrating viruses from seawater and waters with high amounts of organic matter and turbidity (Gerba *et al*., 1978).

Electropositive filters may be composed of fiberglass or cellulose containing a positively charged organic polymeric resin (1MDS), or nano alumina fibers (NanoCeram), which create a net positive surface charge to enhance adsorption of the negatively charged virus. These filters adsorb viruses efficiently over a wide pH range without a need for polyvalent salts. The 1MDS are less efficient with seawater or water with a pH exceeding 8.0−8.5 (Sobsey and Glass, 1980). The electropositive 1MDS Virozorb is especially manufactured for virus concentration from water.

VIRADEL filter methods suffer from a number of limitations. Suspended matter in water tends to clog the filters, thereby limiting the volume that can be processed and interfering with the elution process. Dissolved and colloidal organic matter in some waters can interfere with virus adsorption to filters, presumably by competing with viruses for adsorption sites. Finally, the concentration efficiency varies depending on the type of virus, presumably because of differences in the isoelectric point of the virus, which influences the net charge of the virus at any pH.

8.2.2.2 Sample Elution and Reconcentration

Adsorbed viruses are usually eluted from the filter surfaces by pressure filtering a small volume (1−2 liters) of an eluting solution through the filter. The eluent is usually a slightly alkaline proteinaceous fluid such as 1.5% beef extract adjusted to pH 9.5 (Figure 8.7B). The elevated pH increases the negative charge on both the virus and filter surfaces, which results in desorption of the virus from the filter. The organic matter in the beef extract also competes with the virus for adsorption on the filter, further aiding desorption. The 1- to 2-liter volume of the elutant is still too large to allow sensitive virus analysis, and therefore a second concentration step (reconcentration) is used to reduce the volume to 20−30 ml before assay. The elution−reconcentration process is shown in detail in Figure 8.8. Overall, these methods can recover enteroviruses with an efficiency of 30−50% from 400- to 1000-liter volumes of water (Gerba *et al*., 1978; Sobsey and Glass, 1980).

8.2.2.3 Virus Detection

Several options are available for virus detection and are described in detail in Section 10.7. Briefly, virus can be detected by inoculation of a sample into an animal cell

