Chapter 11

Physiological Methods

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11.1 INTRODUCTION

A great deal of recent focus in environmental microbiology has been on developing rapid or even real-time techniques for analyzing microbial communities using nonculture-based techniques. Some of these techniques involve analysis of the community DNA extracted from a sample (see Chapter 13) or novel spectroscopic techniques (see Chapter 9). While these techniques have revolutionized the amount of information that we can obtain from a sample, it has also become apparent that these techniques provide information about "who" is in the community but not about "what" activities the community carries out. For this reason, environmental microbiologists have realized that a complete analysis of an environmental sample requires physiological measurements that are made in combination with the newer molecular approaches. This is of particular current interest for assessment of possible impacts that climate change will have on ecosystem function or vice versa.

In an undisturbed environment, the activity measured will reflect a baseline level of activity. This baseline activity level is dependent on a variety of biological, chemical and physical parameters—especially important is the nutrient status of the environment. In an ecological sense, measurement of microbial activity in an undisturbed environment allows one to determine the microbial contribution to nutrient cycling (see Chapter 16) within an ecosystem. For example, in the case of carbon cycling, the focus of microbial activity measurements is to determine accurately the production of new biomass by each microbial component of the community, and estimate the amount of energy that is being stored in a particular group of micro- or macroorganisms. This information allows determination of the transfer of energy between trophic levels in the food chain within a particular ecosystem (see Figure 6.6).

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Measurement of microbial activity can also be used to evaluate the response of a microbial community to a variety of stimuli or disturbances, including naturally variable parameters such as moisture level, and anthropogenically imposed variables such as the addition of fertilizers or contaminant spills. In this case, measurement of microbial activity can provide an indication of the general health of the environment and can be used to evaluate the impact of a disturbance on the microbial community. For example, microbial activity has been used to evaluate the impact of soil quality on certain land use practices such as farming, logging and mining. Microbial activity is also an important indicator for evaluating the process of restoration of disturbed sites. One example of this is natural attenuation, the process by which indigenous microbial populations degrade spilled pollutants within a natural environment (see Section 17.7). Other areas where microbial activity measurements are often used include: engineered systems such as municipal wastewater treatment systems; compost systems; and biological reactor systems; all of which utilize microbial processes for specific purposes.

The goal of this chapter is to examine different types of microbial activity measurements in both pure culture and environmental samples. Although activity measurement in pure culture is relatively straightforward, environmental communities contain diverse microorganisms and physiological types, including aerobic and anaerobic heterotrophs and autotrophs (see Chapter 2). Thus, any measurement of microbial activity in an environmental sample can be designed to be more or less inclusive of different classes and physiological types of microorganisms. As a result, it is important to understand the different activity measurements and their relative specificity. In this chapter, activity measurements in pure culture will be discussed first, including: measurement of substrate disappearance; terminal electron acceptor (TEA) utilization; cell mass increase; and carbon dioxide evolution. Following this, activity measurements in environmental samples will be addressed. Environmental activity measurements have been divided into five broad categories: (1) carbon respiration; (2) incorporation of radiolabeled tracers in cellular macromolecules; (3) adenylate energy charge; (4) enzyme activity assays; and (5) stable isotope probing. Frequently, the nature of the research question being asked, and the resources available, determines which technology is utilized.

11.2 MEASURING MICROBIAL ACTIVITY IN PURE CULTURE

As discussed in Chapter 3, growth in pure culture can be described by the generalized equation representing respiration:

$$\text{Substrate} + \text{nitrogen} + \text{TEA} \rightarrow \text{cell mass} + \text{carbon dioxide} + \text{water} \quad \text{(Eq. 11.1)}$$

In this equation, the TEA (terminal electron acceptor) is oxygen for aerobic conditions or one of several alternate TEAs for anaerobic conditions (see Table 3.3). Examination of this equation shows that there are several ways in which microbial activity can be measured. These include substrate disappearance, TEA utilization, biomass production and carbon dioxide evolution. Each of these approaches is discussed briefly in the following sections.

11.2.1 Substrate Utilization

11.2.1.1 Heterotrophic Substrates

For heterotrophic activity the substrate is carbon based. Such substrates can be measured in many different ways, depending on the chemistry of the substrate molecules, and whether a single component or multiple components are present. For example, in a single-solute system, aromatic compounds such as benzene and all benzene derivatives can be measured easily and sensitively by UV spectrophotometry (Figure 11.1). A UV spectrophotometer measures how much light is absorbed while passing through a sample. The spectrophotometer can be set to a particular wavelength of light that is chosen on the basis of the absorbance spectrum of the compound. For instance, the absorbance spectrum of benzoate, shown in Figure 11.2, indicates that the optimal detection wavelength is 224 nm (note that 224 nm is chosen because there is a great deal of interference by salts such as NO_3^- and SO_4^{2-} below 200 nm). Analogously, a fluorimeter can be used to measure fluorescent molecules such as polyaromatic hydrocarbons (PAHs) within single-component systems (Figure 11.3). A fluorimeter offers increased sensitivity over UV spectroscopy.

Often, samples from pure cultures and especially environmental samples contain compounds that interfere with

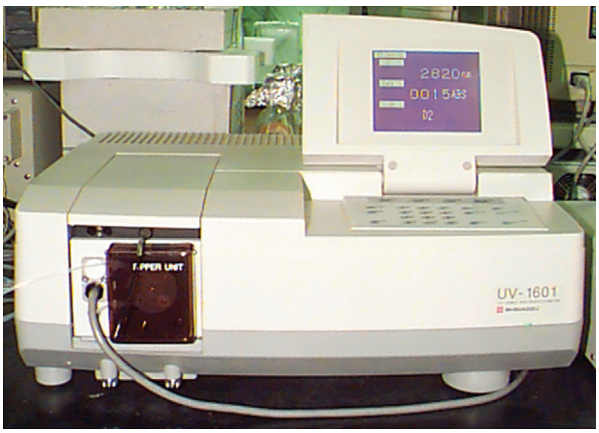

FIGURE 11.1 UV spectrophotometer. Photo courtesy R.M. Maier.

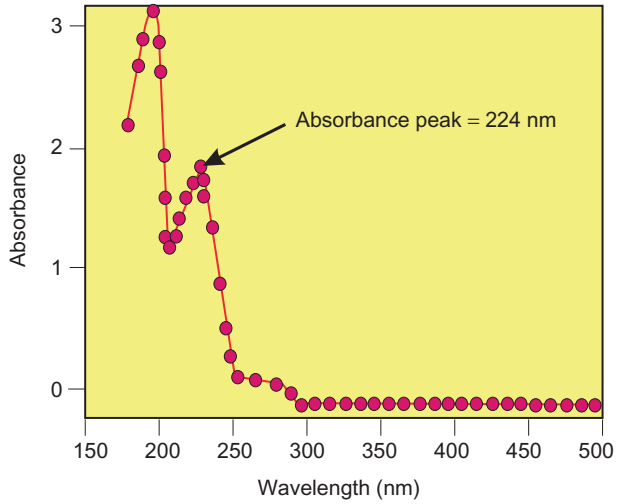

FIGURE 11.2 Absorbance spectrum of benzoate.

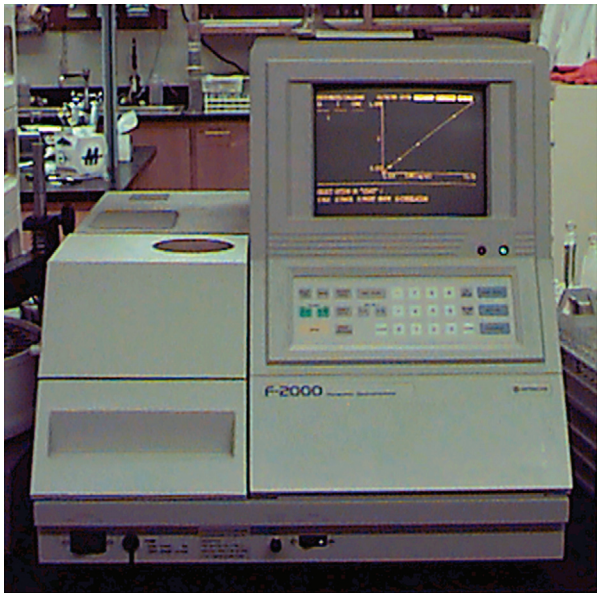

FIGURE 11.3 Fluorimeter. Photo courtesy R.M. Maier.

FIGURE 11.4 High performance liquid chromatograph. Photo courtesy T.R. Sandrin.

the quantitation of the target compound. This can be considered a multiple-component system. For example, humic compounds present in soil and water samples contain many aromatic molecules that interfere strongly with measurement of aromatic substrates. Alternatively, there may be multiple components of interest in a sample containing a complex mixture of compounds such as might be found in gasoline or other fuel oils. In this case, a separation or chromatography step is required prior to detection of the target compound. There are different types of chromatography, including liquid chromatography and gas chromatography. One common instrument based on liquid chromatography is the high-performance liquid chromatograph (HPLC) (Figure 11.4). The HPLC forces liquid solvent through a packed column under high pressure to separate the components within a mixture. The separation is dictated by the type of column packing, and is generally based on charge or hydrophobicity. The resulting chromatogram can be analyzed to provide quantitative information about the amount of each component present in the sample (Figure 11.5). For the example chromatogram shown, a UV detector was used for analysis; however, several other types of detectors can be used. These include the photodiode array detector, which is based on measuring light wavelength absorption, but uses a detector array to rapidly measure the entire wavelength spectrum instead of just a single wavelength. Other detectors used in HPLC include refractometers, which are primarily for sugar analysis; evaporative light scattering detectors, used for surfactants and polymers; conductivity meters, used for ionic or charged compounds; and fluorescence detectors, used for analysis of polyaromatic hydrocarbons and fluorescent dyes. A related instrument is the ultra performance liquid chromatograph (UPLC) (Figure 11.6). The UPLC is similar to the HPLC, but uses smaller-sized particles ($<2\,\mu m$) for analyte separation.

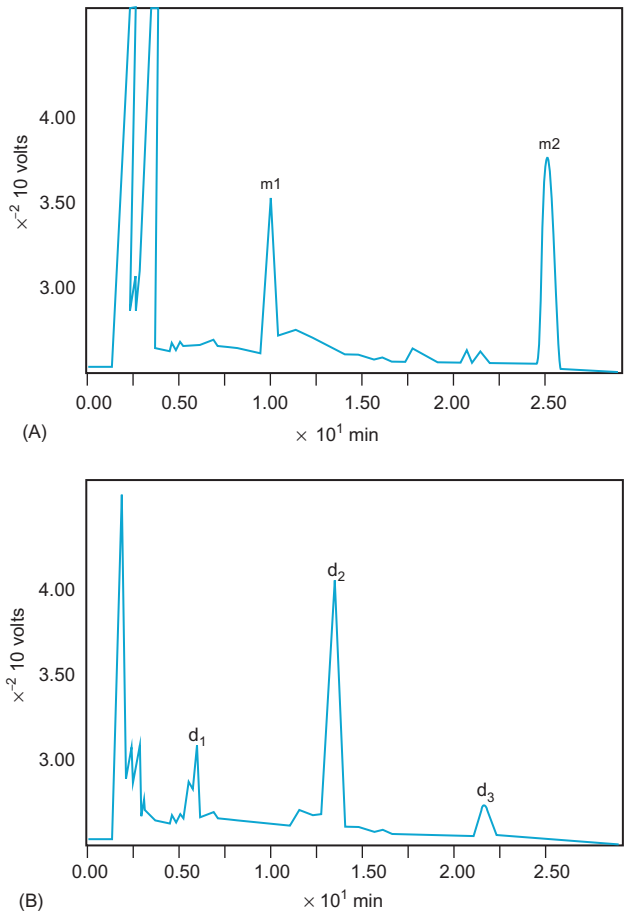

FIGURE 11.5 HPLC chromatogram. These are typical chromatograms of a microbial surfactant, rhamnolipid, produced by *Pseudomonas aeruginosa*. (A) Monorhamnolipid (one rhamnose sugar per molecule) and (B) dirhamnolipid (two rhamnose sugars per molecule). Note that each chromatogram has more than one peak. This is because microbes make a mixture of surfactants that vary in the length of the fatty acid tails. The longer the tail, the more hydrophobic the molecule is, and the longer it is retained on the column. Thus, for monorhamnolipid, the peak labeled m2 has longer lipid tails than the monorhamnolipid in the peak labeled m1. A reverse phase C_{18} column was used for sample separation. Elution was isocratic (meaning the mobile phase composition remained constant during analysis) using a mobile phase of acetonitrile-water (40:60, v/v) at a flow rate of 1 ml/min. A UV detector set at 214 nm was used for detection of the surfactant. Chromatograms courtesy Y. Zhang.

FIGURE 11.6 Ultra performance liquid chromatograph-mass spectrometer (UPLC-MS). Photo courtesy L. Dykes.

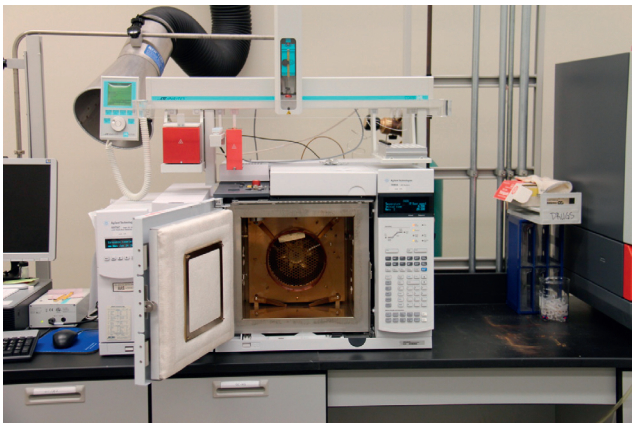

FIGURE 11.7 Gas chromatograph-mass spectrometer (GC-MS). Photo courtesy S. Williams, Office of the Texas State Chemist.

This enables much greater speed, resolution and sensitivity than can be achieved with an HPLC, and is very useful for detection of low-level environmental contaminants, such as antibiotics and pharmaceuticals (Tamtam *et al*., 2009).

Another instrument based on chromatographic separation of solutes is the gas chromatograph (GC) (Figure 11.7). In gas chromatography, the mobile phase is a mixture of gases instead of liquids. Because the mobile phase is gaseous, compounds amenable to GC analysis must be volatile so that they can move through the GC column with the mobile phase. Therefore, compounds that can be analyzed by GC are characterized by lower boiling points and higher volatilities. The GC can be equipped with a variety of different detectors. Two of the most commonly used are the flame ionization detector (FID) and the electron capture detector (ECD). The FID is suitable for analysis of hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, xylene), polyaromatic hydrocarbons or aliphatic hydrocarbons. The ECD

detector is used for analysis of halogenated materials, primarily chlorinated compounds such as trichloroethylene (TCE). It should be noted that for some of these hydrocarbons both HPLC and GC can be used for analysis but for others, such as the aliphatic hydrocarbons, which do not absorb in the UV range, only GC can be used. In other instances, such as for organic acids that have low volatility, HPLC is the method of choice.

Finally, it should be mentioned that the GC, HPLC and UPLC can all be used in conjunction with a mass spectrometer (MS) to allow further confirmation/identification of the detected compound (Figures 11.6 and 11.7). Mass spectrometers bombard a compound with electrons, causing it to break up and fragment into smaller pieces. The fragmentation pattern can then be interpreted to determine the composition of the original molecule. For routine analyses, most mass spectrometers contain libraries of fragmentation patterns that can be used to provide a best fit match or prediction of the target compound being analyzed. This is especially powerful for detection and characterization of unknown compounds such as metabolites that may be produced during biodegradation, and can be invaluable for elucidation of biodegradation pathways.

11.2.1.2 Chemoautotrophic Substrates

In contrast to chemoheterotrophic activity, for which energy is provided by oxidation of organic or carbon-based substrate, for chemoautotrophic activity the energy providing substrate is an inorganic compound. Important microbial activities that contribute to the cycling of inorganic materials in soil and water environments include chemoautotrophic oxidation of ammonia (nitrification) and sulfur (sulfur oxidation). The following sections discuss the approaches used to measure microbial transformation of these two chemoautotrophic substrates.

Nitrification

Nitrification, the oxidation of ammonia to nitrite and nitrate (see Section 16.3.4), can be measured in several ways. A direct assay involves addition of ^{15}N-labeled ammonia and measurement of the formation of ^{15}N-nitrite and ^{15}N-nitrate as the ^{15}N-ammonia is oxidized (Paerl, 1998). These ^{15}N-labeled products are measured by mass spectroscopy. However, the sensitivity of this measurement is low, and thus large quantities of ammonia that far exceed those found naturally may be required in an experiment. A second less direct approach is to divide the samples being measured into two groups and incubate one group with a nitrification inhibitor such as N-serve (in general, nitrifiers are very sensitive to chemical inhibitors). After incubation, the differences in ammonia and nitrate concentrations between the nitrification inhibited and uninhibited systems can be compared. Finally, a third approach to the measurement of nitrification is again to divide the samples into two groups, one of which is treated with a nitrification inhibitor. The samples are then incubated with $^{14}CO_2$ to measure CO_2 fixation. The amount of carbon dioxide fixation is then compared in the presence and absence of the nitrification inhibitor to determine nitrification activity.

Sulfur Oxidation

Sulfur oxidation (see Section 16.4.3.1) can be measured by addition of elemental sulfur (S^0) to a sample and measuring the amount of sulfate (SO_4^{2-}) produced during incubation. However, this is a complicated process. One such assay measures sulfate as a precipitate with barium chloride in acid solution (Kelly and Wood, 1998). Following precipitation, the barium in solution is measured by inductively coupled plasma mass spectroscopy (ICP-MS), an instrument that is used to measure metals. While the ICP-MS can distinguish between different metals, it cannot distinguish between the different species of a given metal in solution (see Section 18.8.2 for metal speciation methods). In this assay, as the amount of sulfate increases as a result of sulfur oxidation, the amount of barium in solution decreases through precipitation with the sulfate. A second approach to measurement of sulfur oxidation is to determine O_2 utilization (Padden *et al.*, 1998) or CO_2 uptake in the presence of sulfur compounds. As described above for nitrification, samples can be incubated with $^{14}CO_2$ to measure CO_2 fixation as an estimate of sulfur oxidation.

11.2.2 Terminal Electron Acceptors

A variety of TEAs are available for microbial use and levels of microbial activity are reflected in the disappearance of the TEA and the appearance of the reduced TEA. Measurement of several common TEAs is discussed in the following sections.

11.2.2.1 Oxygen Uptake

The majority of environmental activities measured are under aerobic conditions where oxygen is the required TEA. Oxygen, unlike many other nutrients, is relatively insoluble in water and can become limiting to growth unless it is constantly replenished (Information Box 4.7). Thus, aeration is an important consideration in both pure culture and environmental systems. Normal dissolved oxygen levels in an aqueous sample that is equilibrated with air range up to 10 mg/L, depending on sample temperature and atmospheric pressure. In general, aerobic activity is maintained until the dissolved oxygen concentration in the sample falls below 1 to 2 mg/L. Because oxygen is a limiting nutrient, it is a good measure of

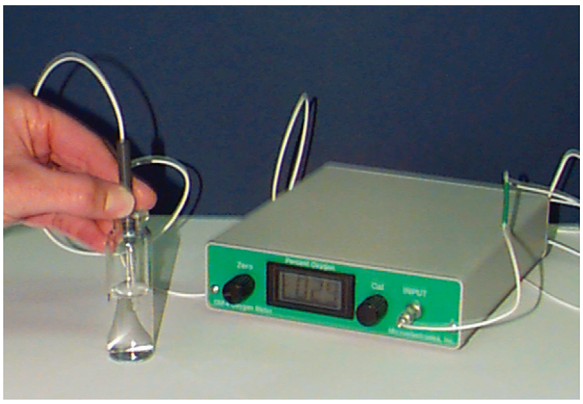

FIGURE 11.8 Oxygen microprobe. Photo courtesy R.M. Maier.

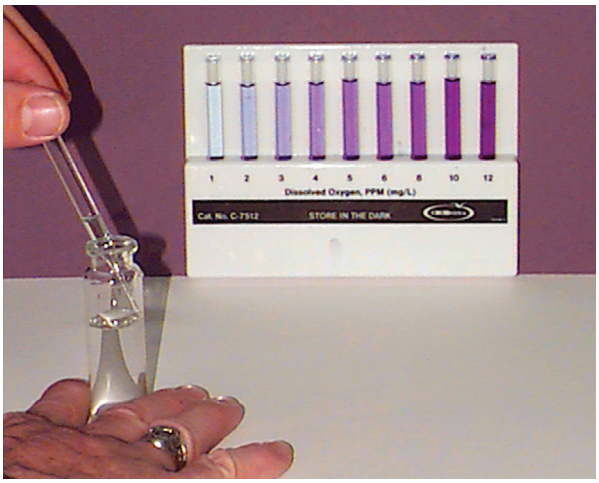

FIGURE 11.9 Oxygen measurement using a Chemets Kit. Photo courtesy R.M. Maier.

growth and activity. Measurement of oxygen in a pure culture is relatively rapid, routine and cheap to perform. This technique is perhaps used most extensively in the wastewater treatment industry to determine the biological oxygen demand (BOD) of wastewater samples (see Section 11.4.2.4) (see also Chapter 25). As discussed in the following sections, oxygen levels in an aqueous sample are generally measured using an oxygen probe or by colorimetric methods.

Oxygen Probes

An oxygen probe is basically an electrode covered by a gas-permeable membrane combined with a meter that converts the electrical signal into an analytical measurement. Oxygen probes do not measure the absolute amount of oxygen in a sample; rather, they measure oxygen amounts relative to an oxygen-saturated solution. A variety of commercially available oxygen probes can be used to measure dissolved oxygen. These include probes that are used for routine BOD measurement, and microprobes that can be used for small sample amounts or can be inserted into a soil column through a port (Figure 11.8). Advances in technology have produced microprobes for the detection of O_2, as well as nitrogen compounds (N_2O, NO_3, NH_4), sulfide, hydrogen and pH (Revsbech and Jørgensen, 1986). The oxygen microelectrodes can be as small as 20 μm diameter, allowing the determination of gas flux across critical interfaces.

Colorimetric Assays

Alternatively, a variety of colorimetric assays are available. In this case, a sample is mixed with a series of reagents to produce an oxygen-dependent color. A titration method known as the Winkler method can be used to determine dissolved oxygen in water samples. Winkler titrations are based on the precipitation of dissolved oxygen using manganous sulfate and a potassium hydroxide–potassium iodide mixture (Wetzel and Likens, 1991). The oxygen precipitate, manganic basic oxide, reacts with sulfuric acid to form a manganic sulfate, which in turn reacts with potassium iodide to liberate iodine. The number of moles of iodine liberated is equivalent to the number of moles of oxygen present in the water sample. The liberated iodine can be determined spectrophotometrically in clear water samples, or can be determined by titration with sodium thiosulfate. The basic Winkler method may be affected by the presence of oxidizing or reducing materials that can occur in natural waters and especially in polluted waters. Modifications of the basic method are described in the APHA, AWWA, WEF (2005) manual. There are also several kits available for oxygen measurement that are based on a colorimetric reaction. An example of such an assay is shown in Figure 11.9.

11.2.2.2 Alternative Terminal Electron Acceptors

Although not nearly as commonly measured as oxygen, perhaps because the methodologies are much more complex and time consuming, there are methods available to quantitate alternate TEAs. For example, in denitrification, nitrate (NO_3^-) serves as the terminal electron acceptor and is reduced sequentially to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2) (see Section 16.3.5.2). One common assay for denitrification involves the use of acetylene (C_2H_2) which blocks the reduction of nitrous oxide to nitrogen (Paerl, 1998). Thus, nitrous oxide accumulates in the system and can be measured using a gas chromatograph with an electron capture detector. A disadvantage of using this method in environmental samples is that acetylene also inhibits nitrification. Since nitrification and denitrification are closely coupled (e.g., the nitrate produced by nitrification is used in denitrification), this inhibition may reduce or eliminate denitrification in the sample. Therefore, in some instances,

denitrification is estimated by simply measuring nitrification (see Section 11.2.1.2). A second problem with this technique is that it is not effective at low nitrate concentrations (less than 0.63 mg/L).

Similarly, there are methodologies available for measuring sulfate (SO_4^{2-}) reduction (Tabatabai, 1994a), iron and manganese reduction (Ghiorse, 1994) and methanogenic activity (Zinder, 1998).

11.2.3 Cell Mass

Cell mass in a pure culture is often quantified by performing culturable plate counts or direct microscopic counts (see Chapter 10). This approach is also often used to estimate the cell mass in environmental samples. Other common approaches to estimating cell mass include measurement of the turbidity or the protein content in the culture being studied. Although these measurements are not suitable for environmental samples, they are easy, rapid and reproducible when used for measuring cell mass increases in pure culture.

11.2.3.1 Turbidity

A rapid estimate of cell mass can be obtained by measuring the turbidity of a bacterial suspension. Turbidity measurements are generally made using a colorimeter or spectrophotometer, both of which work by directing a light beam through the sample. The bacteria in the sample scatter the light beam and lower the intensity of the light coming through the suspension. At low bacterial densities, there is a direct linear relationship between the number of bacteria and the amount of light scattered; thus, as the number of bacteria increases, the turbidity of the suspension increases. At high bacterial densities, this relationship becomes nonlinear. Therefore, a standard curve must be constructed to determine the linear range for turbidity measurement for each organism being measured. In theory, a wide range of wavelengths can be used to measure turbidity, although the practical range is between 490 and 550 nm. The sensitivity of the measurement increases at lower wavelengths, but there are often yellow or brown pigments produced during growth that can interfere with turbidity measurements at lower wavelengths.

11.2.3.2 Protein

A variety of methods are available for measurement of protein (Daniels *et al*., 1994). Currently, the most commonly used assays are based on the Folin reaction (Lowry assay; Lowry *et al*., 1951); the Coomassie Blue reaction (Bradford assay; Bradford, 1976); and the bicinchoninic acid–copper reaction (Smith *et al*., 1985). Each of these assays quantifies the amount of protein present by measuring color produced by the reaction of the protein present with the assay reagents. For example, the Lowry assay is based on the development of a blue color resulting from the reaction of the Folin reagent with aromatic amino acids contained in the protein sample. The sample is measured in a spectrophotometer at 550 nm. Similarly, the Bradford assay measures the binding of Coomassie Brilliant Blue G dye to protein amino groups, which causes the development of a blue color that can be measured at 595 nm. The bicinchoninic assay is based on the fact that proteins react with Cu^{2+} to produce Cu^{+}, which then reacts with bicinchoninic acid to form a purple product that is measured at 562 nm.

The method chosen for a particular application depends on the types of samples and potential for interference by sample components. The basic procedure for protein analysis is first to lyse cells in order to release the protein. This can be done by placing an aliquot of cells into 1 *N* NaOH, and heating the suspension at 90°C for 10 minutes. It is a good practice to wash the cells prior to lysis to minimize the presence of materials that may interfere with the protein assay. Once the cells are lysed, the protein assay is performed and the resulting sample measured in a spectrophotometer to determine protein quantity.

11.2.4 Carbon Dioxide Evolution

In general, the CO_2 trapping methodologies described here have been used to measure aerobic mineralization. However, the methods could be adapted to allow for measurement of CO_2 evolved under anaerobic conditions as well.

11.2.4.1 CO_2 Trapping

An alkaline trap, usually composed of sodium hydroxide or another strongly basic solution, can be used to trap CO_2 produced during mineralization (Figure 11.10A). The alkaline solution traps carbon dioxide by transforming it into the bicarbonate (HCO_3^-) form:

$$CO_2 + OH^- \rightarrow HCO_3^- \qquad \text{(Eq. 11.2)}$$

The trapped carbonate can be detected by using titrimetric, gravimetric or conductimetric measurements. Perhaps the most common approach to quantifying trapped CO_2 is titration of the alkaline solution that was used to trap CO_2 with a standardized acid solution. The HCO_3^- that was formed in the trapping solution is first precipitated with barium chloride. Then the solution is titrated with hydrochloric acid in the presence of a pH-sensitive dye such as phenolphthalein to indicate the end point of the titration. The amount of CO_2 trapped is then calculated from the reduction of the original hydroxyl ion

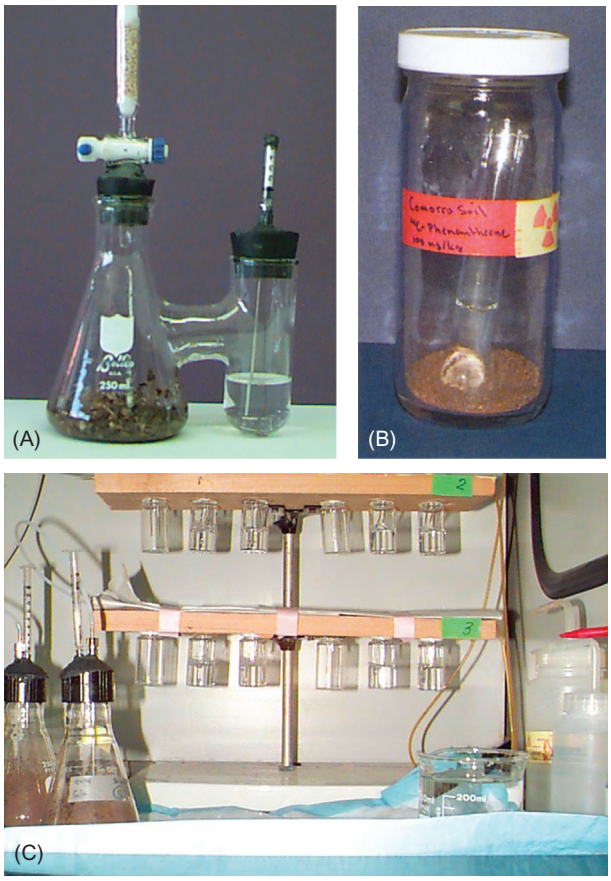

FIGURE 11.10 (A) Biometer flask for trapping CO_2. At intervals over the incubation period, the CO_2 trapping solution is withdrawn from the sidearm, and then replaced with fresh alkaline solution. As the trapping solution is withdrawn, air is drawn into the flask to replenish the oxygen supply within the sealed flask. The replacement air is drawn through a CO_2 absorbing filter (ascarite). To assay respiration, the amount of CO_2 generated is determined by titration of the trapping solution, and then the cumulative CO_2 production over the incubation period can be calculated. (B) Flask with a test tube trap containing alkali. (C) Stripping chain for $^{14}CO_2$ collection. Photos courtesy R.M. Maier.

concentration in the trapping solution. Alternatively, a simple gravimetric method is to precipitate the carbon dioxide as barium carbonate by the addition of an excess of $BaCl_2$. The precipitate can be collected, dried and weighed. A second gravimetric approach is to trap carbon dioxide in granular soda lime and monitor the gain in mass. Conductimetric quantification of CO_2 trapping is based on the reduction of the number of hydroxyl ions (OH^-), which is directly related to the amount of CO_2 trapped, and can be quantified by a decrease in conductivity of the trapping solution.