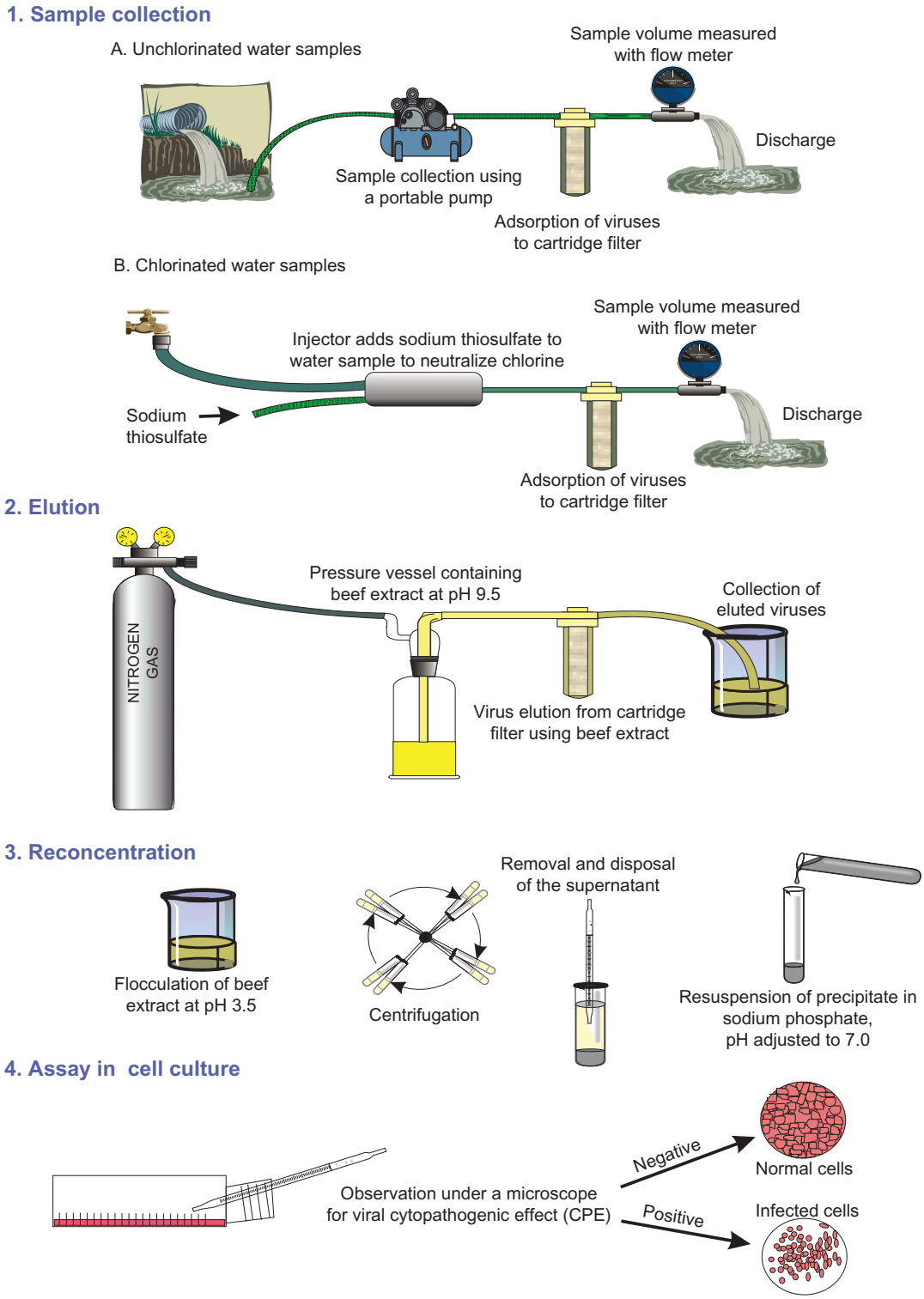

FIGURE 8.8 Procedures for sampling and detection of viruses from water.

culture followed by observation of the cells for cytopathogenic effects (CPE) or by enumeration of clear zones or plaque-forming units (PFU) in cell monolayers stained with vital dyes (i.e., dyes that stain only living nonvirus-infected cells). The PFU method allows more adequate quantitation of viruses because they can easily be enumerated. PCR can also be used to detect viruses directly in either the sample concentrates or the animal cell culture. The overall procedure for sampling and detecting viruses in water is shown in Figure 8.8.

8.2.3 Processing Water Samples for Detection of Bacteria

Processing water samples for bacteria is much simpler than the processing required for viruses. Typically, bacteria are collected and enumerated by one of two different procedures: the membrane filtration and most probable number (MPN) methodologies. Membrane filtration, as the name implies, relies on collection and concentration of bacteria via filtration. In the MPN method, samples are generally not processed prior to the analysis. In both procedures, bacteria are detected via cultural methods using MPN or membrane filtration techniques, described in Chapter 10.

8.2.4 Processing Water Samples for Detection of Protozoan Parasites

As with enteric viruses, it requires ingestion of only a few protozoan parasites to cause infection in humans. As a result, large volumes of tapwater (10 liters or more) or surface water (10 to 100 liters) need to be sampled in order to detect low numbers. The first step usually involves collection of the cysts or oocysts by filtration on pleated cartridge or foam filters (Schaefer, 2007). During filtration, the cysts or oocysts are entrapped on the filters by size exclusion (Figure 8.9). Usually, a pump running at a flow rate of 2 liters per minute is used to collect a sample. The filter is placed in a plastic bag, sealed, stored on ice and sent to the laboratory to be processed within 72 hours.

In the laboratory, the cysts and oocysts are extracted. In the case of the cartridge filter (Envirocheck, Pall Filter, Ann Arbor), an elution buffer (a solution of laureth-12, Tris buffer, EDTA and antifoam) is added to the filter cartridge, and it is placed on a shaker and agitated for five minutes to release the cysts or oocysts. This is followed by pelleting the protozoa by centrifugation and resuspension in a buffer. In the case of the foam filter (Filta-Max, IDEXX, Westbrook, ME), an elution solution of phosphate buffer and 0.01% Tween 20 is added, and the protozoa are squeezed from the flexible filter with a plunger. A great deal of particulate matter is often concentrated along with the cysts and oocysts, and requires further purification by immunomagnetic separation (IMS). In this process, the cysts and oocysts attach to specific antibodies that are associated with magnetic beads (Dynal, Inc., Lake Success, NY), and the beads (with the organisms attached) are removed from solution. After dissociation of the cysts and oocysts from the beads, they are suspended in a small volume of buffer, placed into wells, stained with fluorescent monoclonal antibodies and viewed with an epifluorescence microscope (Figure 8.10). Fluorescent bodies of the correct size and shapes are identified and examined by differential interference contrast microscopy for the