11.2.4.2 $^{14}CO_2$ Trapping

The use of ^{14}C-radiolabeled substrates allows for very sensitive and specific measurements of $^{14}CO_2$ evolution. In this case the CO_2 evolved is specific for the substrate

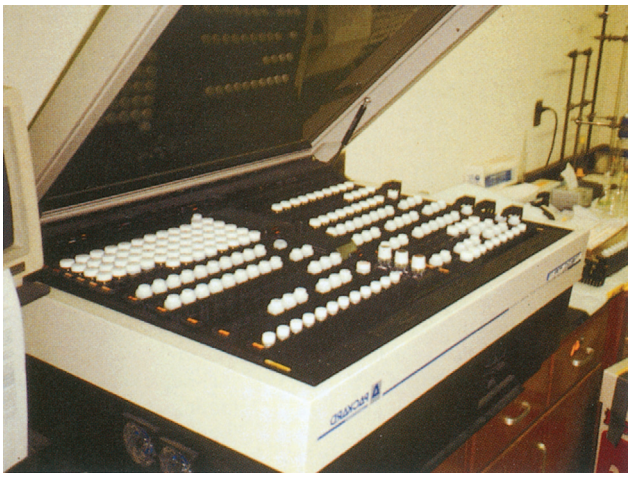

FIGURE 11.11 Liquid scintillation counter. Photo courtesy R.M. Maier.

added. This technique is often used to evaluate the feasibility and rates of biodegradation of organic contaminants. The generation of $^{14}CO_2$ offers confirmation that it is actually the organic contaminant in question that is being degraded. In this case, a biometer flask such as shown in Figure 11.10A can be used, and the alkali within the sidearm trap is assayed for radioactivity using liquid scintillation counting (Figure 11.11). Alternatively, a simple alkaline trap can be made by inserting a test tube containing alkali into a flask (Figure 11.10B). A slightly more complex system is the stripping chain shown in Figure 11.10C. Each flask is connected to a series of six traps. The first and fourth are empty to prevent backflow contamination. The second and third traps are filled with a general scintillation cocktail to remove any ^{14}C-labeled volatile organic compounds that may have been produced during biodegradation. The fifth and sixth traps are filled with a phenethylamine-based cocktail that selectively traps CO_2. Although this system is more complex than those shown in Figures 11.10A and B, it offers confirmation that the radioactivity assayed is actually $^{14}CO_2$.

11.3 CHOOSING THE APPROPRIATE ACTIVITY MEASUREMENT FOR ENVIRONMENTAL SAMPLES

Measuring the activity of a diverse population of microbes within an environmental sample is a very different prospect from measuring the microbial activity of a single isolate in the laboratory setting. As discussed in Section 11.2, the microbial activity of a pure culture in a defined medium can easily be determined by measuring cell number (culturable plate counts or direct counts) or

biomass (turbidity or protein). However, these measurements are not always practical or even realistic for environmental samples. For example, protein analysis of an environmental sample reflects sources of biomass other than the microbial population, including plant debris or microscopic animals.

Although both culturable and direct counts are commonly used to enumerate bacteria at a particular location, they are less helpful for measuring microbial activity. Culturable plate counts underestimate bacterial numbers in an environmental sample because this process enumerates only the cells compatible with the growth medium and culture conditions (see Chapter 10). Further, although many bacteria are viable in their environmental setting, they are nonculturable in the artificial environment of the laboratory. It should be noted that despite the drawbacks of culturable counts for measurement of total activity within a sample, this technique is still useful for estimation of specific physiological groups, such as sulfate-reducing organisms or hydrocarbon-degrading organisms. Direct counts as a measure of total microbial activity are problematic because they include dormant and moribund cells. Also, it is often a small portion of the total population that is responsible for a given activity, and it is difficult to estimate the activity of this part of the population with direct counts. Finally, both culturable and direct counts fail to enumerate cells that adhere tightly to soil particles or other surfaces. As a result of these problems, the measurement of microbial activity in environmental samples has focused more on the quantification of metabolic activity, such as respiration or synthesis of cellular macromolecules, which provides a more direct reflection of the level of metabolic activity within a sample (Table 11.1). These types of measurements are explained in detail in the following sections of the chapter.

11.4 CARBON RESPIRATION

A primary activity of the microbial community in undisturbed environments is the utilization of naturally occurring organic materials. Heterotrophic activity is a measure of the extent to which microbial populations utilize these organic materials as a carbon and energy source. This process is called respiration, and involves the consumption of oxygen (under aerobic conditions) or an alternate terminal electron acceptor (under anaerobic conditions), with production of carbon dioxide as shown in Eq. 11.1. As discussed in Section 3.4, the carbon from a substrate will be partially evolved as CO_2 and partially utilized to build new cell material. For heterotrophic activity under aerobic conditions, one can use the general assumption that 50% of the carbon goes to CO_2 and 50% goes to cell mass. Under anaerobic conditions a smaller portion of the carbon is used to build cell mass, and a larger portion goes to CO_2 and methane (see Section 3.4.2). Thus microbial activity within environmental samples can be monitored by measuring TEA consumption or CO_2 evolution. However, for both CO_2 and TEA, the relationship between the measured parameter and the amount of new cell material formed is specific to the organism, the particular substrate and environmental conditions that influence growth rate. Therefore, a conversion efficiency must be estimated to quantify bacterial production absolutely. Even if an absolute value for microbial activity cannot be determined, measurement of the flux of respiratory gases can provide a relative level of microbial activity.

11.4.1 Measurement of Respiratory Gases, CO_2 and O_2, in Laboratory and Field Studies

As described in Sections 11.2.2 and 11.2.4, there are several approaches to measuring CO_2 and in particular O_2 in pure culture. Most of these approaches can also be adapted for use with environmental samples. The presence of respiration gases can also be determined by gas chromatographic analysis, typically using a thermal conductivity detector. Chromatography requires that an air sample is collected in a gastight syringe, and then injected into a stream of inert gas, which carries the sample through a column that has been packed with selective material. The column packing material acts to separate the individual gaseous components of the sample, and allows simultaneous monitoring of O_2 and CO_2 within the same sample. Carbon dioxide in atmospheric samples can also be monitored using an infrared gas analyzer, which detects CO_2 by the absorption of a specific electromagnetic wavelength. Some specific approaches for measurement of respiration gases in terrestrial and aquatic environments are detailed in the following sections.

11.4.1.1 Terrestrial Environments

Microcosm Studies

Microbial activity within soil or subsurface materials can be measured under controlled conditions in a laboratory, or can be measured *in situ* (in the field). In a laboratory study, a sample of the porous medium is typically incubated in a sealed, airtight enclosure, usually referred to as a microcosm. Some examples of microcosms are shown in Figure 11.12. This approach allows one to design complex experiments that can be performed and replicated under relatively controlled conditions. For example, microcosm studies performed in the laboratory allow for the standardization of environmental parameters such as soil moisture content and temperature, for all samples. In the establishment of laboratory microcosms, a common practice is to sieve the soil through a 2-mm sieve to homogenize the sample and to remove large stones or plant debris. The soil

TABLE 11.1 Several Common Methods Used for the Measurement of Microbial Activity in Environmental Samples[a]

Test	Basis of Test	Application	Advantages	Disadvantages
Measurement of respiration gases	Measurement of oxygen utilization or CO_2 production in an environmental sample. The flux of respiration gases provides an indication of overall metabolic activity, which can reflect the level of microbial activity.	Basal respiration measurements reflect microbial metabolism of organic substrates present in the environment. However, respiration by other components, such as plant roots in soil or algae in water, will also be included, depending on the environment.	Field chambers can be built and installed *in situ* to monitor flux of respiration gases in relatively undisturbed samples. The addition of an organic substrate to the sample can be used to indicate the level of potential microbial activity.	Incubation of samples in a closed chamber (microcosms) in order to monitor the flux of respiration gases can create an artificial environment. CO_2 production can be underestimated due to pH-dependent retention of inorganic carbon as bicarbonate.
Respiration of radiolabeled substrates	Metabolism of a radiolabeled substrate is monitored by measuring the evolution of labeled CO_2.	This method is used to determine the potential for metabolism of a foreign substrate, such as an organic pollutant. Also, overall heterotrophic potential can be estimated by determining the turnover of organic substrates that occur naturally in the environment.	The use of radiolabeling results in high sensitivity of the measurement, thus short incubation periods can be used. The use of specific radiolabeled compounds shows the potential for degradation of that specific substrate.	The concentration of substrate added is often greater than the concentration present in the environment, thus the rate of metabolism may be overestimated unless corrective procedures are used.
Microelectrodes	Probes with tips $<20\ \mu m$ in diameter can be inserted into environmental samples to provide a continuous monitoring of activity.	Microelectrodes have been designed to measure specific respiratory activities, including oxygen utilization and nitrate respiration.	Can monitor real-time activity at critical interfaces in biological systems. Especially useful to monitor the interdependence of aerobic and anaerobic processes in the environment.	The instrumentation is delicate and relatively expensive. The small probe size can result in large variations in observed measurements when used on spatially heterogeneous environments such as soil.
Incorporation of radiolabeled thymidine into cellular DNA	Microorganisms will scavenge DNA precursors, such as thymidine, from their environment. By radiolabeling thymidine, the rate of incorporation into DNA can be measured.	The rate of DNA synthesis provides a reasonable estimate of the rate of cell division, thus providing an estimate of microbial biomass production.	When using a short incubation time, thymidine incorporation is thought to measure bacterial DNA production because the rate of bacterial incorporation of thymidine is thought to be much faster than for other organisms which may be present in environmental samples.	Not all bacteria will incorporate exogenously supplied thymidine into DNA. Also, estimating microbial activity requires the development of a conversion factor relating thymidine incorporation to biomass production.
Adenylate energy charge (AEC)	AEC is a weighted ratio of ATP to total adenylates. ATP is quantified using a luciferin–luciferase substrate–enzyme system.	AEC values reflect a continuum between an active microbial community ($AEC > 0.8$), and a community with a high proportion of dead or moribund cells ($AEC < 0.4$).	AEC can establish the presence of a metabolically active community without the need to incubate the sample or add a surrogate substrate.	AEC is not necessarily a direct measure of microbial activity because adenylates are present in all living organisms and can also be released by decaying cells.
Dehydrogenase assay	This assay measures the rate of oxidation–reduction reactions (electron transport chain activity) by monitoring the reduction of	The rate of reduction of tetrazolium salts reflects the overall activity by all respiring microorganisms.	Actively respiring microorganisms can be visualized microscopically by the deposition of pigmented tetrazolium salts reduction products within	The amount of tetrazolium salts reduced depends on many factors including sample incubation conditions. Direct comparison of activity

(*Continued*)

TABLE 11.1 (*Continued*)

Test	Basis of Test	Application	Advantages	Disadvantages
	a tetrazolium salt by actively respiring microorganisms.		the cell. The assay has a high sensitivity and can be used to measure activity in low productivity environments.	between samples can be made only when the assay is performed under identical conditions.
Hydrolysis of fluorescein diacetate	Hydrolysis of fluorescein diacetate is performed by a variety of enzymes including esterases, proteases and lipases.	The assay measures enzymatic activity, thus providing an estimate of total microbial activity in an environmental sample.	Fluorescein diacetate hydrolysis produces a highly fluorescent product, fluorescein, which is easily detected.	Enzymes involved in hydrolysis can be intracellular or extracellular.
Stable isotope probing	An isotopically labeled, e.g., ^{13}C, substrate is added to a sample. Analysis of ^{13}C distribution into DNA (DNA-SIP) or cell lipids (PLFA-SIP) provides information about the active populations in the community.	This technique identifies the populations within a community that are actively metabolizing the labeled substrate added.	Links phylogeny with function even if the functional populations cannot be cultured. Can be used to measure carbon flow through trophic levels in the environment.	PLFA-SIP is more sensitive in terms of numbers but gives only very general taxonomic information. DNA-SIP is less sensitive in terms of number but gives precise phylogenetic information. SIP is technically difficult and not yet standardized.
Microarray[b]	A matrix of immobilized nucleic acid probes is used to query the nucleic acid makeup in a sample.	Allows high throughput screening of gene expression in a sample.	Allows direct and rapid inquiry of how microorganisms respond to and interact with their environment.	Cost prohibitive in many cases although technological advances are decreasing costs associated with microarray fabrication. Data interpretation can also be challenging.
Proteomics[b]	Analysis of the proteins expressed by a microbial community under a prescribed set of environmental conditions.	Allows identification of proteins that are differentially expressed and thus likely important in microbial responses to environmental conditions.	Provides the best information available describing the microbial community response to a stimulus.	Analysis is expensive, difficult and time consuming. The technique has not yet been fully developed for use with complex, environmental samples.

[a]*This table is meant to highlight advantages and disadvantages of these techniques, and should not be considered as comprehensive.*
[b]*Microarrays and proteomics are discussed in Chapters 13 and 21, respectively.*

can then be dried (if necessary) and adjusted to a set moisture content. However, the disadvantage of preparing the soil in this manner is that soil structural features are altered, such as soil aggregation and pore size distribution, and this may have an effect on microbial activity.

The use of sealed microcosms allows for the measurement of microbial activity within the sample as determined by the flux of CO_2 and/or O_2 within the headspace atmosphere. Headspace gas samples can be withdrawn using a gastight syringe, and the CO_2 or O_2 concentrations can be determined using gas chromatography. An alternative method is to trap the CO_2 produced in a basic solution using a trap such as that shown in Figure 11.10. One problem that can arise during long-term incubation periods is the depletion of oxygen within the headspace of sealed, airtight flasks. To address this problem, flow-through incubation systems have been devised to allow headspace gases to be replenished, while still allowing quantification of microbial respiration. In this case, CO_2-free air is used as the flow-through gas, and any CO_2 in the air exiting the flask is a direct result of microbial activity. The CO_2 in the effluent air can be trapped in alkali and quantified or fed directly into a CO_2 detection device, such as an infrared detector (Brooks and Paul, 1987). A second problem in some alkaline soils is that CO_2 evolution may be underestimated, because the equilibrium shown in Eq. 11.2 is dependent on pH. In soils with a pH above 6.5, a significant portion of the CO_2

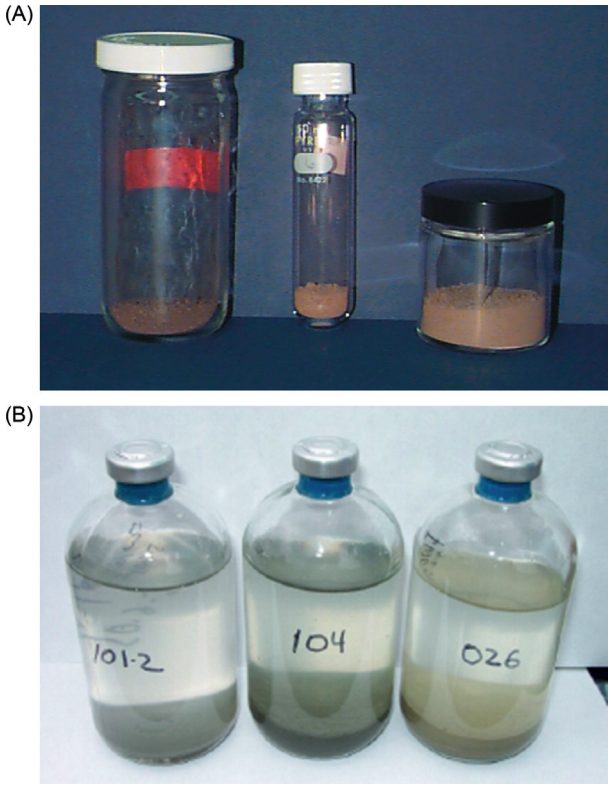

FIGURE 11.12 (A) Examples of microcosms used in studies under aerobic conditions. As shown, a microcosm can be any shape or size depending on the size of the sample and the headspace required. In some cases much larger microcosms may be used so that several samples can be removed and analyzed during the experiment. In other cases, volatile substrates are added to the microcosm. When volatile substrates are used it is important that the cap seal be airtight, and also that the cap seal does not absorb the volatile material. To address these problems a Teflon liner can be placed into the cap. (B) Serum vials or bottles are often used as microcosms for studies under anaerobic conditions. The vials or bottles are sealed with a septum and a crimp-top cap. To impose anaerobic conditions, a needle is inserted into the septum in the crimp top and the flask is flushed exhaustively with an inert gas (e.g., N_2) to drive out the oxygen. Photos courtesy (A) R.M. Maier and (B) A. Somenahally.

produced by mineralization will be retained in the soil in the bicarbonate (HCO_3^-) form. Flow-through systems, which provide continuous replenishment of the atmosphere within the incubation flasks, reduce the retention of CO_2 in the soil by maintaining a low concentration of CO_2 in the atmosphere of the microcosm.

Respiration measurements to determine microbial activity in soil should be corrected for possible nonbiological sources of gas exchange. The nonbiological, spontaneous generation of CO_2 can occur in certain soils, especially when moisture is added to a dry soil, or in soils containing free calcium carbonate. This is a particular problem with arid soils that are high in calcium carbonate ($CaCO_3$). Nonbiological oxygen utilization can occur in the spontaneous oxidation of certain chemical elements, such as iron or copper. The separation of chemical reactions from biological production requires the use of sterile controls. The most common approach to sterilization of soil is to autoclave the soil three times on three consecutive days, or to treat the soil with a chemical agent such as mercuric chloride ($HgCl_2$) to inhibit microbial activity (Rozycki and Bartha, 1981).

Field Studies

Alternatively, field studies can be performed by placing a field chamber over a plot of surface soil and using this setup to make *in situ* measurements (Figure 11.13). In this way the soil structure is not altered by the sampling procedure and field respiration rates of the indigenous population are more reliably determined. In some cases it may be desirable to measure respiration gases below the soil surface. In this case, a gas sampling probe can be inserted beneath the soil surface and used to withdraw gas samples at discrete depths in the soil profile. However, care must be taken to avoid the creation of preferential flow paths, which could result in the introduction of aboveground atmosphere into the subsurface.

Samples of atmosphere from within the field chamber can be withdrawn using a gastight syringe and then injected into a gas chromatograph for CO_2 and O_2 determination (Figure 11.13A). Alternatively, gas from the chamber can be analyzed in real time in the field. This has most commonly been done for CO_2 with instruments based upon infrared absorption, with at least one company offering a system which can be set up to automatically take in-field measurements (Figure 11.13B; LI-COR; Lincoln, NE, U.S.A.). In addition, newer technologies such as photoacoustic- and Fourier transform infrared spectroscopy (FTIR)-based methods are now being tested which promise the ability not only to measure CO_2 but also simultaneously to measure a large number of other gases in the field (Griffith *et al*., 2012; Iqbal *et al*., 2013).

There are several difficulties inherent in field studies. For example, because environmental parameters, such as soil moisture and temperature, cannot be readily controlled, field respiration measurements have more variability than laboratory microcosm measurements. Temperature variations can be especially problematic if the project is so large that it requires several hours (or even days) to collect samples from all of the sites. It is also difficult to perform control studies in the field because sterilization of a soil plot is difficult. Finally, in many environments the respiration of plant roots may contribute significantly to the flux of respiration gases. Because the contributions of heterotrophic bacteria and plant roots cannot be separated in field measurements, CO_2 production has been viewed as a measure of the gross soil metabolic activity. Additional discussion of these issues and suggested gas sampling protocols are

FIGURE 11.13 Examples of field chambers that allow measurement of respiration gases. (A) Gas in the chamber is sampled via a syringe inserted into a septum. (B) A commercial chamber (LI-COR®, Lincoln, NE, U.S.A.) is inserted over a PVC collar (green) in the soil and used to sample and test the gas in the field (via an attached instrument). Figure 11.13A from Pepper *et al.* (2006). Figure 11.13B courtesy J.O. Storlien.

available from the Greenhouse gas Reduction through Agricultural Carbon Enhancement network (GRACEnet) (USDA-ARS, 2013).

11.4.1.2 Aquatic Environments

Oxygen depletion is a common means of determining microbial activity in aquatic environments, in part because of the ease with which dissolved oxygen can be determined. However, many aquatic systems are oligotrophic, especially marine environments, and thus the number and activity of heterotrophic bacteria are limited. Therefore, water samples must be incubated in sealed microcosms for extended period of 12 hours or even longer, in order to detect significant oxygen depletion. Incubation of water samples for the extended periods required to record oxygen demand may create an artificial environment because of alteration of several time-dependent variables in the system. These include: changes in the quantity and the form of organic carbon substrate; changes in grazing pressure resulting from the presence of bactivorous zooplankton; and the exclusion of larger forms of grazing animals from the microcosm (Pomeroy *et al.*, 1994). With time, these effects can create deviations from the initial respiratory rate. To avoid this type of error, incubation times should be kept as short as possible.

Another factor that must be considered in aquatic systems is the close association between photosynthetic and heterotrophic microorganisms. In order to prevent photosynthetic generation of oxygen, which would interfere with the measurement of heterotrophic activity using oxygen depletion, incubations should be performed in the dark. However, in this case the oxygen demand measured will include both heterotrophic activity and oxygen utilization in the dark respiration cycle of photosynthetic organisms. Because of these inherent difficulties in the measurement of respiration gases in aquatic systems, a better choice of activity measurement is usually one of the tracer methods described in the following sections. These techniques have the advantage of requiring much shorter incubation periods and are more specific for heterotrophic bacteria.

11.4.2 The Application of Respiration Measurements in Environmental Microbiology

11.4.2.1 Basal Rate of Microbial Activity in Soil Samples

Concern about the transport of organic pollutants into subsurface environments and recognition of the potential role of microorganisms in the remediation of pollutants in the subsurface have led to an exploration of the microbial life in subsurface environments. In one study described by Kieft *et al.* (1995), the existence of microorganisms was investigated in sediments ranging in depth from 173.3 to 196.8 m below the surface at a site in south–central Washington State. To determine the existence and distribution of microorganisms, a battery of tests was performed on each subsurface sample, including acridine orange direct counts (AODCs), the mineralization of glucose and basal respiration. Basal respiration was measured as the biological CO_2 production in samples that had received no nutrient amendment. In a parallel experiment, the soil was amended with glucose to determine if nutrient addition was required to detect microbial activity. Basal respiration was determined by transferring 5.0 g of subsurface sediment into 70-ml vials or microcosms, which were then sealed with rubber septa. After 3, 7 and 14 days of incubation (22°C), a sample of headspace gas was withdrawn and analyzed for CO_2 content using gas chromatography. The possibility of nonbiological CO_2 production was

investigated using poisoned controls in which $HgCl_2$ (250 μg/ml) was added to preclude microbial activity. Basal respiration in the deep subsurface samples ranged from < 0.001 to 0.664 μg CO_2/g dry soil/h. The highest rates of basal respiration were detected in subsurface samples with the highest total organic carbon content, which were fine-grained sediments originating from lake (lacustrine) deposits. Subsurface deposits originating from buried soil (paleosol) or river (fluvial) sediments were found to have much lower basal respiration. There was strong agreement in the pattern of microbial distribution as revealed by basal respiration, cell number determined by microscopic counts (AODCs) and glucose mineralization. It should be noted that basal respiration is not an *in situ* measurement, and it should be expected that the results obtained will differ from *in situ* respiration rates, which are affected by the physical and environmental parameters of the particular site.

Basal respiration has also been used as an indicator of soil "health" or condition. Dulohery *et al.* (1996) used large (0.5 by 1.0 m) field chambers to determine CO_2 production in forest soils that had been affected by logging activity. Tree harvesting involves the use of heavy equipment, which can alter forest soils in many ways, including removal of topsoil and compaction of the soil profile. A comparison of soil respiration was made between pristine sites and sites where logging activities had taken place. Basal levels of CO_2 production ranged from 52 to 257 mg/m^2/h in undisturbed forest soil sites, depending on the season. CO_2 production was significantly reduced in sites affected by heavy equipment. For example, in areas that had been used as skid trails to haul logs from the site, soil respiration had declined an average of 34%. Attempts to improve soil conditions after logging using fertilizer application and soil tillage were evaluated.

11.4.2.2 Substrate-Induced Microbial Activity

Microbial respiration has been used to evaluate the response of microbial populations to the introduction of organic pollutants, such as petroleum hydrocarbons. In this case, the desired response is that the hydrocarbons be used as a carbon source by microorganisms. This response can be measured as an increase *in situ* respiration above the basal respiration level of the indigenous microbial community. Hinchee and Ong (1992) used subsurface probes to monitor microbial respiration at a number of sites where the subsurface was contaminated with a variety of different hydrocarbon mixtures, including jet fuel and crude oil. The soil probes (Figure 11.14) were installed to monitor atmospheric gases between 1 and 5 m below the ground surface, depending on the site. Probes were installed in areas already contaminated with hydrocarbons and, in order to estimate the basal respiration rate, in areas where there was no contamination. Prior to measurement, air was injected through the probe into the subsurface for 24 hours to ensure that the subsurface contained an adequate amount of oxygen. The air injection was then turned off, and the microbial utilization of O_2 and production of CO_2 was monitored by withdrawing gas samples at intervals of 2 to 8 hours. Respiration rates were calculated on the basis of O_2 utilization (% O_2/h), and revealed that more than 50% of the available oxygen was utilized within 20 to 80 hours in the hydrocarbon-contaminated sites. This was a significant increase over the approximate 10% reduction in the available oxygen in a comparable but uncontaminated site.

As discussed in Section 11.4, conversion factors are needed to determine the rate of substrate utilization from oxygen uptake. In this study, an estimate of the rate of hydrocarbon biodegradation based on O_2 utilization was made using a hexane as reference hydrocarbon. The stoichiometric relationship for the oxidation of hexane indicated that 9.5 moles of O_2 were required for the complete mineralization of 1 mole of hexane. Based on this relationship, the rate of hexane degradation ranged from 0.4 to 13.0 mg hexane/kg soil/day for sites contaminated with jet fuel, and 3.6 to 19.0 mg hexane/kg soil/day for sites contaminated with crude oil. The results provide an approximation of the rate at which petroleum hydrocarbons are biodegraded within this particular subsurface site if adequate aeration is provided. Such an estimate is important in determining the rate at which the site can be remediated biologically, and whether this rate is high enough to contain the contaminant spill and prevent further migration of the hydrocarbons.

Hydrocarbon biodegradation rates based on CO_2 production were also determined but were less consistent than rates based on O_2 utilization. CO_2 production (percent CO_2/h) was unexpectedly low in contaminated sites

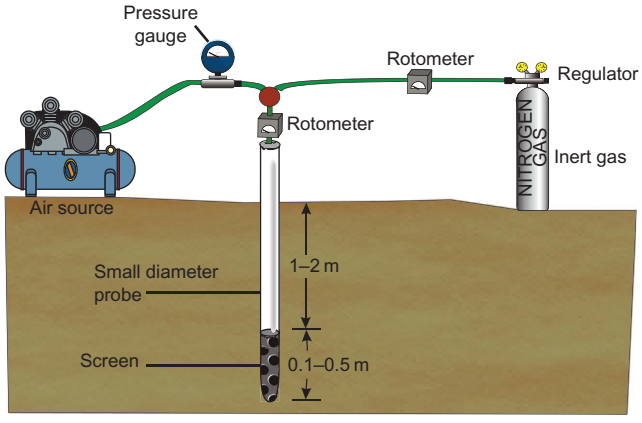

FIGURE 11.14 A subsurface probe used to remove gas samples from various depths within the soil profile. This setup was used to measure *in situ* respiration in a hydrocarbon-contaminated site. Adapted with permission from A.R. and Waste Management Association; Hinchee and Ong (1992).

where the soil pH was above 7.0, indicating the possibility of CO_2 retention in the bicarbonate form by the soil.

11.4.2.3 Microbial Biomass Determination

A measurement of microbial biomass in a soil is commonly performed to give an indication of soil condition and the potential for metabolic activity. This measurement can be made using two different approaches, both based on CO_2 evolution. One approach is the chloroform fumigation–incubation method (see Information Box 11.1; Figure 11.15). A soil sample is first fumigated with chloroform to kill the indigenous microorganisms. Upon reinoculation of the soil, the dead microbial biomass becomes available for microbial consumption. The amount of CO_2 produced by the consumption of this organic material is monitored and used to calculate the initial quantity of microbial biomass. In this procedure, a sample of fumigated soil and a sample of nonfumigated soil, which serves as a control to determine the basal respiration level, are incubated for 10 days in a sealed microcosm. The microorganisms responsible for the respiration will be the very small proportion of microbes that survived the fumigation treatment, or the fumigated soil will be inoculated with a starter culture from the nonfumigated soil. The amount of CO_2 released by mineralization is quantified using an alkaline trap or by GC analysis of headspace gases. The production of CO_2 in the nonfumigated soil represents the basal rate of mineralization, and CO_2 production in the fumigated soil represents primarily the mineralization of microbial biomass. Therefore, the amount of respiration, corrected for the basal rate, is a measure of the amount of microbial biomass present prior to the fumigation step. The amount of carbon held in microbial biomass is calculated as:

$$\text{Biomass C} = \frac{\text{Fc} - \text{Ufc}}{\text{Kc}} \qquad \text{(Eq. 11.3)}$$

where:

biomass C = the amount of carbon trapped in microbial biomass
Fc = CO_2 produced by the fumigated soil sample
Ufc = CO_2 produced by the nonfumigated soil sample
Kc = fraction of biomass C mineralized to CO_2

The value of Kc is of considerable importance because it expresses the proportion of total metabolized organic carbon that is mineralized. Literature values for Kc range from 0.41 to 0.45. These estimates are usually based on tracer experiments in which the mineralization of radiolabeled substrates, such as glucose or bacterial cells, by microorganisms isolated from the soil is determined in a liquid culture. The proportion of radiolabeled substrate that is mineralized to $^{14}CO_2$ or assimilated into microbial biomass can thus be determined.

Information Box 11.1 Measurement of Microbial Biomass in Soil — The Chloroform Fumigation Method

Step 1. Collect a representative soil sample (see Chapter 8).
Step 2. Perform a fumigation experiment as illustrated in Figure 11.15 as outlined:

To determine Fc:
1. Fumigate a portion of the soil sample with chloroform.
2. Inoculate.
3. Incubate for 10 days.
4. Determine CO_2 as an estimate of biomass mineralization = Fc.

To determine Ufc:
1. Incubate a second portion of the soil sample for 10 days.
2. Determine CO_2 as an estimate of basal mineralization levels = Ufc.

Step 3. Estimate Kc by performing a mineralization experiment using ^{14}C-labeled glucose and a soil inoculum (see Section 11.2.4).

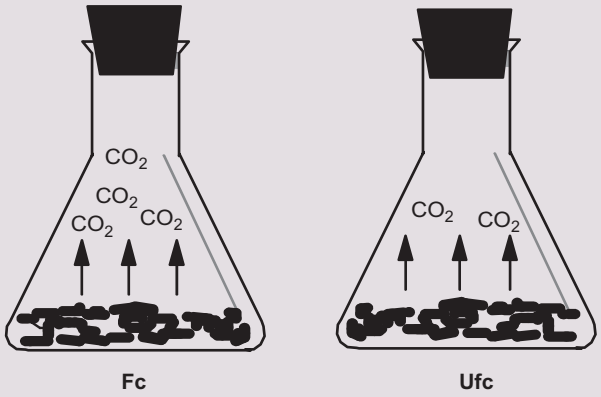

FIGURE 11.15 Microcosms for measurement of microbial biomass in soil. Fc, fumigated with chloroform; Ufc, not fumigated with chloroform.