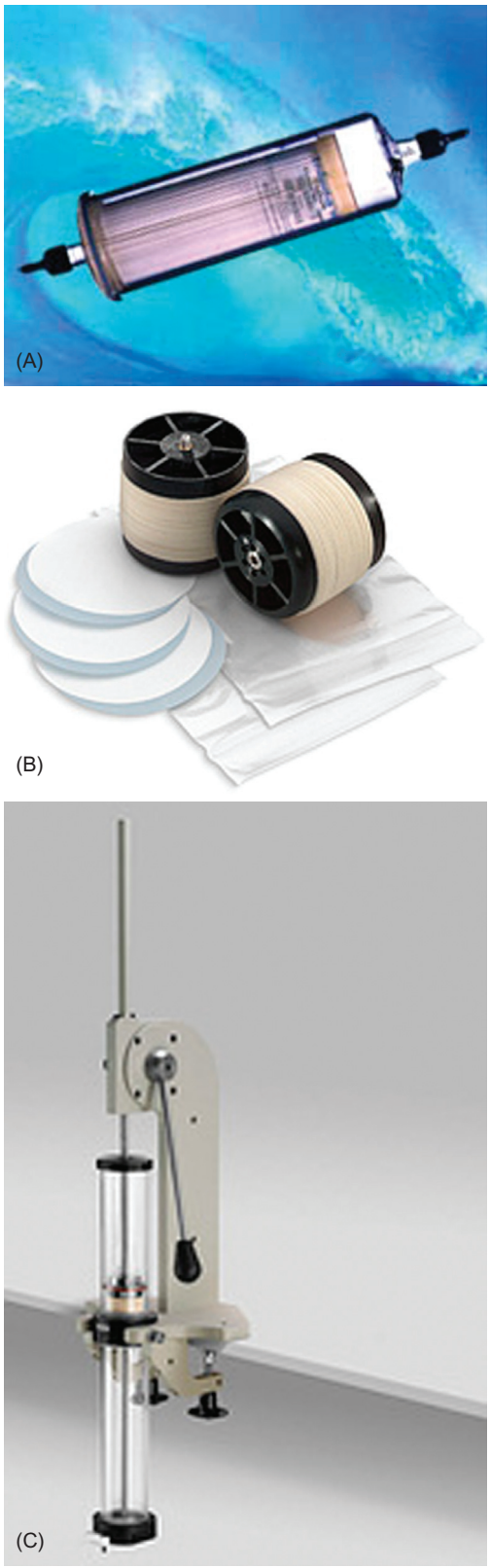

FIGURE 8.9 Filters used for *Giardia* and *Cryptosporidium* concentration from water. (A) Environcheck® Pleated Cartridge filter; (B) Filtra-max® foam filter; (C) plunger system for elution of Filtra-max filter. (A) Photo courtesy Pall Filter, Ann Arbor, MI; (C) Photo courstesy IDEXX, Westbrook, ME).

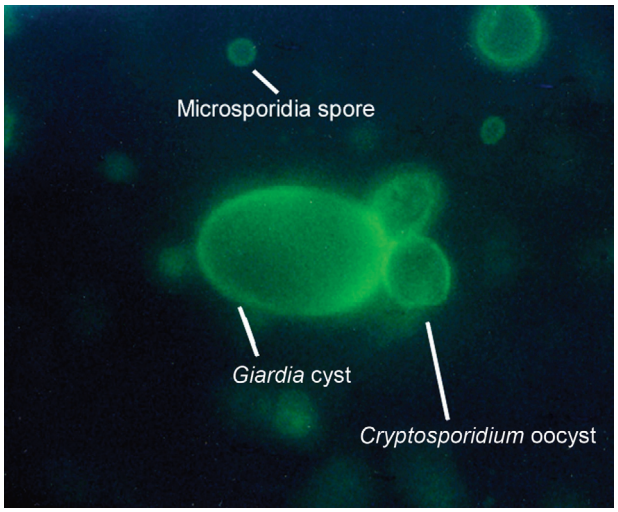

FIGURE 8.10 Immunofluorescence of *Giardia* cysts, *Cryptosporidium* oocysts and *Microsporidia* spores, representative of waterborne pathogenic protozoans.

presence of internal bodies (i.e., trophozites or sporozites). The entire procedure is shown in detail in Figure 8.11.