An alternative method for the estimation of microbial biomass is the substrate-induced respiration method. This method estimates the amount of carbon held in living, nonresting heterotrophic cells by determining the initial respiration response when glucose, a readily metabolized organic carbon source, is added to the soil. The incubation time is limited to several hours, with samples of the mineralized CO_2 taken at regular intervals. The rate of glucose respiration in the soil is expected to increase with time, reflecting both the initial metabolism of the substrate and the fueling of cell division, which results in an increase in the number of metabolizing cells. Thus, the lowest and usually initial rate of glucose respiration is used as an indicator of the microbial biomass present in the soil sample. Calculation of microbial biomass is based on a correlation between the substrate-induced respiration

method and the chloroform fumigation-incubation method, described earlier, or other methods for determining microbial biomass in soil. A commonly used relationship was provided by Anderson and Domsch (1978):

$$y = 40.04x + 0.37$$

where:

y is biomass C (mg/100 g dry weight soil), and
x is the respiration rate (ml CO_2/100 g dry weight sediment/h)

The main advantage of the substrate induction method, compared with the chloroform fumigation method, is that microbial biomass can be estimated in a much shorter time. To implement this method, preliminary studies are required to determine the appropriate glucose concentration to add to the soil. Increasing glucose concentrations saturate the initial uptake capacity of the microbial population, resulting in a plateau in glucose mineralization. The lowest glucose concentration that yields the maximum respiratory response must be independently determined for each soil type, and then applied to that soil in order to standardize the substrate induction method between different soil types. Examples of estimates of microbial biomass range from 12.8 to 203 mg biomass C in 100 g dry soil, depending on soil type and also on the method used to determine microbial biomass (Martens, 1987).

The substrate-induced respiration method has been modified to determine the individual contribution of bacteria and fungi to total heterotrophic activity within soil by the use of selective antibiotics. Glucose-induced respiration is determined in the presence of either streptomycin, which inhibits prokaryotes, or cycloheximide, which inhibits eukaryotes. Dominance of fungal glucose-induced respiration was evident in a soil sample from a semiarid region (Johnson *et al*., 1996), and in the early stages of mineralization of plant litter (Beare *et al*., 1990). In contrast, bacteria are thought to dominate in the rhizosphere and in subsurface sediments. However, it must be recognized that the glucose-responsive, antibiotic-sensitive microbial population may not represent the entire microbial population within an environment, and the use of antibiotics can only yield estimates of the relative contribution of fungi and bacteria to glucose-induced respiration (Johnson *et al*., 1996). In addition, others have modified the substrate-induced respiration approach to detect microbial activity via heat generation instead of CO_2 production (Information Box 11.2; Figure 11.16).

11.4.2.4 Biological Oxygen Demand (BOD)

The 5-day BOD test was developed as a means of monitoring wastewater quality. The BOD test quantifies the oxygen required to metabolize dissolved organic carbon present after wastewater treatment. Thus, the BOD test can provide an indication of the impact that wastewater treatment plant effluents may have on the receiving waters following discharge. This test has become an industry standard in terms of evaluating water quality and has an established protocol (APHA, AWWA, WEF, 2005). Water samples, usually 250 to 300 ml, are incubated in sealed bottles for 5 days at a constant temperature, $20 \pm 1^{\circ}C$. The BOD is calculated from the difference in dissolved oxygen concentration measured at the beginning and at the completion of the incubation period. The emphasis of the BOD test is on the determination of the oxygen demand created by the presence of dissolved organic material. Hence, it may be necessary to add inorganic nutrients or even a "seed" solution of heterotrophic bacteria to ensure that the dissolved organic material is in fact degraded.

The oxygen demand within the sediment of freshwater and estuarine environments can have a major impact on oxygen levels in surface waters, especially in shallow lakes and in rivers receiving organic-based waste material. The oxygen demand is created by microbial activity, as well as invertebrate respiration and nonbiological oxidation. Dissolved oxygen standards for the protection of fish and the aquatic ecosystem as a whole have been instituted for lakes and rivers affected by organic waste generated by human activity. Thus, the implementation of water quality standards necessitates quantification of sediment oxygen demand. Bowman and Delfino (1980) described an experimental apparatus for the determination of sediment oxygen demand in which water circulates over a layer of sediment in a sealed container (Figure 11.17). The closed loop of circulating water passes by a dissolved-oxygen probe, which continuously monitors dissolved oxygen levels.

11.4.2.5 Micro-level Measurements of Microbial Activity

The development of microelectrodes has allowed the *in situ* measurement of respiratory gases (and other environmental parameters) at the micro-level in biofilms and environmental samples (Figure 11.18). Mixed community microbial mats, or biofilms, are formed on many different surfaces as bacteria attach to and subsequently proliferate into a densely packed film (see Section 6.2.4). Biofilms can be found on almost any surface imaginable, from rock surfaces in streambeds to filter systems used in wastewater treatment to the surfaces of our teeth. The flux of respiratory gases within and around biofilms has revealed their importance in terms of organic carbon and nutrient cycling.

Use of microelectrodes to measure different respiration gases within biofilms has allowed us to better understand how microorganisms function within biofilms. For example, depth-dependent activity in a microbial mat was studied by Nielsen *et al*. (1990) using a combined O_2 and N_2O

Information Box 11.2 Measurement of Microbial Activity using Infrared Thermography (IRT)

Historically, scientists have primarily measured the substrates and products listed in Eq. 11.1 (e.g., disappearance of substrate and electron acceptors and production of cell mass and CO_2) as indicators of microbial activity. However, depending upon the process and microorganisms involved, there may be other potential indicators of microbial activity, such as temperature. Microbial generation of heat occurs as a by-product of microbial metabolism, and is perhaps best known for its role in the composting process. Advances in temperature detection technology have enabled high-resolution detection of slight changes in temperature with potential application for determining microbial activity. For example, Kluge *et al.* (2013) used infrared thermography (IRT) to monitor the temperature in soil microcosms amended with glucose (≈1% w/w). Within 18 h, there was a significant increase in temperatures of the glucose-amended microcosms as compared to unamended controls, with the temperature increase peaking at 40 h (Fig. 11.16). By 70 h, the temperature of the amended microcosms had decreased to that of the unamended microcosms. The IRT results mirrored CO_2 production (i.e., respiration) in the microcosms, presumably corresponding to metabolism of the added glucose. Potential advantages of using IRT for measuring microbial activity include: (1) it is a non-destructive technique; (2) measurements are easy to conduct; and (3) it allows observation of spatial patterns in activity.

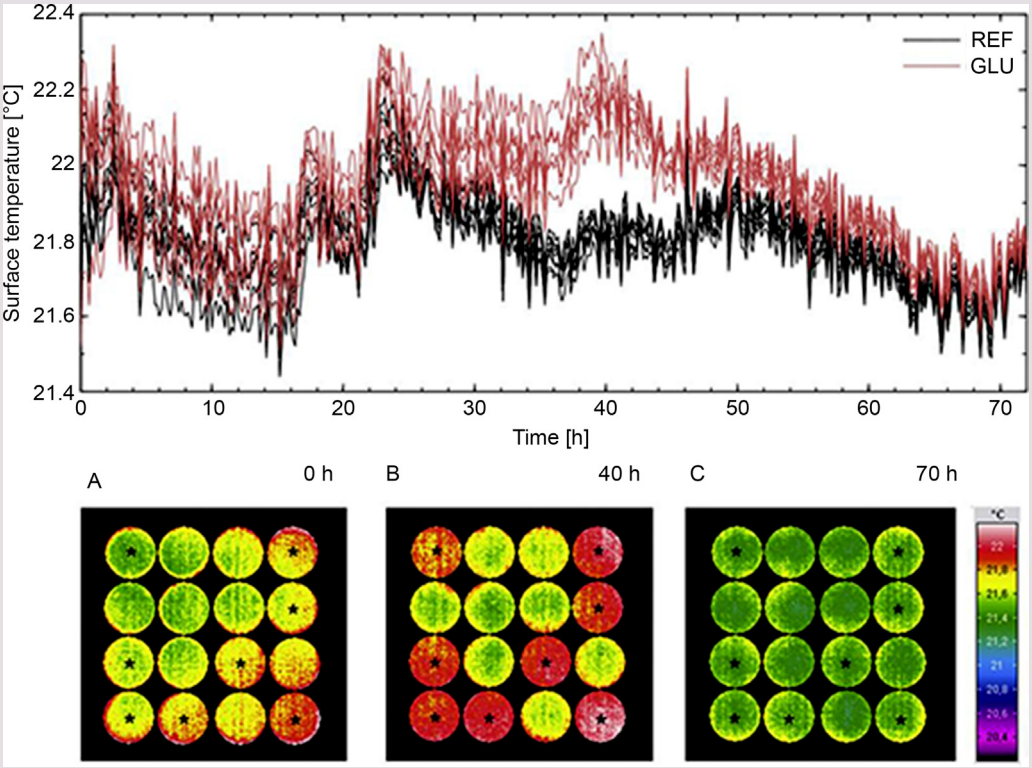

FIGURE 11.16 Surface temperature of 16 soil microcosms 0, 40 and 70 h after either being amended with glucose (indicated by "*") or left unamended (controls). From Kluge *et al.* (2013).

microelectrode with a sensor tip that was 20 μm in diameter (Figure 11.18a). In contrast, the biofilms being studied were several centimeters thick. The microelectrode was lowered into the biofilm at intervals as small as 50 μm to record specific respiration activity. Results revealed that oxygen was depleted by microbial activity in the surface layers of the mat, and that beneath the surface, conditions were anoxic. This allowed the development of denitrifying bacterial populations in the interior of the biofilm. These bacteria can respire under anaerobic conditions using NO_3^- as a terminal electron acceptor in place of O_2. Anaerobic respiration of NO_3^-, referred to as denitrification, was estimated using a N_2O microelectrode and acetylene gas. The addition of acetylene gas blocks the reduction of NO_3^- during denitrification so that the intermediate N_2O is formed (see Section 11.2.2.2). Thus, the rate of N_2O production provides an estimate of the rate of anaerobic respiration of NO_3^- (i.e., μmol NO_3^- utilized/cm^3/h).

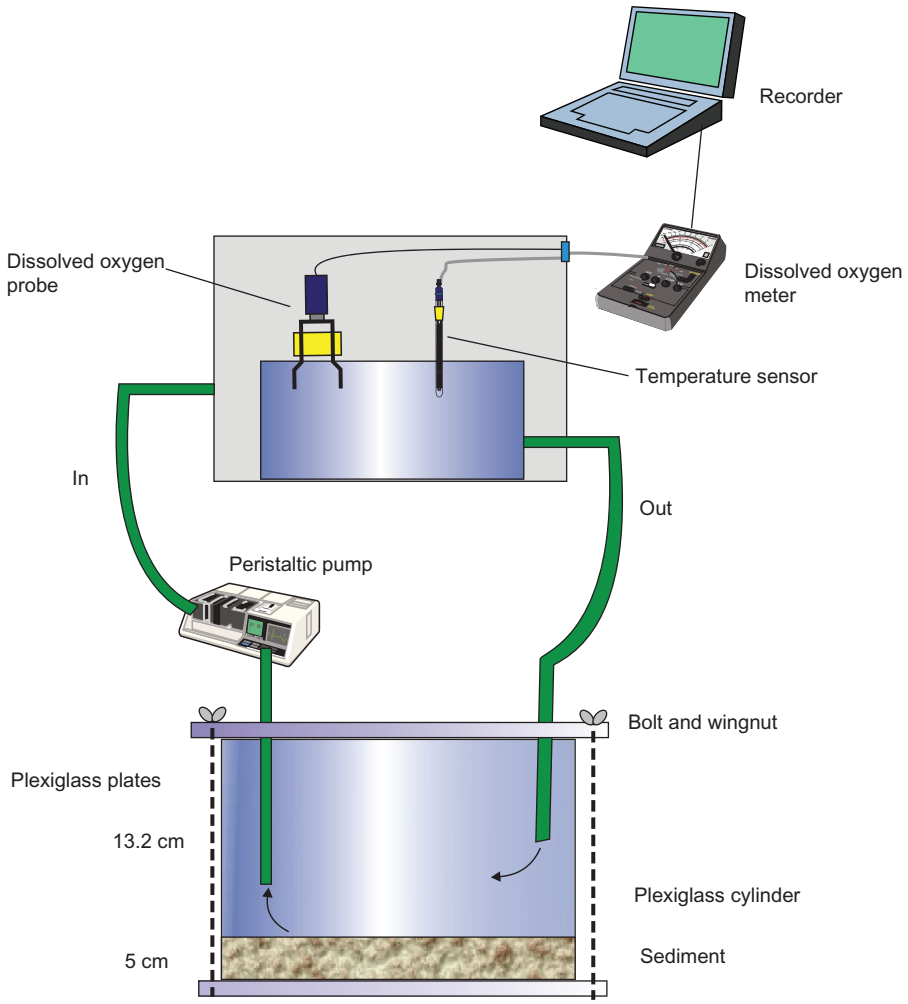

FIGURE 11.17 Apparatus used to determine sediment oxygen demand. Adapted with permission from Bowman and Delfino (1980).

Schramm *et al.* (1996) also revealed the anaerobic nature of microbial activity in the interior of a microbial biofilm using an N_2O microelectrode. In this case, the N_2O microelectrode was coated with immobilized bacteria, which were selected for their ability to reduce NO_3^- metabolically, but only as far as N_2O. Insertion of this microelectrode into the interior of a biofilm recorded the respiration of NO_3^-, a process which could occur only in the absence of oxygen. Using these tools, it has been possible to better understand how the structure of a biofilm affects its function. Specifically, the rapid utilization of oxygen by the abundant bacteria in the upper layers of the biofilm creates an anaerobic environment in the deeper layers that supports the anaerobic respiration of NO_3^-.

11.4.3 Tracer Studies to Determine Heterotrophic Potential

The use of ^{14}C-labeled carbon substrates was discussed in Section 11.2.4.2 as a sensitive way to measure biodegradation of a specific organic compound. This approach can also be used to determine heterotrophic potential in environmental samples. Many radiolabeled substrates are commercially available, including sugars such as glucose, organic acids such as lactic acid, amino acids and even many representative organic pollutants. Mineralization of these substrates can be quantified by measurement of the evolution of $^{14}CO_2$.