8.3 AIR

8.3.1 Sampling Devices for the Collection of Air Samples

Many devices have been designed for the collection of bioaerosols (see Chapter 5). Choosing an appropriate sampling device is based on many factors, such as availability, cost, volume of air to be sampled, mobility, sampling efficiency (for the particular type of bioaerosol) and the environmental conditions under which sampling will be conducted. Another factor that must be taken into account, especially when sampling for microorganisms, is the overall biological sampling efficiency of the device. This factor is related to the maintenance of microbial viability during and after sampling. In this section, several types of commonly used samplers are described on the basis of their sampling methods: impingement, impaction, centrifugation, filtration and deposition. Impingement is the trapping of airborne particles in a liquid matrix; impaction is the forced deposition of airborne particles on a solid surface; centrifugation is the mechanically forced deposition of airborne particles using inertial forces of gravity; filtration is the trapping of airborne particles by size exclusion; and deposition is the collection of airborne particles using only naturally occurring deposition forces. The most commonly used devices for microbial air sampling are: the all glass AGI-30 impinger (Ace Glass, Vineland, NJ); the SKC impinger (SKC-West Inc., Fullerton, CA, U.S.A.); and the Anderson six-stage impaction sampler (6-STG, Andersen Instruments Incorporated, Atlanta, GA).

8.3.1.1 Impingement

The AGI-30 (Figure 8.12) and the SKC glass impingers (Figure 8.13) operate by drawing air through an inlet that is similar in shape to the human nasal passage. The air is transmitted through a liquid medium where the air particles become associated with the fluid and are subsequently trapped. The impingers usually separate at a flow rate of 12.5 L/min at a height of 1.5 m, which is the average breathing height for humans. They are easy to use, inexpensive, portable, reliable, easily sterilized and have high biological sampling efficiency in comparison with many other sampling devices. The impingers tend to be very efficient for particles in the range of 0.8 to 15 μm. The usual volume of collection medium is 20 ml, and the typical sampling duration is approximately 20 minutes, which prevents evaporation during the sampling in warm climates, or the freezing of the liquid medium when sampling at lower temperatures. The SKC biosamplers are more expensive due to the delicate nature of the blown glass which reduces damage to microbes during impingement (Brooks *et al.*, 2005). Another feature of the impingement process is that the liquid and suspended microorganisms can be concentrated or diluted, depending on the requirements for analysis. Liquid impingement media can also be divided into subsamples in order to test for a variety of microorganisms by standard cultural and molecular methods such as those described in Chapters 10 and 13. The impingement medium can also be optimized to increase the relative biological recovery efficiency. This is important, because during sampling the airborne microorganisms, which are already in a stressed state due to various environmental pressures such as ultraviolet (UV) radiation and desiccation, can be further stressed if a suitable medium is not used for recovery. Sampling media range from simple to complex. A simple medium is 0.85% NaCl, which is an osmotically balanced, sampling medium used to prevent osmotic shock of recovered organisms. A more complex medium is peptone (1%), which is used as a resuscitation medium for stressed organisms. Finally, enrichment or defined growth media can be used to sample selectively for certain types of organisms. The major drawback when using these impingers is that there is no particle size discrimination, which prevents accurate characterization of the sizes of the airborne particles that are collected.

8.3.1.2 Impaction

Unlike the impingers, the Andersen six-stage impaction sampler (Andersen 6-STG) provides accurate particle size

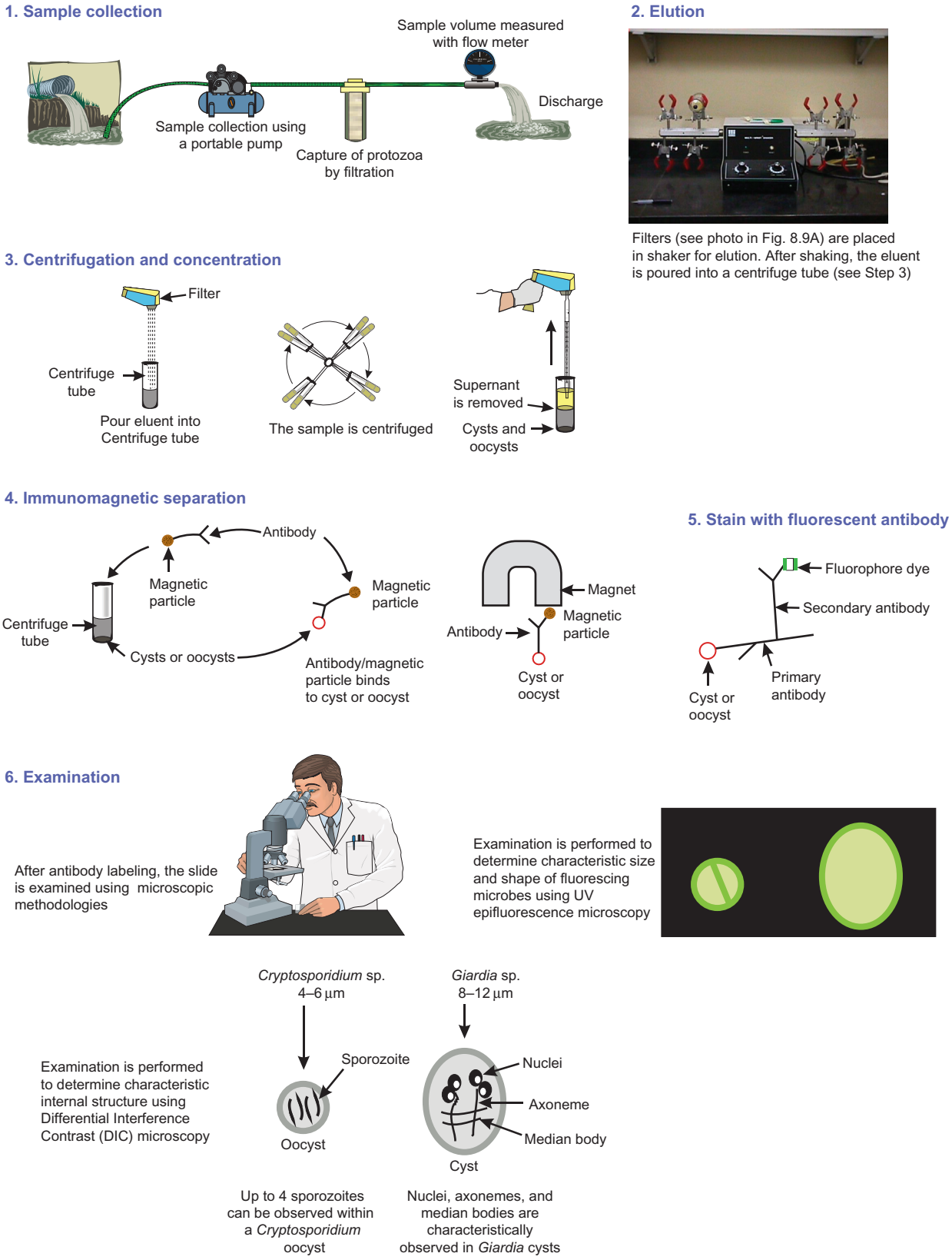

FIGURE 8.11 Procedure for processing and staining water samples for detection of protozoan parasites.

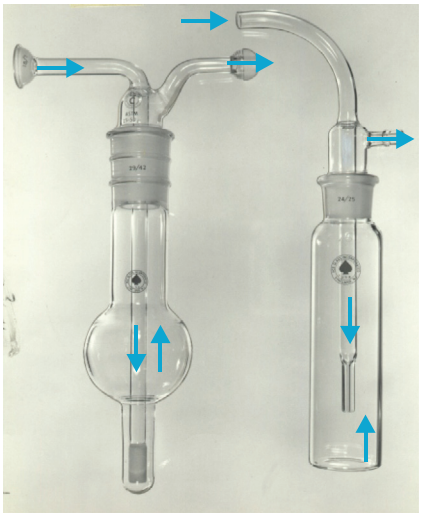

FIGURE 8.12 Two all glass impingers (AGI). The impinger on the right is the classic AGI-30 impinger. Arrows indicate the direction of air flow. The air enters the impinger drawn by suction. As bioaerosols impinge into the liquid medium contained in the bottom of the impinger, the airborne particles are trapped within the liquid matrix.