The addition of a ^{14}C-labeled substrate to an environmental sample reveals the presence of a degrading population in that sample, and also indicates the rate at which the substrate can be mineralized. However, the rate of substrate mineralization is related to the amount of added substrate up to a saturation limit (see Figure 3.7). Thus, depending on the level of substrate added, this measure may represent basal metabolism, or may represent the potential for a microbial response to higher levels of added substrate. So the dilemma associated with the use of radiolabeled substrates is that although they can provide a very sensitive measurement of substrate utilization, the rate of utilization is usually proportional to the amount of labeled substrate added to the environmental sample. This is especially true for compounds such as

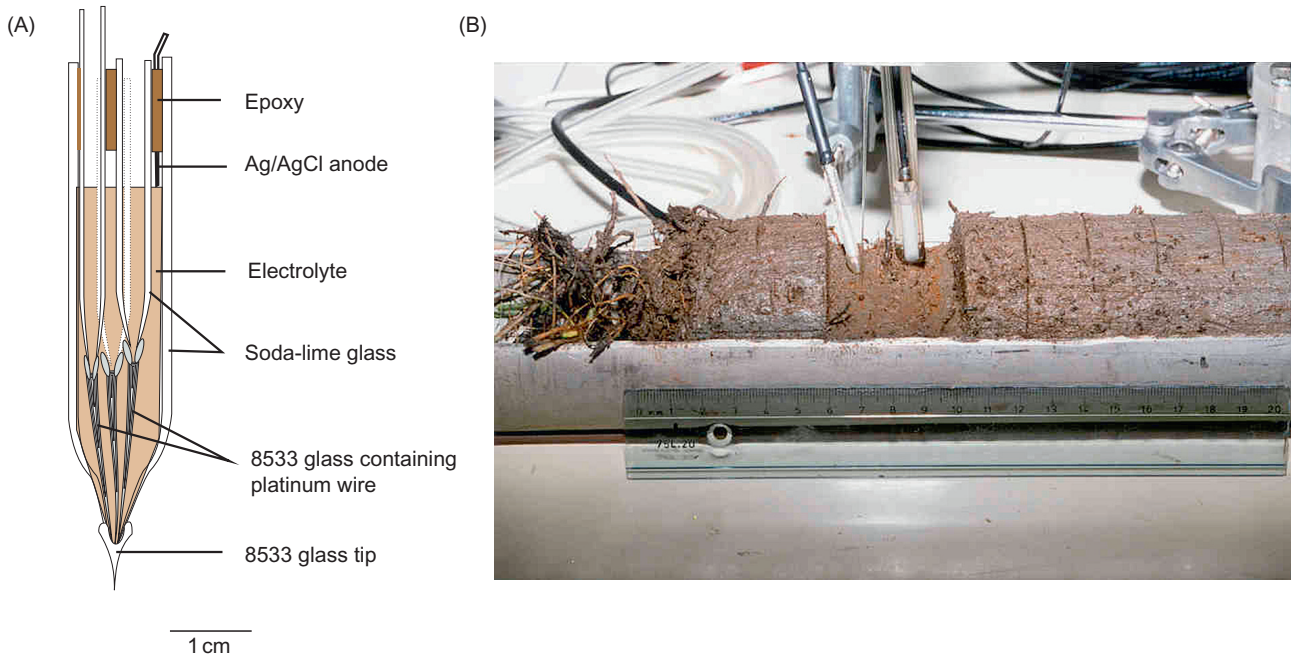

FIGURE 11.18 Microelectrodes used to study biofilms and environmental microsites. (A) On the left is the microsensor used for analysis of O_2 and N_2O. There are three cathodes within the outer casing; one is shown behind the plane of the two others. On the right, the tip of the microsensor is enlarged 250 times. (B) Use of microelectrodes to measure porewater constituents in salt marsh sediment. (A) Adapted with permission from Revsbech and Jørgensen (1986). (B) Courtesy B. Sundby.

glucose, which are very rapidly metabolized by microbial populations. If the fundamental concern of the environmental microbiologist is to determine the rate at which naturally occurring organic substrates are utilized, care must be taken not to overestimate basal levels of activity by adding levels of a carbon source that exceed those already present in the system.

To address this problem, a kinetics-based analysis of labeled substrate mineralization has been developed to calculate the indigenous rate of glucose mineralization in environmental samples. A saturation kinetics model was adapted so that the stimulated rates of substrate mineralization can be extrapolated back to the rate at which a substrate is mineralized in an undisturbed sample. This analysis is referred to as the determination of heterotrophic potential (Wright and Burnison, 1979; Ladd *et al.*, 1979). When a radiolabeled substrate, such as ^{14}C-labeled glucose, is added to a soil or water sample, the rate at which $^{14}CO_2$ is evolved is monitored using an alkaline trapping technique. In the case of a water sample, the amount of radiolabeled substrate assimilated into biomass can be monitored by filtering the bacterial cells from the water sample, and then measuring the radioactivity incorporated into the biomass. Combining the mineralization with the assimilation data provides a measure of the total amount of substrate utilized by the cell. If a single concentration of ^{14}C-glucose is considered, then the rate of uptake by a microbial community can be expressed as:

$$v = \frac{f(S_n + A)}{t} \qquad \text{(Eq. 11.4)}$$

where:

- v = the rate of uptake (or respiration) by the microorganisms (mass/volume/time)
- t = the incubation time
- f = the fraction of labeled substrate taken up (or respired) in time t
- A = the amount of added substrate (mass/volume)
- S_n = the naturally occurring concentration of the substrate (mass/volume)

If S_n is known and A is added such that $A << S_n$, then the assumption can be made that the natural rate of uptake is not significantly altered by the presence of A. Using a short incubation time, the fraction of labeled substrate taken up (or respired) can be determined, and can be expressed as:

$$T_n = \frac{S_n + A}{\nu_n} = \frac{t}{f} \qquad \text{(Eq. 11.5)}$$

where:

- ν_n is the natural rate of substrate uptake (or respiration), and
- T_n is the substrate turnover time due to uptake by the natural population at the natural substrate concentration.

However, in most cases S_n is not known, and A will be added at a concentration much greater than S_n. A situation in which $A >> S_n$ would result in the stimulation of substrate utilization rates above what would be considered the natural level of microbial activity by the indigenous population. Therefore, a single concentration tracer study would not be appropriate for determining T_n. However, T_n can be calculated by measuring the substrate utilization rate with added substrate, and then extrapolating back to the natural level of microbial activity. The basis of this extrapolation technique is Michaelis–Menten kinetics, which state that as the concentration of a substrate increases, the rate of activity of an enzyme will also increase until a plateau is reached. An analogy can be drawn between the transformation of a substrate by an enzyme and the uptake of an organic substrate by bacteria. Therefore, the rate of uptake of an organic substrate by bacteria increases as the concentration of the added substrate is increased, until the mechanism of substrate uptake has been saturated. Michaelis–Menten kinetics can be adapted for heterotrophic potential studies using the modified Lineweaver–Burk transformation, which states that:

$$\frac{S_n + A}{v_n} = \frac{1}{V_{\max}} A + \frac{K + S_n}{V_{\max}} \qquad \text{(Eq. 11.6)}$$

where:

$V_{\max}$ is the theoretical maximal rate of substrate uptake by the microbial population, and
K is a transport constant defined as the substrate concentration at which $v = \{1/2\}\ V_{\max}$

Combining Eqs. 11.5 and 11.6 will give:

$$\frac{t}{f} = \frac{1}{V_{\max}} A + \frac{K + S_n}{V_{\max}} \qquad \text{(Eq. 11.7)}$$

The value of (t/f) can be plotted over several levels of added substrate A, and fitted with a regression line (Figure 11.19). The line is extrapolated back to the x-axis. The slope of the line is $1/V_{\max}$, the y-intercept is $(K + S_n)/V_{\max}$, and the x-intercept is $K + S_n$.

This kinetic approach generates parameters that describe the *in situ* rate of heterotrophic activity. The natural turnover time, T_n, can be calculated when $A = 0$ using Eqs. 11.4 and 11.6:

$$\frac{t}{f} = \frac{K + S_n}{V_{\max}} = \frac{S_n}{v} \qquad \text{(Eq. 11.8)}$$

Other useful parameters include $V_{\max}$, the theoretical maximum rate of substrate uptake, which reflects the abundance and activity of the microbial population. The quantity $(K + S_n)$ may be used as an estimate of the natural substrate concentration, S_n, assuming that K is very small $(K << S_n)$, which is not necessarily true in all cases.

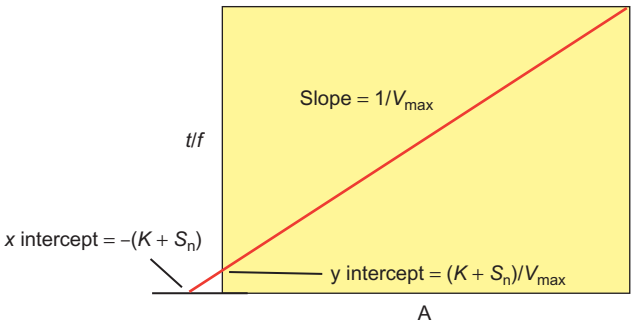

FIGURE 11.19 A Lineweaver–Burke plot.

These parameters can be used to compare the heterotrophic activity of different microbial populations in different environments. However, certain assumptions and limitations must be considered. First, the kinetic approach assumes no significant change in microbial number and no significant depletion of the added substrate during the incubation period. To meet these assumptions, short incubation periods are required. In highly productive environments, incubations of 1 hour may be adequate, but the incubation period must be increased in oligotrophic environments. The temperature of incubation will also affect the measured parameters. All samples should be incubated at the same temperature, preferably at *in situ* temperatures, so that the results are environmentally relevant.

It should also be noted that the heterotrophic potential approach measures only a single substrate and analyzes a diverse microbial population by calculating kinetic parameters as if they were uniform in the population. An assumption is made that the uptake of a substrate by a natural community follows saturation kinetics as if it were a single enzymatic reaction that is not influenced by the presence of other substrates in the environment (Van Es and Meyer-Reil, 1982).

Measurement of heterotrophic activity has been particularly important in understanding how carbon is cycled through the aquatic environment. Heterotrophic bacterial populations have been shown to dominate the utilization of dissolved organic carbon in aquatic environments. Determination of dissolved organic carbon utilization rates revealed that the production of new biomass through heterotrophic processes can equal the amount of biomass formed through primary production by photosynthetic microorganisms. Heterotrophic activity measurements have increased our understanding of the distribution of microbial communities in different ecological zones within the aquatic environment. It has been shown that bacterial abundance and the rate of carbon cycling are greatest in freshwater sediments, as compared with the planktonic, or free-floating, environments. From this, we now understand the ecological importance of sediment-associated bacterial communities in carbon and energy cycling.

11.4.4 Anaerobic Respiration as an Indicator of Microbial Activity

As discussed earlier, soil respiration under aerobic conditions can be monitored using CO_2 production or O_2 utilization as an indicator of carbon utilization. However, many environments are completely anaerobic or contain anaerobic niches, and there are situations in which monitoring the activity of anaerobic respiration is critical. For example, whereas anaerobic environments can support the biotransformation of organic pollutants, in some cases this results in the formation of more toxic metabolites (e.g., the production of vinyl chloride from TCE; Figure 17.2). Anaerobic respiratory pathways include the use of NO_3^-, Fe^{3+} (and other metals), SO_4^- and CO_2 as terminal electron acceptors. For some of these terminal electron acceptors, a gaseous intermediate or end product is formed that can be used as a measure of anaerobic respiration. For example, nitrous oxide (N_2O) is an intermediate in the bacterial reduction of NO_3^- to N_2 (denitrification). Acetylene gas is used to inhibit the complete denitrification of NO_3^-, resulting in the accumulation of N_2O gas (see Section 11.2.2.2). Production of N_2O in samples treated with acetylene gas is proportional to the rate of denitrification. In terms of the soil condition, the presence of N_2O gas suggests a reducing environment in which the indigenous bacteria can utilize NO_3^- as an alternative electron acceptor. The production of N_2O also indicates that nitrification processes at some point resulted in an adequate amount of NO_3^-.

The reduction of CO_2 during anaerobic respiration also produces methane (CH_4) (see Section 3.4). The utilization of CO_2 as a terminal electron acceptor is limited to a group of archaea called the methanogens, and this process requires a strongly reduced environment. The carbon substrates compatible with methanogenesis are simple one- or two-carbon compounds that are produced as a result of the activities of several different groups of microorganisms, including fermentative and acidogenic bacteria. Despite the complexity of the microbial interactions leading to methane production, methanogenic environments are common in soils and are also well characterized for use in the treatment of large volumes of organic waste. Methane production can be a problem in some instances, such as within municipal landfill sites. As landfills age, they often develop highly productive methanogenic populations, and the release of copious amount of CH_4 can create a fire hazard. The most accurate means of determining N_2O and CH_4 is by gas chromatography, although other methods such as photoacoustic-based gas analyzers are also available (see Section 11.4.1.1). Microelectrodes specific for N_2O have also been used to investigate the location of denitrifying activity in inhibited samples, but they are generally less sensitive than gas chromatography (Nielsen *et al.*, 1990).

11.5 INCORPORATION OF RADIOLABELED TRACERS INTO CELLULAR MACROMOLECULES

Quantification of cellular constituents, such as protein or nucleic acids, can be used to monitor the increase in biomass of a microbial population. This approach is often used to monitor the growth of a pure culture on defined media in the laboratory. In the natural environment, numerous sources of these constituents, including plant debris and soil animals, can contribute to the total protein or nucleic acids, and, therefore, these assays would not be specific to microorganisms. A way to make these assays more specific to microorganisms is to measure the incorporation of radiolabeled tracer molecules into cellular macromolecules. This technique is based on the fact that many species of heterotrophic microorganisms can scavenge preformed molecules such as nucleotides or amino acids from their environment, incorporating them directly in cellular constituents. Monitoring the rate at which radiolabeled nucleotides or amino acids are incorporated into essential cellular macromolecules can be used as a means of determining microbial activity. Examples of specific molecules used as tracers are the nucleoside thymidine, labeled with tritium (^3H) which is incorporated into DNA, and the amino acid leucine, labeled with either tritium or carbon-14 (^{14}C), which is incorporated into protein.

There are several advantages of measuring microbial activity using radiolabeled tracer molecules. This technique requires a short incubation period, which is more convenient for field studies and which reduces artifacts created by extended incubation of environmental samples. Further, the use of radiolabeled tracers increases the sensitivity of the measurements, allowing the quantification of very low levels of microbial activity such as those found in extreme environments such as Antarctica (Tibbles and Harris, 1996). There are also several potential sources of error in the tracer method including: (1) the fact that not all bacteria are capable of assimilating tracers into macromolecules; (2) the possible nonspecific incorporation of the label into cellular macromolecules other than the intended target; and (3) the extent of isotope dilution, which may vary both spatially and temporally within a sample site.

11.5.1 Incorporation of Thymidine into DNA

The rate at which a tracer is incorporated into a cellular macromolecule such as DNA provides an indication of the rate of formation of that macromolecule. The measured rate of formation will provide an accurate measure of microbial growth only if the formation rate is directly related to cell division. In a state of growth known as

balanced growth, all cell constituents increase at the same rate. Therefore, the doubling time of any cellular macromolecule would equal the doubling time of the whole cell. Although balanced growth can be achieved under controlled laboratory conditions, sustained balanced growth is unlikely under environmental conditions. However, the synthesis of DNA and protein is strongly related to cell division, so that even when growth is unbalanced, their synthesis is expected to provide a reasonable reflection of cell division.

The scavenging of the thymidine nucleoside and its incorporation into DNA by heterotrophic bacteria have been reviewed by Azam and Fuhrman (1984), Moriarty (1986) and Robarts and Zohary (1993). Once transported across the cell membrane, the thymidine is converted into thymine monophosphate by the action of the enzyme thymidine kinase. Further phosphorylation results in incorporation into DNA as the thymine base. In very general terms, the procedure involves adding [^{3}H]thymidine to a water sample containing planktonic bacteria or to a slurry prepared by mixing soil or sediment with water. The incubation time is usually limited to several hours, and the incorporation of labeled thymidine by the cells is terminated by the addition of a chemical inhibitor or by placing the sample in an ice bath. DNA is then extracted from the cells, and the radioactivity quantified to determine the amount of label that has been incorporated. One problem with this technique is that during the labeling and uptake procedure, nonspecific labeling of other macromolecules, such as proteins and RNA, can occur. In this case, purification of the DNA may be required to improve the accuracy of the activity measurement.

One aspect of this assay that makes it useful for measurement of bacterial heterotrophic activity is that the rate of thymidine incorporation into bacterial DNA is much higher than the rate for other organisms tested, such as algae, fungi or protozoa. Therefore, if the uptake study is limited to short incubation periods (several hours), it is generally believed that the incorporation of these precursors into cellular macromolecules will reflect the activity of growing heterotrophic bacteria. However, not all growing bacteria will incorporate exogenously supplied thymidine into DNA. Exceptions have been noted, particularly within the genus *Pseudomonas*, which may limit the usefulness of this technique in certain environments.

A further complication in the interpretation of the results of this assay is that there are both external and internal pools of thymidine or thymidine metabolites that can compete with the added [^{3}H]-thymidine for incorporation into DNA. The internal pool is created by cells in a *de novo* process in which the nucleotides that form DNA are synthesized from cellular components. If this internal nucleotide pool is large, it may compete with scavenged labeled thymidine for incorporation into DNA. There can also be an external pool of thymidine available for uptake. This external pool is composed of extracellular thymidine probably released from dying organisms. This is especially true for sediments, where thymidine becomes sorbed to sediment particles. The existence of the external and internal pools of thymidine metabolites will result in dilution of the added [^{3}H]thymidine, and cause underestimation of the rate of DNA synthesis. Isotope dilution, which refers to the size of the pool into which [^{3}H]thymidine is diluted, can be estimated (Moriarty, 1986; Robarts and Zohary, 1993) and used to correct estimates of DNA synthesis. Further, the addition of sufficient quantities of exogenous labeled thymidine may cause competitive inhibition of the *de novo* synthesis route and thereby limit the size of the internal thymidine pool.