FIGURE 8.13 An SKC impinger. Photo courtesy John Brooks.

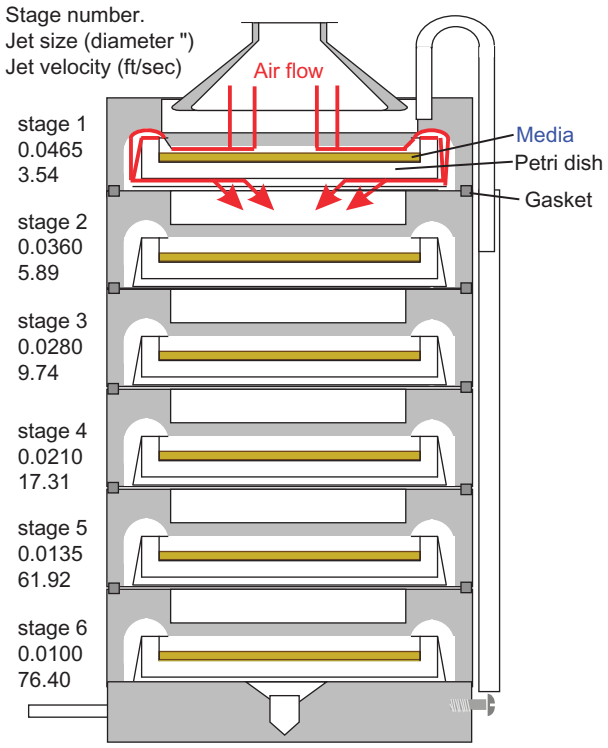

FIGURE 8.14 This is a schematic representation of the Andersen six stage impaction air sampler. Air enters through the top of the sampler and larger particles are impacted upon the surface of the Petri dish on stage 1. Smaller particles, which lack sufficient impaction potential, follow the air stream to the subsequent levels. As the air stream passes through each stage the air velocity increases, thus increasing the impaction potential so that particles are trapped on each level based up on their size. Therefore, larger particles are trapped efficiently on stage 1 and slightly smaller particles on stage 2, and so on, until even very small particles are trapped on stage 6. The Andersen six stage thus separates particles based upon their size.

discrimination (Figure 8.14). It is described as a multi-level, multiorifice, cascade impactor. The Andersen 6-STG was developed by Ariel A. Andersen in 1958 and operates at a input flow rate of 28.3 L/min. The general operating principle is that air is sucked through the sampling port and strikes agar plates. Larger particles are collected on the first layer, and each successive stage collects smaller and smaller particles by increasing the flow velocity and consequently the impaction potential. The shape of the Andersen sampler does not conform to the shape of the human respiratory tract, but the particle size distribution can be directly related to the particle size distribution that occurs naturally in the lungs of animals. The lower stages correspond to the alveoli and the upper stages to the upper respiratory tract. The Andersen sampler is constructed of stainless steel with glass Petri dishes, allowing sterilization, ease of transport and reliability. It is useful over the same particle size range as for the impingers (0.8 to over 10 μm), corresponding to the respirable range of particles. It is more expensive than the impingers, and the biological sampling efficiency is somewhat lower because of the method of collection, which is impaction on an agar surface. Analysis of viruses collected by impaction is also somewhat difficult, because after impaction, the viruses must be washed off the surface of the impaction medium and collected before assay. In contrast, bacteria or other microorganisms can be grown directly on the agar surface. Alternatively, these microbes can be washed off the surface and assayed using other standard methodologies as described in Chapter 10. The biggest single advantage of the Andersen 6-STG sampler is that particle size determinations can be obtained. Thus, the

two reference samplers (impingers and the Andersen 6-STG) complement each other's deficiencies.

8.3.1.3 Centrifugation

Centrifugal samplers use circular flow patterns to increase the gravitational pull within the sampling device in order to deposit particles. The Cyclone, a tangential inlet and return flow sampling device, is the most common type (Figure 8.15). These samplers are able to sample a wide range of air volumes (1−400 L/min), depending on the size of the unit. The unit operates by applying suction to the outlet tube, which causes air to enter the upper chamber of the unit at an angle. The flow of air falls into a characteristic tangential flow pattern, which effectively circulates air around and down along the inner surface of the conical glass housing. As a result of the increased centrifugal forces imposed on particles in the airstream, the particles are sedimented out. The conically shaped upper chamber opens into a larger bottom chamber, where most of this particle deposition occurs. Although these units are able to capture some respirable-sized particles, in order to trap microorganisms efficiently, the device must be combined with some type of metered fluid flow that acts as a trapping medium. This unit, when used by someone proficient, can be effective for microbiological air sampling. It is relatively inexpensive, easily sterilized and portable, but it lacks high biological sampling efficiency and particle sizing capabilities. Analysis is performed by rising the sampler with an eluent medium, collection of the eluent and subsequent assay by standard methodologies.

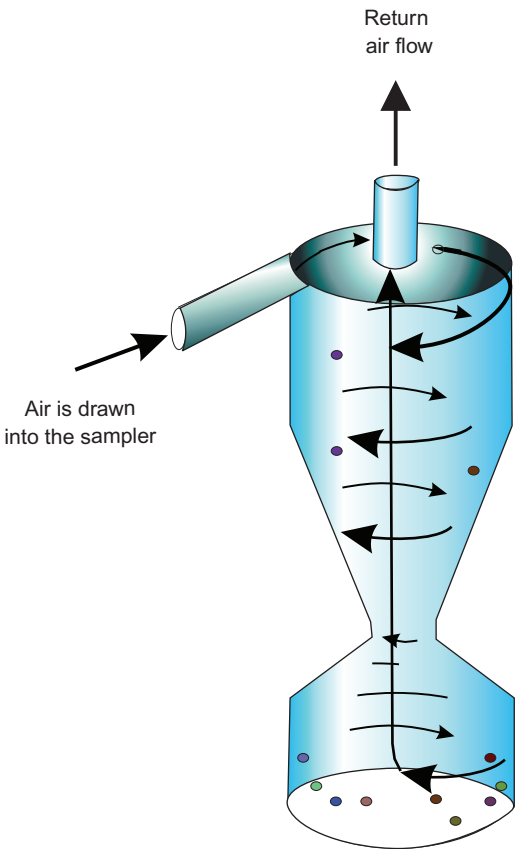

FIGURE 8.15 This is a schematic representation of a tangential inlet and returned flow centrifugal air sampler. Air is drawn into the sampler at an angle (tangential) to the walls of the device so that it circulates around and down the walls. As it circulates the decrease in the diameter of the sampling body causes a dramatic increase in the velocity of the air and subsequently on particle's terminal velocity. This increase in gravitational settling potential causes the particles to be trapped in the lower collection chamber because their "centrifugally increased" mass prevents them from existing with the return air flow.

8.3.1.4 Filtration and Deposition

Filtration and deposition methods are both widely used for microbial sampling because of cost and portability reasons. Filter sampling requires a vacuum source and involves passage of air through a filter, where the particles are trapped. Membrane filters can have variable pore sizes that tend to restrict flow rates. After collection, the filter is washed to remove the organisms before analysis. Filtration sampling for microorganisms is not highly recommended because it has a low overall sampling efficiency and it is not portable. However, in many cases the low cost makes it an attractive method.

One case where filtration is routinely used is in sampling for airborne lipopolysaccharide (LPS). The sampling and analysis procedure for airborne LPS levels is slightly different from methods used for analysis of airborne microorganisms. The most efficient means of sampling is usually filter collection using polyvinyl chloride or glass fiber membrane filters. Quantification analysis is usually done using a chromogenic Limulus amebocyte lysate assay (Hurst *et al*., 2007). This system uses a Limulus amebocyte lysate obtained from blood cells of horseshoe crabs (Brooks *et al*., 2006). The lysate contains an enzyme-linked coagulation system, which is activated by the presence of LPS. With the addition of a substrate, and using luminescence, the system is able to quantitate the amount of environmental LPS by comparison with a standard curve.

Deposition sampling is by far the easiest and most cost-effective method of sampling. Deposition sampling can be accomplished merely by opening an agar plate and exposing it to the wind, which results in direct impaction, gravity settling and other depositional forces. The problems with this method of sampling are: low overall sampling efficiency because it relies on natural deposition, no defined sampling rates or particle sizing and an intrinsic difficulty in testing for multiple microorganisms with varied growth conditions. Analysis of microorganisms collected by depositional sampling is similar to impaction sample analysis.