The thymidine incorporation rate can be converted into a measure of microbial activity using a conversion factor that relates the number of new cells formed to a given amount of thymidine incorporated (i.e., cells formed per mole thymidine incorporated). This conversion factor is derived on the basis of measurements, or best possible estimates, of the amount of DNA per cell, the thymidine content of a cell, and the extent of dilution of the labeled thymidine. An alternative way to determine the conversion factor is to relate the [^{3}H]thymidine incorporation rate directly to cell division based on a separate and independent measure of the increase in bacterial numbers. Common estimates of this conversion factor range from 1.3 to 2.0×10^{18} cells/mole [^{3}H]thymidine incorporated.

Based on thymidine incorporation, bacterial growth can range from less than 1×10^6 cells/g soil/day in an oligotrophic aquifer environment to greater than 1×10^9 cells/g soil/day in a marine sediment (Thorn and Ventullo, 1988). The rate of thymidine incorporation can also be used without conversion to estimate cell division. The thymidine incorporation rate provides a relative measure of microbial activity that can be used in controlled experiments to evaluate the impact of a specific factor, such as the toxicity of heavy metals to microbial activity in environmental samples (Diáz-Raviña *et al.*, 1994).

11.5.2 Incorporation of Leucine into Protein

The rate of leucine incorporation into cellular protein has also been used as a measure of microbial activity (Kirchman *et al.*, 1985; Chin-Leo and Kirchman, 1988). Studies have indicated that a majority of bacteria scavenge leucine from their environment and that most of the assimilated radioactive label will be incorporated into proteins. Further, the *de novo* synthesis of leucine is inhibited by sufficient quantities of exogenous leucine.

11.6 ADENYLATE ENERGY CHARGE

Adenosine triphosphate (ATP) is a compound synthesized by actively growing cells as a means of short-term energy

storage and transfer. ATP captures metabolic energy in the form of high-energy phosphate bonds, and is transported to sites within the cell where energy is required to drive a biochemical reaction. Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) are precursors of ATP, and together the three forms represent the cellular adenylates. ADP and AMP cycle between the sites where high-energy phosphate bonds are added to form ATP and sites where the phosphate bonds are broken to transfer energy to a metabolic process. The ATP content of the cell varies depending on its level of activity, with rapidly growing cells having a higher ATP content than stressed cells.

ATP has been used to measure microbial biomass in sediments and soils. A fairly constant relationship between ATP and cell biomass (10−12 mol ATP/g biomass C) has been measured for soils incubated under specific conditions (Jenkinson, 1988). However, a strong correlation between ATP content and microbial biomass may not hold for microbial populations under conditions of environmental stress, such as in dry or excessively wet soils (Inubushi *et al*., 1989; Rosacker and Kieft, 1990). Thus, the detection of total adenylates (ATP, ADP and AMP) may provide a better indicator of microbial biomass.

Measurement of the cellular adenylates can also be used to determine microbial activity. The relative abundance of ATP compared with its precursors, ADP and AMP, indicates how rapidly the highest energy state (ATP) is formed. The ATP/ADP/AMP ratio provides a biochemical basis for assessing the physiological and nutritional status of organisms. A measure of the adenylate energy charge (AEC) ratio is the weighted ratio of cellular adenylates:

$$\mathrm{AEC} = \frac{\mathrm{ATP} + \frac{1}{2}\mathrm{ADP}}{\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP}}$$

High AEC values (>0.8) reflect an active community, intermediate values (0.4 to 0.8) reflect cells in a resting state and low values (<0.4) reflect a high proportion of dead or moribund cells (Kieft and Rosacker, 1991).

Quantification of AEC as a measure of bacterial biomass has some advantages and disadvantages in comparison with approaches already discussed. A major benefit is that AEC provides a measure of microbial activity in environmental samples without the requirement to introduce a substrate and/or the need to incubate the sample for a period of time, as required in respiration or radiolabeled tracer activity measurements. It should be noted, however, that cellular adenylates are present in all living organisms, and its measurement is not selective for microbial populations. Rapid degradation of cellular adenylates from organic debris will minimize nonmicrobial sources. A preincubation of the soil under aerobic conditions may be necessary to ensure that cellular adenylates from sources such as plant root fragments are given an opportunity to be degraded (Sparling *et al*., 1985).

The quantification of ATP is based on the transfer of energy to a luciferin−luciferase substrate−enzyme system. This system was originally isolated from the abdomen of the common firefly. With the energy supplied by ATP, the enzyme luciferase acts on the substrate luciferin to produce radiant energy that can be detected and quantified and is proportional to the ATP present. Quantification of ATP from environmental samples requires an extraction procedure, followed by the concentration of cellular components into a buffer solution (Martens, 2001). The amount of ATP present is then determined directly using the luciferin−luciferase assay. ADP and AMP in the sample are then converted to ATP by enzymatic reactions and quantified.

AEC has been used to characterize microbial activity in both soil and subsurface environments. AEC analysis in subsurface samples from various depths revealed a wide range of values from relatively high values (0.76), which reflect an active population, to much lower values (0.23), which reflect dead or moribund microbial populations (Kieft and Rosacker, 1991). In surface soils, AEC analysis has indicated a range of microbial activity from inactive (0.5) (Rosacker and Kieft, 1990) to active (0.8) (Brookes *et al*., 1983; Ciardi *et al*., 1991). Drying or saturating the soil can cause a decrease in AEC, to below 0.4 in some soils (Inubushi *et al*., 1989; Ciardi *et al*., 1991). However, Rosacker and Kieft (1990) reported that air drying caused only a temporary decrease in AEC due to a transient increase in cellular AMP concentration. Increased AMP may have been the result of cellular catabolism of RNA, indicating that stressed cells may utilize endogenous metabolism of cellular macromolecules in order to survive. Such transient increases in AMP by stressed cells may affect the interpretation of AEC results.

The value of AEC measurement is that it can establish the presence of metabolically active microbial populations in environmental samples. For example, AEC has been used to characterize microbial populations in environments contaminated with pollutants, such as aviation fuel (Webster *et al*., 1992). AEC measurements in polluted environments can reveal the presence of microorganisms that are resistant to the adverse effects of the pollutants. These microorganisms may also be actively metabolizing the pollutants, thus removing them from the environment.

11.7 ENZYME ASSAYS

Enzymes are specialized proteins that combine with a specific substrate and act to catalyze a biochemical reaction. In the soil and sediment environments, enzymatic activity is essential for energy transformation and nutrient cycling reactions. For example, enzymes catalyze the hydrolysis of certain nitrogen-, phosphorus- or sulfur-containing organic compounds, releasing the ammonia, phosphate or sulfate constituents, which are then available

for assimilation by other organisms. Also of note are enzymes that catalyze the hydrolysis of plant constituents, such as cellulose, starch and other polysaccharides, releasing the monomeric sugar units, such as glucose, which provide important energy sources for microorganisms. Several reviews have examined enzymatic reactions in the soil environment, and discussed their importance in terms of soil fertility and ecosystem function (Burns, 1978; Tabatabai, 1994b; Morra, 1997; Burns *et al*., 2013).

One means of monitoring enzymatic reactions is to measure the conversion of a specific substrate into a product. An example is monitoring nitrate reductase activity through the disappearance of nitrate and the formation of elemental nitrogen. More commonly, enzymatic reactions are detected using a bioassay procedure that is specific for a particular class of enzymes. A bioassay utilizes a surrogate substrate that is transformed by a specific class of enzymes, producing a product that has specific properties for detection. An example is the conversion of *p*-nitrophenol phosphate by phosphatase enzymes, producing *p*-nitrophenol and phosphate. The indicator product, *p*-nitrophenol, can then be quantified spectrophotometrically. Alternatively, fluorescent compounds (e.g., 4-methylumbelliferone) can be used to label substrates for a variety of enzymes, including phosphatases. Use of a fluorimeter to detect the fluorescent reaction product allows greater detection sensitivity than can be achieved using spectrophotometric-based approaches (see Section 11.2.1.1).

The enzymatic activity within an environmental sample can originate from a variety of sources. Enzymatic activity can be directly associated with actively growing microorganisms, including bacteria, fungi and actinomycetes. These enzymes may be contained within the cell and are often located within the cell membrane. Enzymes may also be released from the cell, in which case they are called extracellular enzymes. Extracellular enzymes are released from actively growing cells to hydrolyze large polymers, such as the plant polymers, cellulose, hemicellulose and lignin, in order to facilitate their uptake for further metabolism. Alternatively, enzymes may be found outside cells as a result of the decay and disintegration of bacterial, animal or plant cells. Enzymes may be associated for a short time with moribund cells. These enzymes may also become stabilized on clay or humic particles within the soil structure and can remain viable for a period of time (Tabatabai, 1994b). Therefore, any bioassay of enzymatic activity in environmental samples will measure activity from all sources, and provide an indication of total enzymatic potential within that sample.

Characterizing and quantifying enzyme activity in environmental samples can reflect the health of the environment in terms of nutrient cycling and can also reflect soil fertility parameters, such as crop yield. However, there are problems associated with using enzyme assays to directly quantify microbial activity (Nannipieri *et al*., 1990). First of all, enzymatic activity associated with actively growing microorganisms cannot be easily separated from the activity of extracellular enzymes stabilized in the soil environment or enzymes associated with decaying cells. Another problem is that enzyme assays often require the addition of a surrogate substrate, and as a consequence, the assay determines the potential enzymatic activity and not the actual level of activity in the sample. Enzyme assays are also specific for a particular substrate−enzyme combination, and may not reflect the overall activity of all types of microorganisms. The simultaneous determination of a large number of enzyme assays may be more representative of overall microbial activity, but this approach is more labor intensive.

Many different enzyme assays have been developed to detect either specific or general microbial activity in environmental samples. Some examples are given in Table 11.2. The first several assays in this table measure general microbial activity, and the latter ones are assays for specific activity. One commonly used assay for general microbial activity is the dehydrogenase assay.

11.7.1 Dehydrogenase Assay

Dehydrogenases are intracellular enzymes that catalyze oxidation−reduction reactions required for the respiration of organic compounds. Because dehydrogenases are inactive when outside the cell, this assay is considered a measure of microbial activity. Dehydrogenase reactions can be detected using a water soluble, almost colorless, tetrazolium salt, which when reduced forms a reddish-colored formazan product that can be detected in a variety of ways. Tetrazolium salts compete with other electron acceptors for the reducing power of the electron transport chain. Thus, measurements of the reduction of tetrazolium salts will reflect electron transport chain activity. Hence, this measurement is an index of the general level of activity of a large part of the microbial community, but it is not a direct measure of microbial growth in terms of the production of new biomass. It should also be recognized that all respiring organisms have an electron transport chain, including both aerobic and anaerobic microorganisms. Also included in the measurement are eukaryotic populations, such as algae and fungi. Therefore, the measurement of tetrazolium reduction provides an overall indication of electron transport chain activity.

A commonly utilized tetrazolium salt is 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is transformed into an intensely colored, water-insoluble formazan (INT-formazan). INT has been used to measure microbial activity in surface waters (Posch *et al*., 1997), soil and sediment samples (Trevors *et al*., 1982; Songster-Alpin and Klotz, 1995), subsurface sediments (Beloin *et al*., 1988) and biofilms (Blenkinsopp

TABLE 11.2 General and Specific Enzyme Assays that can be used to Measure Microbial Activity

Enzyme	Substrate	Description of Assay
Dehydrogenase	Triphenyltetrazolium	Dehydrogenases convert triphenyltetrazolium chloride to triphenylformazan; the triphenylformazan is extracted with methanol and quantitated spectrophotometrically.
Phosphatase	*p*-Nitrophenol phosphate	Phosphatases convert the *p*-nitrophenol phosphate to *p*-nitrophenol, which is extracted in aqueous solution and quantitated spectrophotometrically.
Protease	Gelatin	Gelatin hydrolysis, as an example of proteolytic activity, can be measured by the determination of residual protein.
Amylase	Starch	The amount of residual starch is quantitated spectrophotometrically by the intensity of the blue color resulting from its reaction with iodine.
Chitinase	Chitin	Production of reducing sugars is measured using anthrone reagent.
Cellulase	Cellulose Carboxymethylcellulose	Production of reducing sugars is measured using anthrone reagent. Cellulases alter the viscosity of carboxymethylcellulose, a quantity that can be measured.
Nitrogenase	Acetylene	Nitrogenase, besides reducing dinitrogen gas (N_2) to ammonia (NH_3), is also capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4); the rate of formation of ethylene can be monitored using a gas chromatograph, and the rate of nitrogen fixation can be calculated using an appropriate conversion factor.
Nitrate reductase	Nitrate	Dissimilatory nitrate reductase can be assayed by the disappearance of nitrate or by measuring with a gas chromatograph the evolution of denitrification products, such as nitrogen gas and nitrous oxide, from samples; denitrification can be blocked at the nitrous oxide level by the addition of acetylene, permitting a simpler assay procedure.

From Atlas and Bartha (1993).

and Lock, 1990). Environmental samples are suspended in a solution containing INT and incubated for a matter of hours. Incubations between 1 and 12 hours are usually sufficient with shorter incubation times reducing the chance that the microbial community will undergo significant changes in activity. The production of INT-formazan can be detected by microscopic examination of the red INT-formazan deposits that form within the cells, or by quantifying total INT-formazan production. Microscopic examination gives an indication of the physiological status of the microbial population by determining the percentage of total cells that are actively respiring (Zimmermann *et al.*, 1978). Total cell number can then be determined using a counterstain, such as acridine orange, which stains all cells. The difference between the INT-formazan-containing cells and the total cell count is the proportion of the population that is metabolically active. This method is very sensitive, and can be used to detect electron transport chain activity even when low numbers of microorganisms are present or when samples are incubated at low temperatures (Trevors, 1984). For example, Posch *et al.* (1997) studied planktonic bacteria in a high mountain lake that was covered by a layer of ice at the time of sampling. The lake water was exposed to INT and incubated at the *in situ* temperature, 2°C, in order to determine the physiological status of the planktonic community. They obtained the surprising result that as much as 25% of planktonic bacteria in this oligotrophic lake were actively respiring. However, when using this method, it should be recognized that some of the cells considered inactive may in fact be respiring very slowly, or not utilizing INT as an electron acceptor (Posch *et al.*, 1997).

An alternative to microscopic examination of cells is quantification of the amount of INT-formazan produced. An environmental sample can be exposed to INT for a period of time, and then a solvent, such as methanol, is used to extract the INT-formazan from the cells. INT-formazan can be detected spectrophotometrically, and the total production calculated from a standard curve. In terms of quantifying total INT-formazan production, this measurement provides a relative index by which electron transport chain activities in different samples can be compared. However, INT has a low efficiency as an electron acceptor, and formazan production can be affected by numerous factors including: the concentration of INT used; incubation time; incubation temperature; pH of the sample; and whether the sample was incubated under aerobic or anaerobic conditions. Therefore, a direct comparison of activities of different samples can be made only if identical methods and experimental conditions are used (Trevors, 1984).

Several other forms of tetrazolium salts are available and provide certain advantages. For example, tetrazolium salt sodium 3′-{(1-[(phenylamino)-carbonyl]-3,4-

tetrazolium}-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) has the advantage of producing an orange-colored, water-soluble XTT-formazan product (Roslev and King, 1993). Because XTT-formazan is water soluble, its production can be quantified spectrophotometrically without the need to use solvent extraction. Another tetrazolium salt is 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The reduced form of CTC is a water-insoluble, red-fluorescent CTC-formazan, which forms deposits within the cell (Rodriguez *et al*., 1992). The fluorescence of CTC-formazan allows respiring cells to be highly visible when viewed under epifluorescence microscopy. This provides for easier enumeration of respiring cells in environmental samples. Using CTC, Winding *et al*. (1994) determined that actively respiring bacterial cells accounted for only 2 to 6% of the total population in an agricultural soil, and Schuale *et al*. (1993) determined that between 1 and 10% of bacteria in samples of drinking water were actively respiring. The fluorescent properties of CTC-formazan also provide a means of determining the location of physiologically active cells within attached biofilm communities without having to disrupt the biofilm structure (Yu and McFeters, 1994a). Schuale *et al*. (1993) used CTC to examine the viability of thin biofilms formed by microorganisms present in drinking water (Figure 11.20). Their results revealed that between 5 and 35% of total sessile bacteria were actively respiring. Yu and McFeters (1994b) reported that CTC provided a sensitive measure of the efficacy of biocidal compounds in disinfecting biofilms formed by a potentially pathogenic waterborne bacterium. A disadvantage of the use of CTC is that it is redox sensitive and in a low redox environment can be reduced by an abiotic chemical reaction (Schuale *et al*., 1993). Therefore, anaerobic pockets within a biofilm formation or within the soil environment may interfere with

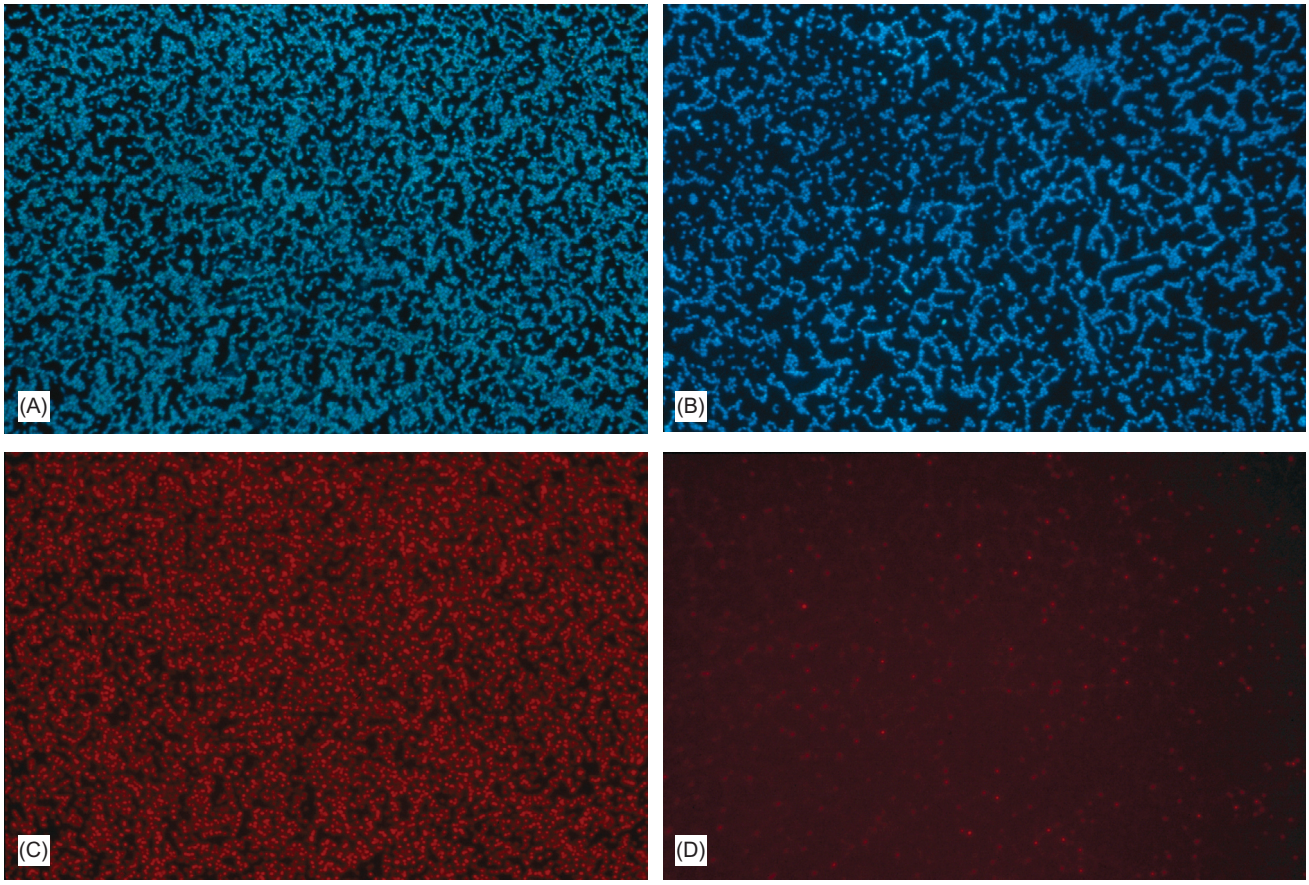

FIGURE 11.20 CTC-staining can be used to detect respiring cells in a biofilm. In this experiment, UV-sterilized, plastic, uncoated, microscope slides were placed in a sterile 1-liter beaker containing 750 ml of 10% R2A medium. The beaker was inoculated with 2 ml of a *Pseudomonas putida* strain 54G cell suspension, and after 24 hours, the slides were taken out and placed into mineral salts medium (starved cells) or R2A medium (unstarved cells). After a further period of incubation, each slide was stained with CTC and then counterstained with DAPI (see Section 9.4). The micrographs that are stained with CTC-DAPI (which stains all cells regardless of physiological state) show that comparable biofilms were formed on both slides (A, unstarved cells and B, starved cells). Examination of the slides stained only with CTC show that nearly the entire biofilm of unstarved cells is respiring (C), while only a few of the starved cells are respiring (D). The results of this experiment were enumerated, and showed that cells that were actively respiring comprised 76.8% of unstarved biofilm cells, but only 9.6% of starved biofilm cells. Data and photos courtesy G. Rodriguez and H. Ridgway, Orange County Water District, Fountain Valley, CA, U.S.A.

the detection of respiring cells, although these are issues for further research.

11.8 STABLE ISOTOPE PROBING

Stable isotope probing (SIP) is a relatively new technique that has great promise for identifying microorganisms involved in specific biogeochemical processes. SIP is similar to the radiolabel-based detection methods discussed previously in this chapter, but it involves the use of a stable (non-radioactive) isotope-labeled substrate to follow the fate of the substrate as it is metabolized by an intact microbial community (Kreuzer-Martin, 2007). The two most commonly used isotopes for SIP are ^{13}C and ^{15}N. As microorganisms metabolize the ^{13}C- or ^{15}N-labeled substrate, they will incorporate the isotope into their biomass. Extraction and analysis of biomarker molecules can then provide information about the fate of these isotopes within the microbial community, and thus insight into which microorganisms were involved in degrading the labeled substrate. In the first demonstration that SIP could work, Radajewski *et al.* (2000) grew the bacterium *Methylobacterium extorquens* in pure culture on either $^{12}CH_3OH$ ("light" methanol) or $^{13}CH_3OH$ ("heavy" methanol), extracted the DNA from both cultures and subjected the DNA to a density-gradient centrifugation. As can be seen in Figure 11.21, the light and heavy DNA clearly separates into distinct bands.

This technique can also be used to identify which populations within a microbial community are involved in the degradation of a specific compound. In theory, if you provide $^{13}CH_3OH$ to a microbial community instead of a pure culture only DNA from the populations within the community that utilized the $^{13}CH_3OH$ would be labeled with ^{13}C and appear in the heavy isotope band. The remaining populations that did not participate in utilization of the $^{13}CH_3OH$ would appear in the light isotope band. This means that one can separate or target "active" portions of the community from "inactive" ones. In this example, the active portion of the community includes the populations that utilize $^{13}CH_3OH$, while the inactive portion of the community does not. However, in reality, there is the potential for cross-feeding to occur where metabolites produced by microorganisms, which are degrading the labeled compound, are taken up by other microorganisms that did not directly participate in conversion of the added substrate. This can be particularly challenging if working with recalcitrant substrates that require long incubation times for degradation to occur.

In SIP, the stable isotope most often used is ^{13}C, and the biomarker molecule most often used is DNA, although biomolecules such as 16S rRNA, lipids and proteins are also used (Figure 11.22). The choice of stable isotope and biomarker is important because each alternative has advantages and disadvantages (Neufeld *et al.*, 2006).

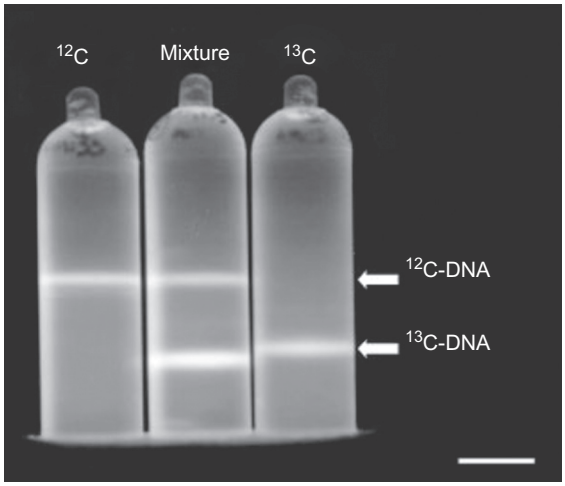

FIGURE 11.21 Centrifugal separation of ^{13}C-labeled from unlabeled DNA for stable isotope probing (SIP) experiments. The three centrifuge tubes were loaded with isotopically distinct DNA. The tube on the left contains only ^{12}C-DNA, the tube on the right contains only ^{13}C-DNA, and the middle tube contains a mixture of ^{12}C-DNA and ^{13}C-DNA. The pure fractions and a mixture of the DNA were extracted from a *M. extorquens* AM155 culture utilizing either ^{12}C- or ^{13}C-methanol as the sole carbon source. Bar, 1 cm. From Radajewski *et al.* (2000).

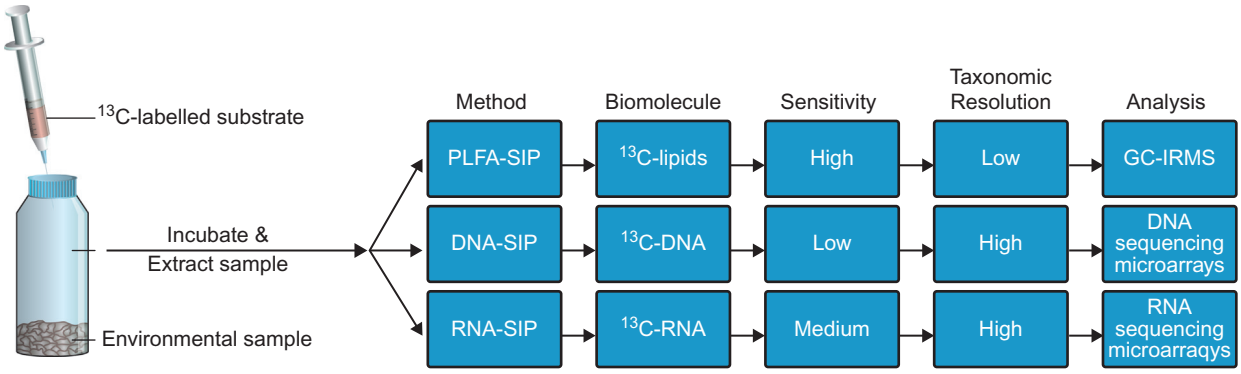

FIGURE 11.22 Example methods and biomolecules used for stable isotope probing of environmental microorganisms. GC-IRMS, gas chromatography-ion ratio mass spectrometry. Adapted from Gutierrez-Zamora and Manefield (2010) and Neufeld *et al.* (2006).

The use of DNA-SIP or RNA-SIP provides the most complete phylogenetic information but requires the separation of ^{13}C-labeled from unlabeled DNA or RNA (Figure 11.21) before being used for downstream analysis such as DNA sequencing (Chapter 13). This reduces the sensitivity of the technique in terms of microbial numbers that are required and the amount of growth that must occur to incorporate sufficient isotope label into the biomarker. In contrast, the use of lipids, called phospholipid-derived fatty acids or PLFA-SIP, does not require separation of the ^{13}C-labeled from unlabeled fatty acids before being analyzed by gas chromatography-ion ratio mass spectrometry (GC-IRMS). Therefore, it can be a more sensitive technique when microbial numbers and growth rates are low. On the other hand, PLFA-SIP does not give good phylogenetic information, just a gross assignment to a domain (e.g., archaea or bacteria) or a large division within a domain.

The development of new methods that directly combine mass spectrometry with DNA- or RNA-based analyses offers the potential to retain the phylogenetic information provided by nucleic acid-based methods while avoiding the need to first separate the labeled and unlabeled nucleic acids. One such example is the use of microarrays combined with high-resolution secondary ion mass spectrometry imaging (NanoSIMS). Mayali *et al.* (2012) used this approach, which they termed Chip-SIP, to characterize metabolism of labeled substrates by microorganisms in a natural estuarine community. Isotope (^{13}C and ^{15}N)-labeled substrates including amino and fatty acids were added to microcosms containing the microbial communities. After a short incubation ($\leq$24 h), microorganisms were recovered from the microcosm via filtration. Microbial RNA was then extracted and hybridized to a microarray which was designed to work specifically with NanoSIMS, and that targeted organisms found in the estuarine community and related marine environments. Scanning the microarray revealed which phylogenetic probes bound isotopically labeled RNA, and thus identified which microorganisms likely metabolized the added substrate. The ChipSIP analysis found that the labeled substrate was partitioned into a wide variety of microorganisms (81 distinct microbial taxa), likely due to the relatively simple structure of the added compounds (e.g., amino acids) and their ubiquitous presence in natural environments. Researchers are also developing methods that combine NanoSIMS with electron microscopy-based methods (e.g., scanning transmission X-ray microscopy; STXM) that potentially allow detection of individual microorganisms (at the cellular level) which have incorporated an isotope label into their biomass (Behrens *et al.*, 2012). These new approaches that combine physiological methods with other approaches (e.g., nucleic acid-based methods) have the potential, over the next decade, to revolutionize our understanding of environmental microorganisms and the processes they conduct.

QUESTIONS AND PROBLEMS

1. You are given a pure culture of four different benzoate-degrading microbes and asked to evaluate which has the fastest degradation rate (μ_{max}). Design an experiment to answer this question. In your experimental design be sure to specify the culture medium to be used and how you will analyze benzoate degradation.
2. Next you are given four different soils and asked to evaluate the capacity of each soil to degrade the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D). Design an experiment to answer this question. Be sure to specify how the experiment will be set up and how you will analyze 2,4-D degradation.
3. One of the soils that you tested in problem 2 shows rapid rates of 2,4-D degradation. How would you determine whether there is one or more than one different population degrading the 2,4-D?
4. You are given an assignment to determine the impact of agricultural practices on microbial activity in soil. You collect a series of soil samples from plots that are undisturbed (no crops, no fertilizer), cropped with normal tillage, cropped with reduced tillage and cropped with no tillage. In addition, a high and low fertilizer application rate was used. Thus, there is a combination of seven different treatments. What microbial activity tests would you choose to run and why? How would you set up these tests?
5. You are assigned to restore a site in a national park that has been severely disturbed by overuse. The site currently has no visible plant growth. You would like to add an organic amendment to stimulate microbial activity. You test several amendments for their effect on activity and for their longevity. These include a manure amendment, wood chips and a manure−wood chip mixture. Describe how you would determine the effect of these different amendments on (1) microbial biomass and (2) microbial activity including nitrification and heterotrophic activity.

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