8.4 DETECTION OF MICROORGANISMS ON FOMITES

Fomites are inanimate objects that may be contaminated with infectious organisms and subsequently serve in their transmission. Clothing, dishes, toys, tabletops and hypodermic needles are examples of common fomites. Fomites can range in size from as small as a particle of household dust, to as large as an entire floor surface. Complexity can vary from a flat tabletop, to a delicate medical instrument. The involvement of fomites in disease transmission was recognized long before the identification of some pathogenic microorganisms. Over 100 years ago, the spread of smallpox among laundry workers was not uncommon. In 1908, outbreaks of smallpox were traced to imported raw cotton contaminated with variola virus in crusts or scabs (England, 1984). Fomites are also believed to be important in the transmission of respiratory viruses, such as rhinovirus. An outbreak of hepatitis B virus, typically a bloodborne virus associated with blood transfusion, was associated with computer cards as the probable agents of transfer. These cards, when handled, inflict small wounds on the fingertips, allowing transmission and entry of the pathogen into a new host (Pattison *et al.*, 1974). Growth of enteric pathogenic bacteria in household sponges and on utensils or surfaces used for food preparation has also been recognized as an important route for transfer of these organisms to other foods or surfaces. Self-inoculation can also occur when the fingers that have handled a sponge or utensil are brought to the mouth.

Fomites may become contaminated with pathogenic microorganisms by direct contact with infectious body secretions or fluids, soiled hands, contaminated foods or settling from the air. For fomites to serve as vehicles of microbial disease, the organisms must be able to survive in association with the fomites, and be successfully transferred to the host. Survival of organisms on a surface is influenced by temperature, humidity, evaporation, desiccation, light, ultraviolet radiation, the physical and chemical properties of the surface and the substance in which the organism is suspended. Enteric and respiratory pathogens may survive from minutes to weeks on fomites, depending on the type of organism and the previously listed factors (Boone and Gerba, 2007).

Sampling of fomites is essential in the food manufacturing industry to assess sanitation practices, and is in common use in the food service and healthcare industries to evaluate cleaning and disinfection efficacy. It is also useful in epidemiological investigation and evaluation of hard surface disinfectants. The approaches most commonly used for detection of bacteria on fomites involve Rodac agar plates and the swab–rinse technique. Rodac dishes are Petri dishes in which the agar fills the entire dish to produce a convex surface, which is then pressed against the surface to be sampled. Selective media can be used for isolation of specific groups of organisms (e.g., m-FC media for fecal coliforms). After incubation, the colonies are counted and reported as colony-forming units (CFU) per cm^2. The swab–rinse method was developed in 1917 for studying bacterial contamination of eating utensils (England, 1984). The method is also suitable for sampling of viruses. A sterile cotton swab is moistened with a buffer or other solution and rubbed over the surface to be sampled. The tip of the swab is then placed aseptically in a container with a sterile collection solution, the container is shaken and the rinse fluid is assayed on an appropriate culture medium, or by a molecular technique such as the PCR method. Other approaches for surface sampling are the use of sponges, vacuum systems using HEPA filters and agar films (Peti-flim, 3M Corporation, Minneapolis, MN), and even laboratory Kimwipes (Yan *et al.*, 2007). Sponges, vacuum systems and wipes allow for sampling of much larger areas than swabs. In practice, usually 100 cm^2 is sampled with a swab or sponge.

QUESTIONS AND PROBLEMS

1. How many samples and what sampling strategy would you use to characterize a portion of land of area 500 km^2? Assume that the land is square in shape with a river running through the middle of it which is contaminating the land with nitrate.
2. Discuss the reasons why deep subsurface sampling is more difficult than surface sampling.
3. A soil is extracted for its community DNA and is found to contain 0.89 μg DNA per g soil. How many bacterial cells does this theoretically involve?
4. When would one utilize electropositive filters for concentrating viruses from environmental samples?
5. When would one utilize electronegative filters for concentrating viruses from environmental samples?
6. In what ways is it easier to sample water for subsequent microbial analysis than it is to sample soil?
7. If you collected a surface soil sample from a desert area in the summertime, when daytime temperatures were in excess of 40°C, how would you store the soil, and how would you get the soil ready for microbial experiments to be conducted 1 month later? Discuss the pros and cons of various strategies.
8. What sampling strategy would you use to give the most complete picture of all bacteria found in a soil sample?
9. How do electropositive filters concentrate viruses from water? Why are they not effective in concentrating viruses from seawater or from water with a pH above 8.5? What is the principle behind eluting viruses from filter surfaces?

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